Past Endemic Malaria and Adaptive Responses in the Fens and Marshlands of Eastern England

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ABSTRACT

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Changes in climate have increased concerns over the return of temperate malaria to the United Kingdom. Hence, studies of ancient disease are becoming more relevant for future health predictions in areas which are under threat of disease re-emergence. Conditions were likely ideal for *Plasmodium vivax* malaria from at least the Roman period, and recent research on Anglo-Saxon Fen populations has suggested an indigenous malarial presence. The primary aim of this project was to investigate the presence of English malaria in archaeological Fen cemetery populations from the Roman, Anglo-Saxon, and later medieval periods (c. AD 40-1600), using biomolecular analyses of human bone, and palaeopathological analyses via extant published data. A further aim was to investigate Masters' (1987) hypothesis concerning preferential survival of non-collagenous proteins (NCPs) within archaeological bone.

Indirect evidence for malaria was sought by reassessing archaeological reports for osteological evidence of the genetic anaemia β thalassaemia at 13 cemetery sites (five Roman, seven Anglo-Saxon, one late medieval) closely associated with the Fens and marshlands of Lincolnshire and Cambridgeshire. A palaeodemographic comparison of 30 Fen (five Roman, 21 Anglo-Saxon, four late medieval) and 31 non-Fen (nine Roman, 18 Anglo-Saxon, four late medieval) cemetery populations was also undertaken to assess any impact of *vivax* malaria on mortality. Osteological evidence does not support the presence of past thalassaemia, with palaeodemographic analysis suggesting an acquired, rather than genetic immunity in the Fens. Possible evidence emerged for 'healthy adaptation' to the increased stresses of Fenland life, and one population provided tentative evidence of intrauterine growth restriction, a condition strongly linked to endemic *P. vivax*. Direct evidence was sought by attempting to extract and test anti-malarial antibodies from human bone samples from 13 Fen-associated cemetery sites (five Roman, seven Anglo-Saxon, one late medieval), encompassing 24 individuals. Bone preservation was assessed in over 200 samples to provide a baseline for sample selection for biomolecular analysis.

Analysis of bone samples proved unsupportive of Masters' (1987) hypothesis. However, a range of endogenous proteins and a possible pathogenic disease marker were revealed, as was a correlation between bone preservation and NCP content. Evaluation of extraction protocols failed to yield antibodies which, if present, were consistently masked by collagen. Consequently, a novel antibody extraction technique has been developed. If successful, this could lead to a replicable technique of ancient, reactive antibody isolation, which would offer an invaluable new tool in biomolecular palaeopathology.

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DECLARATION

I, Ross Kendall, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

STATEMENT OF COPYRIGHT

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For Ellen, Lucie, and Elias - my lights.

"For sartain, tha wor mostly shakin i' they toimes; for tha agur an' fever were terrible bad, an' thar wor poor weak crysoms, fit for nowt but to soop gin an' eat op'um" (Balfour, 1891:150).

CHAPTER 1: INTRODUCTION

"Severe agues, which the inhabitants are rarely without, whose complexions from those distempers become of a dingy yellow colour, and if they survive, are generally afflicted with them till summer, and often for several years, so it is not unusual to see a poor man, his wife, and whole family of five or six children, hovering over their fire in their hovel, shaking with an ague fit all at the same time" (Hasted, 1797:144).

Malaria is a protozoan disease caused by inoculation of *Plasmodium* parasites into the bloodstream through the bite of an infected *anopheline* mosquito (Warrell, 2002). Malaria is endemic in many regions of the world, with up to 3.4 billion people living at risk of infection (Hay et al., 2004). The disease kills over 600,000 people worldwide per year World Health Organization, 2013), and has thus been described as being "one of the top three killers among communicable diseases" (Sachs and Malaney, 2002:680).

Concerns over the return of temperate malaria to the United Kingdom have recently been raised based on a number of ecological and environmental changes. These include an increased presence of indigenous mosquito species, particularly in urban settings, a recent push for wetland restoration projects (e.g., The Great Fen Project), and the elevated average temperatures associated with global warming (Lindsay and Thomas, 2001; Willott, 2004; Lindsay et al., 2010; Medlock and Vaux 2011; Townroe and Callagham, 2014). Studies of past disease are becoming important to future health predictions in areas under threat of disease re-emergence resulting from climatic and environmental changes (Greenblatt and Spigelman, 2003).

The presence and likely endemicity of temperate English malaria between the 16th and 19th centuries is well established (Dobson, 1997; Lindsay and Thomas, 2001), supported in part by the increased prevalence of seasonal mortality rates in marshland parishes (Dobson, 1997). *Plasmodium vivax* is the most widely distributed species of human temperate malaria, with up to 2.6 billion people living at risk of *vivax* malaria infection (Hay et al., 2004; Baird 2007). In antiquity, prior to the availability of prophylactic and curative medications, the disease would have had a profoundly negative impact on the health and well-being of affected populations, being chronic, debilitating, and potentially fatal, especially when comorbid with other commonly encountered pathogens (Dobson, 1997). Despite the reservations of some authors (e.g., Bollet 2004; Pinello, 2008) to the contrary, it

is highly likely that *P. vivax* malaria was present, if not endemic, in the marshes and Fens of south east and eastern Britain for many centuries prior to the post-medieval period. It seems naïve to assume otherwise, given that the climatic, geographic, epidemiological, and demographic conditions were all suitable for the transmission of *vivax* malaria prior to the 16th century. This study proposes to investigate a number of sources of indirect and direct evidence for malaria in British Fenland populations from the Roman to medieval periods.

There has been little traditional palaeopathological research performed on *vivax* malaria. This may be surprising, given the likelihood that the disease undoubtedly affected so many people in the past. Yet the reason for this dearth of research is relatively simple: although *vivax* malaria can be a chronic, recurrent condition, the disease elicits no direct osseous response. There are, therefore, no macroscopically observable skeletal changes that can be unequivocally attributed to malarial infection. Research on putative English malaria prior to the 16th century has, therefore, concentrated on identifying either direct evidence of infection, such as residual ancient *P. vivax* DNA in bone (e.g., Pinello, 2008), or on indirect evidence of malaria, such as attempts to correlate prevalence rates of non-specific skeletal stress markers with likely malarious locations (e.g., Gowland and Western, 2012). Indeed, the latter has provided compelling evidence for an indigenous malarial presence in British antiquity, as suggested by an increased *cribra orbitalia* prevalence in Anglo-Saxon Fenland populations. This condition, which may result from haemolytic anaemia, is one of the most commonly observed sequelae of *vivax* malaria. The aetiology of *cribra orbitalia*, however, is complex. For instance, megaloblastic anaemia resulting from concurrent parasitic infection may also influence its development (Bathurst, 2005; Walker et al., 2009).

The lack of malaria-specific skeletal changes forces a reliance on biomolecular analysis in the search for direct evidence of past temperate *Plasmodium vivax* malaria. The clinically observed longevity of anti-malarial antibodies in life (Wipasa et al., 2010) and the high affinity of antibodies for adherence to, and concentration within bone mineral (Omelyanenko et al., 2013), suggests that immunological analysis may be the most likely method of successfully detecting the disease in bone. Extraction of endogenous antibodies has advantages over the search for exogenous antigens or pathogenic ancient DNA (aDNA), since malaria-specific antibodies can circulate for an extended period (Wipasa et al., 2010), long after pathogenic biomolecules have been removed from the body. As long as antibodies

are retained in the bone, they represent a far more stable target for analysis than pathogenic molecules, which are, by nature, transitory. This assumes, of course, that the antibodies are indeed extant in the archaeological samples, and that they can be successfully extracted and identified.

1.1: Research aims

The main aim of this study was to detect direct evidence of malaria by extracting immunoreactive antibodies from archaeological human bone of individuals inhabiting likely malarious areas. These areas comprise the Lincolnshire and Cambridgeshire Fens and associated marshlands from the Roman to late medieval periods (*c*. AD 40 to 1600). Successfully extracted antibodies could then be tested against *vivax* malaria antigens using commercially available immunological tests (e.g., Enzyme-linked Immunosorbent Assay, or ELISA) in order to detect the presence of the disease.

Reliable and replicable immunological testing of the type proposed in this study requires the extraction of well-preserved, immunoreactive archaeological antibodies. This represents a considerable challenge, with only a handful of publications reporting successful antibody/antigen reactions (e.g., Kolman et al., 1999; Torres et al., 2002; Schmidt-Schultz and Schultz, 2004). Each of these used different protein extraction and characterisation techniques, demonstrating a clear lack of standardisation in methodologies aimed at extracting immunoglobulins from ancient bones. A major problem with utilizing archaeological antibodies, which affects all biomolecular techniques, is diagenesis. This is the term for the complex set of intrinsic and extrinsic factors which degrade organic material (including bone) following the death of an organism. Although advances have been made in understanding protein diagenesis, degradation processes in archaeological human bone remain unpredictable and often idiosyncratic, dependent upon a multitude of factors. These also often result in little correlation between macroscopic and microscopic bone preservation. Assessing target bone sample preservation through histological analysis may, therefore, aid in screening out those affected by diagenesis, and has been advocated by studies as an important precursory step to protein extraction (e.g., Hanson and Buikstra, 1987; Schoeninger et al., 1989; Schmidt-Schultz and Schultz, 2004). Recent technological improvements in protein mass spectrometry (proteomics), which have led to ground-

breaking and innovative characterisation of the extant protein content (or proteome) of ancient bones (e.g., Buckley et al., 2011; Cappellini et al., 2012; Wadsworth and Buckley, 2014), offer the potential to aid in characterising antibody preservation following extraction.

This research will also attempt to investigate the claim that non-collagenous proteins (including antibodies) may preferentially survive the diagenetic changes encountered within the burial environment, due to their high affinity with bone mineral (after Masters, 1987). Analysis of bone samples displaying varied levels of histological preservation may reveal different patterns of surviving exogenous and endogenous proteins. This would provide important information which may be used to direct future studies into archaeological protein survival, or in the targeting of specific proteins for analysis.

Research on indirect evidence of putative past English malaria will also be conducted to complement the direct biomolecular line of inquiry. This will be attempted by two methods. Firstly, published osteoarchaeological reports on populations from likely malarious areas will be examined, in order to identify phenotypic evidence of genetic responses to the long term presence of endemic malaria. This is in response to the recent identification of possible cases of thalassaemia in non-adult skeletons from Poundbury Camp, Dorset (Lewis, 2010). Thalassemia is one of the most commonly observed polymorphisms in modern populations. High prevalence rates of the condition are seen in areas with strong historical ties to endemic malaria, since the condition confers increased protection against malaria infection (Duffy and Fried, 2006). Diagnosis of thalassaemia in archaeological human remains has been hampered firstly by the non-specific nature of possible skeletal sequela resulting from the condition, and secondly, by the unpredictable nature of phenotypic expression of the disorder, particularly in heterozygous genotypes. Additionally, the severity of thalassaemia in homozygous form would frequently have resulted in early infant mortality, often before the development of diagnostic skeletal changes (Ortner, 2003; Lagia et al., 2007; Lewis, 2010). Conversely, heterozygous thalassaemia may result in no skeletal changes at all. The majority of cases in antiquity would, therefore, likely be undetectable in the burial record by traditional palaeopathological analysis. However, pathological changes suggestive of thalassaemia at Poundbury (Lewis, 2010) provide additional diagnostic criteria which may not have been previously recognised in palaeopathological reporting. Additionally, since thalassaemia may not be expected to be

encountered in British archaeological populations, there is the potential for skeletal changes to be misinterpreted.

Secondly, a basic analysis of Fen and non-Fen cemetery demographic profiles will be attempted. A comparison of the age-at-death profiles of cemetery populations from likely malarious and non-malarious areas has not previously been performed, and the analysis of demographic differences between locations may prove useful in highlighting possible epidemiological influences on mortality. *P. vivax* infection, for instance, places a particularly heavy burden upon the pregnant and the very young, resulting in increased morbidity and mortality in these groups (Desai et al., 2007). It is possible that the presence of *P. vivax* malaria in past Fenland environments may have resulted in increased mortality rates for these groups. The difficulties and limitations inherent in extrapolating demographic trends from skeletal information will also be discussed.

1.2: Research questions

The study proposed above can be summarised into the following research questions:

1. Can direct evidence for malaria infection in past populations be detected through extraction and testing of anti-malaria antibodies from archaeological human bone?
2. Do non-collagenous proteins, such as immunoglobulins, preferentially survive within archaeological bone due to their high affinity to bone mineral?
3. Can indirect evidence for past malaria presence be obtained through the identification of skeletal changes suggestive of genetic anaemia in skeletal populations from potentially malarious areas?
4. Can the presence of putative malaria be evidenced by comparing mortality and survivorship patterns from cemetery populations associated with Fen and non-Fen/marshland locations?

1.3: Thesis structure

The thesis begins with an exploration of the historical and environmental evidence pertaining to the presence of malaria vectors and the *P. vivax* parasite in the British Fens and marshlands from the Roman to post-medieval periods. The chapter discusses how climatic

conditions, landscape modification, and resource utilisation may have influenced changing human settlement patterns, mosquito habitats, and parasite transmission. The chapter also discusses how documentary evidence, usually in the form of symptomatic descriptions, may indicate the presence of *vivax* malaria prior to the post-medieval period, while also examining outside perceptions of the Fens and Fen inhabitants. This is followed by a brief discussion concerning the eventual withdrawal of malaria from England in the late 18th to early 19th centuries.

Chapter 3 presents an introduction to malaria in terms of the different vectors and parasite species responsible for transmission and infection of human hosts. It then concentrates specifically on the temperate malaria species *Plasmodium vivax*, detailing its lifecycle and pathophysiology. Following this is an overview of the human immune system, its response to *Plasmodium* infection, and the status of immunity in temperate areas. Finally, the possible implications of the presence of *vivax* malaria for British populations in antiquity is assessed. Chapter 4 introduces the most commonly employed clinical methods of detecting malaria in modern settings, before discussing the history of palaeopathological attempts at detection. An exploration of potential methods of indirectly observing the disease in skeletal material follows. Biomolecular archaeology and the survival and interpretation of ancient biomolecules is then discussed, followed by an assessment of how clinical techniques may be adapted for the biomolecular detection of the malaria in ancient skeletal samples.

The material and methods (chapter 5) details and justifies the criteria for the selection of sites and individual samples for analyses of indirect and direct evidence of *vivax* malaria. It also discusses reasons for choosing specific methodologies for these analyses, before detailing each technique. This is followed by the results (chapter 6), which details the data obtained from both the direct and indirect lines of enquiry employed in the search for *vivax* malaria, while highlighting potentially informative patterns in the data. The discussion (chapter 7) interprets the results of the aforementioned analyses, taking into account the limitations of each chosen methodology. The extent to which each applicable line of evidence supports the hypothesis that *vivax* malaria played a significant epidemiological role in the pre-16th century Fens is evaluated. Finally, the conclusion provides a brief summation

of the project in relation to the research questions posed in Section 1.2, and addresses potential implications of the research.

1.3: Summary and research implications

Little palaeoepidemiological research has been conducted on Fenland populations, whose lives were intertwined with a unique environment of "…moory soil…watry atmosphere, with damp, unhealthy moisture... thick, stinking fogs, and noxious vapours" (Anonymous, quoted in Darby, 1940:117). An indigenous malarial presence in British antiquity has been strongly suggested by recent research on Anglo-Saxon Fen and marshland populations (Gowland and Western 2012), and epidemiological and demographic conditions were likely ideal for temperate malaria transmission from at least the Roman period (Knotterus, 2002). Despite the likelihood that *vivax* malaria exerted a significant epidemiological burden upon Fen populations for many centuries, evidence for its existence prior to the modern period has always been circumstantial. Direct observation of the parasite, or parasite products in archaeological contexts has not yet proven possible. This study is the first to take a different approach by attempting to identify evidence of the natural human biological response to *Plasmodium* infection, rather than the parasite itself, which is less likely to survive in the archaeological record.

The potential impact of this research upon the fields of palaeopathology and palaeoepidemiology should not be underestimated: a reliable antibody extraction protocol would provide a powerful new biomolecular tool for the study of ancient diseases, particularly where a disease is suspected, but not confirmable by traditional analyses. The identification of latent ancient diseases offers great potential for advancing our understanding of their evolution alongside their hosts, as well as for aiding in the construction of modern policies aimed at confronting potentially reemerging pathogens, such as *vivax* malaria. This research also has the potential to shed light on the interaction between environment and disease in these relatively unstudied ancient Fenland populations.

CHAPTER 2: MALARIA AND ENVIRONMENT IN BRITAIN: HISTORIC AND PREHISTORIC CONTEXTS

"*They live out their brief, miserable existences on the edge of a tomb…Good health is a blessing unknown to them. Born among the sources of insalubrity, they suffer its disastrous influence from an early age…They live in a state of permanent ill health, and go to sleep amid suffering only to wake to their pain…Everything conspires against their health: their dwellings, their habits, their rough, unhealthy, insubstantial food and the indifference with which they choose their drinking water* " (Bossi, 1808, quoted in Goubert, 1989:216).

This chapter will examine the documentary evidence which alludes to the presence of malaria in Britain, along with the environmental contexts in which the disease flourished, from the prehistoric to post-medieval periods. The majority of the earlier sources, particularly the Anglo-Saxon and later medieval documentation, are in the form of medical texts which describe treatments for the symptoms of agues and intermittent fevers.

There is no definitive answer regarding when malaria first reached British shores within its human host(s). Whenever this occurred, a native and highly effective mosquito vector was already in place to spread the parasite to the local population. *Anopheles atroparvus* is the only indigenous British mosquito species that "breeds in sufficient numbers and in close association with man to be an efficient vector amongst human population" (Dobson, 1980:376), and hence was almost certainly the vector that transmitted the original *Plasmodium* parasites. *A. atroparvus* retains a relatively widespread distribution in modern United Kingdom and Ireland (Snow, 1998). There seems to exist a latent assumption that the relative dearth of documentary evidence specifically describing English malaria means that the disease was not present prior to the 16th century (e.g., Bollet, 2004; Pinello, 2008; Neghina et al., 2010). This study will explore the available historical, environmental, and physical evidence for the presence of English malaria prior to the post-medieval period.

2.1: Prehistory and the Roman period to *c.* AD 400

The absence of pre-Roman documentary evidence dictates that attempts to trace the presence of British malaria into prehistory must rely either on inference from continental sources, or on alternative methodologies, such as the analysis of past environments, the

physical remains of the *Plasmodium* parasite, and its infected hosts. Ancient south Asian Vedic and Chinese medical writings, dating from 2700 to 600 BC, contain some of the earliest references to malaria-type intermittent fevers and their symptoms, including splenomegaly (enlarged spleen, referred to as 'ague-cake' in the post-medieval period), a condition strongly associated with malaria infection (Carter and Mendis, 2002; Packard, 2007; Neghina et al., 2010). By at least the fifth century BC *Plasmodium vivax* is considered to have been endemic in China, the Indian sub-continent, and Greece (Carter; 2003). The Hippocratic Corpus of medical texts, written between 450 and 350 BC, frequently, though somewhat simplistically, describe symptoms of a variety of intermittent fevers and their physiological impacts on patients living in proximity to marshland environments (De Zulueta, 1973; Grmek, 1989; Sallares, 2002).

Malaria in the forms of *P. vivax and P. falciparum*, quickly established itself in the populations of the eastern and southern Mediterranean around the 5th century BC (Carter, 2003). Anthropophilic *falciparum*, a relative new-comer in the evolutionary history of malaria, likely spread out of west sub-Saharan Africa within the last few thousand years (Carter and Mendis, 2002; Hay et al., 2010). Malaria was possibly spread to Italy through militaristic contact with Carthaginians and/or immigration from Greece (Sallares, 2002; Packard, 2007). *P. falciparum* malaria became the dominant species in central and southern Italy, out-competing *P. vivax* due to its higher reproduction rates in hot climates (Sallares, 2002). However, it was the ability of temperate *P. vivax* to survive at lower temperatures and to 'hibernate' through cold winters inside the human host, that propagated a migration to northern Europe and beyond, eventually reaching the United Kingdom, northern Russia, and the New World (Packard, 2007). The mosquito vector *A. atroparvus* is an opportunistic feeder on both livestock and humans (Sinka et al., 2010), and capable of overwintering in human habitations (Medlock and Vaux, 2011), close to the blood-meals required for *P. vivax* parasite sporogeny, and infective in temperatures as low as 3ºC (Dobson, 1980). The modern day presence of *A. atroparvus*, along with other *anopheline* vectors, suggests that the species are indigenous to the British Isles (Snow, 1999), and represent the most likely vectors in antiquity.

Given the understanding of the life-cycle and habitat requirements of *P. vivax* (discussed in sections 3.3 and 3.4), it is almost certain that it was this malaria parasite that

first established a foothold in the British Isles, although the exact timing of its arrival remains unclear. It has been suggested that the parasite was introduced into Britain around the first century AD by the influx of immigrants from the Roman Empire (Sallares, 2002), although this remains unsubstantiated (Pinello, 2008). If malaria *was* introduced into Britain from the Roman Empire, it is likely that *P. falciparum* entered simultaneously. It remains unclear whether British *anopheline* species are capable of transmitting *falciparum* malaria. Many studies have suggested a lack of capability (e.g., James et al., 1932; Schute, 1940; Ramsdale and Coluzzi, 1975; Ribeiro et al., 1989), although laboratory tests have confirmed the ability of *A. atroparvus* to transmit a particular Italian strain of *P. falciparum* (Dobson, 1997). One species of tree-breeding mosquito, *A. plumbeus*, is capable of its transmission (Marchant, 1997); this indigenous species was the likely vector for a small outbreak in post-medieval London (Kitching, 1971; Dobson, 1980). The climatic conditions in Britain would not, however, have allowed for stable transmission of *P. falciparum* to occur. Ambient temperature, aridity, and suitable vector presence are some of the most important determinants in the transmissibility of malaria (Gething et al., 2012); a parasite that requires a sustained temperature of over 20°C for more than twenty days for sporogenesis to occur (Dobson, 1997), along with the year-round presence of a transmission vector, would struggle to maintain a presence, let alone virulent endemicity, in past or present Britain.

Malaria may have been present in Scotland by AD 208, when the army of Septimus Severus was severely affected by a disease associated with marshlands (Sallares, 2002). It is, of course, possible that the parasite was already present in Britain prior to the Roman Conquest, perhaps transmitted through contact and trade with continental Europe. Sallares (2002) suggests that the recovery of archaeobotanical remains of opium poppies (*Papaver somniferum*) from East Anglian Iron Age sites may provide indirect evidence for the treatment of *vivax* malaria symptoms, since it points to a coordinated effort to cultivate a source of medicinal opium. It is, however, unknown whether opium poppies were initially cultivated, if at all, in Britain solely for treatment of malarial symptoms. The drug was certainly effective for the temporary relief of the symptoms of *vivax* malaria, although it is also known to have been used in later periods for conditions as diverse as insomnia, rheumatism, and neuralgia (Bonser, 1963; Dobson, 1997). The cultivation of opium poppies dates back to at least the 3rd millennium BC in Mesopotamia and opium was widely traded

throughout the Mediterranean and southern Europe by 1000 BC (Kritikos and Papadaki, 1967; Brownstein, 1993). It is likely that the medicinal properties of opium were known to the native British population before the possible Roman timeframe for the introduction of *P. vivax*. Beyond this tentative, indirect link to malaria, evidence for pre-Roman malaria in Britain remains elusive.

Iron Age settlement distribution on Fenland terraces, such as in the localities of Ely and Chatteris in Cambridgeshire, and around the Fen edge margins near Peterborough suggests the prevalence of contemporary utilisation of Fen resources (Hooke, 1998; Evans and Hodder, 2006). Such settlement is also noted around the Humber Estuary (Creighton, 1990) and in Romney Marsh, Kent (Eddison, 2000). Archaeological and aerial photographic evidence demonstrate that by the middle of the first century AD, the siltlands of Lincolnshire, in particular, were heavily colonised (Darby, 1983). Such environs were mainly utilised for the seasonal grazing of livestock and for salt production. The latter activity has been archaeologically recorded as occurring as early as the late Bronze Age (Chowne et al., 2001), and there is extensive evidence (in the form of salterns and briquetage) for Iron Age and Roman salt production throughout the coastal Fenlands, marshlands, and major estuaries of eastern England (Cracknell, 1959; Darby, 1983; Eddison, 2000; Rippon, 2000). Salt making evidence strongly suggests that people were working in close proximity to brackish marshes, and hence close to ideal *anopheline* breeding grounds, long before the arrival of the Romans. If these mosquitoes carried the *Plasmodium* parasite, then the local workforce would certainly have been frequently exposed, stimulating localised endemicity.

Fenland occupation intensified during the Roman period (Figure 2.1), partly in response to an increasingly favourable climate, which led to a falling water table and the exposure of extensive areas of fertile siltlands suitable for summer pasture grazing (Hooke, 1998). Areas of Romney Marsh in Kent were "sufficiently elevated and well-drained to allow Roman settlement to take place" (Cunliffe, 1988:83). Roman settlement has also been noted along the south bank of the river Humber (Ellis and Crowther, 1990) and on Canvey Island in Essex (Cracknell, 1959). This period saw the first attempts at large-scale Fen landscape modification projects (Darby, 1932), including the still-extant 85 mile Carr Dyke, which runs from the River Cam in Cambridgeshire to the Witham near Lincoln (Thorpe and

Zeffert, 1989). It is likely that the majority of these projects were aimed at providing convenient access routes, rather than representing determined attempts at drainage and land reclamation, such as occurred in late Anglo-Saxon and Post-medieval periods. The effects of improving climate and attempts at landscape management on the potential transmission and endemicity of malaria is unknown. On one hand, the increasing population and land exploitation would naturally intensify contact between the mosquito vector and its human prey, promoting a subsequent increase in parasitism. Conversely, modification of the environment, both through natural and human action, may have disturbed some of the *anopheline* breeding grounds, thus reducing the local mosquito population. This is one example of the highly complex relationship between environment, vector and human host. Towards the end of the Roman period, many sites seem to have been abandoned and subsequently buried by flooding events during the late 3rd and early 4th centuries (Darby, 1932).

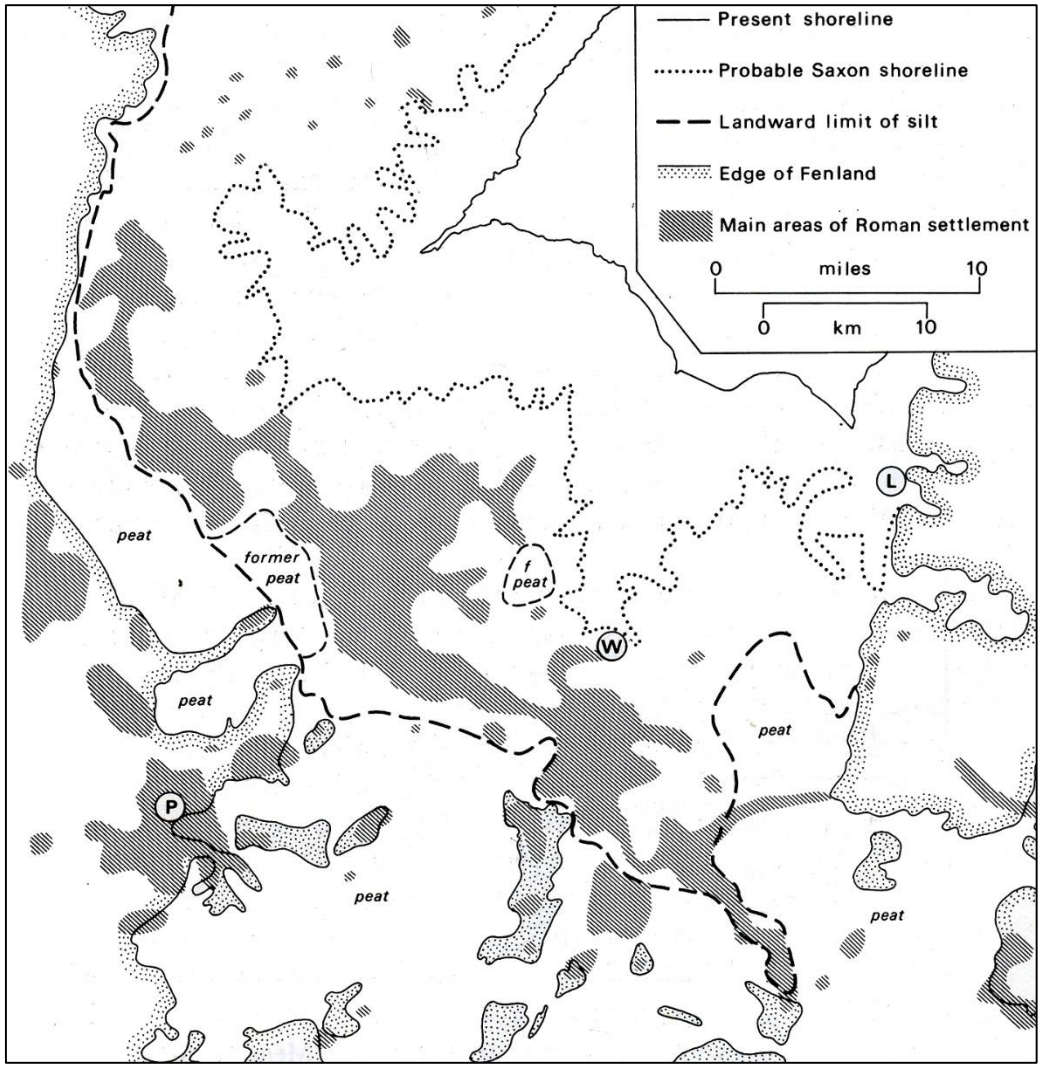

Figure 2.1: Roman settlement areas in the Lincolnshire, Cambridgeshire, and Norfolk Fenlands. L – King's Lynn; P – Peterborough; W – Wisbech (after Darby, 1983:4).

2.2: The Early Medieval (Anglo-Saxon) period *c*. AD 400 – 1066

If we are to accept the proposal that *Plasmodium vivax* first entered Britain with the conquering Romans and that, by this time, the siltlands and marshlands of eastern England were relatively well populated and utilised, then we may also assume that malaria infection was probably widespread by the start of the Early Medieval, or Anglo-Saxon period. Unfortunately, as with the prehistoric and Roman periods, documentary evidence for the presence of malaria in the early and middle Anglo-Saxon periods is virtually non-existent. Sallares (2002) suggests that writings by Gregory of Tours provide strong evidence for the presence of malaria in 5th century France, but it is not until Bede's *Historia ecclesiastica*

gentis Anglorum (Ecclesiastical History of the English People), compiled around AD 731, that reference to symptoms of putative English malaria is first made. Bede's accounts concern the miraculous healings of a young Lincolnshire boy and the sick of London suffering from '*febricintantes*' (Bonser, 1963). It is uncertain if Bede's '*febricintantes*' actually represented true malaria, since the word may have been used to refer to any acute fever (Howe, 1997).It should be noted, however, that Bede's original Latin word for fevers was replaced in the 9th century Anglo-Saxon translation by a potentially more convincing descriptor for malaria, '*lencten ádl'*, or 'Spring ailment' (Bonser, 1963; Howe, 1997). A progression from Bede's potentially ambiguous 'fevers', '*lencten ádl*' is also encountered in Bald's famous *Leechbook*, which was probably compiled no later than the late 9th century (Cameron 1993).

Numerous types of fevers are mentioned in Anglo-Saxon literature, including descriptions of burning fever (*bryne-ádl*), epidemic fever (*drif*), and shaking fever (*hriõian*). The Anglo-Saxon authors were clearly attempting to define different fevers by symptom, but it remains unclear which (if any) of these terms could refer to true malaria, as the disease can be characterised by all of these symptoms. However, Cameron (1993:10) suggests that "it seems clear that this name [*lencten ádl*] most commonly described an endemic form of tertian malaria [hence, *P. vivax* malaria], one in which the parasite remained dormant during much of the year, becoming active in spring about the same time as the adult forms of the mosquito vector emerged." It is also likely that the 9th century translator of Bede's work was drawing on knowledge of the different types of fevers when changing '*febricintantes*' to '*lencten ádl*', especially since Bede describes cases of fever occurring in potentially malarious areas.

Bruce-Chwatt (1976) suggests that '*lencten ádl'* was the precursory term for 'ague', which was in common usage by the 14th century. It has long been argued (e.g., by Creighton et al., 1965) that 'ague' was often used as a catch-all term to describe a number of diseases that are symptomatically similar to true malaria. Certain enteric diseases, such as typhoid fever, for instance, result in the classic malaria-type symptoms of high fever and swollen abdominal organs, which, as with *P. vivax* malaria, can relapse should the patient survive the initial infection. However, it can equally be argued that many of these early periodic fevers described as 'tertian' were probably true malaria, particularly if manifesting in patients

located near marshland environments (Reiter, 2000; Sallares, 2002). Even in modern clinical literature, the most commonly observed malarial symptom, febrile paroxysm, is sometimes referred to as 'ague attack' or 'ague fit', such is the longevity and ingrained nature of the word (Warrell, 2002).

The word 'mal'aere' (literally 'bad air') likely made its first appearance in 14th century Italian writings by Marco Cornaro (Neghina et al., 2010) and was transferred into English usage in 1740 by Horace Walpole to refer to the unhealthy summer conditions encountered in Rome during his travels (Sallares, 2002). During the 18th century, 'malaria' quickly became the term of choice for fevers resulting from contact with miasmic emanations (Bruce-Chwatt, 1976), although 'ague' remained in common usage into the early 19th century, particularly when discussing fevers associated with marshland environments. It was during this period that the two terms were often used interchangeably in both clinical and non-clinical literature. It was not until the turn of the 20th century that 'ague' was finally replaced by 'malaria' as the clinically favoured term (Bruce-Chwatt, 1976).

If Bald's Leechbook and Bede's translator were describing true English malaria, rather than conditions exhibiting malaria-type symptoms, what of the status of malaria in the intervening centuries since the end of the Roman occupation to the 8th century? As with the Roman period, the dearth of documentation for the Early and Middle Anglo-Saxon periods forces a reliance on alternate, and often indirect, methodologies of tracing the disease. Even working on the assumption that malaria was indeed present, and possibly endemic, in the Fens and marshlands of eastern England by the end of the Roman occupation, evidence for its presence is as ephemeral as for the people themselves. It is in these periods that possible indirect skeletal and environmental evidence (e.g., Gowland and Western, 2012), alongside potential biomolecular markers (e.g., Pinello, 2008), must be more heavily relied upon.

Environmental evidence from the early first millennium AD suggests deteriorating climatic conditions during the late third, fourth, and fifth centuries. Rising sea levels caused more frequent marine and fluvial flooding events, resulting in the deposition of silt and formation of fresh peat (Hooke, 1998). It has been suggested that the sea level in proximity to some areas of East Anglia (including the Fens) rose by as much as four metres during the early to mid-first millennium AD (Dark, 2000). The impact of this climatic change on Anglo-Saxon settlement patterns in the Fenland is not yet fully understood. Darby (1983:5)

suggests that at this time much of the Lincolnshire and Cambridgeshire Fenland "seems to have become a land of choked drains and abandoned settlements," while both Roffe (2005) and Rippon (2009) suggest that there was a discontinuity of settlement in the worst affected areas as people gradually relocated to higher ground. A comparison of Figures 2.1 and 2.2 does suggest a shifting Fenland settlement pattern from the Roman to Middle Anglo-Saxon periods, encompassing a general movement away from siltland area occupation.

By the 8th century, according to Bede, the southern Fen island of Ely in Cambridgeshire was both populous and prosperous (Darby, 1983), thus supporting the suggestion that the Fen islands were becoming heavily populated at the expense of the lower siltlands. The latter may have become largely unsuitable for continuous habitation due to poor climatic conditions, although the silt and marshland areas probably dried sufficiently in the summer months to allow for activities such as livestock grazing, salt production, and resource gathering. Recent archaeological work suggests that exploitation of the rich Fenland and marshland resources continued throughout the period (Rippon, 2009), although evidence for continuous siltland settlement remains ephemeral. Despite the inherent risks, remaining siltland occupation would probably have focused on raised roddons (elevated silt ridges), upon which small-scale production of salt-tolerant crops may have taken place (Murphy, 2010).

Despite the deteriorating climate, the Fenlands and marshlands would have continued to provide a rich variety of resources and economic opportunities, certainly enough to keep populations from completely abandoning the area. The Tribal Hidage, a document describing 7th-9th century areas and their hidage assessments, lists numerous communities situated in and around the Lincolnshire Fens, such as the *Spaldas* and the North and South *Gyrwe* (people of the Fens). Both Bede and the Tribal Hidage seem to provide "a clear picture of a peopled landscape" which was "organised into communities with recognisable identities" (Roffe, 2005:265) in the 8th century. The enormity of the Fens and marshlands of Eastern England, in terms of both scale and economic opportunity therein, meant that "the environment was a condition rather than a determinant of land use throughout the period" (Roffe, 2005:265).

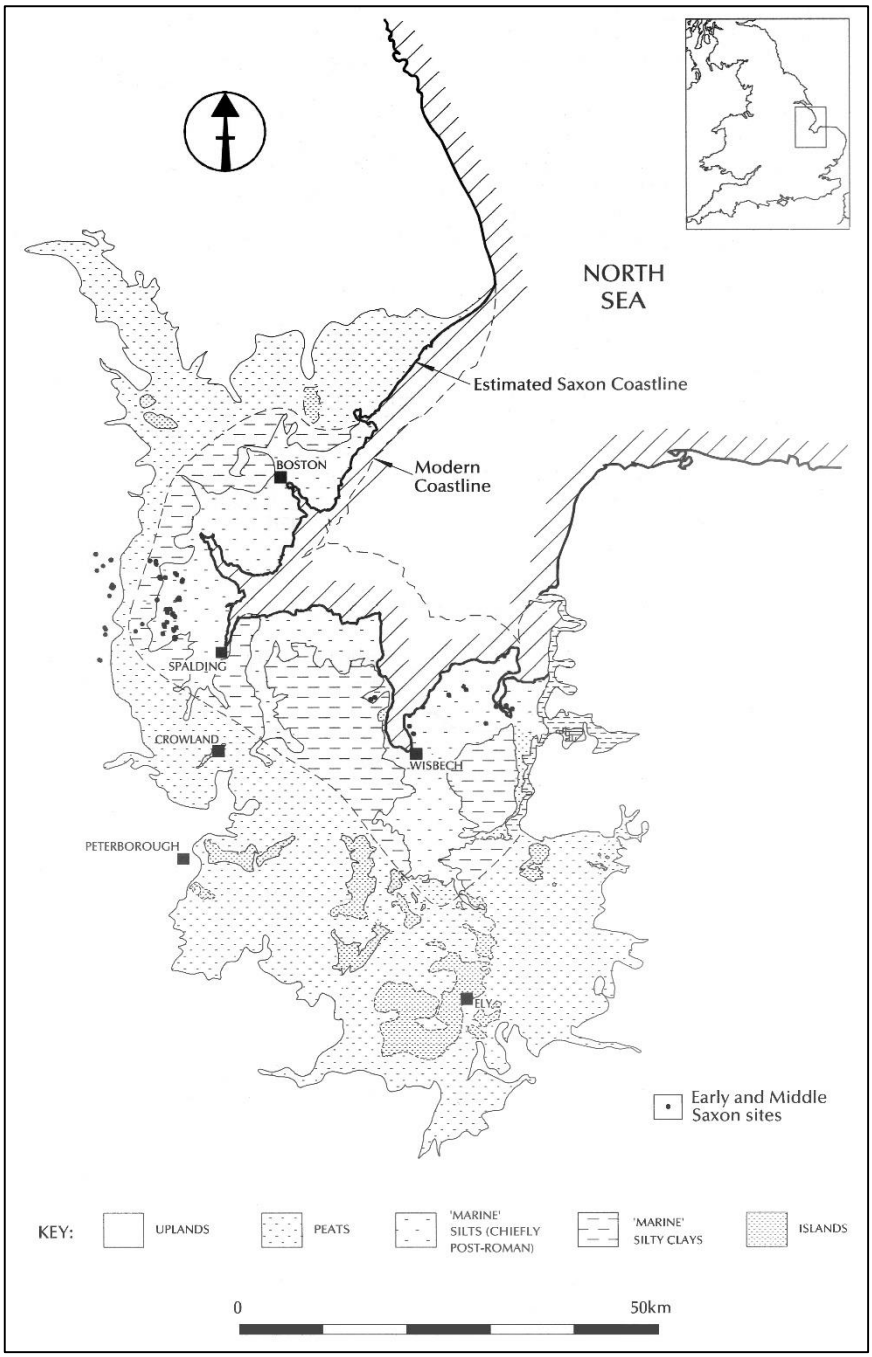

Figure 2.2: Distribution of Early and Middle Anglo-Saxon sites identified by the Fenland Survey in the Lincolnshire, Cambridgeshire, and Norfolk Fenlands (adapted from Crowson et al., 2005:5).

It is likely that the changing environmental conditions also impacted the *anopheline* breeding grounds and consequently, in areas which saw sufficient human depopulation, any

stable endemicity of malaria would probably have been interrupted. It would seem, therefore, advantageous to seek certain physical effects of the disease (e.g., possible biomolecular evidence, osteological pathological markers, or genetic resistances in human populations) in areas of continuous settlement, and hence, long-term, stable exposure to malaria. These areas include the southern Fenland islands and higher silt coastal regions of Lincolnshire, Cambridgeshire and Norfolk, the raised islands of southeast England, such as the Isles of Canvey, Sheppey, Grain, and Oxney, and the roddons, ridges and margins of North and South Kent and Essex marshes.

One of the earliest and most famous literary descriptions of the Fenland environment dates from the mid-9th century. Felix's exposition on the life of Saint Guthlac, who relocated to the Fen island of Crowland, Lincolnshire, and founded a monastery there in 716, describes the general area:

> "There is a midland district of Britain a most dismal Fen of immense size, which begins at the banks of the river Granta not far from the camp which is called Cambridge, and stretches from the now south as far north as the sea. It is a very long tract, now consisting of marshes, now of bogs, sometimes of black waters overhung by fog, sometimes studded with wooded islands and traversed by the windings of tortuous streams" (Colgrave, 1956:87, translating Felix).

Felix describes Crowland itself as:

> "…an island in the middle of the marsh which on account of the wildness of this very remote [spiritual] desert had hitherto remained untilled and known to few. No settler had been able to dwell alone in this place before Guthlac the servant of Christ, on account of the phantoms of demons which haunted it" (Colgrave, 1956:78, translating Felix).

A similar description can be seen dating from the late 7th century, when St. Boltoph arrived near Boston in the north Lincolnshire Fens, a place he found to be "infested with devils of various kinds" (Darby, 1934:191). Beowulf's monster, Grendel, also supposedly inhabited the Fenlands.

These highly illustrative accounts provide fascinating insights into the 8th century physical and metaphysical perceptions of the Fenland environments. The Fens and marshlands of England represented the interface of land and sea, and were a mysterious, remote wilderness supposedly inhabited by demons and mythical creatures (Rippon, 2009). The Fens were "spurned and held in fearful contempt" (Darby, 1934:192), particularly by

outsiders who would likely have heard and perpetuated tales of the heavy miasmic fogs and lurking evil creatures. These early descriptions must, of course, be taken in context. Felix, for instance, probably would not have been aware that the island of Crowland was home to continuous settlement from as early as the Bronze Age (Cope-Faulkner et al., 2010).

Narratives pertaining to the 'phantoms of demons' and 'devils' which haunted the Fens may well have alluded to the ever-present spectres of death and disease which permeated the Fenland environs (Dobson, 1997). Anglo-Saxon ideas concerning diseases and their effective treatments were based upon a complex amalgamation of "classical texts, Judeo-Christian belief and 'native' northern European lore…[which] complement each other to produce a truly rich and fascinating *mélange* of herbal plant lore, *materia medica*, spells, charms, rites and superstitions" (Pollington, 2000:27). This is hardly surprising, given the complicated, dynamic, and interspersed nature of the formation of the English population since the Roman Conquest. A full exploration of the corpus of Anglo-Saxon medicinal thought is beyond the scope this study, yet it is pertinent to touch upon these perceptions if we are to understand how the Anglo-Saxons viewed and understood the causes, symptoms and treatments of malaria.

Although the ancient Chinese seemed aware that malaria was contracted through the bite of an insect, there is no evidence that the classical Greek and Roman world had made this connection (Sallares, 2002). A common perception, witnessed, for example, in the construction of a temple to *Dea Febris* (Fever deity) in Rome and discovery of a Romano-British inscription from Risingham, Northumberland, dedicated to the same deity, was that intermittent fevers were of divine or demonic origin, a concept refuted by earlier Hippocratic and later Roman writers (Sallares, 2002; Neghina et al., 2010). Despite these refutations, the notions of supernatural causation remained entrenched outside of the educated elite for many centuries to follow. It is likely that the Anglo-Saxon understanding of malaria was as heavily influenced by classical mythological ideologies as it was by indigenous folklore and religion. Indeed, Sallares (2002:54) suggests that, in Rome at least, "the shift from pagan religions to Christianity probably made no significant difference to popular perceptions of malaria, which was frequently the target of healing miracles in Christian literature." For instance, Felix's own understanding of disease causes and

processes would have been heavily influenced by Christian ideology and native traditions/superstitions, as evidenced by his descriptions of the demon-haunted Fens.

Early medieval medical understanding was also influenced by the classical Greek theory of the four humours: blood, phlegm, red bile, and black bile. These humours worked in tandem to keep bodily function in a state of equilibrium. Deficiencies in, or imbalance of certain humours would lead to disease, which could only be cured by humoural balancing (Cameron, 1993). Strong backing by the Roman physician/philosopher Galen ensured that the theory of the four humours remained "a basic tenet of medicine for almost another two thousand years; it finally died out in the last half of the nineteenth century" (Cameron, 1993:160). Due to the fundamental misunderstanding of the causes and processes of malarial infection, early medieval treatments would have inevitably been ineffective at parasite elimination, although temporary relief of symptoms may, in some cases, have been achievable. Such treatments included herbal preparations and, failing this, complex magical rites (Bonser, 1963; Cameron, 1993), intended to cast out the underlying demonic influences that upset the balance of humours. Despite an intimate understanding of the clinical pathophysiology of *vivax* malaria, even modern treatment of the disease sometimes fails to prevent later episodic relapses (Baird et al., 2007). Thus, earlier medieval efforts (if they existed) beyond simple symptomatic alleviation would have almost certainly failed, leaving the patient to suffer repeated debilitating relapses.

Environmental evidence suggests that the late 8th century through to the end of the Anglo-Saxon period saw an improvement in climatic conditions. Known as the Medieval Warm Period (MWP), the three centuries preceding approximately AD 1250 saw a trend towards climatic warming and drying, and falling sea levels (Darby, 1983; Dark, 2000; Mann et al., 2009). Increasingly favourable conditions propagated a slow repopulation of the siltlands, although the continuity of such activity remains difficult to establish. Settlement would probably, at least in the early stages, have continued to be sporadic and very much dictated by short-term climatic variability. It is in this period that concerted efforts at landscape management within the Fens and marshes, with the general aim of removing excess water from the landscape (Rippon, 2009) can be seen. Some of the most impressive endeavours were the construction of the Lincolnshire Sea and Fen Banks sometime around the 10th century. The former was particularly impressive: this massive earthwork was

constructed around the Wash in order to protect siltland settlement, pasture and agriculture from marine inundation (Hooke, 1998). The Sea Bank held "the key to future reclamation of the Fens…permitting campaigns of drainage and reclamation" (Hall, 2005:xii). The introduction of watermills, flood gates, sluices, and the canalisation of major watercourses, such as the River Nene, further aided in the control of water (Hooke, 1998; Rippon, 2009).

The Cambridgeshire Fens, mostly under monastic ownership by the ninth century, were modified by a network of canals, which functioned as drainage and a method of linking the Fen-island monastic communities (Hall, 1988). Such improvements in the transport network would have been of great benefit to the populace and economy, although they may also have facilitated the Danes in their increasingly frequent ninth century raids (Darby, 1940). Similar contemporary attempts at marshland reclamation were also occurring in Romney Marsh (Hooke, 1998), most of which was occupied year round by the end of the Anglo-Saxon period (Eddison, 2000). The beginnings of Fenland management, reorganisation and subsequent increasing population density can be traced to these Late Anglo-Saxon attempts to manipulate and improve their surrounding environment.

2.3: The high and late medieval periods *c.* AD 1066-1600

Reclamation, repopulation, and socio-political organisation of the Fenlands and marshlands of eastern England were well underway by the time of the Norman Conquest. The Domesday Book, first assembled in 1086, provides the first detailed illustration of the settlement and population patterns of the Fenlands and marshlands (see Figure 2.3 for Lincolnshire, Cambridgeshire and west Norfolk). The Domesday Book records that by the late 11th century the coastal marshlands had been resettled quite extensively, including much of Romney Marsh (Eddison, 2000; Rippon, 2009). The importance that the extant Lincolnshire Sea Bank played in the increasing number of settlements cannot be underestimated, since all of the recorded Fenland Domesday settlements are found on the landward side of the Bank (Darby, 1983).

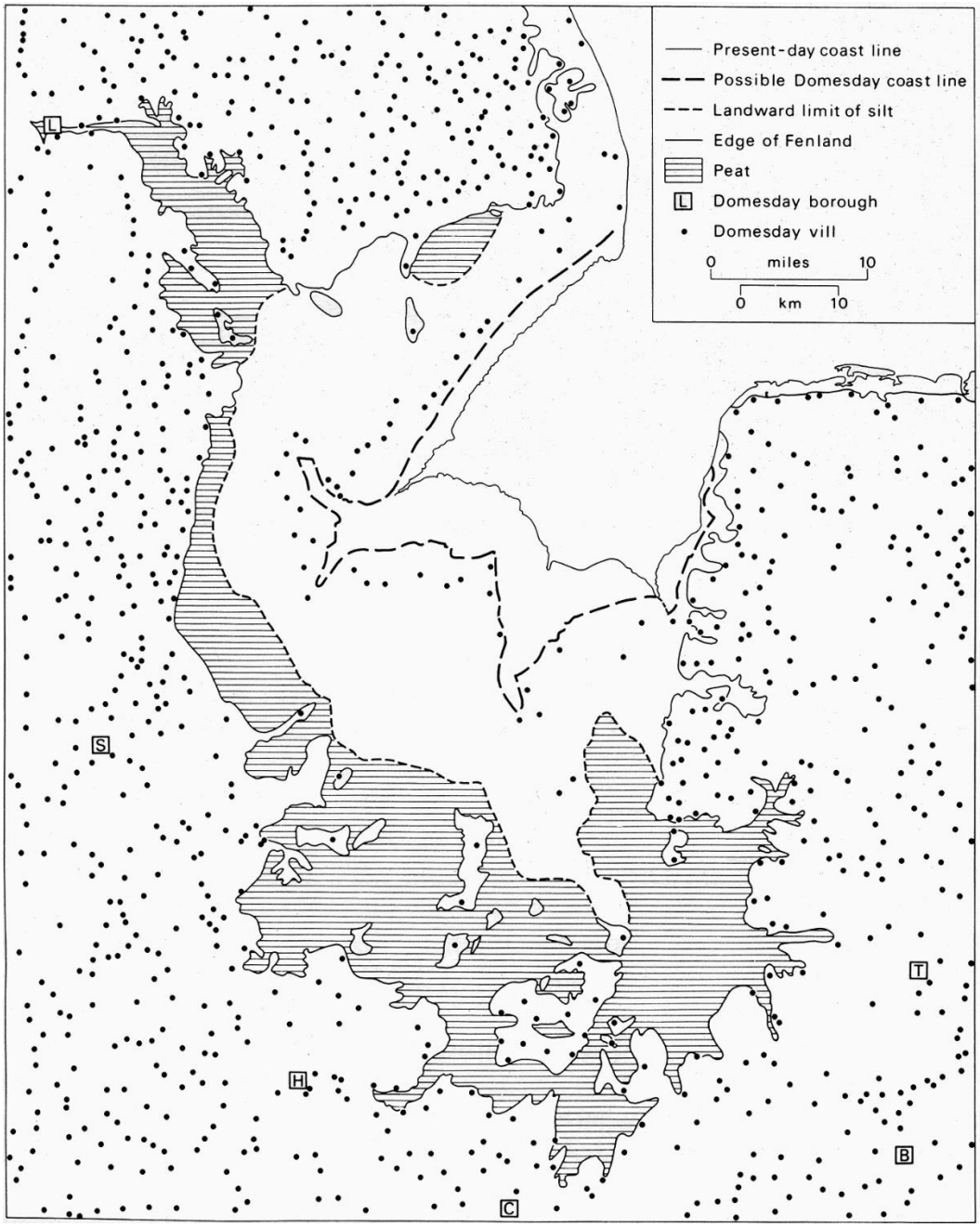

Figure 2.3: Distribution of Domesday settlements in the Lincolnshire, Cambridgeshire, and Norfolk Fens and marshlands (after Darby 1983:9).

Siltland settlements were generally well populated and prosperous in 1086, but their relative isolation within a flood-threatened environment meant that overall population density in the Lincolnshire and Cambridgeshire Fenlands remained moderately low (Figure 2.4), especially in comparison to some upland communities (Darby, 1983). There can be little doubt, however, that these Fenlands were about to experience something of a boom,

both in terms of population and economic prosperity. The coastal regions of Sussex, Essex, and marshland areas of Kent were already experiencing exponential population increase by 1086 (Cracknell, 1959; Smith, 1988). Despite the frequent records of disastrous flooding events over the following centuries (particularly during the mid-13th century) the Fens and marshlands continued to attract an increasing number of inhabitants. The imminent dangers, both environmental and epidemiological, of living and working in such habitats must have been offset by the attractive economic benefits.

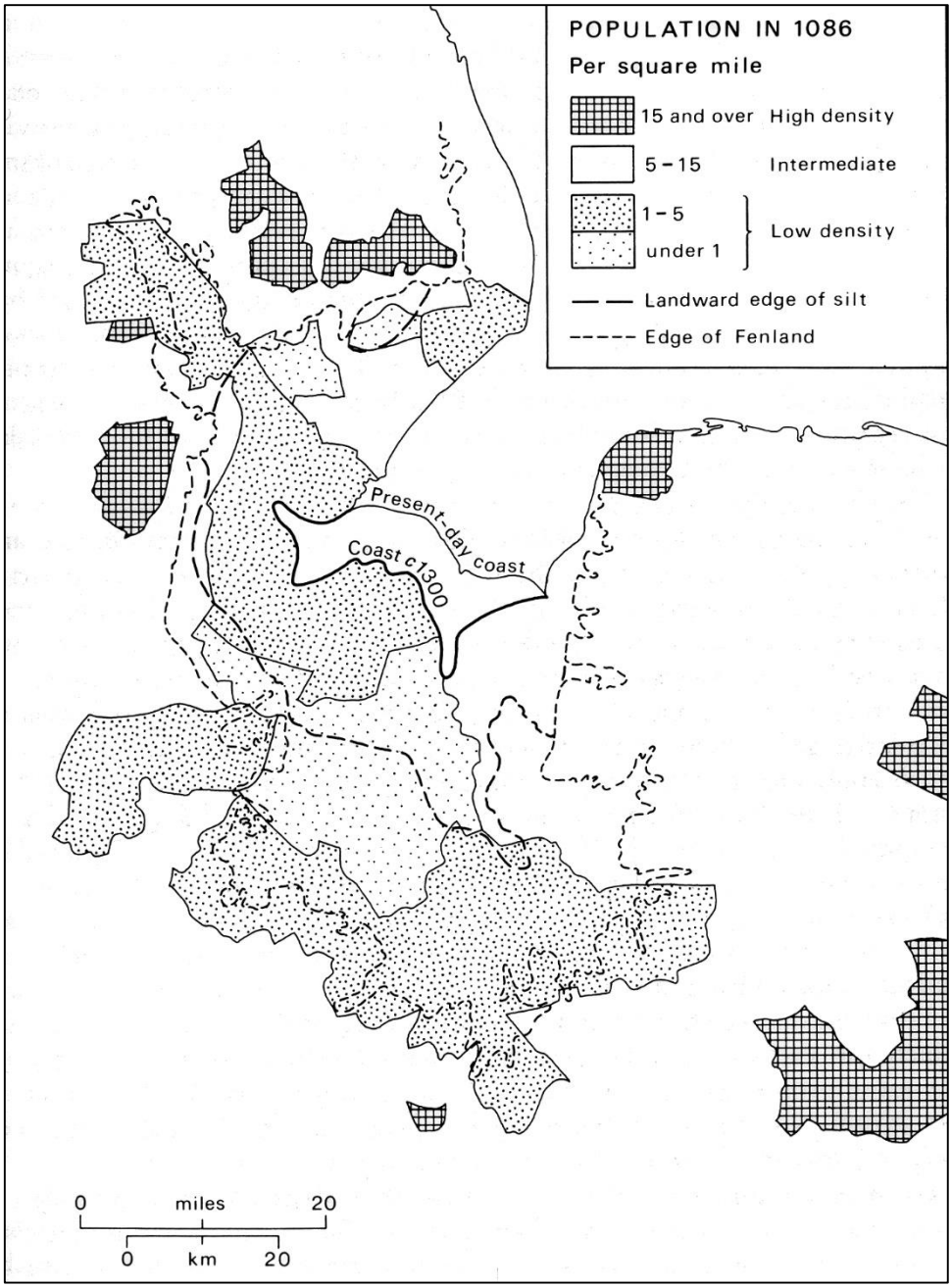

Figure 2.4: Lincolnshire, Cambridgeshire, and Norfolk Fenland population densities in 1086 (adapted from Darby, 1983:10).

Regional and national demand for salt propagated one of the earliest Fenland post-Conquest 'industrial' booms in the 12th century, which saw intensification of salt making activities to supply the North Sea fisheries. This was particularly evident around the Wash (Astill and Grant, 1988). Demand for salt rose so high that by the 13th century, continental sources of this vital commodity were being imported (Astill and Grant, 1988). Salt and

freshwater fishing industries blossomed, and mainstays such as the collection of reeds, rushes and sedges for thatching continued apace. Waterfowl represented an important commodity and the rearing of large flocks of geese became particularly prevalent in certain marshier areas of the Fens, such as around Lincoln and King's Lynn (Grant, 1988). Peat cut from the freshwater areas of the Fens was also becoming increasingly important as a fuel source for the growing local populations, particularly given the limited availability of alternative fuels (Darby, 1983).

The most important economic prospect offered by the siltland environment arguably came in the form of extensive pasture lands, which were rendered extremely fertile for summer livestock grazing by yearly winter flooding. Sale of much sought-after English wool was a driving force behind the Fenland economic and population boom in the three to four centuries post-Conquest. Grant (1988) states that during the last two decades of the 13th century, the wool from approximately three million sheep was exported from the Lincolnshire port of Boston. The number of extravagant Fenland parish churches constructed between the 12th and 15th centuries are testament to the wealth of the area (Darby 1940) - fortunes derived heavily from the wool trade. Sheep and wool were also important commodities in the medieval marshlands of Norfolk and Essex (Cracknell, 1959; Rippon, 2000). The boom in Fen exploitation can be seen in late 13th and 14th taxation records, which show that the resource-rich siltlands of south Lincolnshire were by this time "crammed with people" (Rippon, 2000:198), with population density higher than in the early 19th century (Hallam, 1961). Increasing 14th century population densities are also evident in Norfolk, Suffolk, Essex and Kent (Figure 2.5).

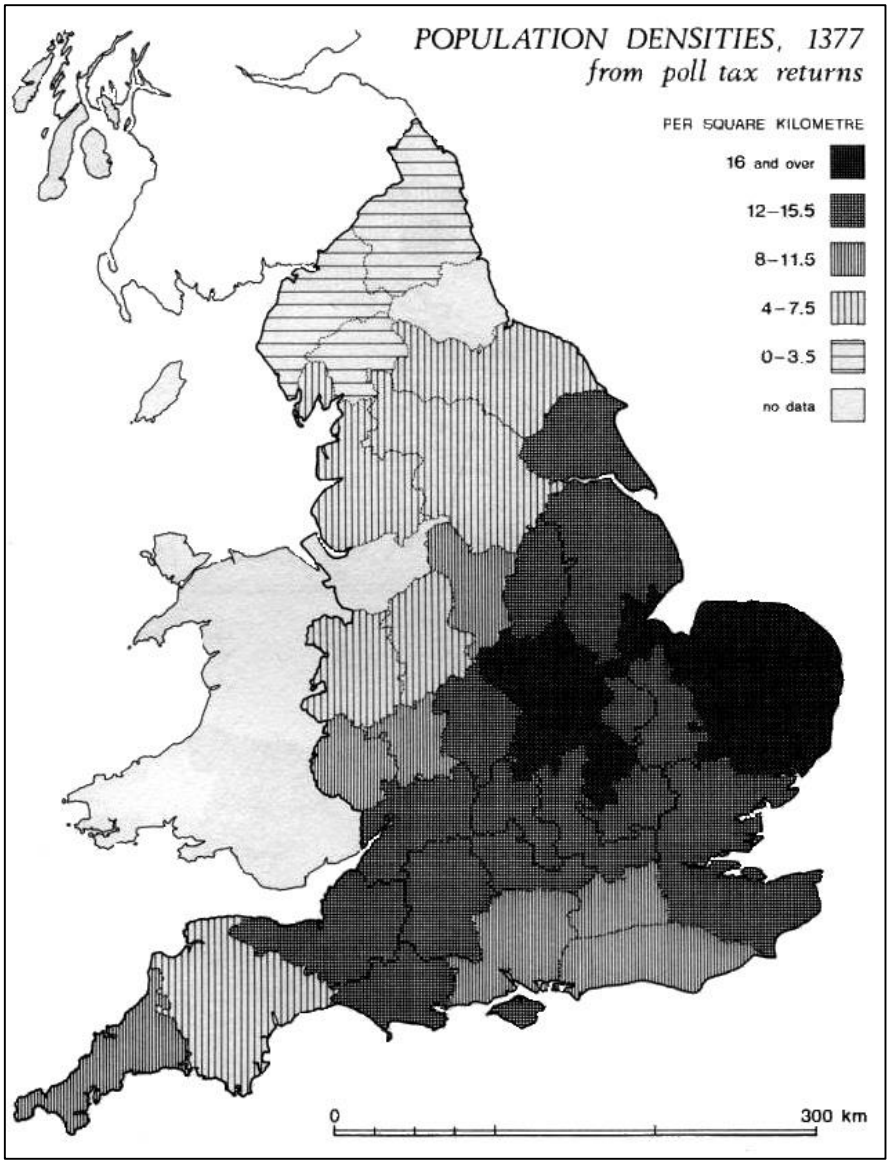

Figure 2.5: National population densities from the 1377 poll tax returns (adapted from Smith, 1988:199).

With increasing population levels and interaction within Fenland and marshland environments, a subsequent increase in contact with indigenous mosquito species should also be expected, yet the extent to which modification and reorganisation of the landscape would have affected *anopheline* breeding grounds, and hence the vector's general distribution, remain unclear. As previously stated, it is likely that any malarial transmission continued uninterrupted in those areas which experienced continuous settlement (e.g., the raised Fen islands), whilst areas experiencing temporary or fluctuating population levels

may not have been able to maintain any endemicity. The climatic data, settlement patterns and population densities from 1066 to the mid-13th century, however, suggest that there was potential for endemicity over a much wider range than during the middle centuries of the first millennium AD. It was not just large-scale land management projects that contributed to this; local smaller-scale changes may also have played an important role in facilitating the spread of malaria to new populations. Such an example can be found at Sedgeford in northwest Norfolk, which saw intentional, controlled flooding through the building of a dam (Faulkner, 2001), hence creating potentially new *anopheline* breeding grounds beyond the 'traditionally' accepted Fen/marshland malarial areas. Improving transport, trade, and communication links throughout the Fenlands may have propagated the spread of malaria from potentially endemic to non-endemic areas. Further to this, inter-continental trade and contact, particularly with southern Europe, an area described as being 'hyperendemic' with malaria in the medieval period (Gasper 2004), may have repeatedly introduced new strains of *P. vivax* into the existing Fenland populations.

It is easy to assume that drainage projects would reduce the mosquito population and the subsequent prevalence of malaria. It has, however, been suggested that wetland drainage and reclamation efforts lead to an *increase* in *anopheline* mosquito populations, at least in the short term (Horn, 1987). In a stable marshland environment mosquito population numbers are checked by predators. Disruption of the local environment through drainage efforts may spur an increase in *anopheline* numbers through the removal of both predators and competing *aedes* mosquito species which predominate in marshlands (Horn, 1987), but may be less adaptable to sudden environmental changes than their *anopheline* counterparts. This may explain the reported increase in biting insects immediately following post-medieval drainage efforts (Darby, 1956). Smaller scale Anglo-Saxon and medieval attempts may, therefore, have propagated an increase, rather than an assumed decrease, in malaria transmission.

Documentary evidence for malaria in the early centuries of the high medieval period remains confined to sparsely-encountered medical descriptions of malaria-type symptoms, despite the increasingly favourable environmental and demographic conditions for disease transmission. This can be seen as very much carrying on the medical traditions of the Anglo-Saxon period. Evidence that the theory of the four humours remained at the core of the

medieval understanding of malaria-type fevers can be seen in the *Ramsey Scientific Compendium*, which was probably compiled around 1110 (Cameron, 1993). The *Compendium* contains a detailed description of the perceived causes of various contemporary malaria-type fevers, each influenced by a humoural imbalance:

> "Quotidian fever comes from phlegm, tertian from red bile, quarten from melancholic, that is, black bile…Quotidian comes from cold humour, because phlegm is cold and moist. Tertian from hot, because red bile is hot and dry. Quarten from melancholy, because it is dry and cold" (Singer, 1917, quoted in Cameron, 1993:54).

Gasper's (2004) research on late 11th century letters to and from Anselm of Canterbury provides possible evidence of malaria in Romney Marsh. The letters contain a detailed description of the symptoms of a patient, Osbern:

> "First of all a sudden chill violently afflicts his whole head and then his heart and whole body; and soon his heart, pounding uncontrollably, fails along with all the senses and powers of his body…After he appears to have revived from this fainting fit, he immediately burns with heat throughout his whole body, as if becoming feverish. A headache also follows this and great discomfort, as if he had to vomit" (Anselm and Frohlich, 1990, quoted in Gasper, 2004:255).

The condition presented here makes a compelling case for possible 'benign tertian' *vivax* malaria, especially since it occurred in close proximity to Romney Marsh, a likely malarious area. Gasper goes on to suggest that tentative evidence of 11th century malaria may provide an important bridge to later periods and that "there is no reason to think that the situation [endemic malarial infection] had changed dramatically between the tenth and fourteenth centuries" (2004:258).

As previously mentioned, by the 14th century 'ague' was in common usage as a descriptor for a wide range of illnesses exhibiting febrile symptoms. It is during this time that early authors and poets began making relatively frequent references to ague (Bruce-Chwatt, 1976). Chaucer, for instance, wrote in 1386:

> "You are so very choleric of complexion,
> Beware the mounting sun and dejection,
> Nor get yourself with sudden humours hot;
> For if you do, I dare well lay a groat,
> That you shall have the tertian fever's pain,
> Or some ague that may well be your bane" (Chaucer, quoted in Reiter 2000:3).

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This passage suggests that by the late 14th century people were aware of the differences between 'benign tertian' fever, the term most likely describing *vivax* malaria, and ague, which could refer to any acute febrile condition. It also demonstrates a continuing belief in the influence of the humours on health.

Franklin (1983) provides the thought-provoking insight into a possible malaria epidemic striking the 14th century inhabitants of Thornbury Manor, Gloucestershire. In a study akin to Dobson's (1980; 1997) analyses of post-medieval parish registers, Franklin examines the court rolls of the Manor from between 1328 and 1352. These rolls recorded, for tax and tenancy purposes, the numbers of deaths each year. Although incomplete and selective in which cases were reported, the records reveal an unusually high death rate for the years 1333-34, following a long, hot summer in 1333. The tithing area within the Manor reporting the highest death rate was the marshland area, suggesting a local, rather than regional disease epidemic. This is a strikingly similar pattern to that discovered by Dobson in post-medieval marshland communities in south-eastern England. Later sources strongly suggest that this area of Gloucestershire was subject to malarial endemicity in the 17th and 18th centuries, so it is highly likely that malaria was also present here in the 14th century (Dobson, 1997). This combined evidence is highly suggestive of an outbreak of epidemic malaria in this community, possibly with a newly introduced strain of *P. vivax* (or even *P. falciparum*) that may have detrimentally affected a population with a resistance to local strain(s). Conversely this could represent the introduction of malaria into a 'virgin' population.

The mid-13th to 14th centuries were a time of profound change in terms of climate, population, epidemiology, and more specifically for the Fenlands and marshlands, economy. Monastic documents suggest that the decades of the mid to late 13th century were some of the most tempestuous ever recorded in terms of climate (Eddison, 2000), with surviving records showing that the weather was particularly severe in southeast England. Numerous marine inundations destroyed flood defences and settlements, and salted agricultural fields, resulting in repeated crop failure and food shortages (Eddison, 2000). Salt production, being reliant on a steady supply of local labourers and dry fuel (invariably peat), would likely have been particularly badly affected by persistent heavy rains and flooding (Jordan, 1996). Unstable climatic conditions and major national epidemics of disease and famine, as well as

problems associated with over-grazing and rain-spoiled feed, greatly afflicted livestock populations. In some areas sheep flock size was reduced by two-thirds (Grant, 1988). These epidemics may have proven partly responsible for the Fenland and marshland economic decline in the 14th and 15th centuries. However, it was not only livestock that were suffering the ravages of disease: in 1348, the Black Death entered Britain and subsequently raged through country, leaving up to 50% of the human population dead (Goldberg, 1996).

The effects of the Black Death on Fenland/marshland populations and potential malarial transmission are unclear. Although local population numbers may have been booming by the mid-14th century, Darby (1940:152) suggests that "the Black Death did not produce great consequences generally" for the Lincolnshire/Cambridgeshire Fenlands. The Fen town of Ramsey, for instance, recorded surprisingly few losses to plague (DeWindt and DeWindt, 2006). There were some deleterious effects, however. For instance, loss of people resulted in the neglect of local watercourse management, culminating in the south Lincolnshire siltland town of Spalding being "in danger of being submerged by the flow of the sea and by the descent of fresh waters" (DeWindt and DeWindt, 2006:152). Flooding events such as these may have increased the available territories for *anopheline* mosquitoes, although this may have been offset by loss of human and livestock prey. Intriguingly, research (Wake et al., 1974; Ell, 1984) has suggested that those suffering with iron deficiency, a symptom of active malarial parasitism, may have had an increased resistance to plague. This is due to the requirement of plague organisms, such as *Yersinia pestis*, for exogenous iron for replication (Ell, 1984). Additional research by Williams et al. (1997) suggested a strong link between infection with *P. vivax* and child malnutrition, the latter of which would likely cause iron deficiency and increased immunity to plague. Further research on this possible immunity is required, particularly considering the incredible complexities of bodily usage and metabolism of iron, including in immunological mechanisms (Weiss, 2002). It does, however, provide a fascinating potential explanation for Darby's assertion concerning the reduced effect of Black Death on certain Fenland populations.

Literary references to agues and fevers appeared increasingly frequently during the 15th and 16th centuries. Shakespeare, for instance, makes numerous mentions of the conditions in his works, such as in the following quote from King Lear:

> "They told me I was everything; 'tis a lie, I am not ague-proof" (Shakespeare 1800:79).

The growing frequency of references to possible malaria is probably due to the increasingly frequent survival of such manuscripts and an advancing knowledge of epidemiology, rather than a growing prevalence of malaria itself. Pinello (2008:34) confidently assumes that "despite the extremely high population densities of the 13th and 14th centuries, this time period was relatively free from malaria", although she offers no support for this statement. This is an easy trap for researchers to fall into: as is often the case in archaeological and historical research, absence of evidence is not always evidence of absence. The increased survival of primary sources from the 15th century onwards seems to have influenced assumptions that malarial infection rates suddenly soared during this period. It is far more likely that malaria was already present throughout the Fens and marshlands of eastern England, perhaps with areas of stable endemicity that expanded in response to favourable demographic and climatic conditions. A short-term period of warming in the early 16th century, for instance, may also have stimulated an increased *anopheline* population density, hence higher malaria infection rates (Reiter, 2000). Environmental and demographic conditions were certainly in place for stable malaria transmission in many areas, well before this period of warming.

The year 1538 proved particularly important for those studying demography and population history through documentary sources. It was in this year that Oliver Cromwell ordered that each parish must keep records of all births, marriages and burials (Dobson, 1997). Although sometimes incomplete and non-continuous, these extensive records allow researchers to track fluctuations in mortality rates over an extended period of time, hence aiding our understanding of population health on local and regional scales. Wrigley and Schofield's (1981) and Dobson's (1997) monumental and exhaustive syntheses on national and regional scales, respectively, are prime examples of the usefulness of parish registers to researchers of historical demography and epidemiology. The burial records of the parish registers are of particular interest to the malaria researcher, as they can be used to illustrate seasonal fluctuations in mortality which, in turn, can be compared to climatic data. Knowledge of the life-cycles of mosquito vectors and their parasites is applied to the historic data in order to infer past events of severe malaria epidemics.

2.4: The post-medieval period *c.* AD 1600-1900

The post-medieval period was a time of intellectual, environmental and socio-cultural change in the Fens and marshes of eastern England, which brought an eventual end to sustained malaria transmission by the late 19th century. Despite these changes, late medieval and post-medieval perceptions of Fenland/marshland environments and their inhabitants remained overwhelmingly negative. John Norden, upon visiting the Essex marshlands in the mid-16th century, commented that he could not

> "comende the healthfulness of it: And especiallie nere the sea coastes…and other lowe places about the creeks which gave me a most cruell quarterne fever" (Norden and Ellis, 1840, quoted in Dobson, 1989:3).

By the late 17th century the Fens were still perceived by outsiders to be a hostile, isolated place, under the constant threat of flooding (Nicholls, 2000) – a threat exacerbated by neglect of drainage systems following the late 16th century Dissolution of the Monasteries (Whyte, 2002). The Fenlanders themselves were generally viewed less than favourably. In 1695, William Camden described the locals:

> "the inhabitants of this and the rest of the Fenny country…Fen-men, a sort of people (much like the place) of brutish uncivilised tempers, envious of others whom they call Upland men, and usually walking aloft upon a sort of stilts; they all keep to the business of grazing, fishing and fowling. All this country in the winter time, and sometimes for the greatest part of the year, laid under water" (Camden, 1701, quoted in Whyte, 2002:41).

Despite relatively successful 19th century attempts at Fenland reclamation, resulting in a "tamed, drained and productive landscape" (Williamson, 2006:211), external perceptions of the Fens remained of an unhealthy, unwholesome "region of bogs and swamps, of fever-haunted marshes, and ague-infested lowlands" (Balfour 1891:1). The perceptions of the older generations of Fenlanders of their own environment were still strongly influenced by superstitions and legends, although attitudes of the younger generations were beginning to change:

> "The people themselves are not easy to make friends with, for they are strongly suspicious of strangers; but once won over, are said to be staunch and faithful. They are grave, long-featured, and rather melancholy in face, touchy and reserved in disposition, and intensely averse to change or innovation of any sort; many of them live and die within the limits of a narrow parish, outside of which they never set foot.

> The younger generations are changing; but they show less disbelief in the old legends than indifference to them" (Balfour, 1891:5).

The mystical and dreadful reputation of the Fenlands, particularly in relation to miasmas, made "men fearful of entering the Fens of Cambridgeshire, lest the Marsh Miasmas should shorten their lives" (Watson-William, 1827:108), while the Fen water "poisons the circumambient air…and sickens and frequently destroys many of the inhabitants" (Parkinson, 1811:21). During much of the post-medieval period agues and fevers were largely attributed to marshland emanations from decaying animal and vegetable materials, and 'miasmic fever' specifically attributed to the inhalation of gases released through the decomposition of organic matter in marshy environments, or the intake of 'infected' waters (Brown, 1867; Holmes, 1891; Nicholls, 2000). In the late 17th century, Dr. Thomas Sydenham, often cited as the "English Hippocrates" (Dobson, 1997), commented that:

> "…if one spends two or three days in a locality of marshes and lakes, the blood is in the first instance impressed with a certain spirituous miasma, which produces quartan ague" (Sydenham and Wallis, 1788, quoted in Creighton 1965:312-3).

There is, however, tentative evidence that a link between insects and illness had been noticed as early as the 1690s. Merret (1695:342–3) described how "midges [mosquitoes] are in some places very troublesome" and that ague was "very rife, with few strangers escaping without a seasoning." Sydenham also noted that fevers and agues appeared in the autumn months after the summer swarming of insects (Dobson, 1997), although he was still satisfied to attribute ague itself to a spirituous miasma. Superstitions and legends concerning the character of the Fens persisted well into the 19th century. Tiddy Mun, for instance, was a Lincolnshire bog spirit who was believed to have control over Fenland mists and waters. Locals were known to call upon Tiddy Mun for help during flooding episodes (Balfour, 1891). Malaria, agues and fevers were also personified and given names such as the Bailiff of the Marshes and Old Johnny Axey, so high was their prevalence (Nicholls, 2000).

Dobson's (1997) review of mortality records for Essex, Kent and Sussex strongly suggests that endemic malaria represented a significant threat to the marshland populations of these areas. In the twenty four parishes examined, burials outnumbered baptisms for nearly every decade during the 17th and 18th centuries. Wrigley and Schofield (1981) found a

similar rate in the Fen parish of Wyberton, Lincolnshire. Crude death rates and infant mortality rates in the Lincolnshire, Cambridgeshire and Norfolk Fenlands during this period were also consistently higher than in upland and even urban parishes (Nicholls, 2000). More specifically, seasonal fluctuations in burial rates often seemed to be timed with the life-cycles of mosquito and parasite, suggesting that malaria was indeed an important factor in these elevated rates.

Although it seems that there can be little doubt that malaria was a significant influence on the mortality of post-medieval marshland populations, there is contention as to exactly how significant. Modern day temperate *P. vivax* strains are generally considered to be 'benign', especially when compared to the often-deadly tropical *P. falciparum*. For instance, no deaths were attributed specifically to *vivax* malaria during the 1969 Sri Lanka epidemic, in which half a million people were infected (Warrell, 2002). The impact of *vivax* malaria on morbidity and mortality in the post-medieval period has recently been heavily downplayed (Hutchinson, 2004; Hutchinson and Lindsay, 2006; Lindsay et al., 2010). These authors instead attribute Dobson's (1997) observed seasonal fluctuations in burial rates to misdiagnosed cases of acute enteric and respiratory disease. There can be little doubt that conditions such as typhoid, leptospirosis, gastroenteric diseases, and tuberculosis greatly affected Fen/marsh dwellers, particularly considering the potential difficulties of procuring fresh, clean drinking water in these environments (Hutchinson and Lindsay, 2006). Some of these diseases, as previously discussed, are symptomatically similar to malaria, particularly in their tendency to cause cyclical paroxysm (Karunaweera et al., 2003), which may have resulted in automatic diagnoses of 'ague.' It is highly likely that patients often suffered from more than one disease (comorbidity), possibly being predisposed by active *vivax* infection (White and Plorde, 1991). Sydenham, for instance, wrote that:

> "whensoever I have observed any one long labouring under one of these agues...I could certainly foretell that he could not long after be taken with some dangerous distemper...infants often-times after these autumnal intermittents become rickety, having swollen and hard bellys being hecticall, troubled with a cough and other symptoms of being in a consumption" (Sydenham and Wallis, 1788, quoted in Dobson, 1997:330).

Although modern *vivax* malaria is assumed to be benign in that the disease itself rarely causes death, it still exacts a debilitating toll upon its host (Vogel, 2013a). Clinical

research involving patients suffering from *vivax* malaria demonstrates that the disease increases the rates of malnutrition (Williams et al., 1997), low infant birth weight (Sina, 2002), anaemia, hepatosplenomegaly, and splenic rupture (Warrell, 2002). The debilitating effects of the disease also undermine the ability of individuals and communities to contribute productively to economies on a local, regional, and national scale (Mendis et al., 2001; Carter and Mendis, 2002; Vogel, 2013a). Desowitz (1997:4) described *P. vivax* as an "economic" pathogen due to its repeated attacks over a long time period: "It is the unproductive labourer who is repeatedly feverish, aching, and anaemic".

It is unknown whether past strains of *P. vivax* were as 'benign' as modern strains are perceived to be (although this perception has been challenged recently, as discussed in Section 3.5). If we assume that past English *vivax* malaria was indeed benign, we should also assume that the effects of the disease on past communities were at least as severe as witnessed in modern populations with access to medications. Nicholls (2000) suggests that past populations exposed to *vivax* malaria would have suffered a chronic state of ill-health. The increased post-medieval marshland mortality rates and reduced life expectancies certainly support this suggestion, particularly when considering the interaction of malaria with other conditions, such as enteric diseases (Mendis et al., 2001; Nicholls, 2000). Social status in the post-medieval period may well also have compounded the effects of *P. vivax* infection on the local populations of the Fens and marshlands. Dobson (1980, 1989) discusses the social inequality inherent in such populations. Affluent land owners, for example, could afford to live outside of the perceived 'unhealthy' locations and hire labourers to work within these areas. The poorer employees, which often included whole families, would have had no choice but to take on the work, thus running the risk of repeated exposure to malaria (Nicholls, 2000).

To add to the burdens of living in such an unhealthy environment, there developed a widespread dependence upon narcotic drugs to counteract the acute symptoms of malarial infection. During the 18th and 19th centuries, the Fenland districts of East Anglia exhibited the highest consumption rate in England of both opium and laudanum (Berridge, 1977). Hemp was also regularly dried and smoked. An 'ague drop' containing arsenic was often given to young children, leading to numerous child fatalities in the 19th century (Dobson, 1997), and in Kent, narcotism was recorded as a major cause of infant mortality in the mid-

19th century (Hunter, 1864). The doping of babies by their carers to keep them quiet seemed to have been a popular, albeit potentially lethal strategy (Dobson, 1997). If not dangerous enough by itself, opium was often "mixed with everything imaginable: mercury, hashish, cayenne pepper, ether, chloroform, belladonna, whiskey, wine and brandy" (Hodgson, 2001:104). The beneficial effects of Peruvian, or Jesuit's, bark (which contains quinine) in malaria therapy was known to English physicians as early as the late 17th century, but prohibitive expense and limited availability put the remedy out of reach of the common Fenlander until the late 19th century (Dobson, 1997). By this time, the shadow of malarial infection was withdrawing from England, leaving a need for opioid narcotics and alcohol based on habit, rather than necessity, particularly among the older generations:

> "…it is only here and there that one can find traces of the poor ague-shaken, opium-eating creatures of earlier times. Many an old woman eats opium openly, and I fear all the men who can get it will drink gin. But the days are gone by when the one or the other was in constant and daily need, to still the shaking or deaden the misery born of the fevermists and stagnant pools" (Balfour 1891:147).

2.5: The withdrawal of English malaria

Partial reclamation of areas of the English Fens and marshlands had occasionally been attempted from the Roman period onwards. As previously discussed, the late Anglo-Saxon/early Norman Sea Bank represented the first truly successful protective barrier, allowing for increasing medieval settlement and exploitation of the fertile siltlands of Lincolnshire and Cambridgeshire. Earlier canalisation had also improved access and trade routes in these areas. Further local attempts at drainage, mostly associated with the monasteries, took place during the medieval period, but it was not until the 17th century that concerted efforts to drain and reclaim the Fenlands were mounted (Williamson, 2006). Spurred by the General Drainage Act of 1600 and the promise of reclaimed land as a reward, wealthy outside businessmen employed Dutch engineers (Cornelius Vermyden being the most famous) to improve drainage courses throughout the Fenlands, East Anglia and southeast England (Cracknell, 1959; Williamson, 2006), with varying degrees of success. These projects were, quite understandably, often met with considerable resistance from local inhabitants, who perceived that the changes would destroy their traditional ways of life. For the most part, such protestations went unheeded, although local resistance in some instances forced delays well into the 18th century (Williamson, 2006).

However successful these projects happened to be, certain areas of the Fens and marshlands remained undrained and continued to be used as pasture until the steam-driven revolution of the early 19th century finally conquered the most poorly drained areas. An excursion through the modern day Fenlands of Lincolnshire and Cambridgeshire very much reinforces the impression of a tamed, highly productive agricultural landscape. The modern Fenlands boast some of the most fertile and highest quality arable farmland in Britain. In some areas up to 92% of the landscape is now under arable cultivation (Cope-Faulkner et al., 2010). The shift from the largely pastoral-based economies of the medieval and early post-medieval periods to almost exclusively arable-based economies was accelerated by steam-powered drainage in the early 19th century (Darby, 1956).

Major drainage schemes in Romney Marsh took place a little later than in the Fenlands of Lincolnshire, Cambridgeshire, and Norfolk. One of the most impressive projects was the 1804-6 cutting of the Royal Military Canal around the northern margins of the marsh. Where upland waters once passed through the Marsh, the Canal now diverted them around (Robinson, 1988; Eddison, 2000). During the 19th century the medieval Dymchurch Wall underwent considerable repair and strengthening and continues today to serve as an important defence against marine incursion (Robinson, 1988). As with the Fenlands to the north, the introduction of steam technology in the late 19th century and, later, diesel- and electric-powered drainage engines, complemented and essentially completed the task of drainage. Improvements in water quality in the Thames and Medway rivers, and increased availability of fresh water, would have reduced instances of comorbidity of *vivax* malaria and enteric diseases associated with contaminated water supplies (Hutchinson and Lindsay, 2006).

So what of the effects of these dramatic post-medieval drainage schemes and shifting economies on the epidemiology of malaria in the Fenlands? Malaria was certainly still present and likely endemic in some areas by the start of the 19th century. However, hospital records and parish registers suggest that the disease was losing its grip on the Fen/marsh populations by the mid-19th century and was all but eliminated by the turn of the 20th century (Nicholls, 2000). This represents a startlingly rapid decline in the transmission of the disease.

The reasons behind the withdrawal of malaria from the Fens and marshes of England during the 19th century are complex and multi-factorial, yet it is tempting to cite drainage as the direct, causative factor. Authors have, quite understandably, often made this assumption (Packard, 2007). However, Williamson (2006) notes that much of the siltland of Lincolnshire had been relatively densely populated for centuries prior to the great post-medieval drainage schemes, yet mosquito populations would have continued to thrive due to the presence of stagnant water in drainage ditches and channels. Prevalence of 'ague' in the siltlands remained common through the 17th and 18th centuries. The effects of widespread, increasingly successful drainage schemes did influence a gradual shift from pastoral to heavily arable farming throughout the Fenlands and marshlands. The separation of livestock from human settlements, as well as the improvement of human dwellings, would have reduced the available over-wintering habitats of *anopheline* mosquitoes, thus lowering the vector population and infection rates below the level required for endemicity and the continued survival of the parasite (Carter and Mendis, 2002; Hay et al. 2004; Williamson, 2006) and heralding the beginning of the end for malaria in Britain. By the end of the 19th century, when the role of mosquito vectors in the transmission of malaria was finally, scientifically understood, "the disease was not even of concern to the Fenlanders themselves" (Nicholls 2000:530).

The following chapter will explore the different human malaria parasites and vectors, concentrating on *Plasmodium vivax* and *Anopheles atroparvus*. The lifecycle and pathophysiology of *P. vivax* will be also discussed, followed by the human immune response to infection and the immune status of temperate populations exposed to the disease.

CHAPTER 3: HUMAN MALARIA: CLINICAL ASPECTS AND IMPLICATIONS OF INFECTION

"*Agues and coughs are epidemicall;*
Hence every face presented to our view
Looks of a pallid or a sallow hue"
(Anonymous author of the poem 'The Inundation, or Life of a Fenman',
quoted in Dobson 1989:3)

Malaria is a eukaryotic protozoan disease caused by the introduction of *Plasmodium* parasites into the human bloodstream through the bite of an infected *anopheline* mosquito (Warrell, 2002). Over 200 species within the genus *Plasmodium* have been identified, four of which commonly infect humans. These are *P. falciparum, P. vivax, P. ovale, and P. malariae*. Malaria is widespread throughout tropical and subtropical regions and also occurs in many temperate areas (Warrell and Gilles, 2002). The disease is contracted by up to 250 million people per year, killing over half a million of its victims (World Health Organization, 2010; 2013), and it has been estimated that between 2.6 and 3.4 billion people currently live at risk of infection (Hay et al., 2004; Baird, 2007). Despite an intimate scientific knowledge of the genomic structure and pathophysiology of human *Plasmodium* parasites, the development of an array of prophylactic drugs, and long-term global eradication efforts, malaria continues to persist and thrive (Doolan et al., 2009).

This chapter will begin with an introduction to the four human malaria parasites, followed by a brief overview of the evolutionary origins of malaria. The main mode of transmission will then be discussed along with the lifecycle, pathophysiology, and supposed 'benign' status of *Plasmodium vivax*. This is followed by a brief overview of the human immune response to infection and a discussion of the implications of the presence of the disease in both modern temperate populations and past British populations.

3.1: Human malaria parasites

The primary method of malaria transmission is via the bite of an infected female *anopheline* mosquito, the only mosquito genus capable of transmitting the disease to humans. Infection by *Plasmodium* parasites can occur congenitally (e.g., Rai et al., 2013) or through transfusion with contaminated blood (Kitchen and Chiodini, 2006), but introduction

of the parasite through a mosquito vector is by far the most common method of transmission. Of the four human malaria parasites, *P. falciparum* is commonly considered to be the most lethal, being responsible for the majority of deaths attributed to malaria. *P. falciparum* is, therefore, also sometimes referred to as 'malignant malaria,' whereas *P. vivax,* for instance, is often considered 'benign', since it has not traditionally been associated with high mortality rates (Gilles, 2002). However, as discussed later, researchers (e.g., Mendis et al., 2001; Beg et al., 2002; Baird, 2007; Rogerson and Carter, 2008; Anstey et al., 2009) have recently questioned the labelling of *P. vivax* as 'benign', emphasising in particular the neglected status of this potentially deadly disease.

Each species of *Plasmodium* was historically characterised by the periodicity of associated fevers suffered by the infected host. These fevers result from the body's immune response to the synchronised eruption of parasites from infected red blood cells, possibly in an attempt to raise bodily temperature above the threshold of parasitic tolerance (Gravenor and Kwiatkowski, 1998). *P. falciparum*, *vivax*, and *malariae* were known as 'tertian' malaria, since the periodic febrile paroxysm (or sudden fever) occurs every 48 hours, while *P. ovale* was referred to as 'quartan' since paroxysm occurs every 72 hours (Table 3.1). These terms are rarely used by modern researchers, who prefer to use species name as the identifier, since these periodicities can be complicated (or non-synchronised) by comorbidity or infection by multiple *Plasmodium* generations (Sallares, 2002). It is, of course, important to be familiar with the historical usage of malaria terminology if modern researchers are to analyse the presence of the disease in antiquity.

***Plasmodium* species**	**Periodicity (hours)**
P. falciparum	48
P. vivax	48
P. ovale	72
P. malariae	48

Table 3.1: *Plasmodium* species and the periodicity of fevers (Perez-Jorge and Herchline, 2014)

3.1.1: *Plasmodium falciparum*

P. falciparum infection most commonly occurs in sub-Saharan Africa, but has been responsible for large-scale epidemics in India, Sri-Lanka, and Haiti (Gilles, 2002). This species requires sustained temperatures of at least 20°C for a minimum of twenty days for

successful sporogenesis (the production of sporozoites, or *Plasmodium* protozoa) and development within the mosquito vector, and is therefore mostly confined to tropical and subtropical latitudes (Dobson, 1997). *P. falciparum* malaria predominates in these areas due to its high rate of reproduction within the human host and the year-round presence of mosquito vectors (Sallares, 2002). This species of parasite is particularly dangerous as it is the only one that causes sequestration (or clumping) of infected red blood cells in blood vessels and organs, including the brain. Symptoms of *falciparum* malaria may include acute fever, vomiting, diarrhoea, splenomegaly (enlarged spleen), jaundice, anaemia, renal failure, hypoglycaemia, neurological impairment, cerebral and pulmonary oedema, and coma (Ellis, 1989; Gilles, 2002). *Falciparum* malaria is especially dangerous to the non-immune, pregnant women, the unborn and young children (Molyneux, 1989; Lindsay et al., 2000).

3.1.2: *Plasmodium ovale*

This species is less widespread and therefore less commonly encountered than *P. falciparum* or *P. vivax*. *Ovale* malaria is generally confined to sub-Saharan Africa and is most often found in the western region of the continent, although sporadic prevalences of infection have been recorded in the western Pacific region, China, and Southeast Asia (Gilles, 2002). The *P. ovale* parasite can be suppressed and out-competed by other *Plasmodium* species, although mixed infections (particularly *ovale* alongside *falciparum*) are relatively common. The lifecycle and pathophysiology of *P. ovale* are similar to that of *P. vivax*, other than the extended 72 hour period between febrile paroxysms (Table 3.1). Patients with *ovale* malaria usually suffer slightly milder symptoms and fewer relapses than with those with *vivax* malaria (Gilles, 2002). As with *P. vivax*, the *P. ovale* parasite is able to remain dormant within the host liver in the form of hypnozoites (or "sleeping parasites"), to later emerge and cause delayed disease relapses.

3.1.3: *Plasmodium malariae*

P. malariae occupies a similar geographic range to *P. falciparum*, but is far less commonly encountered, perhaps being out-competed by the latter. As with *P. ovale*, *P. malariae* exhibits a similar lifecycle and pathophysiology to *P. vivax*. *Malariae* infection usually results in less severe sequelae than *vivax* malaria, although cases of severe, often

fatal kidney damage (nephrosis) have been reported in *malariae* infections (Carter and Mendis, 2002).

3.1.4: *Plasmodium vivax*

Plasmodium vivax is the most widely distributed species of malaria, occurring across tropical, subtropical, and many temperate regions of the world. It is far less commonly encountered in sub-Saharan Africa than *P. falciparum* malaria, which may be indicative of *P. vivax* being out-competed in tropical climates. *Vivax* malaria is particularly rare in the west of the African continent where much of the population lacks the Duffy blood group system, and are hence clinically referred to a being 'Duffy negative'. Individuals with this genetic condition lack alleles controlling certain antigenic properties of red blood (erythrocytes) cell membranes. This confers specific immunity against erythrocytic invasion by *P. vivax* parasites (Marsh, 2002).

P. vivax accounts for between 80-400 million worldwide cases of malarial infection per year, 80-90% of which occur across the Middle East, Asia, the Western Pacific, and Central and South America (Mendis et al., 2001; Hay et al., 2004). The ability of the *P. vivax* parasite to survive and flourish in temperate climates means that up to 2.6 billion people live at risk of infection (Baird, 2007). It is this capacity that makes *P. vivax* the most likely culprit for the spread of malaria across northwest Europe and Britain in past centuries. *P. vivax* is the last species of malaria to be indigenous in Britain's historical past (Kuhn et al., 2003), and hence this is the species of malaria on which this study will focus.

3.2: The origins of human malaria

The *Plasmodium* parasite is a very ancient protozoan organism, originally thought to have evolved around 55 million years ago from Old World small mammal reservoirs, in parallel with early primates (Cockburn, 1963; Dunn, 1965). Fossil remains of *anopheline* and *culex* mosquitoes (vector species capable of infecting mammals and primates) have been recovered from Old World deposits of this period, along with associated *Plasmodia* parasites (e.g., Poinar, 2005). Recent biomolecular and genetic research has, however, extended the *Plasmodium* lineage back to approximately 200 million years ago (Rich and Ayala, 2006). Similar research suggests that *P. falciparum* is closely related to an avian malaria parasite

lineage that possibly diverged from mammalian parasites around 130 million years ago. The lines leading to modern human *P. ovale, malariae,* and *vivax* parasites likely diverged some 100 million years ago (Carter and Mendis, 2002).

Opinion is divided as to the exact location for the origin of *P. vivax*. It has been argued that the parasite may have had its origins in the New World (Ayala et al., 1999). Possible evidence for this can be seen in the modern presence of *Plasmodium simium*, "a malaria parasite of New World monkeys which appeared to be genetically, as well as morphologically, indistinguishable from *P. vivax*" (Carter, 2003:214). However, genetic research strongly suggests an Old World source for New World *P. vivax,* with *P. simium* being a likely vestige of this introduction (Li et al., 2001). Complementary phylogenetic and biomolecular studies suggest that southern Asia was the most likely place of *P. vivax* origin (McCutchan et al., 1996; Qari et al., 1996; Escalante et al., 1998), and its modern form probably diverged around three million years ago from *P. cynomolgi,* a parasite of Old World monkeys (Carter and Mendis, 2002).

The intimate association between humans and malaria parasites can likely be traced to the dramatic deforestation of parts of the sub-Saharan African environment, caused by climatic change and agricultural expansion, and initiated around eight to ten thousand years ago (Packard, 2007). It has been suggested that this clearing of woodland and the subsequent creation of marshy land offered arboreal mosquito species a new ecological niche in which to breed in close proximity to human settlements (Capasso, 1998). This change has been implicated as resulting in the original spread of *P. falciparum* to humans from African arboreal primate reservoirs. The persistent presence of genetic red blood cell polymorphisms such as sickle cell anaemia, the thalassaemias, and glucose-6-phosphate dehydrogenase (G6PD) deficiency in many endemic malaria areas suggests long-term *Plasmodium*-driven selective pressures for such protective polymorphisms to occur and persist (Capasso, 1998; Hume et al., 2003; Carlton et al., 2008).

3.3: The *anopheline* vector

As with all species of malaria, infection of the human host with *P. vivax* begins with the inoculation of parasites into the bloodstream via the saliva of an infected female *anopheline* mosquito, while taking a blood meal from the human host. '*Anopheles*' is a

genus of mosquito within the sub-family Culicinae of the order Diptera. Of the approximately 420 species of *anopheles* mosquitoes, around 70 are recognised as vectors of malaria parasites (Service, 1993). Five species are common to the British Isles: *A. algeriensis*, *A. claviger*, *A. messae*, *A. plumbeus*, and *A. atroparvus*. Of these five, only the latter two species are thought capable of transmitting the *Plasmodium* parasite, and evidence of the role of *A. plumbeus* in transmission is tentative (Snow, 1998; 1999). Thus, it is *A. atroparvus* that is thought to have been the most important vector in the spread of malaria in past British populations. The present geographical distribution of *A. atroparvus* in Britain coincides with those areas worst affected by past episodes of malaria, such as the Fen and marshlands of south east and eastern England (Dobson, 1997). This suggests that *A. atroparvus* was indeed the main vector of past malarial transmission. *A. atroparvus* remains the most commonly encountered vector species throughout northern Europe today (Sinka et al., 2010).

Although generally considered to prefer brackish, saline water for breeding (Snow, 1999), *A. atroparvus* larval sites have also been identified in freshwater habitats, such as river margins, wells, and canals (Hutchinson, 2004; Sinka et al., 2010). However, it is the coastal salt marshes, brackish estuarine marshes, and fenlands of eastern England that provided (and still do, where such terrain is extant) the perfect breeding habitat. The species has been described as opportunistic, both zoophilic and anthropophilic in nature (Sinka et al., 2010). The adult female *atroparvus* mosquitoes semi-hibernate (overwinter) inside livestock shelters and human habitations, remaining in close proximity to food (Medlock and Vaux, 2011). The mosquito can remain infective at temperatures as low as 3ºC (Dobson, 1980), although infectivity is reduced somewhat while overwintering (Snow, 1999). They can, therefore, survive harsh winters with constant access to blood-meals, emerging in warmer periods to return to their breeding grounds (Shute and Maryon, 1974; Hulden et al., 2005). These combined factors provide the perfect intermediary vector for the most widespread of temperate malarial parasites, *Plasmodium vivax*.

3.4: The lifecycle and pathophysiology of *Plasmodium vivax*

Upon inoculation of *P. vivax* sporozoites (see Table 3.2 for definition of terms) into the human bloodstream by the *anopheline* vector, the introduced sporozoites travel to the

liver, inside which they experience several phases of reproduction, known as pre-erythrocytic schizogony (Gilles, 2002). This process produces multiple single-nucleus parasite cells (schizonts), some of which remain sequestered and dormant within the liver in hypnozoite form. After 6-16 days of reproduction, a number of schizonts rupture, releasing merozoites into the bloodstream (Gilles, 2002). The merozoites attach to and invade erythrocytes (red blood cells), within which they reproduce asexually (erythrocytic schizogony), metabolising erythrocytic cytoplasm and up to 75% of endogenous haemoglobin in the process (Pasvol and Wilson, 1982).

Schizont	A cell that reproduces by schizogony
Schizogony	Asexual reproduction of *Plasmodia* sporozoites
Sporozoite	Motile, infective stage of *Plasmodia* that results from sporogeny within mosquito vector; inoculated into host
Merozoite	Schizont that arises from schizogony; erythrocyte-invasive form of the parasite; may enter either asexual or sexual phase of the parasite life cycle
Trophozoite	The asexual schizont of the *Plasmodia* parasite
Gametocyte	The sexual form, male or female, of *Plasmodia;* found in human erythrocytes; produce gametes upon ingestion by vector
Hypnozoite	Exo-erythrocytic 'dormant' *P. vivax* cell in the human liver; responsible for malarial relapse

Table 3.2: Definitions of selected *P. vivax* life-cycle terms (Gilles, 2002)

Within host erythrocytes, the merozoites become large, ring shaped trophozoites, which mature to form new schizonts. These eventually rupture the infected erythrocyte and are released back into the bloodstream. Prior to cell rupture, some schizonts differentiate into gametocytes (a pre-gamete cell form), which are later ingested by *anopheline* mosquitoes as they take blood meals from the infected host, after which they undergo a sporogenic cycle within the mosquito gut in preparation for infection of a new external host (Hadley, 1986; Pinello, 2008). Thus the parasite lifecycle is completed and perpetuated (see Figure 3.1). It is the rupturing of erythrocytes, release of schizonts, further erythrocytic infection and destruction, and the host immunological responses (the ‘blood stage’ of infection) that cause the symptoms of *vivax* malaria (Trampuz et al., 2003).

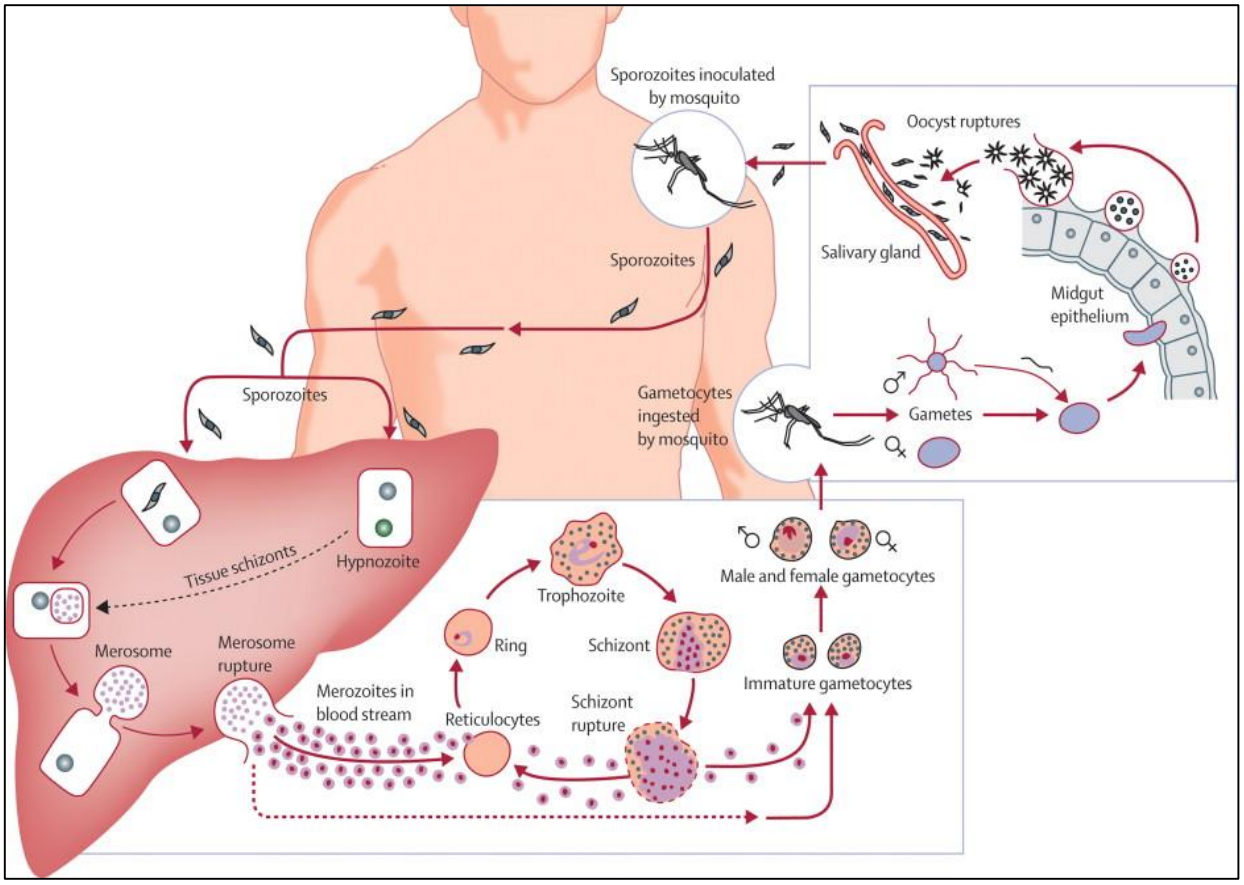

Figure 3.1: The lifecycle of *Plasmodium vivax* (after Mueller et al., 2009:556).

The hypnozoite stage of parasitism is peculiar to both of the *P. vivax* and *P. ovale* organisms and does not occur in *falciparum* or *malariae* infections. Quiescence within the host liver allows the parasite to 'hibernate', thus making it less dependent on year-round populations of mosquito vectors to complete its lifecycle. It is this capability that gives *P. vivax* malaria an advantage over other malaria species in temperate climates, where vectors may not be as prevalent in colder periods of the year. The *P. vivax* parasite is particularly suited to temperate climates as it requires a relatively low temperature of 16ºC (compared to 20ºC for *P. falciparum*) for at least sixteen days in order to reach maturity within the mosquito gut. This required average temperature is regularly reached during most summers in southern Britain (Dobson, 1997). The ability of both mosquito vector and parasite to 'hibernate' through cold winters (the vector within human or livestock habitations, and the parasite within the human host) is a crucial factor in continuing and maintaining malaria transmission in a host population (Dobson, 1980). Research has shown that *P. vivax* ably

survives the harsh winters of northern Europe and the Korean Peninsula, for instance (Oh et al., 2001; Hulden and Hulden, 2011).

The reduced virulence and 'hibernating' hypnozoite stage of *P. vivax* infection are likely adaptive traits borne out of a long evolutionary history of primate parasitisation (Anstey et al., 2009), factors which allow the *P. vivax* parasite to keep its host alive during extended sub-optimal transmission conditions (Ewald, 1994). The circumstances determining activation of dormant hypnozoites are not clearly understood, although recent research (Hulden and Hulden, 2011) suggests that the bite of non-infective *anopheline* mosquitoes may introduce specific salivary proteins into the bloodstream. These proteins may signal the presence of new vectors, thereby triggering hypnozoite activation and disease relapse. One infected bite may trigger multiple relapses within a relatively short time period. Relapses may also be triggered by infection with other diseases, including *falciparum* malaria (Vogel, 2013a).

The persistent presence of hepatic hypnozoites and their capacity to cause relapse, sometimes many months or years following initial blood-stage infection (Ewald, 1994; Adak et al., 1998), can confound medical strategies of disease prevention and treatment (Baird et al., 2007; Price et al., 2007). This is often the case with outbreaks of temperate strains of *P. vivax*, which have a tendency to produce a primary blood-stage infection, before entering an extended period of hepatic dormancy host for at least 9-12 months. Intermittent relapses and dormancy periods are then experienced (Adak et al., 1998; Oh et al., 2001; Price et al., 2007). Such delayed attacks of disease have been recorded in *vivax*-exposed populations and in travellers returning from endemic temperate zones, such as parts of the Korean peninsula and Indian sub-continent (Ellis, 1989; Baird et al., 2007).

Infection by malarial parasites is generally characterised by cyclical paroxysm, which is defined as "an acute fever that is typically preceded by chills and rigor" (Karunaweera et al., 2003:188). Paroxysm is a common host reaction to many bacterial, viral, and parasitic infections and represents a highly complex immunological response. The blood stage of *P. vivax* infection elicits well-defined (in terms of duration), repeated cyclical paroxysmal episodes and is, therefore, one of the most intensely studied host immunological responses to parasite infection. *P. vivax* initiated paroxysmal episodes are usually characterised by chills and rigor, accompanied by the development of a high fever of up to

41° C within three hours of onset. Following these initial symptoms, body temperature falls and profuse sweating occurs, lasting for several hours until normal temperature is regained. Paroxysm is often accompanied by nausea, vomiting, headache, myalgia, and joint pain. The entire episode usually lasts from 4-8 hours, after which the patient is left physically exhausted (Mendis et al., 2001; Karunaweera et al., 2003).

Research has suggested that paroxysm and inflammatory responses associated with *P. vivax* malaria are more severe than in *P. falciparum* (Baird et al., 2007; Price et al., 2007; Anstey et al., 2009), a somewhat surprising implication given the much lower percentage of erythrocytic invasion and preferential targeting of young erythrocytes (reticulocytes). Hence, the *P. vivax* parasite invades only approximately 2% of total blood cells. In comparison, *P. falciparum* invades 80% of all erythrocytes (Pinello, 2008), resulting in a much higher parasite load. Price et al. (2007:80) suggest that this difference in symptomatic severity may be due to the ability of the *P. vivax* parasite to induce "fever at levels of parasitemia lower than those causing fever in *P. falciparum* infection…the host inflammatory response is activated to a greater extent during *P. vivax* infections, with plasma levels of fever-inducing cytokines being higher in *vivax* malaria compared with *P. falciparum* infections". The exact role of cytokines in the immunological response in *P. vivax* infection is not yet clearly understood, although recent research has suggested that they may play a vital role in protecting patients from developing severe symptoms (Gonçalves et al., 2012).

Many cases of *P. vivax* malaria are non-life-threatening if the infection is not complicated by comorbidity, or co-infection with other malaria parasites, and the patient receives suitable treatment. Despite this, the similarity of *vivax* paroxysmal symptoms to other infections can cause misdiagnosis, especially in imported cases where the physician is unfamiliar with the disease (e.g., Hänscheid et al., 2003). Since *P. vivax* is generally assumed to be non-fatal, the disease has acquired the reputation of being 'benign'. However, even uncomplicated infection can occasionally result in 'severe' *vivax* malaria, encompassing life-threatening symptoms, some of which are remarkably similar to those seen in cases of *P. falciparum* malaria. These symptoms can include lung injury, splenic rupture, renal failure, cerebral malaria, acute respiratory distress syndrome (ARDS), severe anaemia, and haematoma (Beg et al., 2002; Baird et al., 2007). Complications of these symptoms by concurrent diseases/infections or nutritional deficiency increases the potential

lethality of severe *P. vivax* malaria, particularly in children (Caulfield et al., 2004). The similarity in symptoms between *P. falciparum* and severe *P. vivax* malaria has led to misdiagnosis and subsequently inadequate treatments, particularly in areas where expensive diagnostic equipment is not readily available. When presented with symptoms of severe *vivax* malaria, clinicians in these areas often ascribe an automatic diagnosis of *P. falciparum* (Baird, 2007), resulting in ineffective control of dormant hypnozoites. The pathophysiology of severe *P. vivax* infection is poorly understood, particularly since studies have sometimes failed to take into account complicating factors such as concomitant infections, access to treatment, individual immunity, and drug resistant parasite strains (Rogerson and Carter, 2008; Anstey et al., 2009).

3.5: The 'benign' status of *P. vivax*

A growing awareness that *P. vivax* infections may be responsible for cases of severe and fatal malaria has prompted a renewed consideration of the assumed 'benign' status of the disease, and has stimulated calls for increased research funding (see Bassat and Alonso, 2011). These calls have been partly driven by recent studies highlighting cases of severe *P. vivax* in Southeast Asia (Kochar et al., 2005; Barcus et al., 2007; e.g., Tjitra et al., 2008). Funding of malaria research has, quite understandably, been focused on *P. falciparum*, the most virulent and deadly species. Due to the perceived benign nature of *P. vivax* malaria, the disease has received "lower priority from researchers, policy makers, and funding bodies" (Price et al., 2007:79), attracting approximately only 3% of malaria research funding (Gething et al., 2012). *P. vivax* is also notoriously difficult to study in a laboratory setting, since it generally operates at low levels of parasitemia and has a more complex life cycle than *P. falciparum*. The low erythrocyte infection percentage and hypnozoite stage of *P. vivax* infection confound in-vitro study of the pathogen, and hence complicate research on drug resistance, mechanisms of attack, and general pathophysiology (Baird et al., 2007). The global burden and potential lethality of *P. vivax* infection combined with a lack of adequate research funding have led to the parasite being described as "perhaps the most neglected, potentially dangerous and highly prevalent infection" (Baird, 2007:533).

3.6: The human immune system and its response to *Plasmodium* infection

3.6.1: The immune system

Much of early 20th century malaria research was focused on the characterisation of certain aspects of the disease, mostly conducted through microscopic analysis of vector, parasite, and infected blood samples (Cox, 2010). By the mid-20th century, revolutionary advances in biotechnology allowed researchers to investigate the disease on a biomolecular level. Much of this work was driven by the search for effective vaccines (Marsh, 2002), some of which proved highly successful in helping to eliminate malaria, particularly in developed, temperate areas. With modern technological advances, understanding of the mechanics of the immunological response to malaria continues to grow.

Early research on malaria immunology, such as that undertaken by Golgi and his contemporaries, hinted at the intricacies of the relationship between the human immune system and its parasitic invader (e.g., Laveran, 1891; Ross, 1910). Building upon this early foundation, researchers now appreciate that the human immune response to infection represents a complex and sophisticated coordination of processes aimed at the control and destruction of invading organisms, achieved without harming the host. Since a discussion of the full complexities of the human immune system is beyond the scope of this study, a simplified overview of the processes involved in the immune response will be presented, before considering the more specific responses to malaria infection. An understanding of the molecules and processes involved in the immune response to malaria infection is important if successful methodologies for biomolecular analysis of archaeological human remains are to be successfully achieved.

The human immune system comprises two distinct, but complementary mechanisms: the innate and adaptive responses. The innate response forms the initial protection against invasion from exogenous pathogens. It is non-specific in that it produces immune factors that do not recognise specific pathogens. Instead, it offers generalised protection through recognising common pathogenic genetic patterns (Kindt et al., 2006). The adaptive response comprises the secondary immune defence system, which produces and utilises specialised immune factors to develop and build up an immunological memory. The adaptive response is split into two mechanisms, the humoral and cellular immune responses. Put simply,

humoral immunity refers to the processes by which protein antibodies are produced and bind to foreign molecules (or antigens), thus acting as a marker of infection. Additionally, the humoral response initiates the production of cytokines. The cellular immune response produces cytotoxic T lymphocytes which bind to and subsequently destroy foreign molecules (Berg et al., 2002; Riley, 2004).

When a new pathogen first invades its host, it will encounter the body's pre-existent, innate defence mechanism. This consists of interactions between non-specific IgM immunoglobulins and phagocytes, the latter consisting of macrophages and neutrophils that ingest foreign bodies. This results in the 'recognition' of exogenous pathogenic microbes and adherence to them, thus marking them for phagocytosis. In this manner, the innate mechanism allows for an immediate response to pathogenic invasion. However, since the pre-existent, circulating phagocytes and immunoglobulins are relatively scarce within the bodily tissues, there may be insufficient numbers to cope with the pathogen load. In this situation, the macrophages initiate an inflammatory response, controlled by the release of certain cytokines (Wood 2001). These proteins serve a number of important functions, such as the activation of extra macrophages and increases to vascular permeability. They also promote increased blood flow to the affected area, which introduces further immune factors to fight the infection. Cytokines are also responsible for the increase in bodily temperature (fever) associated with illness, which is a protective measure aimed at limiting pathogenic replication. The innate immune response of phagocyte replication and pyrexia (fever) may be enough to kill the invading organism (Wood, 2001).

If the innate immune response fails to recognise or destroy a pathogen, the adaptive immune system is required. Lymphocytes represent the most important components of this system. Memory T and B lymphocytes, for instance, are crucial, as they develop long-term immunity to specific pathogens. B lymphocytes are particularly important since they release immunoglobulin antibodies (Parham, 2009). A primary role of these antibodies is to 'recognise' and bind to foreign antigens. The specific binding sites on antibody molecules are known as 'paratopes', while the target sites on the pathogen are known as 'antigenic epitopes'. The binding process (also called 'opsonisation') marks the pathogen for phagocytation (Figure 3.2). Most antibodies consist of two identical heavy (H) chains and two identical light (L) chains, each consisting of approximately 110 amino acids. These

chains together form a Y-shaped structure (Figure 3.2) weighing around 150kDa (Janeway, 2001). They are considered to be relatively large biomolecules.

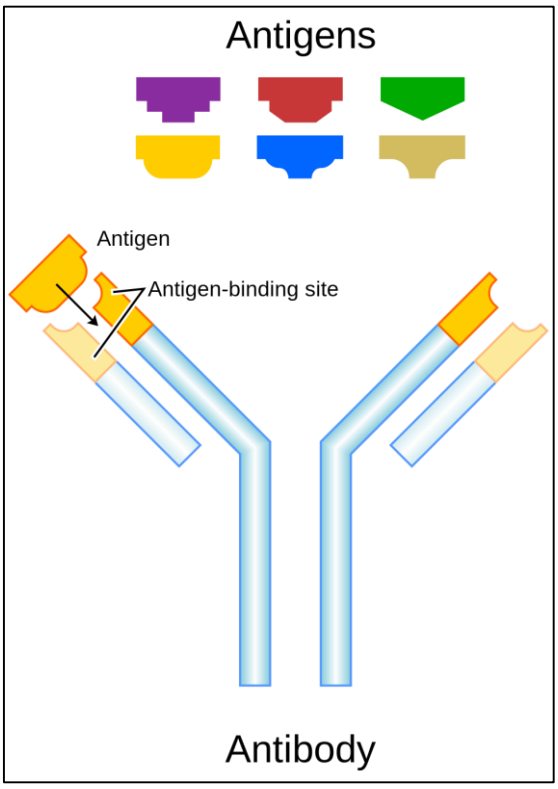

Figure 3.2: Basic antibody structure and interaction with antigenic epitopes. Dark blue – heavy chains; light blue – light chains (adapted from Fvasconcellos, 2007).

The five main classes of immunoglobulin antibodies are IgA, IgD, IgE, IgG, and IgM. Each class has different functions and specific paratope configurations, although they can have identical specificity to antigens (Wood 2001). IgG antibodies are the most abundant class, accounting for approximately 75% of serum immunoglobulins (Nezlin, 1998). They consist of the classic Y-shaped heavy and light chain structure held together by disulphide bonds, and are mostly active in the adaptive immune response. IgGs can remain in the blood serum for extended periods following infection (Meulenbroek and Zeijlemaker, 1996) and are one of the few classes of antibody that crosses the placenta, sharing maternal immunity with the developing foetus (Kane and Acquah, 2009). IgMs are by far the largest antibodies, with a molecular weight of up to 950kDa. This is due to a unique structure in which five pairs (a pentameric form) of heavy and light chains are linked by a heavy central J-chain (Figure 3.3). IgMs are non-specific and free circulating, forming a vital part of the

innate immune response. Their unique structure means that IgM paratopes bond very strongly (avidity) and to a wider range (specificity) of antigenic epitopes. IgMs also act upon up on certain complementary receptors to increase their avidity (Wellek et al., 1976). These are crucial properties in a 'first-response' immunological unit.

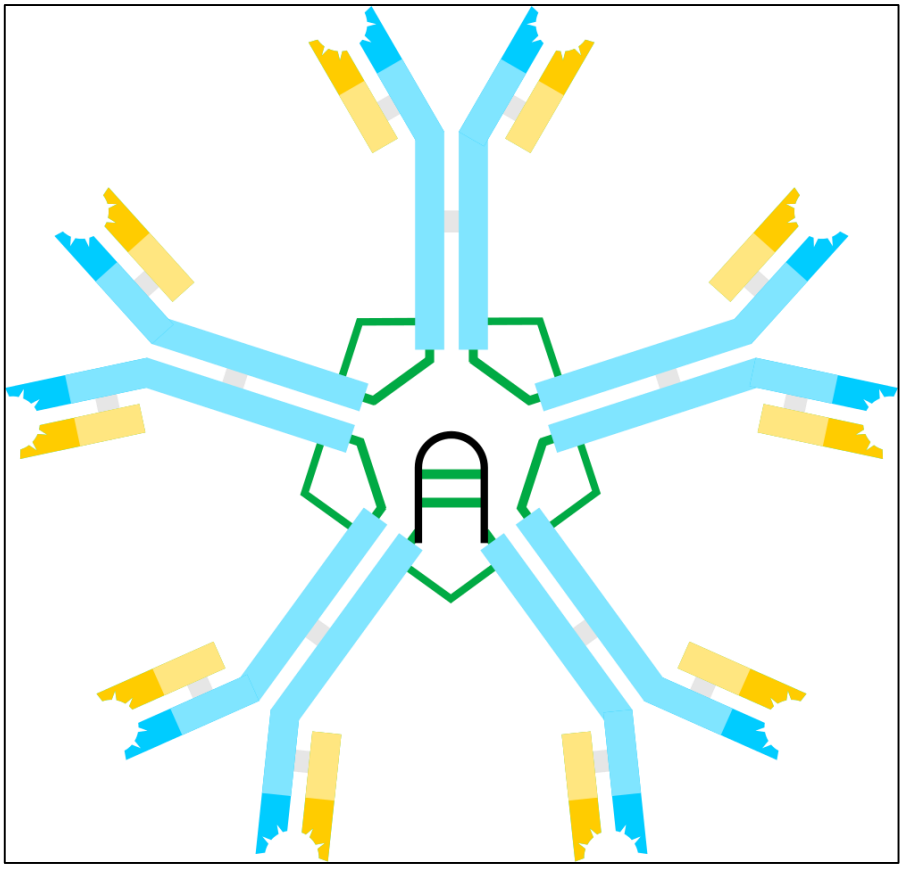

Figure 3.3: Immunoglobulin M (IgM) structure. Blue – heavy chains; yellow – light chains; green - disulphide bridges; black – J chain (adapted from Fijałkowski, 2006).

3.6.2: The immune response to *Plasmodium vivax* infection

In this section, consideration of the immune response to *vivax* malaria infection is given initially under the assumption that the infection is the patient's first contact with the parasite. It is also assumed that the patient is in relatively good health with no pre-existing conditions that would offer protection against or exacerbate the infection. Although this scenario may not prove common in the field, it is important to explore how the *Plasmodium vivax* parasite interacts with a healthy immune system with no immunological 'memory' of

malaria infection, before considering particular factors which may alter the course of the infection.

The immune response to infection by *Plasmodium vivax* parasites begins following the first inoculation into the bloodstream by the *anopheline* vector. Due to the very short period of sporozoites blood stream circulation prior to hepatocytic (liver cell) invasion, the exact mechanism of the primary immune response to inoculation is not clearly understood. However, it is likely that at least some of sporozoite antigens are recognised by the innate immune response, which may prevent some of them from invading the liver cells (Marsh, 2002). The main innate immune response is stimulated by the release of merozoites into the bloodstream by the rupture of infected hepatocytes, which usually occurs some 6-16 days after initial parasite inoculation. In response to the presence of numerous circulating foreign organisms, the immune system reacts very much as it would to any invasion by viruses, bacteria, or other parasites (Clark et al., 2006). As described above, the immediate response is the activation of innate IgM antibodies and phagocytes, and the release of cytokines in an inflammatory response.

Complex parasite cell forms (e.g., merozoites) contain hundreds of antigenic epitopes and will therefore induce the production of further phagocytes and IgM antibodies, in the case of first malaria infection. If the infection is successfully controlled at this stage, these immune factors will remain in circulation for only a few weeks (Marsh, 2002). However, the adaptive immune system will be stimulated if the innate response fails to fully control the infection, or if the patient is later infected with a different strain; cross-strain protection is generally quite poor (White, 1996).

The exact nature of the adaptive response to the presence of *Plasmodium* parasite molecules has only recently begun to be understood, particularly the roles of B- and T-lymphocytes in antibody production and mediation. Of crucial importance are human leukocyte antigens (HLA). These ‘carrier’ molecules are produced by most body cells and determine “which antigens can elicit an immune response in any one individual” (Marsh, 2002:68). Put simply, in the adaptive response to malaria parasite molecules, B-lymphocytes first recognise then absorb specific antigenic molecules. The molecule is broken down within the lymphocyte and its epitope is conjugated with a HLA molecule. This epitope/HLA complex is then transported back to the surface of the B-lymphocyte, where it

is recognised by a T-lymphocyte. This recognition stimulates the T-lymphocyte to produce specific T-helper cells, which in turn stimulate the B-lymphocyte to produce antibodies specific to the original antigenic epitope. This is an important stage in the development of immunological 'memory' (Marsh, 2002).

The lifecycle of the *Plasmodium vivax* parasite presents unique challenges to the immune system upon first infection. Many of these problems are derived from the fact that the parasite spends the majority of its lifecycle within host cells (either hepatic or erythrocytic), which, in theory, offers a degree of protection against blood-borne immune system products. As previously mentioned, the brief time spend circulating prior to hepatocytic invasion allows for only a 'transient' innate immune response (White, 1996). Similarly, the merozoites and schizonts released from infected erythrocytes spend only a very brief exo-erythrocytic period before invading new red blood cells. Therefore, much of the immune response must be focused upon intracellular methods of neutralising *vivax* cells. In the liver, hepatocytes contain HLA molecules, which may allow for the production and presentation of surface antigens, subsequently marking the infected hepatocyte for lysis (Marsh 2002). Conversely, erythrocytes, in which the parasite spends the majority of its active life cycle, contain very few HLA molecules. To counteract this, the immune system is capable of recognising unique parasite-induced changes to the erythrocyte (in particular its cell membrane) and is thus able to mark the infected cell for destruction (Marsh, 2002). Research has suggested that antibody interaction with gametocytes released from infected erythrocytes may work to inhibit the reproductive cycle of the parasite once ingestion back into the *anopheline* mosquito vector has taken place (Mulder et al., 1994; Srikrishnaraj et al., 1995; Lal et al., 2001).

Erythrocytic release of merozoites and their by-products also stimulates the production of cytokines which, as previously mentioned, are responsible for the paroxysm associated with malaria infection. The exact roles of cytokines in the immune response to malaria are not yet fully understood, but have received considerable research focus (e.g., Karunaweera et al., 1992, 2003; Malaguarnera and Musumeci, 2002; Clark et al., 2006; Hemmer et al., 2006). It is known that the cytokine response is particularly high in *vivax* malaria infections, and may be an influence in the development of some of the potentially fatal severe *vivax* symptoms (Sina, 2002; Park et al., 2003) previously mentioned.

3.6.3: Immunity to temperate endemic *P. vivax* malaria: the Republic of Korea as a modern case study

What is endemicity and what constitutes an endemic disease? Before discussing the development of immunity in endemic malarious areas, it is necessary to address these questions. 'Endemic' has been defined as "occurring frequently in a particular region or population", and an endemic disease is one that is "generally or constantly found among people in a particular area" (Martin, 2010:242). Malaria is currently endemic in many tropical and sub-tropical countries. As can be seen in Figure 3.4, transmission is particularly intense in sub-Saharan Africa, south and Southeast Asia, and parts of South America.

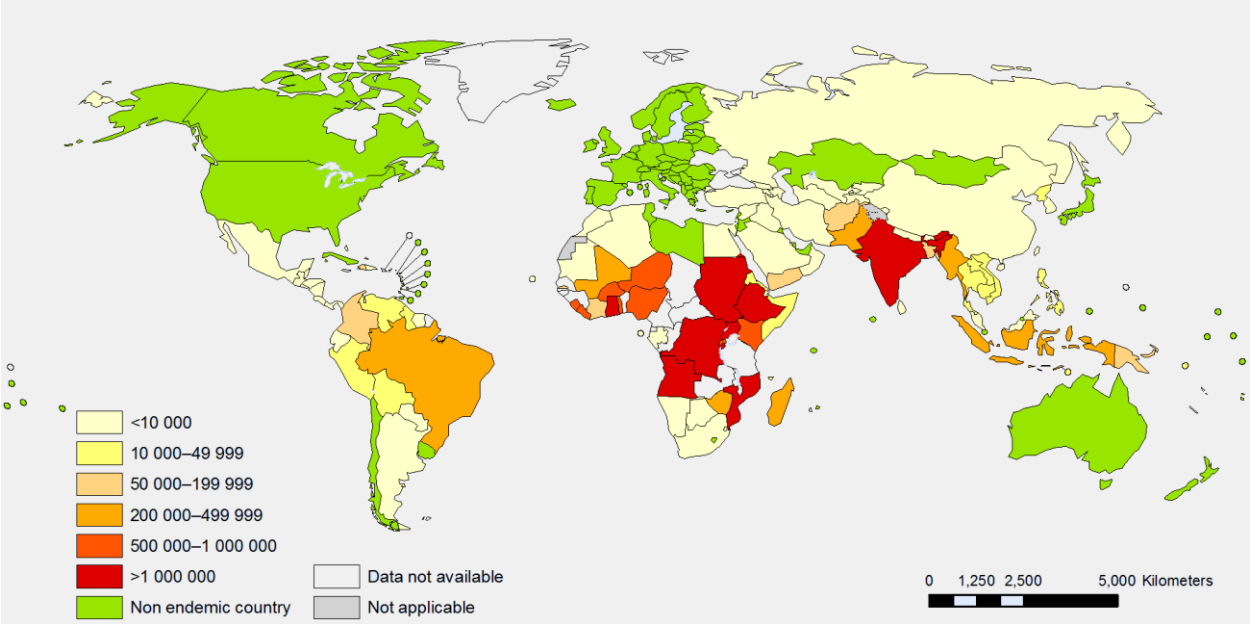

Figure 3.4: Global distribution of reported, confirmed human malaria cases in 2010 (adapted from World Health Organization, 2012).

It is generally agreed that *Plasmodium vivax* was the malaria parasite prevalent in parts of Britain in past centuries, since the temperate climate and vector habitats would have been sub-optimal for survival of other human malaria parasite species (Dobson, 1997). It is pertinent, therefore, to explore the development and status of immunity to *vivax* malaria in modern populations inhabiting temperate areas. In doing so it may be possible to further understand mechanisms of immunity in past British populations. However, there are several important caveats to be made prior to attempting a retrospective analysis here. Firstly, the

status of endemicity of past malaria in Britain is unknown. It is unclear, for example, whether the disease was endemic for the entirety of its presence in the country, or whether there were short periods of endemicity, perhaps tied to climatic changes or population movements. As previously discussed, historical records from post-medieval south eastern counties certainly suggest that *vivax* malaria was endemic for much of the period. Prior to this, the existence of British malaria is disputed by some authors, who assume that the parasite was not present at all (e.g., Bollet, 2004; Pinello, 2008; Neginha et al., 2010), while others cite favourable climatic and environmental conditions for malaria transmission (e.g., Reiter, 2000), or medical accounts of symptoms highly suggestive of the disease (e.g., Franklin, 1983; Gasper, 2004).

Secondly, the development of immunity to malaria is governed by a number of diverse and complex factors. These include individual health status at the time of infection, the presence of certain genetic factors (e.g., blood cell polymorphisms) influencing individual resistance, effectiveness of available treatments, transmission rate (itself being heavily dependent upon vector population/survivorship), and disease virulence. The latter is particularly important, since increased strain virulence would lead to a peak in mortality rates, thus increasing selective pressures. If occurring over an extended period, this may possibly influence the development of certain malaria-mediated genetic resistances, such as thalassaemia and G6PD deficiency. Although retrograde analysis from modern examples may not be ideal, it represents the only method of exploring the development of 'immunity in action' on the individual and population levels.

Plasmodium vivax is the most geographically widespread malaria species, found throughout South America, the Indian sub-continent, Southeast Asia and the western Pacific islands (Mendis, 2001). As previously discussed, *vivax* malaria generally prevails in temperate areas. Due to the unique lifecycle of parasite and vector, *vivax* malaria can be described as somewhat transcending the definition of 'endemic' offered above. Whereas sub-tropical *vivax* fits the definition in that climatic conditions allow for year-long transmission, temperate strains require vectors and parasites that can survive through potentially unfavourable periods. In northwest Europe in antiquity, this mosquito vector was most likely *Anopheles atroparvus*, which commonly over-winters inside animal shelters and human habitations (Dobson, 1997). *Anopheline* vectors have also been observed over-

wintering as far north as Finland (Hulden et al., 2005). This strategy gives the mosquito year-round access to blood meals and potential parasite hosts (Dobson, 1997). This finely adapted vector-parasite relationship and the ability of *P. vivax* to 'hibernate' inside the human host during periods unfavourable to transmission combine to make continuous endemicity a real possibility in temperate areas, both past and present. From the point of view of those studying temperate *vivax* malaria palaeoimmunity, it is somewhat unfortunate that the disease has been eliminated from most temperate areas of the World where it once flourished. This hinders attempts at immunological analogy between past and present temperate populations.

Research on modern populations inhabiting endemic *vivax* malaria areas has generally concentrated on Indonesia, India, and the Korean peninsula. Since much of Indonesia and India are sub-tropical and tropical, this research is usually of less relevance to researchers studying *vivax* malaria immunity in past temperate areas. The Republic of Korea (ROK), however, presents a potentially more useful target. Climatically, the central areas of the Korean peninsula are perhaps the closest modern analogue to temperate north western Europe, with average summer and winter temperatures of approximately 22 to 24º and -4 to -2º Celsius, respectively (Korean Overseas Information Service, 1993). The low average winter temperatures likely account for the prevalence of *vivax*, rather than *falciparum* malaria here.

After centuries of *vivax* malaria endemicity, The World Health Organization declared the ROK to be malaria-free in 1979. The disease, however, re-emerged in and around the Demilitarised Zone (DMZ) on the North/South Korean border in 1993 and has once again become established as a source of temperate malaria. Up to 80% of the re-emerging cases occur in military personnel serving in the DMZ (Feighner et al., 1998; Oh et al., 2001), and a recent resurgence in cases suggests an increase in drug-resistant *vivax* strains (Jun et al., 2009). Malaria morbidity rates in the ROK rise annually in the spring and early summer months, which correspond to the activation of dormant hepatocytic *vivax* parasites in preparation for a short summer season of vector-mediated transmission. The high post-medieval mortality rates recorded in the Fens and marshlands of England suggest a similar pattern of infection and pathology. These record high mortality in the spring and autumn, which Dobson (1980) suggests correspond to deaths associated with relapse of

vivax malaria in the spring, and autumnal mortality influenced by primary infections acquired in the summer months.

The main vector involved in the transmission of the Korean strains of *vivax* malaria is most likely *Anopheles sinensis*. This species is behaviourally similar to *A. atroparvus* in that they are both zoophilic (Ree et al., 1967). The species differ significantly in habitat preference: *A. sinensis* is usually confined to fresh water breeding grounds, while *A. atroparvus* generally prefers a brackish environment. *A. sinensis* is also far less anthropophilic than *A. atroparvus* and is not known to overwinter in human dwellings (Harrison and Scanlon, 1975). The predilection of *A. sinensis* for animal blood has been suggested as a possible cause of the mid-1990s surge in *vivax* malaria cases, which followed widespread crop failures and a forced reliance on livestock as a food source. It has recently been suggested that zooprophylaxis (the movement of livestock in order to attract zoophilic mosquitoes away from potential human targets) may be a more effective *vivax* malaria control than reduction of mosquito numbers (Nah et al., 2010). Behavioural differences between *A. sinensis* and *A. atroparvus* strongly suggest that the latter may be a more efficient vector of *vivax* malaria. If this is indeed the case, transmission of past British malaria may have been more intense than in modern ROK populations.

Korean data is particularly useful when considering the course of disease and host immune reactions in primary *vivax* infection, a situation most likely faced by non-immune migrants to malarious areas in the past. The investigation by Oh et al. (2001), for example, demonstrated an expected behaviour of *vivax* infection in the endemic DMZ, in that no relapses were reported over the winter months, with most infections acquired during the summer becoming evident the following spring. Long-term latency (the time between transmission and emergence of infection symptoms) was typically at least 10 months, although prophylactic treatment in the interim period may have extended this. A common complication of *vivax* infection was thrombocytopenia (abnormally low blood platelet count), a condition encountered in similar studies of *vivax* malaria (e.g., Lim et al., 1996; Yamaguchi et al., 1997; Kumar and Shashirekha, 2006). Symptoms of thrombocytopenia range from minor bruising in mild cases, to uncontrolled, potentially fatal internal haemorrhaging in severe, untreated cases. Non-immune patients with *vivax* malaria tend to exhibit more severe thrombocytopenia than those with other forms of malaria, although the

exact reasons for this are not clearly understood. Research has implicated elevated levels of IgG antibody and certain cytokines (e.g., Yamaguchi et al., 1997), as well as circulating nucleic acids (e.g., Franklin et al., 2011).

Defoe's 18th century account from the Essex marshes concerning the deaths of multiple immigrant wives gains some credibility when considering the evidence presented by research on modern temperate and severe *P. vivax* infections:

> "I took notice of the strange decay of the sex here insomuch that all along the country it was very frequent to meet with men that had had from five and six wives to fourteen and fifteen wives, nay and some more. I was informed that in the marshes on the other side of the river over against Candy [Canvey] Island there was a farmer who was then living with the five and twentieth wife, and that his son, who was then about thirty-five years old, had already had about fourteen...The reason, as the merry fellow told me who said he had had about a dozen and a half wives....though I found afterwards that he fibbed a little...was this: that they, being bred in the marshes themselves and seasoned to the place, did pretty well with it, but that they always went up into the hilly country or, to speak their own language, into the uplands for a wife. That when they took the young lasses out of the wholesome and fresh air they were healthy, fresh and clear and well: but when they came out of their native air into the marshes among the fogs and damps, there they presently changed their complexion, got an ague or two, and seldom held it above half a year or a year at the most" (Defoe 1722, quoted in Cracknell, 1959:25-26).

The local men, having been "bred in the marshes themselves and seasoned to the place" (Defoe quoted in Cracknell, 1959:25), evidently had at least partial immunity (whether acquired or innate is unknown) to malaria infection, which may have lessened the debilitating effects of the relapses they must surely have suffered. The non-immune immigrants, however, had no such fortune. A scenario may have been that young, non-immune women were brought into a malaria-endemic area, after which they "got an ague or two…and seldom held it above half a year or a year at the most" (Defoe quoted in Cracknell, 1959:26). Although Defoe does not specify malaria as an agent of mortality, and acknowledges the likely exaggeration of the account, it is highly likely that the disease played a significant role in the morbidity and mortality of the non-immune.

Given the additional immunological pressures afforded by the insalubrious Fen environment alongside potential *P. vivax* infection during pregnancy, increased Fen mortality for females may be expected over and above that observed in the non-Fen

cemeteries. Short (1750, quoted in Dobson 1997:318) wrote that immigrants into Fen/marshland areas:

> "run a great risk, who having been brought up, and accustomed to a clear healthy air, remove to fenny, wet, sticky soils; for people born in, and inured to a bad air, bear it much better, and find less sensible inconvenience from it, than such as have been bred and familiarized to a good one…though burials in such places may exceed births…There it is evident, that great numbers dying in infancy, are supplied by fresh in-comers, who settle and marry there; and that the endemics of the place are more fatal to them than the natives."

It is well established that women from non-malarial locations or those residing in areas of low endemicity have limited immunity to *P. vivax*, and are therefore more likely to develop severe sequelae in response to infection (Nostern et al., 2004; ter Kuile et al., 2008). Infections in areas of low malaria transmission, where women have little acquired immunity, are believed to be much more likely to result in severe disease and death of the mother or foetus, when compared to populations in *P. vivax*-endemic areas of Africa (Desai et al., 2007). Should pregnancy have occurred, *P. vivax* infection may well have been catastrophic for these women and their infants. Pregnancy increases the risk of both primary malaria infection and maternal anaemia once infected (Diagne et al., 2000). It has been suggested that *vivax* malaria infection is a significant worldwide contributor to maternal and infant anaemia, a condition linked to low birthweight and an associated increase in infant mortality (Nacher et al., 2003; Price et al., 2007).

Should mother and infant survive the pregnancy and immediate post-partum period, acquired immunity to *P. vivax* may develop if transmission rates are high enough to sustain the presence of antibodies. It is known that breastfeeding confers protective immunity to the infant against a number of conditions, including certain respiratory and enteric diseases (Hanson, 1998; Oddy, 2004). Breastmilk has also been shown to contain factors that inhibit the growth of the *P. falciparum* parasite (Kassim et al., 2000). Unfortunately, the vast majority of studies concerning the effects of breastfeeding on infant malaria infection have focused on *P. falciparum*. Subsequently, the extent to which breastfeeding affects the impact of *P. vivax* infection on infant health remains uncertain, although the anti-inflammatory and immunological agents present in human milk are likely to be beneficial (Goldman et al., 1986; Riley et al., 2001; Field, 2005). The presence of protective genetic polymorphisms would also offer some degree of immunity to the infant. Despite the increasing availability

of symptom-suppressing quinine during the late 19th century (Schute and Maryon, 1974), earlier patients had no recourse to effective treatments and were therefore likely at increased risk of suffering severe *vivax* malaria symptoms, such as thrombocytopenia, hypoproteinemia, oedema, and dramatic weight loss (Price et al., 2007).

To further understand implications of the presence of *P. vivax* for past British populations, it is necessary to consider the numerous ways in which the parasite affects modern populations living with the burden of *vivax* malaria. Anstey et al. (2009) cite many factors as being important in influencing the modern burden of *vivax* malaria. Geographic location, genotypes, and socio-cultural factors (e.g., poor access to health care facilities) all play key roles in increasing morbidity and mortality rates associated with normal and severe *P. vivax* infections in contemporary populations (Anstey et al., 2009). Studies in Papua New Guinea, for instance, have demonstrated that some populations are at higher risk of developing severe anaemia depending on their location, and the presence of genetic haemoglobinopathies (Fowkes et al., 2008; Genton et al., 2008). Papuan studies have also shown that non-immune immigrants from non-endemic *vivax* affected regions displayed a much higher probability of developing severe *vivax* malaria (Genton et al., 2008) and associated increased mortality rates. Rogerson and Carter (2008), however, caution that the true mortality rate caused directly by *P. vivax* infection in these studies is unclear, since comorbidities are not taken into account. Repeated infection and relapse have been linked to the development of severe *vivax* malaria, particularly when combined with comorbidity and poor nutrition (Baird, 2007). Indeed, repeated *vivax* infections can result in serious symptoms such as "cachexia, spontaneous abortions, male infertility, developmental arrest, and impaired mental function" (Rogerson and Carter, 2008:877).

Despite increasingly common clinical reports of severe symptoms associated with *P. vivax* infection, the disease remains a relatively rare killer in modern settings. *P. vivax* has evolved the dormant hypnozoite stage and, accordingly, the virulence of temperate strains is tempered in order to keep the host alive through sub-optimal transmission periods (Hulden and Hulden, 2005). Although individuals with uncomplicated infection occasionally develop severe, life-threatening symptoms, access to suitable medications can at least help to control the symptoms. *P. vivax* relapse from the dormant hypnozoite state occurs unpredictably, and for reasons that remain poorly understood. In modern research on Nepal, where *P. vivax* and

P. falciparum share co-endemicity, 17% of those infected with the former experienced relapse within six months of the initial febrile attack (Manandhar et al., 2013). Similar studies from India have shown relapse rates varying between 9% and 40% (Adak et al., 1998; Gogtay et al., 2000; White, 2011), depending upon the length of follow-up study. It should be stated here that these relapses occurred in individuals who had *undergone* anti-malarial treatment. It is likely that relapses occurred more frequently in pre-medicalised antiquity, with the chances of each relapse coinciding with other illnesses increasing with each subsequent febrile episode. For instance, Samuel Jeake (1652-1699) of Rye, Sussex, kept extensive diary records between 1667 and 1693, in which he chronicled 330 personal attacks of ague (Dobson, 1997). This averages over 12 relapses per year, and although not all attacks were severe or debilitating, they often resulted in lengthy episodes of ill health.

As previously discussed, the transmission and relapse rates of *P. vivax* in temperate areas are generally quite low, especially where endemicity is sporadic. As a result, any partial immunity acquired after the primary attack may be lost in the extended period prior to the first relapse. Hence, populations in low-transmission areas do not generally achieve consistent levels of acquired immunity. The disease affects people in every age category and causes long term debilitation in terms of personal health, longevity and productivity (Mendis et al., 2001). Untreated *vivax* malaria eventually becomes a chronic condition, with the individual suffering consistently low-level fatigue and anaemia until the next relapse (Aufderheide and Rodriguez-Martin, 1998). It has been suggested that "every person in a moderately *P. vivax*-endemic area can expect to experience anywhere from 10 to 30 or more episodes of malaria in the course of childhood and working life" (Mendis et al., 2001:101). The deleterious economic impact of an endemic disease that frequently incapacitates working adults should not be underestimated, especially in populations already suffering with poor health and/or inadequate access to healthcare.

There is uncertainty as to the exact reasons why post-medieval marshland mortality rates were so high. While there is little doubt that malaria played an important role, it is unknown whether the disease was the primary contributor. Some researchers have suggested that 16th and 17th century strains of *P. vivax* may have been more virulent than modern strains (e.g., Dobson, 1980; Pinello, 2008). Pinello (2008), for instance, argues that the possible introduction of new *P. vivax* strains into post-medieval Britain through increased

trade links may have altered indigenous parasite genotypes, causing a subsequent increase in virulence. Unfortunately, the exact parasite strains in operation in the past are unknown. While it is possible that highly virulent strains of *P. vivax* attributed to high seasonal mortality, strain-based explanatory models must remain conjectural for the present.

It has been argued that past mortality figures cannot be wholly trusted, since the diagnosis of *vivax* malaria by physicians prior to the 20th century may have been confused by the similarity of malaria symptoms to other common diseases (e.g., Swellengrebel and Buck, 1938; Hutchinson, 2004; Hutchinson and Lindsay, 2006). For instance, water-borne pathogens causing cholera and other enteric conditions undoubtedly played a part in increasing mortality rates in unsanitary areas, where cases may have been misdiagnosed as malaria, or 'ague'. Hutchinson and Lindsay (2006) discuss the deleterious effects of large-scale raw sewage dumping into the Thames and Medway rivers on downstream, marshland communities, citing a mid-19th century cholera epidemic as a major cause of increased mortality rates in these areas. They do not, however, attempt to account for the similarly high post-medieval mortality rates observed in the Lincolnshire and Cambridgeshire Fenlands, which would surely have suffered far less from urban water contamination. They also assume that all *P. vivax* infections were benign, ignoring modern evidence for increased prevalence of severe *vivax* malaria associated with repeated infection and comorbidities in economically deprived areas. Further to this, they present Registrar General Reports from the mid to late 19th century, the time period which heralded the disappearance of malaria from Britain and saw a sharp decrease in malaria transmission.

The interaction of malaria infection with other conditions and diseases is an important factor to consider when studying malaria-related morbidity and mortality in the past. Comorbidity can play an important part in the outcome of malaria infection, dependent on the type of concomitant condition (Sallares, 2002; Anstey et al., 2009). The syphilis bacteria (*Treponema pallidum*), for instance, cannot survive the high temperatures associated with malarial paroxysms (Fraser et al., 1998). *Vivax* malaria, therefore, provides a degree of protection against the development of this disease. Indeed, in the early 20th century, end-stage syphilis sufferers were subjects of 'malariotherapy', which entailed intentional infection with *vivax* malaria as a curative measure (Vogel, 2013 b). However, immune system compromise associated with malaria may leave the sufferer more

susceptible to the development of malnutrition (Williams et al., 1997), severe anaemia (Price et al., 2007), and infection by tuberculosis (Setzer, 2010) and typhoid (Kumar and Katiyar, 1995), the latter of which being particularly dangerous as it can intensify the symptoms of malaria (Pinello, 2008). The effects of combined helminth and malaria infections (a likely combination in past marshland communities) are currently unknown, with modern epidemiological research producing conflicting results (see Mwangi et al., 2006 for a synthesis of such research).

It is unlikely that one agent alone can account for the high mortality rates in marshland and Fen environments. Rather, a combination of many factors must have been responsible. As has been discussed, in areas affected by poor sanitation, poor nutrition, and lack of access to healthcare in the form of diagnostics, prophylactics and medical treatment, one may expect to encounter high morbidity and mortality rates. This is exactly the situation in which many marshland and Fenland populations would have found themselves in the past. Individuals would have been exposed to numerous viral, bacterial, and parasitic infections, probably suffering from more than one at once. Even if *P. vivax* infections were indeed 'benign', the disease has been shown to contribute to child malnutrition (Williams et al., 1997), severe anaemia in infants and children, and maternal anaemia (Price et al., 2007; Anstey et al., 2009). These conditions, perpetuated by relapses and possibly combined with little protective immunity, would serve to weaken individuals sufficiently that co-infection could prove fatal. This is discounting the consequences of widespread post-medieval addiction to alcohol and opiates. Research on modern populations suffering the burden of *vivax* malaria strongly suggests that past populations would indeed have found the disease thoroughly debilitating and commonly associated with death (Tan et al., 2008).

Findings from the previously discussed modern Papuan studies concerning immigrants to endemic malarious areas may go some way to supporting the Daniel Defoe's 18th century account of the repeated, swift demise of extraterritorial wives taken by Essex marshland inhabitants (Defoe, 1722). Two points stand out in this account. Firstly, although the numbers are probably exaggerated, it is quite conceivable that new brides brought into a marshland environment would quickly fall ill after being exposed to *vivax* malaria and other diseases associated with poor sanitation. If pregnancy occurred, then the increased nutritional requirements of the maternal body might lead to severe anaemia, resulting in low

infant birthweight and high mortality rates, both of mothers and infants (Nacher et al., 2003; Price et al., 2007). Secondly, and as previously mentioned, the account refers to the male inhabitants being 'seasoned to the place.' This suggests that there *was* a level of malaria immunity extant in the marshland populations of Essex. This may have taken the form of acquired immunity, or blood cell polymorphisms that conferred immunity to malaria infection. The presence of acquired immunity would suggest that transmission and relapse occurred frequently enough to sustain anti-*vivax* antibodies, while the presence of genetic immunities would indicate that the malaria parasites were sufficiently virulent to select for protective polymorphisms and had been present for sufficient time to drive the selection of these alleles. Although not usually associated with *vivax* malaria outside of tropical areas, new cases of populations exhibiting both glucose-6-phosphate dehydrogenase (G6PD) deficiency and the Duffy-negative blood group system in more temperate endemic *vivax* areas have recently been recorded (Sina, 2002; Louicharoen et al., 2009). This research suggests that long-term exposure to *vivax* malaria, even in temperate climates, can influence the development of protective haemoglobinopathies. It is not beyond the realms of possibility that either acquired or genetic immunities were present in past British populations and it is one of the aims of this study to investigate which type of immunity, if any, may have been active.

The next chapter will discuss the different methods by which malaria is most commonly diagnosed in clinical settings with the aim of assessing the suitability of these for use in detection of the disease in archaeological human remains. This will be accompanied by an exploration of the history of palaeopathological attempts at identifying the malaria either directly through, or indirectly, in skeletal material. The chapter will end with a brief synthesis of the evidence gathered and a proposed method for detecting *vivax* malaria in archaeological contexts.

CHAPTER 4: THE CLINICAL AND ARCHAEOLOGICAL DETECTION OF MALARIA

"It was then that he had the ague. He still remembers the terrible coldness, as he sat over the fire with his teeth chattering, and how his mother used to put rugs round him to try to get him warm. Soon afterwards he would be just as hot…it was severe enough to keep him away from school for six months…The disease…had been in existence for very many years"
(Wilson, 1938:1383).

The accurate diagnosis of malaria in clinical settings is the most important step in ensuring that appropriate treatments are administered to the patient in order to facilitate swift recovery from a potentially fatal disease (Chiodini and Moody, 1989). Clinical diagnosis usually relies on serological identification of malaria parasites, parasite products, or human immune-response proteins. Detection of the parasites themselves is considered diagnostically definitive, since malaria infection can be symptomatically similar to many other conditions (Gilles, 2002). Blood-slide light microscopy is therefore considered the 'gold standard' of diagnostic methodologies, although in certain instances misdiagnosis can occur (Hänscheid, 1999). It is due to these potentially catastrophic errors that biomolecular techniques of detecting clinical cases of malaria have gained in popularity in recent years, each offering alternative or supplementary strategies alongside microscopy. These are generally aimed at detecting the presence of the malaria parasite on a molecular level (e.g., *Plasmodium* DNA), products of the parasite released during infection, or products of the human immune response to infection. Some of these biomolecular techniques have been adapted to use with archaeological human remains, with varying degrees of success, and so it is therefore necessary to provide a brief overview of how each of the most-commonly adapted tests is used in its original, clinical setting. Traditional palaeopathological methods of identifying ancient disease and tracing malaria in human skeletal remains are then discussed, before exploring the use of biomolecular methodologies, including the potential advantages and drawbacks of each. Exploring and evaluating the most relevant clinical and palaeopathological methods are necessary steps prior to identifying the most appropriate analytical tests in the search for *Plasmodium vivax* malaria in archaeological human remains.

4.1: Clinical Detection

There are numerous methods employed in the effective detection and characterisation of malaria infection in clinical settings, ranging from basic light microscopy to highly specialised automated flow cytometry. This section outlines some of the most commonly used clinical detection methods that have the potential to be employed in palaeopathological or archaeological studies of malaria.

4.1.1: Light microscopy

Microscopic detection of *Plasmodium* parasites was first achieved in 1880 by Charles Laveran, who observed active parasites within the erythrocytes of a patient suffering from ‘ague' (White, 1996). Simple microscopic analysis with few modifications has since become the standard method for the detection and diagnosis of malaria in clinical settings (Chipeta et al., 2009). Light microscopy is a relatively fast, inexpensive technique that requires little in the way of specialised equipment. The method is used to directly visualise erythrocytic and exo-erythrocytic parasites within a blood-smear sample, and a competent microscopist should be able to detect very low levels of parasitaemia (approximately 5-50 parasites/μL of blood), as well as differentiate between *Plasmodium* species (Moody, 2002). Diagnosis of malaria based solely on symptomatic assessment can lead to mis-or over-diagnosis, and hence ineffective treatment of the disease, particularly in situations where malaria is rarely encountered, such as in cases of infected travellers returning to a non-malarious area (Chipeta et al., 2009). As has been previously mentioned, the early symptoms of malaria can be remarkably similar to other paroxysmal conditions, such as typhoid or influenza. In view of this, the World Health Organization (2010:36) suggests that confirmation of malaria by either microscopy or Rapid Diagnostic Test (RDT) should always be attempted, and that "treatment solely on the basis of clinical suspicion should only be considered when a parasitological diagnosis is not accessible."

Despite the high specificity of microscopic diagnosis, potential problems with sensitivity in terms of detection of low parasitaemia levels present a drawback, since such diagnosis is dependent on highly trained microscopists. Numerous studies (e.g., McKenzie et al., 2003; O’Meara et al., 2006; Zurovac et al., 2006; Ohrt et al., 2007) have demonstrated high inter-observer error, even among highly experienced technicians. Microscopic

diagnosis can be confounded by the fact that partially immune patients in endemic areas may exhibit no microscopically detectable parasites, particularly during periods of low disease transmission (Gilles 2002). The ability to detect low parasite loads is of crucial importance in an accurate diagnosis of active *vivax* malaria, which is often characterised by low-level parasitaemia (Anstey et al., 2009). Additional analytical techniques may therefore, in certain circumstances, be necessary to provide a definite diagnosis (Moody, 2002; Rosanas-Urgell et al., 2010).

Simple light microscopy cannot currently be employed in the direct detection of malaria in archaeological bone. However, recent research (Setzer et al., 2013) has provided a method of successfully visualizing preserved red blood cells (erythrocytes) in archaeological bone. The technique could also, theoretically, be employed to identify parasitised erythrocytes in infected individuals. Light microscopy could also potentially identify changes in bone structure caused by *P. vivax* infection, should such structures survive within the bone (see Wickramasinghe and Abdalla, 2000 for a clinical discussion of these changes in bone marrow). Light microscopy may well, therefore, play a useful initial role in the detection of ancient malaria, if the corresponding structures survive.

4.1.2: Rapid diagnostic tests (RDTs)

Rapid Diagnostic Tests (RDTs) are a relatively recent addition to the malariologists' armoury and offer a fast, portable, cost-effective method of detecting malaria that require no specialised laboratory equipment and minimal training to use (Drakeley and Reyburn, 2009). Many of the commercially available test kits have recently been approved by national governmental agencies (e.g., The Food and Drug Administration in the USA) for use in the rapid diagnosis of malaria and are now becoming widely available (UNICEF, 2007). The introduction of RDTs has been welcomed by clinicians in remote parts of Africa, where laboratory facilities and experienced microscopists are not always readily available. The introduction of more expensive malaria treatments (e.g., artemisinin-based combination therapies), combined with the common practice of presumptive treatment of malaria (even when the patient tests negative) have prompted the need for cheap, reliable tests (Drakeley and Reyburn, 2009) as an alternative to microscopy. Correspondingly, many sub-Saharan countries now routinely employ RDTs in malaria diagnosis (Maltha et al., 2014).

RDTs are immunochromatographic tests designed to detect parasite antigens in blood using specific monoclonal or polyclonal antibodies against the antigens. More recent versions are usually in the form of a cassette into which the blood sample and buffer solution are introduced. Target proteins are identified "by complexing them with capture antibody embedded in a line on a nitrocellulose strip through which a drop of blood is eluted by a few drops of buffer solution. The buffer solution contains a labelled antibody to complete a visualised complex and that also provides a second control line" (Drakeley and Reyburn, 2009:334). Many of the early tests were designed to detect histidine-rich protein (PfHRP) of *P. falciparum*, pan-malarial *Plasmodium* adolase (an enzyme expressed in the blood stage of infection), or *Plasmodium* lactate dehydrogenase (pLDH), an enzyme produced by all malaria parasites (with different isomers, or forms, for each species) and released from infected erythrocytes (Piper et al., 1999; Kakkilaya, 2003). Some of the newer RDTs are a 'combination' style which, using the same parasite products as before, can detect either *P. falciparum* or one of the other three *Plasmodium* species. Drawbacks of 'combination' RDTs are that they cannot specify parasite species beyond *P. falciparum* and cannot detect mixed malaria infections if *P. falciparum* is present. Non-combination PfHRP and pLDH tests are commonly used, the former being most extensively employed in Africa, where *P. falciparum* dominates, the latter in areas of high *P. vivax* and *P. ovale* prevalence (Drakeley and Reyburn 2009).

P. vivax-specific pLDH RDTs can detect a parasitaemia level of approximately 100-200 parasites/μL of blood, whist also 'capturing' pLDH antigens for up to three weeks post-infection (Kakkilaya, 2003). Although the detection limit is significantly higher than in microscopy, the ability to capture residual antigens gives RDTs an advantage in that the disease can be detected when, potentially, microscopically-observable blood-stage parasites no longer remain. This facility is particularly useful with diagnosing *vivax* malaria, since a positive RDT test can lead to treatment of latent hypnozoites. One problem with RDTs is cross-reactivity, which may result in a false positive test. PfHRP tests, for instance, are reported to have up to a 83% prevalence of cross-reactivity with antibodies to rheumatoid factor, although pLDH tests have a much lower reported prevalence of 3.3% (Bartoloni et al., 1998; Kakkilaya, 2003). The sensitivity of RDTs has also been called into question, particularly in cases of either very low or very high levels of parasitaemia, or in mixed

malaria infections (Mangold et al., 2005). Maltha et al. (2010), for instance, found that nearly 70% of *vivax*-specific RDTs tested using blood samples with high *P. falciparum* parasite density registered a false-positive result for *vivax* - a potentially life-threatening diagnostic mistake.

Although RDTs are proving to be an important and useful tool in malaria diagnosis, they should not be used to provide 'stand-alone' diagnoses. Gillet et al. (2010:1) suggest that "the reliance on RDTs as the primary or the single tool for the diagnosis of malaria….should be avoided."

4.1.3: Indirect/Immuno-fluorescence antibody test (IFAT)

The Indirect or Immuno-Fluorescence Antibody Test (IFAT) has been used in the diagnosis of malaria since the 1960s (e.g., Tobie and Coatney, 1961; Sodeman Jr. and Jeffery, 1966) and represents one of the first purely immunological approaches to malaria detection. Since then, IFATs have become increasingly important in malaria immunoserological diagnosis mainly due to their relative simplicity, replicability, low cost and high sensitivity (Doderer et al., 2007). The principle of the IFAT test is very similar to that of the more modern Enzyme Linked ImmunoSorbent Assay (ELISA) test (discussed later). The former can be seen as a precursor to ELISAs. In simple terms, IFATs use fluorescent microscopes to detect the reaction of fluorescent-conjugated antibodies with specific antigens, from which it is possible to determine the presence and quantity of specific antibodies in human blood (Collins and Skinner, 1972). As with the ELISA, IFAT methodologies in serology are generally based on the introduction of primary and secondary (the latter being fluorescent-conjugated) antibodies along with receptor blockers. These are interspersed with saline washes to remove non-bound proteins. Fluorescent reactions can then be visualised microscopically to determine the presence and quantity of the target antibody (Gotoh et al., 1995; Lee et al., 2011).

Despite the usefulness of IFATs in malaria immunoserology, ELISA may gradually replace this technique. ELISAs overcome the time-consuming IFAT process of multiple slide preparation, since their format is a pre-prepared 96 well plate containing antibody-coated surfaces. Attempts have been made to make the IFAT more suitable for processing high numbers of samples (e.g., Voller and O'Neill, 1971), but ELISAs offer a faster, more cost-

effective alternative in situations requiring the processing of a high volume of samples. However, due to their high sensitivity and established methodological standards, IFATs have proven particularly useful in detecting *P. vivax* in areas of low endemicity and in asymptomatic patients (Lee et al., 2011).

4.1.4: Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) technique is designed to achieve the rapid amplification of unique, targeted regions of the DNA of a selected organism, resulting in the exponential increase in the number of these regions. These can then easily been visualised using electrophoretic fluorescence techniques (Gilles 2002). Since its development in the early 1980s, the use of the PCR technique has become increasingly prevalent in biological, medical, genetic, and forensic laboratory research (Bartlett and Stirling, 2003). PCR is routinely used in the diagnosis of cancer, viral and bacterial infections, and parasitic infections such as Lyme disease and malaria. The technique is recognised as "the most sensitive and specific method to detect malaria parasites" (Hänscheid and Grobusch, 2002:395). PCR offers the ability to detect extremely low levels of parasitaemia (less than 5 parasites/μL of blood) and is up to 1000 times more sensitive than microscopy (Bottius et al., 1996; Moody, 2002). Additionally, precise species differentiation is now achievable, even in cases of mixed malaria infection (Genc et al., 2010). The technique has also successfully been used without blood, as demonstrated by Mharakurwa et al. (2006), who utilised PCR to detect *P. falciparum* DNA in human urine and saliva samples, thus negating the usual requirement of infected blood samples.

Given these impressive facts, it would seem prudent to screen all potential malaria sufferers using PCR. However, this is simply not a viable option in many areas. The process requires sophisticated, expensive laboratory equipment and highly trained personnel, which limits routine, clinical analyses to all but the most developed, affluent areas. Issues with sample contamination, extended processing and reporting times, and protein degradation during transportation further ensure that the routine diagnosis of malaria using PCR remains beyond the scope of most clinicians working in malarious areas (Hänscheid and Grobusch, 2002).

4.1.5: Radioimmunoassay (RIA)

This technique was first described by Yalow and Berson (1960). RIAs utilise radioactively-marked antibodies or antigens. These are mixed with a target blood sample which is assumed to contain non-radioactive versions of the same antibodies or antigens. Should these non-radioactive proteins be present, they displace their radioactive counterparts, thus freeing the radioactive marker. In the case of malaria, for instance, a measurement of the amount of freed marker using isotopic detectors demonstrates the presence of infection and level of parasitaemia. RIAs have proven to be highly sensitive in the detection of *P. falciparum*, especially in cases of low parasitaemia (Gilles 2002). However, their sensitivity in detecting antigens or antibodies associated with *P. vivax* seems to be less reliable (e.g., Avraham et al., 1983).

Much of the research utilising RIAs in malaria diagnosis took place in the 1980s (e.g., Mackey et al., 1980; Avraham et al., 1983; Avidor et al., 1987), yet despite the inherent radioactivity-related risks and requirement for special licensing and equipment, biomolecular research utilizing RIA techniques continues due to its relatively low cost and high sensitivity and specificity. However, the technique has been very much been supplanted by enzyme-linked immunoabsorbent assay (ELISA). This is almost certainly due to the prohibitive nature of utilizing radioisotopes, which severely limits the use of RIAs in field settings (Gilles 2002).

4.1.6: Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA, also referred to as Enzyme Immunoassay, or EIA) was developed in the 1970s as a safer alternative to the radioimmunoassay and a less cumbersome, time-consuming alternative to the IFAT (Doderer et al., 2007). In ELISA the radioactive signals are replaced by colourimetric or chromogenic indicators, thus eliminating the risk issues associated with RIA. ELISAs are commonly employed in medical diagnostic and industrial quality control applications to measure the presence and quantity of a particular antigen or antibody (Jordan, 2005).

As with most immunological tests for malaria, ELISAs were originally developed to detect antibodies to *P. falciparum* (Taylor and Voller, 1993) and suffered from poor sensitivity to other *Plasmodium* species (Doderer et al., 2007). Pan-malarial and species-

specific ELISAs are more recent commercially available additions. Many of these more recent tests are still undergoing evaluative field trials to assess their quality in terms of specificity and sensitivity, particularly in comparison to other immunological diagnostic malaria tests, such as the IFAT (e.g., Doderer et al., 2007; She et al., 2007; Muerhoff et al., 2010; Nam et al., 2010). The use of recombinant antigens, such as circumsporozoite (CSP) and merozoite surface (MSP) proteins, and ELISAs that can detect both immunoglobulin IgG and IgM antibodies, have so far proven particularly advantageous in the detection of *P. vivax* (e.g., Rodrigues et al., 2003; Doderer et al., 2007; Park et al., 2008).

The test is generally performed using 96-well plates, but can take a number of methodological forms. In malaria diagnosis, the most common forms are ‘direct’, ‘indirect' and ‘sandwich' ELISAs. The indirect form detects the presence of specific antigens or antibodies, whereas the sandwich form is used to both detect and quantify the target. The aim of both types is to observe a colour change within the wells that contain reactive, bound antigens/antibodies. A measurement of colour density and intensity reveals the presence and intensity of the reaction. Put simply, the direct method involves the introduction of samples containing antibodies conjoined with enzymes into the plate wells, each of which contain pre-prepared surfaces with affixed antigens: "If the antigen is present, the antibody-enzyme complex will bind to it, and the enzyme component of the antibody-enzyme complex will catalyze the reaction generating the colored product. Thus, the presence of the colored product indicates the presence of the antigen" (Berg et al., 2002:102). In indirect ELISAs the blood samples containing antibodies are introduced into the wells, followed by secondary enzyme-linked antibodies against human antibodies (e.g., enzyme-linked mouse-raised anti-human antibodies). Colorimetric substrate is then added. Colour change associated with a bonding reaction between the introduced secondary enzyme-linked antibodies and human antibodies can then be recorded (Berg et al., 2002; Brown and Brown, 2011). The sandwich ELISA is very similar to the indirect ELISA, but utilises a different secondary enzyme-linked antibody to the indirect test. Sandwich ELISAs are quite commonly used in malaria diagnosis since they provide the ability to directly measure both the presence and quantity of antigens (e.g., Bidwell and Voller, 1981; Chung et al., 2001; Lim et al., 2002).

A further type of ELISA is the competitive ELISA (C-ELISA), which is a highly sensitive assay designed to detect either very small antigens, or antigens present in low

concentrations. In C-ELISAs the sample under analysis is usually mixed with a known quantity of antigen which has been conjugated with an enzyme. This blend is then applied to a protein-binding plate coated with an antibody: "because only the conjugated form of the antigen allows for the colorimetric reaction to develop in the presence of the substrate, the maximal reading occurs when there is no antigen in the sample. The more antigen that is present in the sample, the lower the resulting [colorimetric] reading…the readout is inversely associated with the amount of antigen" (Jordan, 2005:423). C-ELISAs are not commonly used due to a comparatively higher workload and expertise, but they offer an important option when attempting to detect low concentrations of antigen (Jordan, 2005).

Advantages of ELISAs over other standard immunoassay tests, such as IFATs and RIAs, are generally cited to be in terms of higher sensitivity, comparative speed and ease of use, and the ability to test nearly 100 samples per plate. This latter point makes ELISAs especially attractive in situations that require the processing of a high volume of samples, hence their increasingly common use in industrial and medical applications (She et al., 2007). The main disadvantages of ELISAs are the relatively high cost of the constituent materials and the requirement for both laboratory facilities and well-trained personnel (Vidal and Catapani, 2005). These factors may limit the use of ELISAs in malaria diagnosis, particularly in more remote and economically deprived areas.

4.1.7: Western blot test

The Western blot (or immunoblot) test is a well-established and widely used type of immunological test aimed at identifying specific proteins. This is usually accomplished following separation by molecular weight, most commonly by polyacrylamide gel electrophoresis, or PAGE (Burnette, 1981). In PAGE, application of an electrical current induces the proteins to move through the gel, with the smallest moving fastest (thus separating by molecular weight). Once proteins are separated, the gel containing the proteins is transferred to a ‘blot', or membrane, which is usually made of nitrocellulose or polyvinylidene fluoride (PVDF). A separate stage, known isoelectric focusing, is sometimes employed prior to blotting. This is a technique used to separate proteins by electric charge, rather than by size (e.g., Polpanich et al., 2007). When transfer is complete, the membrane can be probed with an antibody to identify the presence of specific protein antigens (Towbin

et al., 1979; Abbas et al., 1991). The use of immunoblotting in clinical diagnosis of malaria is seen less frequently than other types of tests, such as the ELISA or IFA; this is possibly due to sample size limitations. However, the method is certainly viable (e.g., Makler and Hinrichs, 1993) and offers a reasonably swift and cheap alternative to other methodologies when bulk sampling is not required. Western blot tests in their various forms have been employed in the detection of ancient biomolecules in archaeological human remains (see section 4.4.4).

4.1.8: Malaria pigment (haemozoin) detection: automated haematology

Automated haematology analysers have been utilised in the diagnosis of malaria since 1953 and have since proven useful tools in the early diagnosis of the disease (Campuzano-Zuluaga et al., 2010). Analysers were originally designed to facilitate complete blood counts (CBCs), a method of blood screening that is routinely used in medical and clinical settings alongside such methods as flow cytometry. Occasional attempts utilising CBCs were made to diagnose malaria through observing alterations in blood cell populations. However, due to the inability of the analysers to distinguish between malaria- and non-malaria-induced changes to blood cell count, this application was seldom used (Campuzano-Zuluaga et al., 2010). Recent advances in automated haematology have produced analysers and flow cytometers (e.g., Frita et al., 2011) which are capable of detecting a signature unique to infection with haematophagous organisms, including malaria: haemozoin.

Haemozoin (Hz), also known as malaria pigment, is a brown crystalline waste product excreted by all *Plasmodium* spp. parasites during the ingestion of haemoglobin in the erythrocytic stage of infection. Hz was first identified in 1847, although association of the presence of pigment with malaria infection was not achieved until the late 19th century, when Charles Laveran attributed it to *Plasmodium* parasites. In 1897 Ronald Ross observed the pigment within the gut of an *Anopheline* mosquito, and was thus the first researcher to identify mosquitoes as the vector for malaria infection (Sullivan, 2002). Since the 1950s the presence of Hz was usually detected through routine CBCs using automated analysers which are capable of identifying white blood cells (leucocytes) containing phagocytised pigment, although, as already mentioned, these machines were not designed to specifically diagnose

malaria (Hänscheid et al., 2001; Campuzano-Zuluaga et al., 2010). The most recent and advanced analysers utilise lasers to more accurately distinguish not only the presence of Hz, but also the *Plasmodium* species responsible (e.g., Noland et al., 2003; Nyunt et al., 2005). Despite evaluations of specificity and sensitivity yielding some conflicting results (Suh et al., 2003; Rathod et al., 2009), the analyses have so far proven to have high accuracy, particularly in cases of low parasitemia. The relatively high expense of equipment and laboratory expertise required for this type of analysis precludes its routine use in field diagnostics, but the technique has been particularly useful in diagnosing malaria in cases of infected travellers returning from malaria-endemic areas (Campuzano-Zuluaga et al., 2010).

The techniques described above represent the most commonly employed clinical methods of detecting and diagnosing malaria. They also have been, or have the potential to be, utilised in the search for the disease in past populations.

4.2: Archaeological detection

4.2.1: Palaeopathology and macroscopic analysis

Palaeopathology, the study of ancient disease, has a long and sometimes chequered history, fascinating and inspiring researchers since the first mid-18th century analyses of prehistoric faunal pathologies (Roberts and Manchester, 2005). Since then, the discipline has evolved through 19th century descriptive, rather than interpretive, analyses (Steinbock, 1976), through 20th century large-scale population-based epidemiological and statistical analyses, to 21st century biomolecular-based applications. On the smallest scale, palaeopathological research can illuminate the health status of a single individual at the time of death, potentially providing information on nutrition, social status, and in rare instances, activity and behaviour. On a much larger scale such analyses can be vital in developing our understanding of pathogen antiquity, co-evolution with hosts, and the cultural and physical responses to the presence of disease within populations (Mitchell, 2003; Setzer, 2010).

Evidence for disease in past societies can be gathered through inspection of historical documentation, artwork, and artefacts, although interpretational issues may limit the usefulness of these sources (Mitchell, 2011). Coprolites and preserved soft tissues can also provide direct evidence for disease and pathogenic processes (e.g., Aufderheide et al., 2005).

However, since these rarely survive in archaeological contexts, the medium of choice for study is usually skeletal remains, which can preserve remarkably well for extended periods of time in the burial environment. It is understandable, therefore, why skeletal remains have commanded the majority of palaeopathological research.

Chronic diseases can leave diagnostic markers in skeletal remains, (Roberts and Manchester, 2005), whereas highly virulent pathogens, such as plague, often destroy the host before reactionary bone remodelling occurs (Ortner, 2007). Macroscopic identification of such conditions is therefore rendered extremely difficult, if not impossible. Consequently, palaeopathological analyses of skeletal populations have traditionally focused on macroscopically identifiable chronic diseases (e.g., tuberculosis) in individuals that have survived the acute disease phase and have developed responsive bone changes in (Steinbock, 1976; Ortner, 2003).

Working from a thorough knowledge of the morphological appearance of the normal human skeleton, a main task for the palaeopathologist is to clearly describe, record, and diagnose pathological lesions (Grauer, 2007) using standardised (as far as possible), unambiguous terminology based on clinical criteria (Roberts and Manchester, 2005). Macroscopic analysis of skeletal remains "serves as a starting point for virtually all methodological approaches" in palaeopathology, often forming a necessary prerequisite to supplementary biomolecular research (Grauer 2007:57). However, macroscopic diagnosis of disease in human remains presents many difficulties. Firstly, as already mentioned, acute diseases may leave no skeletal markers and therefore remain occult and undetected. The majority of observable disease processes in skeletal remains are the result of long-term chronic conditions (Roberts and Manchester, 2005). Secondly, diagnosis is usually dependent on good skeletal preservation, which is itself heavily dependent on multiple factors. These include but are not limited to: age of the buried individual (juvenile and infant bones often preserve relatively poorly), the effects of an individual's health upon the skeleton, burial customs, taphonomic and diagenetic processes in the burial environment, grave disturbance, care taken during excavation, and post-excavation processing. These factors can all serve to destroy evidence of disease processes (Mays, 2010). Thirdly, bone responds to disease either through destruction or proliferation of bone, or both at once. Consequently, different pathogens may induce similar, if not identical, osseous responses

(Grauer, 2007). Fourthly, many diseases have very complex aetiologies which are poorly understood. These include some of the most commonly observed and recognisable pathologies, such as osteoarthritis and *cribra orbitalia* (Waldron, 2009; Walker et al., 2009). Hence, although these diseases may be frequently recorded, extrapolations concerning their exact causes and subsequent impacts on health are ultimately confounded. Linked to this is the problem of applying clinical diagnostic criteria to dry bone for diseases where the former are not necessarily applicable to the latter (e.g., in diagnosing osteoarthritis) (Waldron, 2009).

Finally, extrapolation of palaeopathological data to form population-wide interpretations of disease prevalence can be confounded by the osteological paradox (Wood et al., 1992). Put simply, this states that a cemetery population is not representative of the actual living population. For palaeopathological analyses, this may present the following problem: the presence of pathological lesions demonstrate that individuals survived their condition long enough to develop a bony response, whereas those displaying no observable pathologies may have died during the acute phase of a disease (Roberts and Manchester, 2005; Pinhasi and Bourbou, 2007). This may lead to the assumption that the latter skeletons represent the 'healthier' individuals in a population, whereas they may actually represent the frailer portion of a population who succumbed, rather than adapted, to disease.

Good skeletal preservation is particularly important, since patterning of bone changes throughout the skeleton may offer valuable diagnostic clues and aid in differential diagnosis (Roberts, 2009).Wherever possible, differential diagnosis should attempted: "by a process of gradual elimination on the basis of known patterning in modern clinical circumstances a most likely diagnosis may be made" (Roberts and Manchester, 2005:9). Differential diagnosis also benefits from accurate age and sex estimation of skeletal individuals, tasks that are not always straightforward (Grauer, 2007). For instance, the expression of certain diseases in bone may be age-dependent (e.g., β thalassaemia intermedia) (Lewis, 2010), or more commonly observed in a particular sex (e.g., rheumatoid arthritis) (Alamanos and Drosos, 2005). Despite the difficulties highlighted above, macroscopic methods remain some of the most commonly employed and useful diagnostic tools in palaeopathological analysis.

4.2.2: The palaeopathology of *Plasmodium vivax* malaria

There has been little traditional palaeopathological research on *vivax* malaria. This may be surprising, given the fact that the disease undoubtedly affected so many people in the past. The reason for this dearth of research is, however, simple: although *vivax* malaria is a chronic condition potentially lasting many years, the disease elicits no direct osseous response. There are, therefore, no macroscopically observable skeletal changes that can be unequivocally attributed to malaria infection. The most common sequela of *vivax* malaria is haemolytic anaemia, which is likely caused by the preference of the parasite for reticulocytes and the repeated haemolysis of these young erythrocytes. In endemic areas, re-infection and relapse may result in continual parasitisation and ongoing chronic anaemia (Collins et al., 2003). It has also been suggested that *vivax* malaria reduces the effectiveness of erythropoiesis and influences phagocytosis of non-parasitised erythrocytes (Wickramasinghe and Abdalla, 2000; Khan et al., 2010). These factors may help to explain the increased prevalence of anaemia associated with *vivax* malaria in comparison to *falciparum* malaria. The clinical focus tends to be on the haematological consequences of anaemia, rather than any effects the condition may have on the skeleton. Radiographic analysis has, however, suggested that certain skeletal markers *may* be associated with anaemia, and therefore may be used as a possible proxy indicator for malaria infection (Setzer, 2010). Two such skeletal markers that are commonly associated, rightly or wrongly, with anaemia in palaeopathological analyses are *cribra orbitalia* and porotic hyperostosis.

4.2.2(i): *Cribra orbitalia* and porotic hyperostosis

Cribra orbitalia (CO) and porotic hyperostosis (PH) are two of the most distinctly recognisable pathological conditions in archaeological human skeletal remains and have consequently received much attention from palaeopathologists (Stuart-Macadam, 1991). Due to the plasticity and increased presence of haematopoietic bone within the non-adult skeleton, both conditions are more frequently observed in juveniles than in adults (Ortner, 2003). CO is commonly encountered throughout Old and New World archaeological populations (Sullivan, 2005). It is characterised by pitting and porosity in the orbital roofs and usually presents bilaterally (Figure 4.1). PH is also encountered worldwide, although is much less frequently recorded than CO in past British populations (Walker et al., 2009;

Lewis, 2010). PH has been commonly recorded in Mediterranean and North American archaeological human remains (e.g., Angel, 1966; El-Najjar et al., 1976; Palkovich, 1987; Keenleyside and Panayotova, 2006) and is characterised by similar morphological changes to CO, but presenting as "circumscribed areas of pitting and porosity on the external surface of the cranial vault" (Walker et al., 2009:109) (Figure 4.2). It is often, but not exclusively, associated with expansion of the cranial diploë, which results in a characteristic 'hair-on-end' appearance when viewed radiographically (Walker et al., 2009).

Figure 4.1: Bilateral *cribra orbitalia* (photograph by Jeff Veitch, Durham University).

Figure 4.2: Porotic hyperostosis evident on parietal and frontal bones (adapted from Lagia et al., 2007).

Cribra orbitalia and PH generally receive little medical attention and are rarely reported in the clinical literature. Palaeopathologists have, therefore, been forced to deduce their aetiologies from studying skeletal material, which is hardly an ideal situation, since few researchers are medically trained. Despite familiarity with the macroscopically and radiographically observable characteristics of both conditions, palaeopathologists have yet to come to a consensus concerning the exact aetiological factors influencing their development (e.g., Walker et al., 2009; Oxenham and Cavill, 2010). The relationship (if any) between the conditions also remains unclear, although it is generally agreed that CO has a more complex aetiology than PH (Ortner, 2003). Undeterred by uncertainty concerning the factors influencing their development, researchers have attributed the conditions to numerous causes. For instance, chronic infection by parasites, especially *Plasmodium* species, has been considered an important factor in the development of CO and PH (e.g., Bathurst, 2005). However, before considering any possible influences of malaria infection on the development of CO and PH, it is useful to understand the history of research concerning the palaeopathological analysis of these important conditions.

Early 20th century researchers were generally content with describing the conditions without in-depth consideration of their aetiologies. CO was first described in the late 19th

century (Steinbock, 1976), but the condition was not considered to be pathological. PH was initially described by Hrdlička (1914) as 'symmetrical osteoporosis.' It was not until 1966 that the term 'porotic hyperostosis' was first coined by Angel in his research paper on prehistoric eastern Mediterranean populations, in which he linked the condition with skeletal manifestations of thalassaemia and sickle-cell anaemia related to chronic malaria infection.

During the 1960s and 1970s a dominant explanatory paradigm for the presence of CO and PH in archaeological populations emerged: iron-deficiency anaemia (Walker et al., 2009). Many of the contemporary, conflicting theories concerning the causes of iron-deficiency anaemia were based on North American prehistoric and historic samples. Moseley (1965), for example, rejected earlier suppositions that PH was caused by malaria-related thalassaemia, citing the lack of residual thalassaemic alleles in modern North American populations. Instead, Moseley attributed the high prevalence of prehistoric PH to iron-deficiency anaemia. This was followed with research by El-Najjar et al. (1976) which suggested a possible link between chronic iron-deficiency anaemia (characterised by a high prevalence of PH) and the heavily maize-based diets of prehistoric and historic Anasazi populations.

Cybulski (1977) was one of the first researchers to suggest a multi-factorial aetiology for anaemia, opposing a single, diet-based cause. His study of historic, sedentary British Columbian populations demonstrated a high prevalence of CO, even though the Northwest coast of North America offers one of the most diverse environments in terms of marine and terrestrial resources. Given the varied resources and abundance of dietary iron available in the biosphere, Cybulski theorised that the aetiology of CO was both inherited and acquired, the latter being caused by parasite (or helminth) infection, the former possibly due to hereditary spherocytosis, a haemolytic anaemia characterised by spheration and subsequent haemolysis of red blood cells. Either condition would result in chronic anaemia, particularly in individuals with high iron requirements, such as pregnant and breast-feeding women, and growing children.

The potential importance of helminth and pathogenic infection in the development of anaemia, and hence CO and PH, was later highlighted by Walker (1986), Kent (1986), and Bathurst (2005). Walker's research suggested that marine-borne parasites and poor sanitation, rather than reduced dietary intake of iron, were the likely culprits for the high

prevalence of CO and PH among historic California Channel Island-dwelling Native Americans. Kent's (1986) research in the southwest of the USA suggested that anaemia associated with high pathogen loads derived from unsanitary living conditions, while Bathurst's discovery of the human intestinal parasites *Diphyllobothruim* spp. (fish tapeworm) and *Ascaris lumbridoices* (round worm) in Canadian Pacific coastal shell middens "supports the hypothesis that parasite burden may be a contributing factor to the incidence of anemia" (Bathurst, 2005:121).

Studies conducted during the last decades of the 20th century show that researchers began to move away from single factor and reductionist theories concerning the aetiologies of anaemia, CO and PH, and towards more varied approaches. Stuart-Macadam (1992 a; b) for instance, suggested that diet may only play a minor role in the development of iron-deficiency anaemia, and that the condition resulting in PH may actually represent an adaptive strategy within populations to protect against parasitic and pathogenic infection, since low levels of endogenous iron may inhibit the growth of invading organisms. It is via this mechanism that Murray et al. had earlier discovered that dietary-derived iron deficiency in Somali nomads prevented "the more serious consequences of potentially fatal infections with malaria, tuberculosis, and brucellosis to which the nomads are constantly exposed" (Murray et al., 1978:1115). Anaemia as a 'healthy' adaptation proved a relatively popular theory, supported by researchers such as Wright and Chew (1998), although some (e.g., Holland and O'Brien, 1997) continued to champion a nutritional-based explanation. The 1990s also saw an increasing appreciation of the potential effects of inherited factors on the development of anaemia, CO and PH, although much research (e.g., Hershkovitz et al., 1991; Grauer, 1993; Mittler and Van Gerven, 1994; Tayles, 1996) remained split between either genetic inheritance or iron deficiency as causative factors, dependent upon geographic location of study.

Iron deficiency as the primary cause of CO and PH was re-evaluated in the late 1990s by Hershkovitz et al. (1997), whose research on skeletal manifestations of sickle cell anaemia conflicted with the commonly-held assumption that iron deficiency stimulates marrow expansion in order to create more haematopoietic bone, thereby increasing red blood cell output. They argued that such action is counter-productive, since marrow expansion would demand increased consumption of available nutrients, thus unnecessarily

stressing an already nutrient-deprived system. This viewpoint was later supported by histological research (Wapler et al., 2004), which demonstrated that less than half of the examined cases of CO exhibited the marrow hypertrophy traditionally attributed to iron deficiency. Ortner and Ericksen (1997) posited that CO-type manifestations in juveniles assumed to be caused by iron deficiency may actually be the result of sub-periosteal haemorrhaging associated with scurvy. They also cautioned that CO and PH may well have different aetiologies, even when both are observed in one individual. Peckmann (2003) even suggested a possible correlation between smallpox and a high prevalence of PH in 19th century South African communities.

The first decade of the 21st century saw the emergence of a new explanatory model for CO and PH. Megaloblastic anaemia was first suggested by Fairgrieve and Molto (2000), who attributed the high prevalence of CO in archaeological Egyptian populations to the condition. As with iron-deficiency anaemia, megaloblastic anaemia is an acquired condition. However, the latter usually results from deficiencies in folic acid and vitamin B-12, and can develop due to dietary insufficiency and/or parasite infection (Sullivan, 2005). Despite this new development, some researchers continued to cite iron-deficiency anaemia as the primary cause of CO and PH (e.g., Keita, 2003; Papathanasiou, 2005; Obertová and Thurzo, 2008). Walker et al.'s (2009) response to the continued uncertainty concerning the aetiological factors behind the development of CO and PH was to prepare a synthesis of the clinical and palaeopathological literature related to the conditions. The synthesis led them to agree with research suggesting that marrow hypertrophy cannot result from iron deficiency; rather, they cite megaloblastic and hereditary anaemias as more likely responsible for the development of PH and CO. The former, they suggest, may be influenced by haemolysis, while the latter can also being influenced by vitamin C deficiency.

Although the long-standing iron-deficiency anaemia hypothesis has suffered a setback, some researchers (e.g., Oxenham and Cavill, 2010) continue to champion the theory. This is the state of modern thinking on the aetiologies of PH and CO: aetiological theories have almost come full-circle before maturation into a more integrated approach, from single causative factors such as Angel's malaria-induced inherited haemolytic anaemias and dietary-driven iron-deficiency anaemia, through iron deficiency as an adaptive strategy and megaloblastic anaemia. As with many palaeopathologically observable conditions, CO

and PH obviously have complex clinical aetiologies that are still not yet fully understood. A full appreciation of the complex factors contributing to their development can only be achieved when palaeopathologists move beyond single causative factors to analyse multiple lines of evidence including clinical, dietary, environmental, and socio-cultural data. This approach is now being taken by palaeopathologists more and more frequently.

In terms of researching malaria in past populations, the aetiological association between CO, PH, and anaemia is less important. Although *vivax* malaria is a certain cause of clinical anaemia, a link between these skeletal markers and anaemia is currently tenuous. A more conservative stance would be to view CO and PH alongside other non-specific skeletal markers of stress, such as enamel hypoplasia and Harris lines (conditions discussed below). Facchini et al. (2004), for instance, demonstrated a positive correlation between enamel hypoplasia and porotic hyperostosis in Roman Ravenna, attributing the high enamel hypoplasia prevalence to nutritional stresses and parasitic infection, both of which would have been influenced by the surrounding swampy environment. The prevalence and distribution of non-specific stress markers can provide valuable information concerning health status on a population level (e.g., Facchini et al. 2004; Buckley, 2006; Gowland and Garnsey, 2010). Research on Anglo-Saxon Fen/marshland-associated populations (Gowland and Western, 2012), for instance, suggests that populations living in the type of environment conducive to malaria infection exhibited significantly higher prevalence rates of stress markers such as *cribra orbitalia*.

4.2.4: Non-specific skeletal stress markers

Two of the most common palaeopathologically recorded non-specific stress markers in skeletal material are Harris lines and enamel hypoplasia. First clinically characterised in 1926, Harris lines "are transverse sclerotic layers in the metaphyseal parts of long bones, reflecting the episodes of delayed or arrested development of the longitudinal growth of the bone" (Piontek et al., 2001:33). The formation of Harris lines has been clinically correlated with different stresses, including dietary deficiencies (e.g., Park, 1964) and general illness (e.g., Acheson, 1959). This correlation has inspired frequent palaeopathological study of Harris lines as potential indicators of episodic, non-specific periods of stress during bone growth in archaeological populations (Mafart, 2009). Their usefulness in this aspect is,

however, debated, and for the following reasons Harris lines will not be analysed in this study: firstly, although clinical research suggests a strong association between stresses and the formation of Harris lines, their predictability is low (Gindhart, 1969). For instance, they have been observed in ‘healthy' children, and not consistently in individuals who have suffered periods of high stress in childhood (Mafart, 2009). Secondly, Harris lines are notoriously difficult to observe in archaeological bone. Radiographic analysis is required for more accurate recording - equipment that is not always available to palaeopathologists. However, since Harris lines can also be difficult to see on radiographs, analyses may suffer from high inter- and intra-observer error (Grolleau-Raoux et al., 1997). Thirdly, since living bone continually remodels, Harris lines formed in childhood may be later remodelled and resorbed (Mays, 1995), and therefore undetectable. Finally, Harris line presence rarely correlates well with other, potentially more reliable non-specific skeletal stress markers, such as enamel hypoplasia (e.g., McHenry and Schulz, 1976; Alfonso et al., 2005; Mafart, 2009).

Enamel hypoplasia (EH) has long been associated with non-specific episodic stresses during the early years of life (e.g., Sarnat and Schour, 1941). These stresses act to "depress the activity of the ameloblasts and to result in the production of a thin and poorly calcified enamel matrix, with the formation of linearly distributed pits or grooves of defective enamel" (Ogden, 2008:284). As with Harris lines, the exact aetiology of EH is uncertain. It has, however, been clinically linked to over one hundred conditions, including malnutrition, anaemia, parasitic infection, premature birth, low birthweight, and general illness in neonates and children (Duray, 1996; Lewis and Roberts, 1997; Palubeckaitė et al., 2002). Although minor enamel defects are frequently observed in outwardly ‘healthy' children (Ogden, 2008), it is generally accepted that the presence of EH is a reliable indicator of a stress-related interruption of amelogenesis (enamel formation) (King et al., 2005; Starling and Stock, 2007).

Enamel hypoplasia has attracted much palaeopathological attention since "the nature of enamel means that defects cannot be remodelled and, therefore, they represent a permanent chronological record of a stressful incident during the first 7 years of life" (Lewis and Roberts, 1997:581). Working on this basis, researchers have attempted to quantify instances of hypoplasia, calculate age of occurrence, and determine the duration of insult of

each defect in ancient populations (e.g., McHenry and Schulz, 1976; Alfonso et al., 2005). On the surface, this sounds like the ‘perfect' pathology to study in ancient skeletal material - a medium that does not remodel (like bone) and usually preserves superbly well in the burial environment. There are, however, issues with EH analysis. For instance, many palaeopathological studies of EH rely on the assumption that all stages of enamel formation (e.g., secretion and later maturation stages) occur at set rates, which is not necessarily the case. Suga (1989:194), for instance, states that "the progressive mineralisation pattern is completely different between the matrix formation and maturation stages." Thus variation in the rate of amelogenesis calls into question the results of any research that does not recognise this. For the purpose of this study, it will only be necessary to record a basic presence/absence of EH. It is not important *when* stresses occur in the early years of an individual, but rather that EH is a reliable indicator of said stresses occurring *at all*.

Although malaria cannot be directly attributed to EH formation, there can be little doubt that the infection would influence its development, particularly when comorbid with other conditions. Skinner and Hopwood (2004), for example, suggest that the development of EH in great apes is influenced by periodic episodes of malaria interacting with malnutrition. Interestingly, one of the malaria parasites recorded in great ape infections, *P. silvaticum*, is a relapsing type of malaria, akin to *P. vivax* in humans. The synergistic interaction of malnutrition and infectious disease has been noted as influencing EH development in humans (Duray, 1996). The immunosuppressive effects of malaria only serve to compound biological stresses by increasing the chances of co-infection with other diseases (Phillips, 1983). Unfortunately, recent research has suggested a lack of correlation between human malaria and EH development (Gowland and Garnsey, 2010; Gowland and Western, 2012).

4.2.5: Inherited conditions

Inherited conditions represent one of a set of complex factors that dictate the outcome of the human host response to malarial infection, alongside host adaptive immunity, health status, comorbidity, and parasite virulence (Chotivanich et al., 2002). High frequencies of specific genetic mutations that confer a degree of protection against *Plasmodium* infection within a population suggest a long history of endemic disease

exposure and the "very considerable force of natural selection applied by malaria" (Sallares and Gomzi, 2001:211). Inherited mutations (also referred to as polymorphisms) that bestow protection against malaria infection can take a number of forms. Examples of such mutations are red blood cell (RBC) membrane abnormalities, such as a lack of the Duffy antigen on the RBC membranes in African populations (protecting against *P. vivax* infection) (Duffy and Fried, 2006), genetic changes in RBC shape such as in ovalocytosis (Cattani et al., 1987), enzyme deficiencies, such as glucose-6-phosphate dehydrogenase (G6PD) deficiency, and haemoglobinopathies, such as the thalassaemias and sickle cell anaemia (Allison, 2002; Williams, 2006).

These inherited conditions all share a common trait in that they have, over an extended time period, become ‘balanced' polymorphisms, "in which the homozygote hematologic disadvantage is balanced by the heterozygote advantage of protection from malaria" (Chotivanich et al., 2002:1172). Haemoglobinopathies, such as thalassaemia, generally have higher frequencies in areas of the world also affected by endemic malaria, since they confer a degree of protection and, therefore, a selective advantage over non-carriers (López et al., 2010). Thalassaemia is a good example of a balanced polymorphism, in that the homozygous disadvantage is balanced by a heterozygous resistance to malaria. Homozygous (thalassaemia major) sufferers usually die before reproducing, and non-carriers may die of malaria infection. This results in an increased frequency of surviving heterozygotes who pass on their increased resistance to malaria.

The detection of inherited conditions associated with malarial infection in archaeological human remains is usually based on either identifying macro- and microscopic osseous changes, or directly testing aDNA for genetic mutations. Of course, neither of these techniques can definitively prove that an individual suffered from malaria, but only that their ancestors most likely had extended contact with endemic malaria (Sallares and Gomzi, 2001). As with *cribra orbitalia*, porotic hyperostosis, and enamel hypoplasia, the detection of inherited polymorphisms offers a possible indirect method of tracing malaria in the archaeological record.

4.2.5.1 The thalassaemias

One of the most commonly observed polymorphisms in modern populations is

thalassaemia, the general term for a group of congenital haemolytic anaemias with high prevalence rates in southern and eastern Mediterranean countries, India, China, and Southeast Asia (Weatherall and Clegg, 1972) - areas all with a long history of malaria endemicity. Sufferers in other parts of the world can usually trace their ancestry to one of these areas. The two main types of thalassaemia result from mutation affecting specific gene code sites of either the α or β polypeptide chain of the haemoglobin molecule. Put simply, both conditions reduce the quantity of haemoglobin within RBCs, subsequently decreasing RBC capacity for oxygen transportation (Aufderheide and Rodriguez-Martin, 1998; Galanello and Cao, 2011). Low haemoglobin levels causes an increase in abnormal RBC production and subsequent lysis of the cells, hence promoting 'haemolytic' anaemia. Subsequent expansion of the medullary cavities (marrow hypertrophy) in response to increased demand for RBCs is commonly observed in sufferers (Olivieri, 1999; Lewis, 2010). Genotypic expression of α or β thalassaemia is classed as 'major' or 'minor', dependent upon homozygosity or heterozygosity (Olivieri, 1999; Perisano et al., 2012). A third class of β thalassaemia, known as β thalassaemia intermedia, can also occur.

Both α and β thalassaemia major cause severe symptoms which often lead to premature death (often in infancy) of the sufferer, whereas minor expressions are much less severe, being either asymptomatic or causing very mild clinical symptoms, with no associated skeletal changes. Patients with β thalassaemia intermedia suffer mild symptoms, but can survive without the intensive medical treatments required by homozygotes (Parano et al., 1999). Skeletal changes associated with intermedia patients often worsen with advancing age (Taher et al., 2006), depending upon the severity of each case. Each genotypic expression of the thalassaemias is thought to confer some protection against malaria infection, although the exact underlying mechanisms of protection remain poorly understood (Duffy and Fried, 2006; Galanello and Cao, 2011). For instance, α thalassaemia seems to confer significant protection against *P. falciparum* malaria, yet increases susceptibility to *P. vivax* malaria infection in young children and babies (Allen et al., 1997). The reason for this is unclear, partly due to a poor understanding of the α thalassaemia phenotype and its interaction with malaria (Rees et al., 1998), but Williams et al. (1997) suggest that early infection with mild *vivax* malaria may offer cross-species protection against *P. falciparum* infection in later life. It has been suggested that in thalassaemics

infected with *P. falciparum*, parasitised blood cells are more effectively targeted by the immune response, thus limiting the impact of the blood-stage infection and the subsequent severity of the disease (Williams et al., 2002; Krause et al., 2012) .

The correlation of endemic malaria with a high prevalence of the thalassaemias has been used in palaeopathological research to infer either the presence of malaria in antiquity, or the migration of sufferers from endemic areas, through the observation of skeletal changes associated with thalassaemia. Homozygous expression of α and β thalassaemia (major) and β thalassaemia intermedia produce osseous changes regardless of genotype (α or β), making differentiation of genotype from macroscopic analysis of archaeological bone impossible (Ortner, 2003). Palaeopathological investigations of thalassaemia in the archaeological record have long been based on macroscopic bone analysis, from Angel's (1964, 1966) seminal Mediterranean investigations to modern analyses of British (Lewis, 2010) and Sardinian (Setzer, 2010) human remains. There have also been a handful of attempts to detect thalassaemia in archaeological bone using PCR (e.g., Filon et al., 1995; Yang, 1997; Hughey et al., 2012) and immunological (e.g., Ascenzi et al., 1991) techniques. However, macroscopic analyses have tended to be more successful than biomolecular techniques. This does not, of course, mean that macroscopic detection of thalassaemia has been faultless. In addition to the problems associated with all macroscopic palaeopathological research already discussed, early studies tended to suffer from a lack of comparative clinical data, relying heavily on historical analogy based on modern living populations, and making somewhat over-simplistic diagnoses of thalassaemia based on skeletal changes now known to have multi-factorial aetiologies (Hershkovitz *et al*., 1991; Lagia et al., 2007; Setzer, 2010). Despite these problems, improvements in modern medical understanding of the thalassaemias and a corresponding increase in comparative clinical data have aided palaeopathologists in establishing more standardised diagnostic criteria for identifying the condition in ancient human bone (Lewis, 2010). Although there remain issues with identifying thalassaemic sequelae in archaeological bone, recent successful research (e.g., Lewis, 2010; Hughey et al., 2012) suggests a more promising future for the detection of the condition in ancient human remains.

4.2.5.2: Sickle cell disease

First clinically observed and characterised in the early 20th century, sickle cell disease is the collective term for haematopathologies resulting from the inheritance of haemoglobin S, a genetic variant of normal adult haemoglobin (Brozovic and Anionwu, 1984; Allison, 2002). Sickle cell disease is the most common worldwide haematopathology, with up to a 15% allelic frequency, and is generally found in populations with a history of exposure to intense endemic *P. falciparum* malaria (Piel et al., 2010). Hence, the highest allelic frequencies are seen in sub-Saharan Africa.

The disease is named after the 'sickling', or transformation of red blood cells into sickle shape during periods of low blood oxygenation. The two major genotypes of sickle cell disease are sickle cell anaemia (or SCA), resulting from the inheritance of the haemoglobin S gene from both parents (homozyosity), and sickle cell trait (or HbAS), resulting from the inheritance of one haemoglobin S gene and one normal haemoglobin A gene (heterozygosity) (Allison, 2002). Sickling and subsequent lysis of affected RBCs in homozygous carriers leads to increased blood viscosity, severe haemolytic anaemia, obstruction of small blood vessels, and eventual organ damage or failure. The severity of the disease in its homozygous state is such that the majority of untreated carriers die before they reach five years of age (Piel et al., 2010). Conversely, sufferers with sickle cell trait are usually asymptomatic and require much more extensive blood deoxygenation to trigger RBC sickling (Allison, 2002), although rare and serious complications such as medullary cancer, renal failure, thromboembolism, and neonatal death have been associated with heterozygosity (Tsaras et al., 2009). Although it is generally accepted that sickle cell carriers (SCA and HbAS) are more resistant to malaria infection, exact mechanisms behind the protection remain unclear, as does the degree to which heterozygosity confers protection (Williams et al., 2005). It has been suggested that the sickling reaction of RBCs upon parasitisation causes rapid haemolysis by the immune system, thus restricting the severity of malaria infection (Balgir, 2006). Conversely, research in India has suggested that homo- and heterozygous expression offer very limited protection against simultaneous *falciparum* and *vivax* malaria infection (Kar et al., 1990).

The vast majority of studies of the interaction of sickle cell disease and malaria resistance have concentrated on populations living in endemic *P. falciparum* malarial areas.

Given the geographic concentration of sickle cell alleles, it is clear that intense *P. falciparum* transmission is a driving force behind the selection of the haemoglobin S allele. Since *P. vivax* was almost certainly the prevalent parasite in past British malarial transmission, it is unlikely that sickle cell disease played a significant (if any) part in the development of malaria resistance. Although high post-medieval mortality rates *may* have been malaria-related, *vivax* virulence and transmission probably would have not been sufficiently intense to apply a selective pressure strong enough to select for sickle cell disease. Skeletal evidence of this condition will therefore not be sought in this study.

4.2.5.3: Glucose-6-phosphate dehydrogenase (G6PD) deficiency

G6PD deficiency is the third most common genetic polymorphism that confers a degree of protection against malaria infection after sickle cell disease and the thalassaemias. The condition affects nearly 500 million people worldwide, the majority of whom are males (90%) living in endemic *P. falciparum* malaria areas (Wajcman and Galacteros, 2004). The deficiency results from one, or a combination of up to 150 different mutations in the gene that codes for G6PD, an enzyme crucial in the removal of oxidants which damage RBCs. These compromised RBCs are quickly destroyed by the body (haemolysis), thus protecting against erythrocytic development of the malaria parasite (Sallares et al., 2004). The majority of sufferers are asymptomatic, only developing symptoms (acute haemolytic anaemia being the most common) in times of oxidative stress or when exposed to certain triggers (Ruwende and Hill, 1998; Mehta et al., 2000). For instance, favism is a well-known condition linked to G6DP deficiency and is characterised by the onset of acute haemolytic anaemia following the consumption of fava, or broad beans. Clinical methods to detect the condition have traditionally been expensive, time consuming, and require freshly collected blood samples. However, rapid diagnostic and ELISA methods are under evaluation (Kuwahata et al., 2010).

Very little research on G6PD deficiency in past populations has been carried out, despite the likelihood that the condition has a long history of affecting populations in endemic malarious areas. However, favism is well documented in antiquity, suggesting the latent presence of G6PD deficiency (Mehta et al., 2000). The dearth of research is likely due in part to a lack of comparative clinical data and to the difficulties of tracing the condition in

archaeological remains: there is little clinical evidence that the episodic nature of acute haemolytic anaemia associated with G6PD deficiency induces macroscopically observable changes in bone, unlike in chronic conditions such as the thalassaemias. Palaeopathological research on the condition has, therefore been confined to the biomolecular analysis of ancient DNA. For example, Sallares et al. (2003) isolated a Mediterranean variant of G6PD deficiency in the ancient DNA of an infant from Lugano, Italy, and, although not working with ancient material, Liu et al. (2002) demonstrated that genetic coding for G6PD mutations survive and can be amplified from archived clinical samples. The identification of G6PD deficiency through next generation aDNA analysis and, potentially, new rapid diagnostic and assay testing, offers an exciting avenue of research for indirectly tracing malaria in past populations. As new, more affordable and practical testing methodologies become available, they may well become part an important area of study in future palaeopathological research.

4.2.6: The nature of temperate *P. vivax* 'endemicity'

When considering the possible development of past genetic resistance tied to putative *P. vivax* in past British populations, it is important to consider the nature of endemicity in the marshlands and Fens of England. Malaria epidemiology and the factors influencing the development of endemicity within any region are extremely complicated. Accordingly, the level of endemicity within a region is measured by the percentage of the local population infected at any one time (Hay et al., 2010). Stable malaria endemicity is often associated with tropical or sub-tropical areas, where the intensity of transmission is high and the disease maintains a constant, stable presence within local populations. Here, malaria endemicity is catagorised as holoendemic (>75% of population infected), hyperendemic (50-75%), and mesoendemic (10-50%). Areas of unstable malaria transmission, where less than 10% of the population are infected at any one time, are classified as being hypoendemic (Hay et al., 2008). As expected with the aforementioned research bias, these classifications are mainly based on areas dominated by *P. falciparum*. Biological differences mean that *P. vivax* malaria does not necessarily fit this model of endemicity. Temperate *vivax* malaria can remain latent at the hypnozoite stage for up to 14 months following initial inoculation (Sattabongkot et al., 2004; Petersen et al., 2013), and

subsequent relapses can be spaced many months apart. Unlike *P. falciparum* malaria, which once cleared by the body requires a new source of infection, individuals with *P. vivax* often remain infected indefinitely unless suitable treatment is administered. This may well have been the situation in the past Fens and marshlands of Britain, with a large proportion of the population retaining the infection, but not being necessarily continuously *infectious*, or suffering from clinical illness. Transmission may have been unstable and outbreaks intermittent, dictated by yearly climatic fluctuations and their impact upon *anopheline* population levels, but endemicity would remain constant due the high percentage of latent *vivax* infections. The adaptation that allows *P. vivax* to survive and flourish in temperate regions of the world is a reason why authors (e.g., Knotterus, 2002; Packard, 2007) confidently assert that malaria was 'endemic' in these areas.

Dobson's (1997) observed seasonal fluctuations in post-medieval marshland mortality rates follow a typical temperate endemic *P. vivax* transmission pattern, similar to that noted by Hulden et al. (2005) in Finland. Infections initiated in late summer and autumn would incubate over winter, and precipitate outbreaks the following spring (hence 'spring fever') and early summer. The intensity of these outbreaks was dictated by the climate of the preceding summer: a hot summer would increase *anopheline* vector numbers, thereby increasing the intensity of parasite transmission. The British mortality figures are, therefore, highly suggestive of the presence of endemic *P. vivax* malaria. Endemicity and transmission frequency are important factors in the development of genetic resistance to malaria. If, as suggested by the mortality figures, *P. vivax* was endemic and exerting a selective pressure upon British Fen and marshland populations, then genetic resistance is more likely to occur. The emergence of haemoglobinopathies (e.g., G6PD deficiency) have recently been observed in temperate endemic *P. vivax* areas (Sina, 2002; Louicharoen et al., 2009), demonstrating that the disease, at least in endemic areas, can exert a significant enough selective pressure to drive the development of responsive genetic anaemia. However, what is not certain is whether the deleterious alleles developed due to spontaneous point mutations, or were imported into the populations from external sources (e.g., migrants from malarious areas).

4.2.7: The interaction of *P. vivax* malaria and thalassaemia

Very little research has been conducted on the mechanisms of genetic resistance relating specifically to *P. vivax*. In a reflection of malaria research on a wider scale, concentration has been on the interaction of haemoglobinopathies with the more deadly malaria species, *P. falciparum* (e.g., Williams et al., 2002; 2005). Research investigating the response of thalassaemic infants exposed to both *P. falciparum* and *P. vivax* has suggested that thalassaemia increases the susceptibility to *P. vivax* infection, which confers an increased protection against *P. falciparum* infection (Williams, 2006). There has also been some suggestion that thalassaemia may actually increase the parasitaemia of *P. vivax* infection (e.g., O'Donnell et al., 2009), particularly in children under five years of age. This seems counter-intuitive, since increasing parasitaemia would increase cell haemolysis rates, thus elevating the risk of developing severe *vivax* symptoms. Price et al. (2007), however, suggest that severe *vivax* malaria is less likely to develop in infants under six months of age, who retain increased levels of protective foetal haemoglobin. Beyond this, however, very little is understood concerning the interaction of temperate *P. vivax* and thalassaemia (Douglas et al., 2012).

4.2.8: Inherited conditions, *P. vivax*, and the palaeopathological literature

Considering the three genetic haematopathologies discussed earlier, it emerges that the most likely polymorphisms to have developed in response to putative endemic *P. vivax* malaria in past British populations are the thalassemias and G6PD deficiency. The development of these polymorphisms would, of course, require a very particular amalgamation of past circumstances. For instance, the selective pressure of the disease would need to have been strong enough to select for these protective genetic traits. The disease also would have to have been endemic within a population for a significant time period (at least a few hundred years, according to Carter and Mendis, 2002) for the polymorphism to persist and become 'balanced', although the sporadic development of skeletal changes associated with genetic haemoglobinopathies is not necessarily dependent upon the 'balanced' nature of the polymorphism; it would, of course, be possible for detectable pathologies to develop during the balancing period. Whether these circumstances ever existed in Britain is currently unknown. The very low modern British prevalence of

thalassaemia (approximately 0.1% prevalence: Hickman et al., 1999) and G6PD deficiency (0% in 1962: Nkhoma et al., 2009; more recent figures unavailable) suggests that the mutations responsible have not persisted within in the gene pool. Whether this is due to the withdrawal of the selective pressure (i.e., *vivax* malaria) or to the fact that the conditions never developed is open to conjecture. Nevertheless, it worth exploring the possibility that such polymorphisms developed as a response to *P. vivax* malaria in British antiquity.

It was decided not to attempt to identify G6PD deficiency or sickle cell disease. Clinical evidence of the skeletal response to G6PD deficiency is severely lacking and the expense of aDNA/PCR (the standard clinical method) detection of the condition also precludes analysis of this disease. However, the recent development of cost-effective screening technologies (e.g., Kim et al., 2011) may enable future attempts at detection in archaeological samples. The uncertain association between *P. vivax* and sickle cell disease and the intense pressure evidently required to select for the polymorphism mean that the chances of it developing in British antiquity are very remote.

This study will attempt to identify the development of thalassaemia as a possible indirect result of endemic malaria within past British skeletal populations excavated from potential malarious areas. This analysis will be based on a review of palaeopathological skeletal reports, since thalassaemia results in generally predictable macroscopic changes in the skeleton (Hershkovitz et al., 1997; Lewis, 2010), which should have been recorded in such reports. There has been a tendency for some diagnostic assumptions to be perpetuated in the palaeopathological literature (e.g., the continuance of periostitis to be attributed to non-specific infection caused by physiological stress) as a result of analysts relying on past osteological reports for diagnostic criteria, rather than attempting to access the latest palaeopathological interpretations and clinical literature (Grauer, 2012). Further to this, many human skeletal analytical reports are produced by palaeopathologists working either freelance or internally for contract archaeology units. The author's experience has invariably been that human remains analysis in the contract environment is often chronically under-resourced in terms of time and funds. These pressures may cause many problems, including insufficient pathological descriptions and poor standardisation in recording between analysts and reports (Roberts and Cox, 2003). Although guidelines stipulating the minimum content of an osteological report have been published (Brickley and McKinley, 2004) to address this

lack of standardisation, problems remain relevant when considering the identification of rarely-observed diseases in British human skeletal remains, particularly when macroscopic analysis alone may be insufficient for a diagnosis. The aforementioned perpetuated assumptions and pressures may facilitate an expectation that certain conditions simply will not be encountered in British skeletal populations.

The palaeopathological diagnosis of thalassaemia in British antiquity may fall into this trap. A disease most commonly encountered in populations with a long history of exposure to endemic *falciparum* malaria, its palaeopathological diagnosis has usually being tentatively based on macroscopic observance of a combination of non-specific pathognomonic skeletal changes, such as *cribra orbitalia*, osteopenia, and porotic hyperostosis (Lewis, 2010). Since the latter condition is rarely observed in British archaeological populations (Roberts and Manchester, 2005), the assumption may follow that thalassaemia must be equally, if not more, rare. As previously mentioned, prevalence of *cribra orbitalia* or porotic hyperostosis was often traditionally explained by iron deficiency anaemia (Waldron, 2009). It is only relatively recently that aetiological reappraisals (e.g., Walker et al., 2009) have questioned this traditional interpretation. It is, therefore, entirely possible that contract analyses contemporary with the 'iron-deficiency hypothesis' may have provided this diagnosis and missed possible cases of acquired (e.g., megaloblastic) or hereditary anaemias (e.g., thalassaemia). Further to this, many such analyses may not necessarily have access to the latest advances in diagnostic techniques.

Lewis (2010) has recently reviewed the clinical literature concerning the osseous sequelae of thalassaemia and identified additional diagnostic criteria that she used to identify probable and possible cases in child skeletons in the Romano-British assemblage from Poundbury Camp, Dorset. Using selected diagnostic criteria of Hershkovitz et al. (1997), Ortner (2003), Yochum and Rowe (2005), Lagia et al. (2007), Lewis (2010), and Perisano et al. (2012) this study will analyse selected palaeopathological reports in an attempt to determine any under-representation of thalassaemia, and whether the disease can be identified in skeletal populations from likely endemic malarious areas in Britain

4.2.9: Skeletal evidence for thalassaemia

Confidence with which palaeopathologists can more accurately identify sequelae that

may be indicative of thalassaemia in archaeological skeletal remains has recently improved (see Lewis, 2010). As previously mentioned, the establishment of new diagnostic criteria highlights the need for re-analysis of selected site reports and inhumation catalogues in an attempt to determine whether any published sequelae of thalassaemia could have been overlooked, or attributed to other conditions due to the perceived rarity of thalassaemia in British antiquity. It is, therefore, possible that the condition is under-represented in the British palaeopathological literature, due to the fact that many of these pathological analyses were conducted prior to the refining of diagnostic criteria. In terms of identifying thalassaemia in the skeletal record in a British archaeological population some key questions need to be addressed:

1. At which British sites are we most likely to encounter thalassaemia?
2. Which individuals are most likely to develop skeletal changes associated with thalassaemia?
3. How likely are these individuals and their sequelae to be sufficiently represented in the archaeological record?

Assuming that *P. vivax* exerted a significant enough selection pressure to facilitate the development of thalassaemia as an adaptive polymorphic response, where would we most likely encounter the condition? A key concern here is the effect of malarial endemicity on the persistence of thalassaemic alleles in the gene pool. Both α and β thalassaemia are rarely encountered in modern, non-endemic areas of the world, and are only usually seen in immigrants from endemic malarious areas (Vetter et al., 1997). The 'malaria hypothesis', first proposed by Haldane (1948) and confirmed by later research (e.g., Allison, 2002; Piel et al., 2010), suggests a close link between the development of haemoglobinopathies and endemic malaria, since natural selection increases the genetic traits which act to protect the carrier from malaria infection. The link is observed most commonly in association with *P. falciparum*, yet Dobson's (1997) synthesis of post-medieval burial records suggest that the potential epidemiological impact of malaria on past British marshland communities should not be underestimated. A link between the selective pressure of *vivax* malaria and the development of haemoglobinopathies may be as valid for pre-medicalised societies as it is in

the modern era.

The Fenlands and marshlands of Lincolnshire and Cambridgeshire were chosen as areas of interest for this study for a number of reasons. Firstly, as established in Chapter 1, the environmental conditions from at least the Roman period would have provided favourable territory for *anopheline* breeding. During the Roman period, the silt Fens of Lincolnshire and Cambridgeshire became relatively well settled (Darby, 1983), although a climatic deterioration towards to the end of the period seemingly resulted in the abandonment of some of these settlements. Total abandonment of the Fens never occurred, however, as people relocated to the higher ground of the Fen islands and around the Fen edge in order to maintain their relationship with the rich resources on offer (e.g., salt production, reed and peat cutting, and seasonal grazing pastures). Despite their growing reputation for disease and danger, the resource-rich Fens drew increasing numbers of people who, in the face of constant epidemiological and environmental threat, eventually tamed the landscape through drainage and reclamation. Consequently, Fen resource exploitation has been a continual phenomenon since prehistory (Oosthuizen, 2012) .

It is not known how the fluctuating Fenland population may have affected malaria transmission and the potential for development of protective haemoglobinopathies. The malaria hypothesis suggests that those communities exposed long-term to endemic malaria should prove most likely to develop resistance in the form of thalassaemia, and that communities with discontinuous exposure are less likely to form genetic resistance. There exist areas within the Fens and around the Fen edge with archaeological evidence for continual occupation from prehistory to the modern day. These areas include the Cambridgeshire Fen islands of Crowland, Littleport, and Ely (Newman, 2007; Woolhouse, 2009; Cope-Faulkner et al., 2010), and Fen edge populations such as those at Sleaford (Dickinson, 2004; Murphy, 2011) and in the locality of Peterborough (Evans and Hodder, 2006). It is perhaps here, in places of continual Fen-associated occupation, that skeletal evidence of genetic resistance to malaria may be found, if at all. Many of these areas also saw influxes of migrants from continental Europe from the Roman period onwards, potentially introducing thalassaemic alleles into native human populations, and perhaps also new strains of *P. vivax* malaria into local *anopheline* populations.

Secondly, the choice of target populations is influenced by the results of research which employed spatial epidemiology to correlate prevalence rates of *cribra orbitalia* (CO) with historically recorded distributions of *A. atroparvus* and malaria cases (Gowland and Western, 2012). The intriguing link between Fen/marshland location and a high prevalence of CO provides perhaps the most compelling argument to date for the presence of English *P. vivax* malaria (likely in combination with comorbidities) prior to the post-medieval period. The study builds upon and supports previous research demonstrating higher CO prevalence in Mediterranean marshland populations likely exposed to endemic malaria (Gowland and Garnsey, 2010), and suggests that *P. vivax* malaria may have played an important role in influencing higher rates of CO in Anglo-Saxon Fenland populations. Although not specifically attributable to malaria infection, elevated prevalence rates of CO in Fenland populations may be indicative of megaloblastic anaemia associated with parasitaemia, be it by *plasmodia*, helminth, or both. Many of the sites selected for this study are classed as potentially 'malarial' by Gowland and Western. Possible inconsistencies in classification can be reconciled by the fact that some sites fell at the very edge of the interpolation map, from which the 'malarial' classification was taken (personal communication, Gaynor Western, January 2014). CO prevalence rates that vary considerably between 'malarial' sites (e.g., 3.5% and 59.5% at Castledyke South and Highfield Farm, Littleport, respectively) support a multifactorial aetiology for the condition, and although the research cannot necessarily (and does not claim to) pinpoint definite malarious sites, the correlation was compelling enough to suggest a malarial influence. Hence, many of these sites (at least, those with available skeletal reports and inventories) will provide a useful starting point for both the search for thalassaemia and the demographic population analysis discussed in Chapter 5 (sections 5.1 and 5.2).

When considering the choice of time periods addressed in this study, the following question may, quite rightly, be posed: why ignore post-medieval skeletal populations from the areas investigated by Dobson (1997), since surely these are the most likely populations to have developed genetic resistance to malaria? The decision to investigate Roman, Anglo-Saxon, and medieval samples from Lincolnshire and Cambridgeshire, rather than post-medieval samples from Kent and Essex, is influenced by two factors. Firstly, very few post-medieval cemetery populations from either area of the country are available for study. Post-

medieval burial usually fell under the remit of the Anglican Church, their cemeteries and churchyards being considered consecrated ground (Sayer, 2011). Excavation of these burial grounds is a relatively rare phenomenon and excavated individuals are often subject to reburial, rather than curation. In rare instances (e.g., St. Peter's Church, Barton-upon-Humber) the cemetery population may remain archived on-site, with potential availability for study. Even so, Church permission is still required for destructive analysis (unfortunately, permission to sample a small number of Barton-upon-Humber skeletons for this study was denied). Consequently, there are simply an insufficient number of cemeteries/individuals from this period for a study of this scope.

Secondly, the elevated post-medieval seasonal mortality rates in Lincolnshire and Cambridgeshire Fen parishes (West, 1974; Reynolds, 1979; Wrigley and Schofield, 1981; Nicholls, 2000) suggest that *vivax* malaria played an important epidemiological role during this period, and the presence of the disease here as suggested by the research of Gowland and Western (2012), is sufficiently compelling to challenge the assumption that malaria was absent from England prior to the 16th century. As previously mentioned, very little palaeoepidemiological research has been conducted on archaeological Fenland populations. This research has the potential to shed light on these relatively unstudied areas and populations.

Thus, the search has been narrowed to settlements and cemetery populations likely to have been exposed to endemic *P. vivax* malaria for an extended period, such as the Fen islands. But who within these populations is most likely to exhibit skeletal sequelae of thalassaemia? This is a difficult question to address, given the idiosyncratic nature of phenotypic expression and the diversity of skeletal sequelae that can develop in response to the condition, particularly in heterozygous individuals. Differential diagnosis of thalassaemia can also be confounded by skeletal preservation. It is, therefore, worth considering how the expression of each genotype might present within the archaeological record, and which individuals might display the resultant phenotypic characteristics.

In a pre-medicalised population homozygous expression of β-thalassaemia (β-thalassaemia major) would almost certainly lead to premature death, most likely during late pregnancy or early infancy, due to a lack of available specialised medical treatment (Lewis, 2010). Homozygous α-thalassemia most often results in late pregnancy death and still birth

due to severely compromised intrauterine oxygenation (Weatherall, 2001). Ortner (2003:365) suggests that skeletal changes associated with β-thalassaemia major "become radiologically obvious after the first year of life." Sequelae are rarely encountered prior to six months of age, but marrow expansion can affect any part of the skeleton after the first year, with the cranium and facial bones often being the first affected (Yochum and Rowe, 2005; Tyler et al., 2006). Untreated infants may survive the first few months of life due to their increased concentration of foetal haemoglobin, but when these levels decrease, severe haemolytic anaemia and splenomegaly occur. Should the individual survive these symptoms (which, in antiquity, is rather unlikely), skeletal changes in the form of marrow hyperplasia occur in response to inefficient production of red blood cells, and excessive production of the hormone erythropoietin (Weatherall, 2001). Subsequent iron sequestration can lead to toxicity, stroke, or cardiac failure (Olivieri, 1999).

Since untreated homozygous β-thalassaemics rarely survive past a few months, this causes a potential difficulty in identifying these individuals in the archaeological record. A differential diagnosis of the condition would be impossible in the majority of affected infants, since death may occur before the development of skeletal sequelae. Lewis (2010:7), for instance, suggests that "in addition to the possible cases of thalassaemia intermedia [at Poundbury]…there may have been a greater number of children who died as the result of T-major". Thalassaemia major may, therefore, remain all but invisible in the archaeological record, regardless of how many infants were affected. Perhaps the only method of identifying the condition would be by observing a significantly elevated prevalence of perinatal and infant skeletons within a target burial population. However, this may also be the case within non-resistant individuals, since *P. vivax* infections can be particularly dangerous for neonates (Poespoprodjo et al., 2009), especially if any maternal immunity was lost during pregnancy (Whitty et al., 2005) and, therefore, not passed on to the infant.

Skeletal changes as a consequence of thalassaemia intermedia are more likely to be encountered in the archaeological record (Ortner, 2003), since homozygotes are unlikely to survive long enough to develop diagnostic sequelae, and thalassaemia minor rarely involves skeletal modification (Ortner, 2003). Individuals with thalassaemia intermedia may remain asymptomatic until adulthood, or can display symptoms from as early as two years, depending upon the severity of the case. In the latter category in untreated individuals, one

might expect to observe growth retardation in early childhood (Taher et al., 2006). Skeletal changes associated with thalassaemia intermedia may include marrow hyperplasia of the facial bones and damage to the maxillary sinuses. Additionally, up to 40% of adults present with scoliosis (Yochum and Rowe, 2005).

4.2.10: The effects of *P. vivax* infection on the foetus and neonate

An avenue of palaeopathological investigation that has yet to receive attention is the effects of *vivax* malaria infection on the skeletal development of the growing foetus and in the first few antenatal months. The burden of *P. vivax* malaria in pregnancy is a neglected area of research (Greenwood et al., 2007; Price et al., 2007; Mueller et al., 2009), with the majority having concentrated on *P. falciparum* infections in sub-Saharan Africa (Desai et al., 2007). It has long been known that pregnant women are more susceptible to *P. vivax* infection, but it is only until relatively recently that the deleterious effects of infection on the mother and foetus have begun to be appreciated (Nosten et al., 2004). Although less severe than in *P. falciparum* infections, the main risks associated with *P. vivax* infection during pregnancy are maternal anaemia and significantly decreased birthweight through intrauterine growth restriction (IUGR) (Nosten et al., 1999; Rodriguez-Morales et al., 2006; Desai et al., 2007). In endemic areas, malaria has been suggested to be responsible for up to 70% of cases of IUGR (Steketee et al., 2001).

Exactly how malaria may influence low birthweight remains poorly understood (Conroy et al., 2011), especially with *P. vivax* parasites, which, unlike *P. falciparum*, do not cytoadhere to the placenta (Greenwood et al., 2007; Umbers et al., 2011). Also in contrast to *P. falciparum*, *P. vivax* infection results in more pronounced reductions in birthweight in subsequent (multigravida) pregnancies (Desai et al., 2007). Although miscarriage and pre-term delivery is quite rare in cases of *P. vivax* infection, some women develop severe sequelae, such as thrombocytopenia and cerebral involvement (Rodrigues-Morales et al., 2006). Should neonates survive the complications associated with IUGR, which include increased risks of diarrhoea, hypoglycaemia, immunological deficiencies, and pneumonia (Ashworth, 1998; Pallotto and Kilbride, 2006), they remain at high risk of developing dangerous symptoms of *P. vivax* infection, such as severe anaemia and thrombocytopenia (Poespoprodjo et al., 2009). One might expect a protective effect of residual maternal anti-

malarial antibodies, yet research (Whitty et al., 2005) suggests that mothers can lose any previously acquired immunity during pregnancy.

If many cases of *P. vivax*-affected pregnancies are carried to term, the effect of IUGR should be reflected in the foetal/neonate skeleton, and care must be taken when recording these individuals from archaeological populations inhabiting potentially malarious areas. For instance, cemetery populations may exhibit unusually high ratios of infant skeletons that may be recorded as 'foetal' based on bone lengths that have been reduced by IUGR. Cemetery populations may, therefore, contain a higher-than-expected ratios of 'small' babies. It would be challenging to infer the presence of *P. vivax* malaria based on gross infant mortality figures, given the difficulties inherent in attempting palaeodemographic analyses of infant mortality based on skeletal numbers (see Lewis and Gowland, 2007). Despite this, it may prove interesting to compare very young infant mortality ratios between suspected malarious and non-malarious sites and to investigate any instances of increased numbers of 'small' babies within populations. A literature search reveals no other attempts at identifying IUGR in in archaeological populations inhabiting potentially malarious areas.

4.2.11: Palaeodemography

A further indirect method of potentially identifying the presence of putative English *vivax* malaria would be to perform basic palaeodemographic profiling of burial populations based in likely 'malarious' and 'non-malarious' areas. As previously discussed, Dobson's (1997) extensive analysis of historical burial rates has provided compelling evidence for the impact of *vivax* malaria on post-medieval Kent and Essex marshland parishes. Fen-associated parishes in Lincolnshire and Cambridgeshire have also demonstrated unusually high mortality rates for this period, particularly for infants (e.g., West, 1974; Reynolds, 1979). Demographic analysis for archaeological, undocumented populations is more difficult, often relying on age-at-death ratios of cemetery populations to make inferences on any variations observed. Although cemetery data have been deemed unreliable for demographic analysis by some (e.g., Hollingsworth, 1969; Bocquet-Appel and Masset, 1982), skeletal material provides an important resource for primary demographic data. Confounding factors to this type of analysis include possible errors or inconsistencies in ageing and sexing skeletal individuals, and differential preservation, deposition, and

recovery of skeletal remains (Hoppa and Vaupel, 2002; Chamberlain, 2006).

With these limitations in mind, it may prove useful to compare mortality (age-at-death) profiles from archaeological Fen/marshland and non-Fen/marshland (henceforth referred to as Fen and non-Fen) cemetery sites, since no such comparison has been performed to date. It could be hypothesised that the presence of *P. vivax* malaria in Fen/marshland environments may result in increased mortality rates for certain groups of individuals when compared to non-Fen/marshland populations (e.g., neonates, infants, and women of child-bearing age). A difficulty may, however, rise in discerning the influence of putative malaria from other disease factors which may have been present in the notoriously insalubrious Fen/marshland environs, especially given that the age groups mentioned are often at risk of higher mortality, regardless of environment.

4.3: Biomolecular analysis and palaeopathology

'So far the achievement of biomolecular archaeology in relation to malaria is only a tiny fraction of its potential' (Sallares and Gomzi, 2001:200).

Biomolecular analysis has its roots in the emergence of the discipline of immunology in the late 19th century. In 1898, for example, the reaction of antibodies and antigens was first observed in animal blood serum experiments (Newman et al., 1996). Since that time knowledge of molecular biology and immunology has increased exponentially. Given the increasing appreciation of the vast potential that human remains offer in furthering our understanding of the past, it was inevitable that biomolecular methodologies would begin to be employed in palaeopathological analysis. However, it is with advances in the past few decades that "modern instrumental chemical and biochemical techniques has provided us with the resources necessary for the effective recovery, detection and characterisation of biomolecules and their decay products in archaeological materials" (Evershed, 1993:75). Analysis of archaeological inorganic residues using techniques derived from chemistry and physics became commonplace during the mid-20th century, yet a general assumption remained that organic residues would not survive in the archaeological record. Radiocarbon dating methods developed since the 1950s using surviving bone collagen in human remains spurred interest in the organic biochemical information stored in ancient bone. Improving radiocarbon technology allowed for the discovery of carbon, and later nitrogen isotopes

within collagen. Since then, isotopic analysis of ancient human bone has become an increasingly important technique in attempting to reconstruct past dietary and migration patterns (Pollard, 2001).

One of the first biomolecular applications specifically concentrating on organic residues in archaeological materials occurred in the late 1970s, when researchers successfully identified lipids surviving within ceramic vessels (Pollard, 2001). This pioneering research demonstrated firstly that various organic ancient biomolecules can indeed survive in archaeological materials, and secondly that such research had the potential to open up a vast resource of information previously considered inaccessible. The archaeological community was particularly excited by two applications of biomolecular research developed during the 1980s. Firstly, biochemical methodologies were employed in the apparently successful detection of ancient proteins in fossil material (e.g., Lowenstein, 1981). This discovery led to a rush of research which concentrated particularly on detecting blood-protein residues on ancient artefacts (e.g., Loy, 1983; Newman and Julig, 1989), with varying degrees of success. Although the methodological validity of many of these studies was hotly debated (e.g., Downs and Lowenstein, 1995; Leach and Mauldin, 1995), they laid important groundwork in the area of ancient biomolecular detection (Malainey, 2011). Secondly, the mid-1980s saw the development of techniques capable of detecting and amplifying fragments of DNA from ancient organic material. This discovery heralded "the true beginning of biomolecular archaeology as a discipline in its own right" (Brown and Brown, 2011:9). As with ancient protein detection, immediate enthusiasm for ancient DNA (aDNA) analysis influenced numerous studies (e.g., Hagelberg and Clegg, 1991; Woodward et al., 1994; Scholz et al., 2000), many of which helped to highlight the methodological difficulties of obtaining valid results from degraded or contaminated samples (O'Rourke et al., 2000). Despite these problems continuing to limit 'traditional' aDNA, the technique has come to dominate biomolecular palaeopathology as researchers strive to develop reliable, replicable techniques (such as new next generation sequencing) for aDNA extraction and characterisation (Cooper and Poinar, 2000). This is largely due to the vast potential offered by surviving nucleic acids as a source of information on topics as diverse as the coevolution of humans and pathogens, kinship, migration, epidemiology, and genetic characterisation (Pinello, 2008).

As the discipline of biomolecular archaeology matures and methods move onwards towards standardisation and replicability, new techniques of detecting human immunological products and pathogens in ancient osseous tissues are currently in development, such as high-resolution proteomic techniques for analysing archaeological bone proteins. These are yielding ground-breaking insights into entire ancient proteomes and disease biomarkers (e.g., Cappellini et al., 2011; Wadsworth and Buckley 2014). Most new methodologies are often expensive and, due to their novelty, may give variable and debatable results; as in any archaeological discipline, "with maturity and widespread application come the complications" (Bentley et al., 2004:365). It is only through collaborative work between molecular biologists and archaeologists that new methodologies can mature and become truly informative and reliable tools. This study should add to the growing corpus of research knowledge through investigation of ancient biomolecules associated with *Plasmodium vivax* infection.

4.3.1: Ancient biomolecules: survival and interpretation

As previously mentioned, numerous biomolecules have been targeted for investigation of ancient human remains. These broadly fall into the categories of lipids, nucleic acids (e.g., DNA and RNA), collagenous proteins, and non-collagenous proteins (Brown and Brown, 2011). Analysis of each class of molecule presents a unique challenge to the biomolecular archaeologist, which must be met and overcome in order to produce reliable, replicable results. Before selection of the appropriate target biomolecule and the extraction/analytical methodology, it is important to first understand the structure and composition of the organic material in which the target molecule is found. Secondly, it is equally, if not more vital to appreciate that from the moment of death numerous complex processes occur within the organism which inevitably alter the properties of endogenous biomolecules, to a greater or lesser extent depending on multiple factors. The processes by which organic materials decay and degrade following death are known collectively as diagenesis (Smith et al., 2007).

Human bone matrix is comprised of approximately one third organic and two thirds inorganic materials. The latter is made up of mineral comprising mostly calcium phosphate in the form of hydroxyapatite. The organic component of bone mainly comprises collagen

and osteocalcin, which account for around 90% of the osseous protein content. The remaining 10% consists of non-collagenous proteins (NCPs), such as haemoglobin, albumin and immunoglobulins (Freundorfer et al., 1995; Collins et al., 2002). Many of these NCPs, along with cellular molecules such as DNA and lipids, are not necessarily intrinsic to the bone matrix itself, but are "associated with fluids and cellular components in the tissue, and their persistence in bone is more difficult to predict than collagen and osteocalcin" (Collins et al., 2002:384-5).

Study of bone diagenesis is a recent phenomenon, born out of the necessity for critical analysis of the ancient biomolecular research boom of the late 20th century. An understanding of the manner in which decay processes affect ancient biomolecules was therefore demanded in order to validate associated research. A considerable share of research on bone and biomolecular diagenesis has concentrated on collagen, which is understandable given the ubiquity of the protein and its potential application within biochemistry and biomolecular archaeology (e.g., Collins et al., 1995; 2002; Tuross, 2002; Dobberstein et al., 2009; Wilson et al., 2012). The degradation of DNA has also received attention (e.g., Colson et al., 1997; Rollo et al., 2002; Harbeck et al., 2004; Pruvost et al., 2008). Diagenesis of non-collagenous proteins is less well understood, although a small number of studies have attempted to rectify this (e.g., Masters, 1987; Freundorfer et al., 1995; Grupe and Turban-Just, 1996; Wiechmann et al., 1999; Brandt et al., 2000).

Two of the main difficulties faced by biomolecular archaeologists are degradation of the target molecules and contamination with modern molecules (Brown and Brown, 2011). Contamination with modern DNA is the most common problem encountered in aDNA studies. However, both DNA and proteins can become contaminated and degraded soon after death. Upon death the breakdown of cell membranes and endogenous organelles immediately commences, a process known as autolysis. This destruction releases acidic enzymes which, in life, are contained within cell lysosomes. Reactive chemical by-products of decomposition are also produced. These enzymes and chemicals attack and degrade biomolecules and can demineralise bone (Child, 1995). Only those biomolecules which survive autolysis can persist in the burial environment (Brown and Brown, 2011). Microbial (e.g., bacterial and fungal) invasion of the bone follows inhumation. This is a much slower process than autolysis, but these organisms can excrete enzymes and acids to further degrade

bone mineral and biomolecules, increase bone porosity, and potentially introduce foreign proteins which may confound results of biomolecular testing (Hedges, 2002). Acids produced by microorganisms can also convert hydroxyapatite to brushite, a mineral that requires increased space, thereby resulting in cracking (Mays, 2010), which further exposes the internal bone structure to external diagenetic factors. Environmental processes also influence the breakdown of biomolecules in the archaeological record. Water and oxygen, for example, are highly significant diagenetic agents (Brown and Brown, 2011).

Diagenetic processes and the survival of ancient biomolecules in the archaeological record are very much mediated by the environment immediately surrounding the skeletal remains (Hedges and Millard, 1995). The most influential diagenetic factor is debated. It has, for example, been argued that hydrological action is "the most significant factor in the production of diagenetic change... [having] a far greater effect than age, soil content, pH or heat" (Cattaneo et al., 1995:271) , whereas Collins et al. (2002:386) suggest that "microbial deterioration is probably the most common mechanism of bone deterioration….optimized at near neutral pH - conditions which would otherwise protect bone". It is clear that factors as diverse as soil pH, geology, microbial concentration, hydrological and oxidative factors, temperature, humidity, post-interment disturbance, burial rituals, and individual health prior to burial are but a few of the variables that determine the successful endurance of biomolecules in the burial environment. Given the many possible variables influencing diagenesis and the difficulties in studying the processes 'in action', it is unsurprising that debate continues over the most important factors (Hedges, 2002).

Despite these difficulties, research into the survival of NCPs in the burial environment has suggested that the acidic nature of NCPs gives them a high affinity for calcium sites on the hydroxyapatite within bone, which should subsequently offer a degree of protection following inhumation (e.g., Masters, 1987; Freundorfer et al., 1995; Grupe and Turban-Just, 1998; Wiechmann et al., 1999; Nielsen-Marsh and Hedges, 2000). These proteins should, accordingly, suffer less deterioration than collagen, which does not bind to the mineral content and is therefore more susceptible to hydrological and microbial agents (Hedges, 2002). This may be surprising, given that collagen is generally considered a more stable organic component of bone (Hedges and Wallace, 1978), and early research by Gürtler et al. (1981) isolated collagen, rather than serum proteins in archaeological samples.

More recent research has claimed success in detecting NCPs, including immunoglobulins, in archaeological bone (e.g., Cattaneo et al., 1992; Wiechmann et al., 1999; Brandt et al., 2000; Schmidt-Schultz and Schultz, 2004, 2007; Wadsworth and Buckley, 2014), including bone that has undergone post excavation treatment (washing, drying, storage, etc.) and museum curation (Tuross, 1991), suggesting that such processes may have less impact on the survival of NCPs than expected. However, many of these studies are careful to highlight the problem of diagenetic protein alteration: although the affinity with hydroxyapatite may lessen the impact of hydrological leaching, for example, it does not necessarily protect the NCPs from other forms of diagenetic alteration, such as microbial and enzymatic attack.

Protein identification through immunological testing may be hampered by diagenetic processes which can alter the epitopic structure, molecular weight, and isoelectric points of the target protein (Brandt et al. 2000). Analysis is also potentially complicated by the tiny quantities of surviving molecules in archaeological remains; this is particularly apparent in the search for ancient serum proteins (including NCPs) which make up less than 10% of the total protein content of fresh bone. Methodologies to detect these elusive proteins must therefore be exceptionally sensitive, which itself may lead to cross reactions and detection of non-specific, degraded protein molecules or exogenous microbial proteins (Child and Pollard, 1992).

The interpretation of the results of ancient protein analysis must, of course, take into account current limitations. Mis- and over-interpretation of data is a problem that plagued the discipline in its early stages, as some researchers were quick to pronounce their findings without a full appreciation of the "unrecognised limitations" (Brown and Brown, 2011:146) of the methods used in such research. This was evident in early studies which claimed to have successfully detected and analysed blood residues on prehistoric tools (e.g., Loy, 1983; Nelson et al., 1986). Negative results, controls, and the general limitations of research should always be reported (Gernaey et al., 2001), and appropriate caveats made when necessary.

So why, considering all of the technical challenges and pitfalls involved in ancient protein detection and characterisation, does such research continue? The answer to this lies in the information stored in ancient biomolecules which offers the researcher valuable clues to so many aspects of the past - information that supplements, and yet is beyond the reach of

traditional palaeopathological methodologies. The potential scholarly rewards for accurately detecting, characterising, and interpreting ancient biomolecules make the recognition and confrontation of the methodological challenges more than worthwhile; after all, no scientific field develops and progresses without addressing and overcoming inherent difficulties as they are met (Brown and Brown, 2011).

4.3.2: Biomolecular palaeopathology

> "*The preservation of immunoglobulins is particularly intriguing, and opens up the possibility that an independent record of disease states remains in the bones of many individuals*" (Tuross, 1991:53).

Biomolecular and biochemical techniques have been used to address a variety of archaeological questions, ranging from the biological profiling of human skeletons using aDNA, to the investigation of the development of prehistoric dairying practices using residual protein analysis. Researchers have also been intrigued by how applications of biomolecular palaeopathology can be used to further our understanding of palaeoepidemiology and disease processes. Many applications have concentrated on attempting to recover bacterial and viral aDNA from archaeological material, such as tuberculosis (e.g., Baron et al., 1998; Braun et al., 1998; Fletcher et al., 2003; Muller et al., 2014), syphilis (e.g., Kolman et al., 1999; Bouwman and Brown, 2005; Barnes and Thomas, 2006), leprosy (e.g., Haas et al., 2000; Likovský et al., 2006; Schuenemann et al., 2013), plague (e.g., Drancourt et al., 1998; Haensch et al., 2010; Wagner et al., 2014), and typhoid (e.g., Papagrigorakis et al., 2006). Ancient DNA recovery has also been attempted on range of ancient helminths and parasites causing diseases such as toxoplasmosis (Terra et al., 2004), trypanosomiasis (Aufderheide et al., 2005), and intestinal roundworm (Oh et al., 2010). Many parasite aDNA studies have utilised either mummified human tissue or coprolites, with far fewer concentrating on bone. Biomolecular studies of ancient malaria in human remains are divided between the search for *Plasmodium* aDNA (e.g., Sallares and Gomzi, 2001; Chilvers, 2004; Nerlich et al., 2008; Pinello, 2008), human immunological reactionary products to malaria infection (e.g., Miller et al., 1994; Massa et al., 2000; Bianucci et al., 2008; Fornaciari et al., 2010), or both together (e.g., Setzer, 2010), in either skeletal or mummified tissues. Since this study will utilise immunological methodologies to

test for evidence of ancient *Plasmodium vivax* infection in archaeological bone, it is pertinent to explore the related research in order to form a viable sampling and testing methodology.

4.4: Malaria and ancient biomolecules

Despite the apparent ubiquity of malaria in the ancient and modern world, the disease is a frustratingly difficult one to identify in the archaeological record. This is partly due to the absence of specific macroscopically-observable skeletal indicators directly associated with malaria infection. On a molecular level, PCR-based research represents the bulk of attempts to identify the disease in archaeological remains, through the detection of malaria parasite aDNA. Yet only a small handful of samples out of hundreds tested have yielded positive results for *P. falciparum* aDNA (see Sallares and Gomzi, 2001, and Nerlich et al., 2008 for examples). *P. vivax* aDNA has yet to be isolated in archaeological bone samples, though attempts have been made (e.g., Pinello, 2008). Reasons for this apparent failure are likely threefold. Firstly, *P. vivax* infection is characterised by low parasitaemia, hence there are comparatively fewer circulating parasites entering the archaeological record within the bone (Brown and Brown, 2011). Secondly, active blood-stage infections do not last a particularly long time (compared to the *vivax* hypnozoite stage). The target individual must, therefore, have been suffering from an active infection at the time of death for the parasites to be present in the blood stream. Thirdly, despite an intimate knowledge of the parasitic genome, researchers simply do not know how well *P. vivax* DNA survives the numerous diagenetic processes following host death and interment. These three factors conspire to make *P. vivax* detection an extremely difficult and potentially expensive endeavour when utilising ‘traditional' PCR techniques. The recent emergence of next generation DNA sequencing techniques (including shotgun sequencing) may prove very useful in the search for elusive *P. vivax* aDNA (e.g., Rizzi et al., 2012). Recent success in confirming the presence of tuberculosis (Chan et al., 2013), leprosy (Schuenemann et al., 2013), and brucellosis (Kay et al. 2014) aDNA fragments in ancient tissue suggests a promising future for the detection of *Plasmodium vivax* aDNA.

A second avenue of biomolecular research that has been used to trace malaria in archaeological remains is the detection of antibodies to parasite antigens or parasite-derived

products of infection. The majority of these studies have concentrated on detecting the presence of *P. falciparum* histidine rich protein-2 (PfHPR-2) or *P. falciparum* lactate dehydrogenase (PfLDH) antigens using RDTs, ELISAs, or IFATs. PfHRP-2 is a water-soluble protein antigen released from parasitised erythrocytes, while PfLDH is "a soluble enzyme found in the glycolytic pathways of the malaria parasite and is produced by sexual and asexual stages" (Fornaciari et al., 2010:584). These biomolecules have been detected in either mummified (e.g., Massa et al., 2000) or osseous (e.g., Fornaciari et al., 2010) tissues. As PfHRP-2 and PfLDH are specific to *P. falciparum* infection, the search for *P. vivax* antigens tends to concentrate on the detection of parasite lactate dehydrogenase (pLDH), an enzyme produced by *P. vivax*, *ovale*, and *malariae* parasite during the erythrocytic stage of infection. Moving beyond parasite antigen detection, immunological methods to trace products of the human immune response (i.e., immunoglobulin antibodies) to ancient malaria infection have also been employed. The most commonly used biomolecular tests for pLDH and/or ancient antibodies are discussed below.

4.4.1: Rapid diagnostic tests (RDTs)

Combination-style RDTs currently offer the cheapest and quickest method of detecting pLDH in clinical settings, with species-specific pLDH RDTs being much more expensive (UNICEF, 2007). In clinical cases, circulating pLDH antigens have been detected for up to three weeks post-treatment, which potentially offers a window of detection in archaeological human remains (if the individual died during or shortly after infection). Unfortunately, a literature search yields no evidence of the use of these tests in the detection of *P. vivax* in such samples. One reason for this may be that combination RDTs cannot currently distinguish between the non-*falciparum* malaria species. However, this does not necessarily negate their use as diagnostic tools by researchers of past British malaria: *P. vivax* was almost certainly the exclusive agent of disease in question, so positive pLDH tests should indicate the presence of this species, rather than *P. ovale* or *malariae.* A second reason may be due to the relative insensitivity of RDTs, most of which require a parasitaemia level of at least 100 parasites/μL of blood for detection; archaeological bone may contain a significantly lower (if any) concentration of surviving, detectable pLDH antigens. Further to this, RDT's can only detect pLDH antigens for up to three weeks post-

infection. As with the detection of *Plasmodium* aDNA, the skeletal individual tested must therefore have died within a short period of infection, especially since antigen levels would drop significantly as those three weeks progressed.

Finally, as with all clinical immunological tests, RDTs are not designed to be used on ancient material. Manufacturers, quite understandably, do not test them for cross reactions with molecules found in association with ancient samples. One instance of RDT detection of ancient *falciparum* pLDH (Fornaciari et al., 2010) required the adoption of a significantly modified methodology to reach this apparently successful result. Even so, cross reactivity resulting in false positives are likely to remain an issue in RDT testing, particularly when poor controls are used, as seems to have been the case in aforementioned study. Strict controls, subsequent supplementary testing or protein profiling may help to resolve these issues, or help confirm a RDT diagnosis. Given the lack of research using RDTs to detect ancient *P. vivax* pLDH, it would be advantageous to attempt such detection using targeted British archaeological samples prior to the use of more sensitive tests, or as confirmatory tests, should positives be encountered elsewhere.

4.4.2 Enzyme-linked immunosorbent assays (ELISA)

ELISAs are successfully and frequently employed in modern medical and forensic fields, as well as in the clinical detection of malaria (e.g., Nam et al., 2010). The use of ELISAs on ancient human remains to detect the presence of malaria has a similar scope to that of RDTs, concentrating mostly on *P. falciparum* antigen detection in mummified tissue (e.g., Massa et al., 2000). The ELISA has been reportedly used successfully in the detection of *P. vivax* in Peruvian mummified tissue (Allison et al., 2009), although this research should perhaps be treated with caution, as it does not seem to have been peer reviewed.

As with RDTs, there is no evidence that ELISAs have been employed in the detection of *vivax* malaria (either pLDH or human antibodies to *vivax* infection) in archaeological bone. The reasons for this may be similar to some of those listed above for RDTs, particularly concerning the cross reactivity with untested molecules. The use of ELISAs in ancient malaria detection may prove to be a double-edged sword for a number of reasons. Firstly, one ELISA kit can test up to 96 samples, meaning that after a relatively large initial financial outlay, they are more economical on a per-sample basis than single-

sample RDT cartridges. Secondly, ELISAs are far more sensitive than RDTs in clinical applications and therefore have a greater potential to detect *vivax* malaria in archaeological samples. This increased sensitivity, however, also means a greater chance of cross reactions occurring with foreign biomolecules (see Brandt et al., 2002 for an example of this).

Despite potential drawbacks, ELISAs probably represent the immunological test of choice in detecting ancient *vivax* malaria due to high sample throughput at relatively low cost, the wide range of commercially available antibody/antigen combinations and high test sensitivity/specificity at low parasitaemia. Careful methodological design would be necessary to limit and characterise any possible cross contamination and false positives. Purification of target proteins may also prove useful prior to ELISA. This was shown in research by Kolman et al. (1999), who successfully purified archaeological antibodies (IgGs) from bone and tested them using ELISA against syphilis antigens, encountering positives with purified samples only.

4.4.3: Radioimmunoassay (RIA) and Immunofluorescence (IFAT)

The high sensitivity of RIAs offers the potential to detect tiny quantities of surviving target proteins. Early attempts were made at the modification of the standard biochemical methodology in order to detect ancient collagen and serum proteins (e.g., Lowenstein, 1980, 1981), although as with many early ancient biomolecular studies, diagenetic factors were only given cursory attention. The method is championed by Lowenstein, who has continued to modify the method (now known as protein radioimmunoassay, or pRIA), with apparent success, in a variety of archaeological and forensic applications (e.g., Lowenstein et al., 1991, 2006). Overall, little RIA research has been conducted on ancient pathogenic biomolecules, including those occurring in *vivax* malaria infection. This is probably due partly to inherent methodological issues, such as risks associated with radioactivity, problems with cross reactivity, and reliability issues in clinical detection of *vivax*-associated proteins (e.g., Avraham et al., 1983). This study will not use RIAs due to these problems.

IFATs are routinely used in the clinical detection of malaria, their high sensitivity proving particularly useful in the diagnosis of low-parasitaemia *vivax* malaria and in asymptomatic patients (Lee et al., 2011). The tests have also been employed in pathogen detection in archaeological remains. Le Bailly et al. (2008), for instance, used both IFAT and

ELISA to detect protozoan *Giardia intestinalis* antigens in ancient coprolites. Bianucci et al. (2008) used IFAT to test the same mummified child remains that Miller et al. (1994) and Cerutti et al. (1999) had earlier analysed using a ParaSight™-*F* test (an early type of RDT). Conflicting results were achieved: some samples testing positive by RDT gave negative IFAT results. Ancient DNA analysis on the same sample has also failed to detect evidence for *P. falciparum* infection (Sallares and Gomzi, 2001). This suggests that either the RDT gave false positive results, or the methodologies used in the earlier studies were suspect (or both). IFATs are certainly more sensitive than RDTs, which increases their chance of detecting low quantities of antigen while also increasing the possibility of cross reactions (RDTs may completely miss samples with low parasitaemia). As with Western blot tests, IFAT methodologies in testing ancient samples are more standardised than newer techniques and should be considered a potentially useful tool in the detection of ancient *vivax* malaria, although as with all immunological tests, the issue of diagenetic alteration must be accounted for.

4.4.4: SDS-PAGE and Western blot tests

SDS-PAGE and subsequent Western blotting are not frequently employed in the clinical detection of malaria, yet they are some of the most commonly used biomolecular techniques in the characterisation of ancient proteins. SDS-PAGE is designed to separate out proteins within a sample by molecular weight and are often used as a fore-runner to down-stream protein identification and characterisation techniques, such as antibody/antigen reactivity immunoblotting (e.g., Wiechmann et al., 1999; Torres et al., 2002; Schmidt-Schultz and Schultz, 2004, 2007), or mass spectrometry (e.g., Ostrom et al., 2000; Boros-Major et al., 2011). SDS-PAGE and Western blot testing are relatively cheap and fast to perform, allowing for the testing of multiple samples at once. The methodologies involved in testing ancient bone samples using SDS-PAGE and Western blots are some of the more standardised within biomolecular archaeology. The main problem for ancient protein separation and analysis using electrophoresis and immunoblotting is that diagenetic processes may alter molecular weights and morphologies, thus giving unexpected results. Degradation may also obscure antibody/antigen interactions, leading to false positives or false negatives in Western blotting. Despite this, the general ease and standardisation of such

tests mean that they should certainly be considered for use when testing archaeological bone for proteins associated with *vivax* malaria infection.

4.4.5: Palaeoproteomics and protein profiling

Palaeoproteomics refers to a collection of relatively new methods derived from biomolecular proteomics for studying ancient proteins. The methods are often based on the separation and subsequent profiling of the entire protein complex (proteome) of a given sample, and are hence known collectively as ‘protein profiling' techniques. Protein profiling offers an advantage over antigen/antibody immunological detection in that the former does not require the survival of functional protein epitopes, thereby avoiding the problem of cross reactions and false negatives/positives. In protein profiling every protein in a sample is isolated, from which single proteins can be selected and studied using amino acid characterisation (Brown and Brown, 2011). Initial profiling can be achieved by traditional SDS-PAGE, Western blot test (either electrophoresis or isoelectric focusing), or by various chromatographic methods, such as ion-exchange, gel filtration (Ó’Fágáin et al., 2011; Karlsson and Hirsh, 2011), and reversed phase high performance liquid chromatography (rpHPLC). Ostrom et al. (2000), for instance, used a combination of Western blot, RIA, and rpHPLC to isolate protein in archaeological bone samples. They followed this by structural characterisation of the protein by matrix-assisted laser desorption ionisation (MALDI), "a relatively new ionization technique that is capable of ionizing a large variety of compounds, particularly large proteins, for analysis by mass spectrometry" (Harvey, 2005:386).

The use of MALDI mass spectrometry to recover a "sequence information from subpicomolar quantities of fragmented proteins and peptides" (Boros-Major et al., 2011:197) means that the technique is potentially an appropriate method for characterising degraded ancient proteins, which can subsequently be identified by matching to specific peptide (amino acid sequences specific to individual proteins) mass fingerprints (PMFs). Such analysis has recently been reported as successful in the identification of tuberculosis and osteogenic sarcoma proteins in archaeological human skeletal remains using MALDI tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS) (Boros-Major et al., 2011). These studies suggest that proteomics offers great potential in the analysis of other pathological organisms in future research. Caution should, however, be taken in the

interpretation of ancient protein profiling results: given the novelty of palaeoproteomics as a field of biomolecular archaeological research, procedural protocols in the profiling of ancient proteins are currently non-standardised and little is known about how diagenetic changes may alter specific PMFs (Brown and Brown, 2011). Cutting-edge methods of palaeoproteomics analysis using extremely sensitive nanoflow liquid chromatography peptide sequencing (nLC-MS) are beginning to address the problem of identifying diagenetic changes to ancient proteins and are opening broad new avenues of biomolecular investigation. These recent technological advances have opened up exciting new avenues of research into ancient non-collagenous proteins (e.g., Cappellini et al., 2012; Wadsworth and Buckley, 2014). This technology provides a potentially important method of detecting and characterising surviving peptides of extremely low-abundance, a category into which many archaeological malaria-related proteins must surely fit. However, as always, "rigorous scientific discipline is needed in the exploitation of new techniques" (Minnikin et al., 2010).

Although proteomic methods could be used in the search for biomolecular evidence of ancient *P. vivax* infection, such as parasite antigenic proteins and different human response immunoglobulin types, the high expense currently precludes an extensive investigation using this technique. Proteomics could, however, provide initial positives for target protein identification, or important confirmatory testing should malarial positives be encountered in other analyses,

4.4.6: Haemozoin detection

The detection of the *Plasmodium* biocrystalline waste product haemozoin as a marker of malaria infection is a relative newcomer to the field of clinical malariology. It is therefore no surprise that only one (unsuccessful) attempt has been made at such detection in archaeological bone (see Setzer, 2010). Given the novelty of the application in palaeopathological analysis, there is no published research on the effect of diagenesis on the survival of haemozoin in the burial environment, although insoluble biocrystals such as haemozoin should, theoretically, be more resistant to some diagenetic processes than proteins and DNA. Clinical techniques, using similar equipment to that used in protein profiling, or flow cytometry, may potentially be adapted for analysis of archaeological bone samples, and it may prove advantageous to attempt such analyses, given the potential for

survival of haemozoin in archaeological bone (Setzer, 2010). Histological examination of bone thin-sections prior to analysis may be useful as this may allow for both an evaluation of the state of bone preservation (as suggested by Schmidt-Schultz and Schultz, 2004 for all biomolecular palaeopathological studies). Thin-sectioning may also allow for direct microscopic visualisation of haemozoin in the ancient bone sample, particularly given the recent advancements in erythrocyte visualisation (Setzer et al., 2013).

4.5: Summary

Biomolecular analysis offers vast potential in the detection and characterisation of ancient diseases that leave no visual evidence of their presence in human bone. This potential has been recognised by researchers who have continued to push the limits of biomolecular palaeopathological techniques in the search for disease-related biomolecules in archaeological remains. The discipline has matured from early, often controversial attempts at ancient organic residue analyses in the 1970s and 80s, to the recent use of high-resolution mass spectrometry technology to give a more complete protein profile from ancient organic samples. Each of the discussed techniques that have been employed in the detection of ancient biomolecules has advantages and disadvantages (see summary Table 4.1). Every methodology is affected, to a greater or lesser extent by uncertainties concerning the degradation of target biomolecules. Diagenetic processes are highly variable, dependent on multiple factors within the burial environment (Nielsen-Marsh and Hedges, 2000), and it is therefore extremely difficult to predict how each individual sample will be affected. Traditional aDNA and immunological methods are particularly vulnerable to biomolecular contamination or degeneration, which can give confounding results. Sallares and Gomzi (2001:199) state that "…immunological methods as applied so far to ancient malaria still seem to suffer from a problem with specificity. When a positive result is obtained, it is impossible to be sure what the antibody is actually reacting with", a difficulty common to all immunological tests of ancient bone for evidence of ancient pathogens. The problem is such that it has even been suggested that immunological tests "should only be regarded as a useful screening technique" and that "confirmatory studies will always be required" (Child and Pollard, 1992:45), sentiments echoed by Gernaey et al. (2001). Modern analytical techniques, such as high-resolution mass spectrometry and haemozoin detection, may be less

susceptible to this complication in that they avoid the cross-contamination issues associated with immunological testing. However, these newest techniques are not invulnerable to the problems of diagenesis and remain somewhat untested on ancient samples.

Method	Target Biomolecule	Advantages	Disadvantages	References
'Traditional' aDNA	DNA - Human or parasite	Established method; high sensitivity and specificity. Next generation sequencing looks very promising.	Expensive; diagenesis; contamination issues	Taylor et al., 1997; Sallares & Gomzi, 2001; Chilvers, 2004; Pinello, 2008;
ELISA	Antigens/ Antibodies	96 samples per test; high sensitivity; high specificity	Diagenesis; untested on ancient *P. vivax*; possible cross-reactivity	Cattaneo et al., 1992; Kolman et al., 1999 Massa et al., 2000; Brandt et al., 2002; Le Bailly et al., 2008
IFAT	Antibodies to parasite antigens	High sensitivity	Cross-reactivity; Diagenesis	Bianucci et al., 2008; Le Bailly et al., 2008
Light microscopy	Parasites/ infected cells	High specificity; may identify poor preservation/diagenetic alternation	Molecules not usually visible microscopically	Wickramasinghe & Abdalla, 2000; Schmidt-Schultz & Schultz, 2004
Haemozoin detection	Haemozoin biocrystals	Potentially stable/ insoluble biomolecule; high specificity and sensitivity	Expensive; relatively untested; no established methodology	Setzer, 2010
Protein profiling	Surviving protein peptides	No false negatives; high sensitivity and specificity	Expensive; relatively untested; no standardised methodologies	Ostrom et al., 2000; Boros-Major et al., 2011; Cappellini et al., 2012
RDT	Parasite antigens	Cheap; easy to perform	Conflicting results; low sensitivity; diagenesis	Miller et al., 1994; Fornaciari et al., 2010
RIA	Antibody/ antigen	High sensitivity	Questioned reliability; hazardous	Gurtler et al., 1981; Lowenstein 1981; Lowenstein et al., 2006
Western blot	Antigens or antibodies	Easy to perform; relatively cheap; established methodology	Diagenesis; expensive if using high antibody quantities to probe	Ascenzi et al., 1985; Grupe & Turban-Just, 1998; Wiechmann et al., 1999; Torres et al., 2002; Schmidt-Schultz & Schultz, 2004 & 2007

Table 4.1: Summary of major methodologies employed in pathogen biomolecular palaeopathology, with advantages and disadvantages.

A possible solution may lie in a multifactorial approach to the search for ancient malaria in Britain, such as attempted by Setzer (2010) in her examination of Sardinian skeletons. Macroscopic palaeopathological analysis should form the basis of sample selection for biomolecular testing, followed by evaluation of the bone preservation through

histological analysis. Macroscopic (and possibly radiographic) identification of conditions that may be indirectly related to malaria infection, such as stress-induced skeletal markers (e.g., enamel hypoplasia), and adaptive genetic polymorphisms (e.g., the thalassaemias) would supply important lines of evidence upon which possible biomolecular testing would be based. The use of a number of targeted techniques such as these will help to identify sample targets of known preservation levels, thus giving the highest chance of detecting and characterizing *P. vivax*-associated biomolecules. This exploration of the most commonly employed methodologies in the fields of palaeopathology and biomolecular palaeopathology is useful in that it presents a firm base from which to choose the most appropriate methodologies for this study of *P. vivax* infection in past British populations.

4.6: Protein extraction from archaeological bone

> *"In a good preservation state, bone conserves intact collagen molecules and many, if not all, NCPs from recent and ancient times"* (Schmidt-Schultz and Schultz, 2004).

A major challenge in the detection and characterisation of archaeological bone proteins is the successful extraction of target proteins from the bone matrix. During life and after death negatively charged non-collagenous proteins (particularly glycoproteins) adsorb to bone hydroxyapatite, where they are afforded additional protection from diagenetic factors following initial decomposition by the hardly-soluble calcium-phosphate mineral (Wiechmann et al., 1999). The challenge lies in the successful removal of these tightly bound proteins from the bone matrix, and their preservation and conservation during the extraction process.

Research over recent decades has attempted to refine protein extraction techniques. This is likely due to an increasing appreciation of the role that diagenesis plays in the degradation of ancient proteins and the associated methodological difficulties in archaeological protein detection (Child and Pollard, 1992; Child, 1995; Collins et al., 2002). Increasingly, methodological refinements have turned to clinical techniques of protein extraction, with an emphasis on the reduction of protein degradation during the extraction process (Schmidt-Schultz and Schultz, 2004). Prior to this, these seems to have existed a latent assumption that any equilibrium reached by archaeological bone and their endogenous proteins with their surrounding burial environment, in terms of diagenesis, would remain in

place during the extraction process (i.e., the target proteins would not further degrade during extraction). It has, however, been suggested that mechanical grinding of bone prior to extraction causes the equivalent damage to collagen of 5000 years of burial (Collins and Galley, 1998). Clearly any processing steps taken that may reduce the likelihood of protein degradation should be considered. Since there remains insufficient research on the deleterious effects of extraction processes on surviving archaeological non-collagenous proteins, a move towards preventative clinically-based methodologies seems advisable.

4.6.1: Selection of target biomolecule(s)

Identification of the most appropriate *P. vivax*-associated biomolecule is crucial in this attempt to detect the disease in archaeological bone. The choice can be quickly narrowed to either endogenous biomolecules (e.g., antibodies synthesised by the body in response to infection) or exogenous biomolecules (e.g., those introduced into the host by the mosquito vector, or associated with the invading parasite, such as DNA or haemozoin). Extraction of endogenous antibodies has advantages over and above the search for exogenous antigenic molecules or pathogenic aDNA. Host antibodies (mostly immunoglobulin G, or IgG) to *vivax* infection have been clinically shown to circulate and remain at a stable titre (serum concentration) for an extended period in the absence of reinfection (Wipasa et al., 2010), long after exogenous pathogenic biomolecules have been removed from the body. This may be one explanation for Pinello's (2008) unsuccessful attempt at detecting *P. vivax* aDNA. If antibodies are retained in the bone, they represent a far more stable target for analysis than pathogenic molecules, which are, by nature, transitory.

Immunoglobulin G antibodies represent the most abundant antibody class, accounting for approximately 75% of serum immunoglobulins in healthy individuals (Meulenbroek and Zeijlemaker, 1996). Non-collagenous proteins, including immunoglobulins, exhibit a high affinity for bone hydroxyapatite, to which they strongly adsorb during life (Masters, 1989; Wiechmann et al. 1999; Schmidt-Schultz and Schultz, 2004). Clinical research has shown that adsorptive nature of hydroxyapatite can concentrate certain non-collagenous proteins, including immunoglobulins, to levels above that found in plasma. This concentration of antibodies within the bone mineral likely provides an

increased resistance to infection (Omelyaneko et al. 2013). IgGs should, therefore, concentrate within the bone mineral and have a chance of surviving the effects of diagenesis after death and burial, making them a potentially viable and valuable target for biomolecular studies of *vivax* malaria. Wipasa et al.'s (2010) confirmation of long-lived anti-*vivax* antibodies strengthens the case for selecting IgGs as the best target biomolecule for this study, since the target individual would not necessarily have to have died during, or immediately following, active *vivax* infection in order to detect the presence of the disease.

CHAPTER 5: MATERIALS AND METHODS

This chapter details and justifies the samples chosen and the various methodological approaches employed in the attempt to identify the presence of *P. vivax* malaria in British antiquity from skeletal remains. It firstly outlines the recently established skeletal sequelae of thalassaemia that may have been misidentified in pathological and skeletal reports from sites selected for this study, before listing these sites and justifying their inclusion. Following this is a brief discussion of the criteria for selecting sites for palaeodemographic analyses of Fen and non-Fen cemetery populations. Thirdly, the selection criteria for skeletal individuals and bone elements for biomolecular analyses are detailed, along with the methods used for initial sample preparation and histological analysis. The methods used in five separate protein and immunoglobulin extraction protocols and subsequent techniques employed in protein characterisation are then detailed. Finally, the procedures used in rapid testing for malaria antigens are presented.

5.1: Identification of cases of genetic anaemia in skeletal reports

In light of the recent additional diagnostic criteria for thalassaemia in skeletal tissue provided by Lewis (2010), it was decided to examine selected palaeopathological reports for any missed or misidentified evidence of the condition. Reports from the sites selected (section 5.3.1) for anti-malaria antibody identification were examined, since these are the populations most likely to develop conditions intimately associated with exposure to malaria (Hume et al., 2003). As discussed in section 4.2.9, thalassaemia intermedia is the most likely encountered form of the disease, since homozygotes (thalassaemia major) would invariably die before developing diagnostic skeletal changes, and thalassaemia minor may not necessarily present with skeletal modifications at all (Ortner and Putschar, 1981; Lewis, 2010). Historically, those with thalassaemia intermedia could potentially survive well into adulthood without medical support, exhibiting varying degrees of sequelae, which often worsen with age (Lagia et al., 2007; Lewis, 2010).

Attempts have been made to identify more specific skeletal indicators of thalassaemia in ancient populations, beyond the 'traditional' non-specific markers (e.g., *cribra orbitalia* or porotic hyperostosis). Ortner and Putschar (1981:252), for instance, suggest that "bone lesions in thalassemia are entirely due to increased spatial demands for

the hyperplastic erythropoietic marrow", which affect the crania and facial bones most seriously, followed by long bone epiphyses, ribs, vertebral bodies, metacarpals/tarsals, and phalanges. Hershkovitz et al. (1997) suggest premature epiphyseal fusion (particularly affecting the distal leg long bones and proximal humerus) as being pathognomonic of thalassaemia, while Lewis (2010) identifies costal osteomas and the 'rib-within-rib' phenomenon as likely criteria for diagnosing the condition. Unfortunately, the latter is only visible radiographically, and would, therefore, not necessarily be recorded during routine palaeopathological analyses. However, records of rib hypertrophy, or of ribs with multiple healing 'fractures' may be suggestive of thalassaemia.

Table 5.1 lists possible sequelae that may, in combination, be suggestive of thalassaemia in skeletal material. Due to the non-specific nature (e.g., scoliosis) and relatively high frequency (e.g., multiple rib fractures) of many of these, only individuals displaying two or more sequelae were marked for further analysis. In cases where a combination of suggestive skeletal changes was encountered in the selected palaeopathological reports (see section 6.1), every effort was be made to obtain appropriate bone samples for further macroscopic and radiographic inspection. It should be noted, however, that at the time of the research, the Cambridgeshire Archaeological Store (archive), which possesses the bulk of samples chosen for this study, was in the process of relocation. Consequently, access to samples of potential interest from these sites was precluded (other than Littleport, which is yet to be archived).

Sequelae
Porotic hyperostosis
Lateral orbit displacement
Sunken nasal bridge
Maxillary swelling
Cranial diploë thickening
'Hair-on-end' appearance in cranial diploë
Osteopenia
Sternal widening
Premature epiphyseal plate closure
Epiphyseal/metaphyseal widening
Femoral fractures
Rib osteomas
'Rib-within-a-rib'
Rib cortical erosion
Multiple rib fractures
Scoliosis

Table 5.1: Selected skeletal sequelae of the thalassaemias, from Ortner and Putschar (1981), Ortner (2003), Hershkovitz et al. (1997), Lagia et al. (2007), Lewis (2010), Yochum and Rowe (2005), and Perisano et al. (2012). Bold sequelae are cited as more specific for thalassaemia.

5.2: Demographic analysis of Fen/marshland and non-Fen/marshland cemetery populations

As briefly discussed in section 4.2.11, palaeodemographic analysis of cemetery populations from potentially malarious and non-malarious areas may prove useful in the search for indirect evidence of *P. vivax* malaria. For these analyses, a number of Fen and non-Fen cemetery sites were selected based on the criteria defined below in section 5.2.1. Total and period specific mortality profiles, survivorship, and probability of death (the latter two after Chamberlain, 2006) were constructed, and statistical tests (chi-square and Kolmogorov-Smirnov) were then applied, where appropriate, in order to investigate any significant relationships between environment and cemetery demographics.

5.2.1: Cemetery selection

Geographic location was of paramount importance for selecting sites, and analysis was dependent upon the availability of published skeletal reports containing sufficient

demographic detail. Fen-associated inhumation cemeteries needed to be either within the Fens, close to the Fen edge, or in close proximity to marshlands, river flood plains or tidal estuaries. The sites chosen were all associated with the historically recorded presence of malaria vector *anopheline* species (Nuttall et al., 1901), and were within an appropriate distance (approximately 3-12km, depending upon mosquito diet) of likely *anopheline* breeding grounds (Kaufmann and Briegel, 2004). Furthermore, many of the selected sites representing the Anglo-Saxon period have been identified as potentially malarious, based on topography and *cribra orbitalia* prevalence (Gowland and Western, 2012). Conversely, non-Fen associated inhumation cemetery sites were selected based on their location away from *anopheline* breeding grounds, and with no recorded vector presence.

A further prerequisite in terms of the type of cemetery selected was population density: only rural and semi-urban (e.g., *Durobrivae*) cemeteries were chosen. It is not valid to compare urban and non-urban cemetery populations, given the differential epidemiological pressures, which may result in markedly different mortality profiles. However, there is a general a dearth of urban centres associated with Fens and marshlands and this forced a natural reliance upon rural and semi-urban cemeteries. The selected Fen-associated cemetery sites are listed in summary Table 5.2, while the summary in Table 5.3 shows non-Fen associated sites. Full tables (A2.1 and A2.2) are provided in Appendix 2. Table 5.4 displays the total number of Fen/non-Fen individuals from each period in these locations.

Site	Period	Site	Period
Durobrivae, Cambs	Roman	Market Deeping Bypass, Cambs	Anglo-Saxon
Hoplands, Sleaford, Lincs	Roman	Monkton, Kent	Anglo-Saxon
Prickwillow Road, Ely	Roman	Quarrington, Lincs	Anglo-Saxon
The Parks, Godmanchester	Roman	Ramsgate, Kent	Anglo-Saxon
Watersmeet, Huntingdon	Roman	Rivenhall, Suffolk	Anglo-Saxon
Barton Bendish, Norfolk	Anglo-Saxon	Snodland, Kent	Anglo-Saxon
Baston, Lincs	Anglo-Saxon	St Peter's, Barton-upon-Humber, Lincs	Anglo-Saxon
Burgh Castle, Norfolk	Anglo-Saxon	Staunch Meadow, Suffolk	Anglo-Saxon
Caistor-on-Sea, Norfolk	Anglo-Saxon	Thetford, Norfolk	Anglo-Saxon
Castle Mall, Norwich	Anglo-Saxon	Ulwell, Dorset	Anglo-Saxon
Castledyke South, Lincs	Anglo-Saxon	Westfield Farm, Ely	Anglo-Saxon
Cleatham, Lincs	Anglo-Saxon	Orchard Lane, Huntingdon	Medieval
Edix Hill, Cambs	Anglo-Saxon	Rivenhall, Suffolk	Medieval
Haddenham, Cambs	Anglo-Saxon	St Peter's, Barton-upon-Humber, Lincs	Medieval
Highfield Farm, Littleport	Anglo-Saxon	Stonar, Kent	Medieval

Table 5.2: Fen sites selected for demographic analysis.

Site	Period	Site	Period
Ancaster, Lincs	Roman	Empingham II, Rutland	Anglo-Saxon
Babraham Institute, Cambs	Roman	Filton, Bristol	Anglo-Saxon
Bainesse Farm, Catterick	Roman	George Street, Aylesbury	Anglo-Saxon
Baldock, Herts	Roman	Great Chesterford, Cambs	Anglo-Saxon
Mangiovinium, Bucks	Roman	Henley Wood, Berks	Anglo-Saxon
Newarke Street, Leicester	Roman	Mill Hill, Kent	Anglo-Saxon
Queenford Farm, Oxon	Roman	Norton, Cleveland	Anglo-Saxon
Rudston Villa, Yorks	Roman	Orpington, Kent	Anglo-Saxon
St. Albans, Herts	Roman	School Street, Ipswich	Anglo-Saxon
Barnstaple Castle	Anglo-Saxon	Sewerby, Yorks	Anglo-Saxon
Buckland, Kent	Anglo-Saxon	West Heslerton, Yorks	Anglo-Saxon
Castle Green, Hereford	Anglo-Saxon	Blackfriars Street, Carlisle	Medieval
Charlton Plantation, Wilts	Anglo-Saxon	Brighton Hill South, Hamps	Medieval
Coddenham, Suffolk	Anglo-Saxon	Corbridge, Northumberland	Medieval
Darenth Park, Kent	Anglo-Saxon	Wharram Percy, Yorks	Medieval
Dunton Green, Kent	Anglo-Saxon		

Table 5.3: Non-Fen sites selected for demographic analysis.

Fen sites		Non-Fen sites	
Period	Individuals	Period	Individuals
Roman	173	Roman	704
Anglo-Saxon	1909	Anglo-Saxon	1385
Medieval	1170	Medieval	859
Total	3252	Total	2948

Table 5.4: Number of Fen/non-Fen individuals from each period.

As can be seen in Tables 5.2 and 5.3, there is a distinct weighting towards Anglo-Saxon sites, particularly in the Fen category. This is, in part, an artefact of the availability of published site reports and skeletal inventories. It is also a reflection of the Fenland settlement patterns briefly discussed in Chapter 2, providing a demonstration of the increasing Anglo-Saxon settlement and utilisation of the Fens. The small number of Fen Roman sites may be as much due to burial practice as it is to a relatively limited presence in Fen environments (discussed in section 7.1.2(iii)). A reduced number of medieval cemeteries may be due to the tendency for churchyard burial in this period, and the associated difficulties in archaeological excavation and analysis of such populations. In both categories there are cemeteries that yielded large numbers of individuals. St. Peter's Church in Barton-upon-Humber, for instance, accounts for 728 Anglo-Saxon and 849 medieval Fen burials, while Wharram Percy yielded 645 medieval non-Fen individuals. The possible skewing effects of these large populations were investigated by running alternate analyses with these two sites included and removed.

It should also be noted that the reports chosen for palaeodemographic analysis were compiled aver an extended time period (the earliest dating back to 1973) by numerous palaeopathologists, using a range of methods for analysing human skeletal remains. A problem (discussed in 7.1.2(iii)) arises when attempting to compare populations which are recorded using non-standardised and poorly reported methods. For instance, some reports chosen for this study (e.g., Waldron, 2007) explicitly state and cite the methods employed for biological profiling of skeletons (e.g., estimating age, sex, stature, etc.), while others (e.g., Anderson, 1996) offer very limited information. Robert and Cox (2003:399) collected data from over 30,000 British skeletons from 324 site reports, and found the reported age-at-death data "too problematic to use in association with the palaeopathological data." The methods used for biological profiling of skeletal remains have evolved over the past few decades in response to calls for standardisation in skeletal analysis. This has resulting in the introduction of texts (e.g., Buikstra and Ubelaker, 1994; Brickley and McKinley, 2004) and initiatives (e.g., The Global History of Health Project: http://global.sbs.ohio-state.edu/), which may help palaeopathologists work towards a goal of standardisation in reporting, and increase the ease and validity of cross-study comparisons. However, since this study will access reports representing a variety of non-standardised reporting methods, it is hoped that

the use of large sample sizes and broad age categories should mitigate for some of the issues arising (see age category Table 6.2, Section 6.2.1)

5.3: Biomolecular testing for evidence of *vivax* malaria

5.3.1: Site selection

The primary focus of this study is the biomolecular analysis of skeletal remains to provide direct evidence for the presence of *Plasmodium vivax* malaria. A number of criteria needed to be met for the selection of sites that may yield individuals suitable for biomolecular analysis. Firstly, site location was based on the criteria for selecting Fen sites listed above in 5.2.1. Figures 5.1 and 5.2 shows the location of the South Lincolnshire and Cambridgeshire, and North Lincolnshire sites, respectively. Secondly, the chosen sites needed to have yielded skeletons that had been inventoried, were accessible (i.e., archived, or in the processes of being archived), and were sufficiently preserved for sampling. Permission for destructive analyses was also required for each site. Sites are listed by period. Each is briefly introduced and the prevalence rates of the non-specific pathologies *cribra orbitalia*, enamel hypoplasia, and porotic hyperostosis are listed, where available. Summary tables 5.5, 5.6, and 5.7 also display prevalences of pathologies (where available) at the end of each period section. Prevalence rates for skeletal pathologies are usually recorded as being either Crude (CPR), or True (TPR). CPR, as its name suggests, is a crude estimate of pathological prevalence, regardless of preservation, within a population based on the percentage of individuals displaying the pathology. TPR gives a more accurate representation of disease prevalence, since it records the number of elements preserved, rather than individuals, affected by a pathology, thereby controlling for skeletal completeness. As can be seen in Tables 5.5, 5.6, and 5.7, CPR is far more commonly reported.

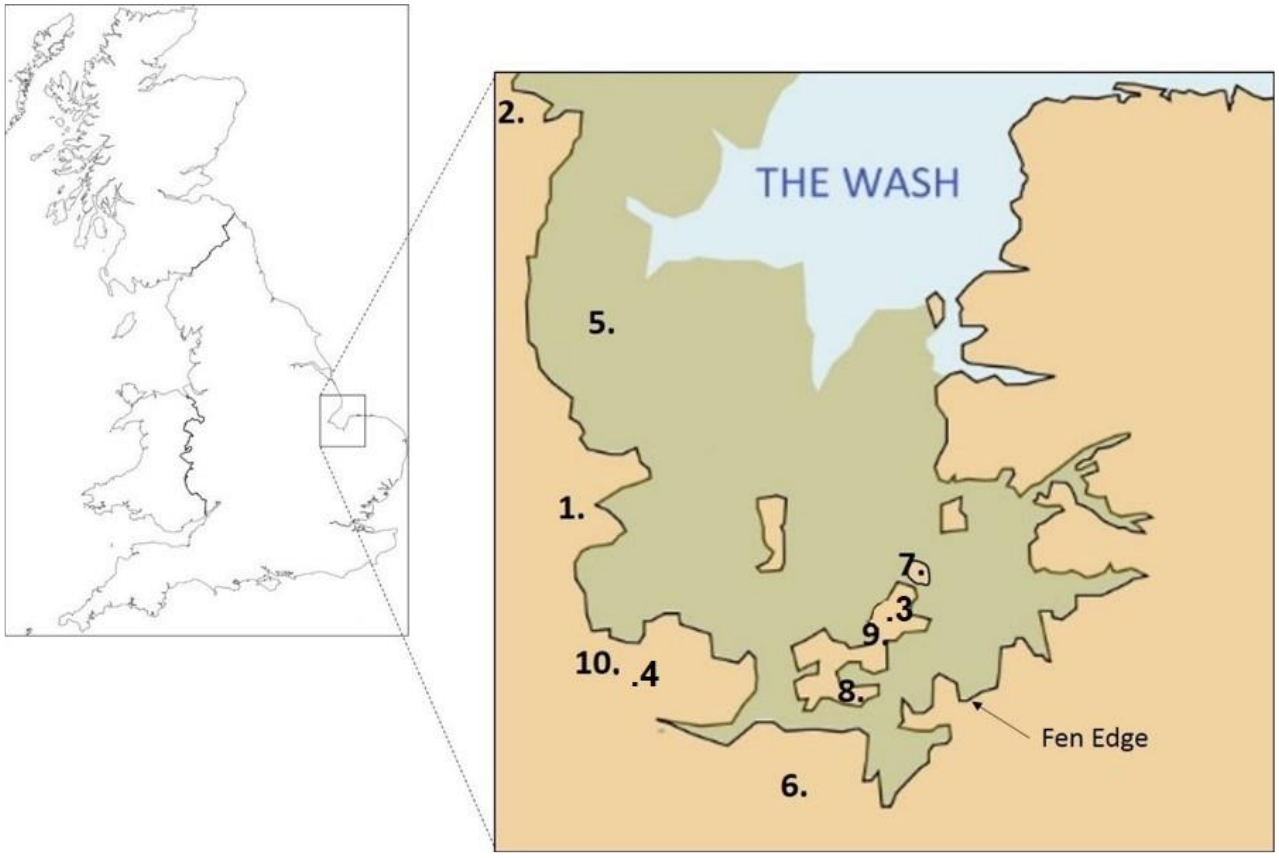

Figure 5.1: Relationship of south Lincolnshire and Cambridgeshire sites to the Fens. 1 – *Durobrivae*; 2 – The Hoplands, Sleaford; 3 – Prickwillow Road, Ely; 4 – The Parks, Godmanchester; 5 – Baston; 6 – Edix Hill; 7 – Highfield Farm, Littleport; 8 – Haddenham; 9 – Westfield Farm, Ely; 10 – Watersmeet and Orchard Lane, Huntingdon.

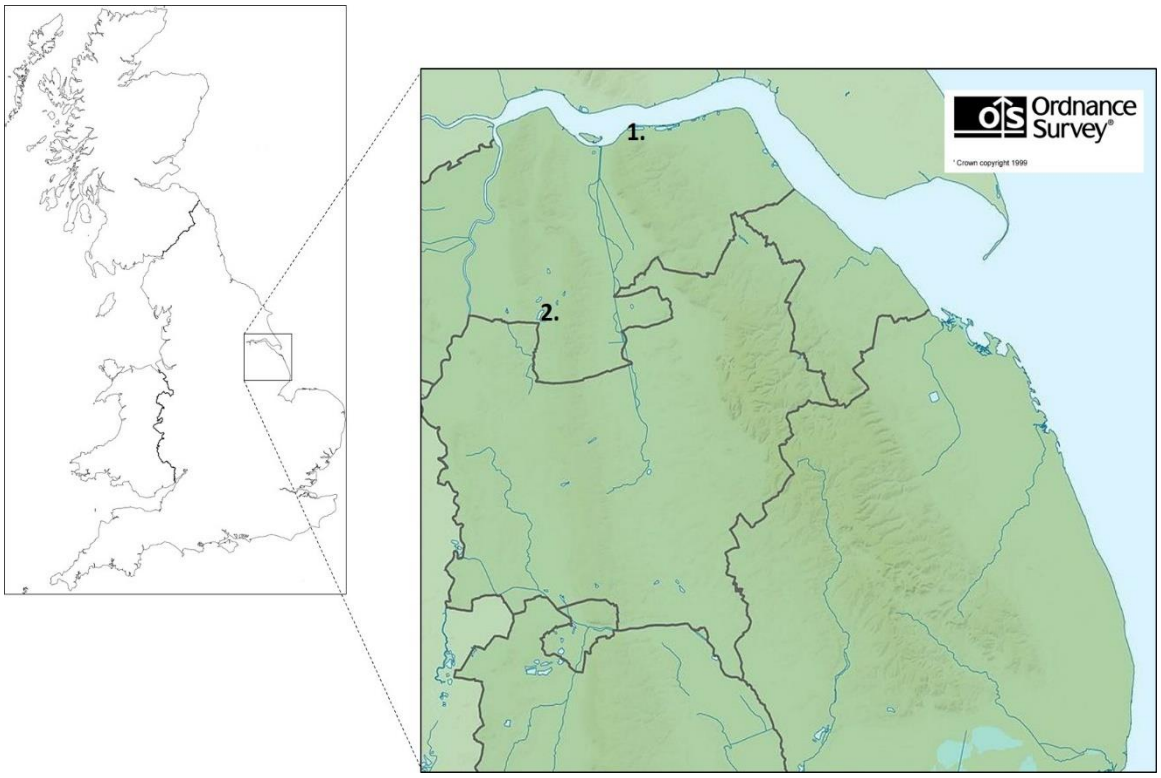

Figure 5.2: North Lincolnshire site locations. 1 – Castledyke, Barton-upon-Humber; 2 – Cleatham.

5.3.2: Romano-British sites

5.3.2(i): *Durobrivae*, Cambridgeshire

The site of the Roman town of *Durobrivae* is situated in the north eastern corner of the modern county of Cambridgeshire to the immediate east of the village of Water Newton, and approximately two miles to the west of modern Peterborough (Figure 5.1). The Cambridgeshire Fen edge lies to the immediate east and northeast of the site.

The town site itself remains largely unexcavated, concealed beneath pastures and protected from disturbance by its scheduled status. Despite this, much has been inferred about the history of *Durobrivae* and its surroundings. The foundation of the settlement likely occurred in the mid-1st century AD in the form of a small fort on the south bank of the River Nene, probably constructed to control an important early crossing point. This later became the point at which Ermine Street crossed the Nene (Fincham, 2004). The Fen Causeway, an important road running eastwards thorough the Fens into Norfolk via the raised Fen islands, was constructed in the late 1st century (Fincham, 2004). This represents the first known

major attempt to access the previously impassable central Fens, and perhaps alludes to the importance placed by the Romans on Fenland resources, such as salt.

Following the withdrawal of military occupation around 100 AD, the *vicus* surrounding the fort rapidly grew along the line of Ermine Street. Its location on a navigable watercourse and major thoroughfare influenced the growth of *Durobrivae* to the extent that it had become an important centre for trade and industry by the late 3rd century (Fincham, 2004). It has even been suggested that *Durobrivae* functioned as a *civitas* capital, given the size of the fortified settlement and importance of its pottery industry (Wacher, 1975). Given its location, it is likely that *Durobrivae* played a very important role in the market economy of the Fens during the 2nd and 3rd centuries. Individuals who harvested the resources of the central and southern Fens would have had close links to *Durobrivae*, possibly even setting up permanent domiciles around the town and working in the Fens on a seasonal basis (Fincham, 2004). It should be expected, then, that the population of *Durobrivae* would have been familiar with the health-related risks associated with Fen-edge occupation, particularly those individuals who made a living accessing the rich Fen resources.

The cemetery of *Durobrivae* was located outside the southwest gate of the town defences, and was originally discovered in the 18th century. A major portion of the cemetery was excavated by Cambridgeshire County Council Archaeological Field Unit in 1999 in advance of disturbance by motorway maintenance work. More than 50 inhumations were revealed, with grave goods dating the life of the cemetery to the later 3rd to early 5th centuries (Casa Hatton and Wall, 1999). The individuals were generally well preserved and exhibited a typical range of pathologies for a semi-urban Roman population, including manifestations of physiological stress in the forms of *cribra orbitalia* (3.7% CPR) and enamel hypoplasia (3.7% CPR) (Duhig, 1999).

5.3.2(ii): The Hoplands Business Centre, Sleaford, Lincolnshire

The town of Sleaford is located in central Lincolnshire, approximately 30km south of Lincoln (Figure 5.1). With little evidence of prior prehistoric activity, Old Sleaford probably had its origins in the middle to late Iron Age. Its position on the navigable Slea River on the western edge of the resource-rich silt Fens would have been a vital factor governing the founding and initial growth of the settlement (Elsdon and Jones, 1997). Archaeological

evidence suggests that by the early Roman period, the town had increased in significance and prospered, possibly continuing into the Anglo-Saxon periods as an important centre for the production and trade of salt and pottery (Elsdon and Jones, 1997; Bradley-Lovekin, 2005; Vince, 2006; Willis, 2006). It has, however, been suggested that Old Sleaford diminished in importance in the 3rd century due to the increasing success and prosperity of *Durobrivae* approximately 40km to the south (Fincham, 2004). Indeed, previous investigations have revealed 3rd to 4th century deposits suggestive of partial abandonment of Old Sleaford (Western, 2011).

The Hoplands site is situated adjacent to a Roman road and close to the centre of the Iron Age and Roman settlement of Old Sleaford, which itself lies beneath the eastern part of the modern town. The site was excavated in 2008/2009 by Archaeological Project Services in advance of commercial development (Murphy, 2011). Excavations revealed part of a nucleated 3rd to 4th century cemetery comprising 53 graves, which yielded 54 inhumed individuals. The skeletal population exhibited a very high prevalence of *cribra orbitalia* (37% CPR/61.5% TPR). This is compared to a 12.5% CPR at neighbouring Ancaster (upland), and the general Romano-British 13.5% CPR reported by Roberts and Cox (2003). This may support the association of Fen locality and high *cribra orbitalia* prevalence suggested by Gowland and Western (2012). The population also displayed a high prevalence of enamel hypoplasia (42.6% CPR/14.2% TPR) compared to 6.7% CPR at Ancaster and a national average of 9.64% CPR (Western, 2011). These elevated figures suggest that the Hoplands population was subject to adverse conditions/environs that promoted severe metabolic stresses during childhood, possibly in the form of parasitic infection and vitamin deficiency (Western, 2011). The proximity of the Fen edge to the settlement may well have had a negative effect on the overall health of this population.

5.3.2(iii): Prickwillow Road, Ely, Cambridgeshire

A Roman mixed cremation/inhumation cemetery site at Prickwillow Road, Ely (Figure 5.1), was located approximately one kilometre north east of Ely cathedral, and was revealed during excavations in 1999 and 2000 (Atkins and Mudd, 2003). From the Roman period until successful drainage schemes in the 17th and 18th centuries, the Isle of Ely was completely surrounded by wetlands, with freshwater peat Fen to the south, east, and west,

and salt marsh to the north (Godwin, 1978; Mortimer et al., 2005). It is perhaps not surprising that the Isle provided a centre for human activity for centuries prior to the Anglo-Saxon settlement at Westfield Farm. Scattered local archaeological evidence of Neolithic and Bronze Age activity precede an increased intensity of activity from the middle Iron Age to Roman period (Lucy, 2007 a). The first documentary evidence for Ely dates to the mid-12th century and concerns the foundation of a monastery by Etheldreda in the late 7th century (Holton-Kreyenbuhl, 2005).

The cemetery yielded 16 inhumed individuals (87.5% adults), dating to the 3rd to 4th centuries (Anderson, 2003) . Gross preservation was reported as reasonably good and the small population displayed a generally low pathological prevalence. No mention was made of *cribra orbitalia*, and no cases of enamel hypoplasia were reported (Anderson, 2003), although the recorded high prevalence of carious lesions may have obscured many hypoplastic defects.

5.3.2(iv): The Parks, Godmanchester, Cambridgeshire

The modern town of Godmanchester is located in the Great Ouse valley, on the southern bank of the River Great Ouse in the Huntingdonshire district of Cambridgeshire (Figure 5.1). With little archaeological evidence of permanent prehistoric settlement, it is likely that settlement at Godmanchester began life with the building of two consecutive Roman forts and associated *vicus* during the mid to late 1st century AD. This was quickly followed by the construction of Ermine Street. The site of the forts demonstrates the importance of the location in controlling an important river crossing along a main Roman route (Jones, 2003). Following military withdrawal from the area, the settlement at Godmanchester was re-planned and continued to grow, with archaeological evidence of 2nd and 3rd century buildings, late 3rd century defences, and a recently discovered 4th century cemetery (Green, 1975; Jones, 2003).

The settlements of both Huntingdon and Godmanchester lie within the Great Ouse valley flood plain and are subsequently liable to flooding events (Huntingdonshire District Council, 2007), the threat of which past occupants must have been very familiar with. As early as 1673, Blome depicted the location of the Great Ouse valley and its proximity to the Fen edge to the immediate east and northeast. The threat of inundation and the health risks

associated with Fenland occupation would most likely have been a burden experienced by the occupants of the Great Ouse valley prior to the 19th century.

The 4th century cemetery comprised 62 relatively well preserved inhumations, with a moderately even sex ratio between males and females. Eighty three percent of the skeletal population were sufficiently well preserved to be assigned an age category, 27% of whom were aged less than 16 years (Brickley, 2003; Jones, 2003). Pathological analysis suggests that the population enjoyed reasonably good health and living conditions, although evidence of poor dental hygiene, rickets, non-specific infection (6.4% CPR), and anaemia in the form of *cribra orbitalia* (1.6% CPR) were also noted (Brickley, 2003). The surprisingly low prevalence of the latter condition may be due to the highly fragmentary nature of many of the extant crania. It should also be noted that enamel hypoplasia was not recorded in this population due to the very poor survival of dentition.

5.3.2(v): Watersmeet, Huntingdon, Cambridgeshire

Huntingdon is the administrative capital of the Huntingdonshire district of Cambridgeshire. Located on the north bank of the River Great Ouse (Figure 5.1), Huntingdon was likely founded in the Roman period as a subsidiary settlement to *Durovigutum*, or Godmanchester (described above), on the opposite south bank of the river (Oakey and Spoerry, 1996). The combined settlements at Huntingdon and Godmanchester controlled the important location where Ermine Street crossed the Great Ouse River. There is, unfortunately, sparse archaeological evidence for the early development of Huntingdon due to major modernisation and poor recording of archaeologically sensitive areas of the town from the 1960s to 1980s (Oakey and Spoerry, 1996). A main source of information concerning Roman Huntingdon comes from the numerous extra-mural cemeteries that escaped modernisation (Nicholson, 2006).

The cemetery site of Watersmeet lies on the southern edge of modern Huntingdon, approximately 50m west of the Great Ouse. The site was excavated by Archaeological Solutions in 2003 prior to development, and revealed part of a mid-4th to early 5th century Roman cemetery, possibly associated with Whitehills villa to the immediate east (Nicholson, 2006). The remains of 72 individuals were recovered, 78% of whom were adults. Preservation was generally quite poor throughout the assemblage, which severely hindered

analysis and interpretation. Very low prevalence rates of *cribra orbitalia* (1.5% CPR) and enamel hypoplasia (1.5% CPR) can likely be attributed to poor cranial preservation (Phillips 2006).

Site	Cribra orbitalia CPR/TPR (%)	Enamel hypoplasia CPR/TPR (%)	Porotic hyperostosis CPR/TPR (%)
Durobrivae	3.7/NR	3.7/NR	0
Hoplands	37/61.5	42.6/14.2	0
Prickwillow Road	0/0	0/0	0
The Parks	1.6/NR	NR	0
Watersmeet	1.5/NR	1.5/NR	0
British average	13.5/NR	NR/9.64	NR

Table 5.5: None-specific pathology prevalence from selected Romano British sites. NR = not reported. British averages taken from Roberts and Cox, 2003.

5.3.3: Anglo-Saxon sites

5.3.3(i): Edix Hill, Barrington, Cambridgeshire

Edix Hill is located close to the villages of Barrington and Orwell in the Cam valley, approximately 12km south-west of Cambridge, close to the edge of the south Cambridgeshire Fens (Figure 5.1). To the immediate south of the Hill lies the river Rhee floodplain, to the north, the expanse of Fens. The cemetery itself sits upon a chalk mound surrounded by low level clay land, which , prior to post-medieval drainage, would have been marshy or flooded for much of the year (Malim et al., 1998). The cemetery may have been associated with a settlement controlling an important crossing site of the river Rhee.

First discovered in the late 1860s during drainage works and coprolite (fossil) extraction, the cemetery underwent numerous antiquarian investigations during the 19th century. The location was rediscovered through metal detecting in the 1980s and partially excavated due to threat from ploughing (Malim et al., 1998). The cemetery dates to the 6th to early 7th centuries. Excavations yielded 148 skeletal individuals, including 48 males and 40 females. Although gross preservation was relatively good, there were numerous cases of extensive plough damage, particularly affecting the crania (Malim et al., 1998). This may partly account for the absence of *cribra orbitalia* prevalence in this population.

5.3.3(ii): The 'Three Kings', Haddenham, Cambridgeshire

The village of Haddenham is located approximately five miles south west of Ely, on the edge of the Isle of Ely (Figure 5.1). Cambridgeshire County Council Archaeology Section undertook salvage excavations in 1990 following the discovery of inhumations during development at the 'Three Kings' Public House in the centre of the village. The inhumations were dated to the early Anglo-Saxon period, the cemetery likely being associated with a nearby settlement (Robinson and Duhig, 1992). Excavations yielded 11 individuals in varying states of preservation, with few pathological conditions being noted. A lack of *cribra orbitalia* may have been influenced by poor preservation of the skulls, many of which were reported as crushed (Robinson and Duhig, 1992). Despite an overall dearth of non-specific pathology, the location of the site on the very edge of the Isle of Ely would have exposed the population to malaria, should the disease have been present.

5.3.3(iii): Highfield Farm, Littleport, Cambridgeshire

Littleport lies close to the eastern border of Cambridgeshire, approximately 10km north of Ely (Figure 5.1). Prior to 17th century drainage and land reclamation, Littleport would have occupied a small raised 'island' of land surrounded by the Cambridgeshire Fenlands. Highfield Farm is located to the southwest of the modern town centre, close to route of the main A10 road. The Farm lies close to the highpoint (approximately 20m OD) of the 'island' (Woolhouse, 2009). This elevation may explain the abundance of archaeology associated with Highfield Farm since the immediate area would likely have remained drier than its surroundings throughout its usage, which evidence suggests lasted from the Mesolithic through to at least the Anglo-Saxon periods (Holt, 2008; Woolhouse, 2009). The latter is characterised mainly by the discovery of 5th to 6th century cemetery occupying the aforementioned plateau of land. The cemetery was excavated in 2004 and 2005 by Archaeological Project Services in advance of commercial construction (Holt, 2008), yielding 86 inhumed individuals (61 adults, 25 sub-adults) and four cremated individuals. Macroscopic preservation of most skeletons was fair to good, although hydraulic action resulted in poor preservation of some individuals (Western, 2007). Analysis of the skeletons revealed high prevalences of non-specific markers of stress (Table 5.6), which may be suggestive of poor health associated with Fen edge habitation.

5.3.3(iv): Westfield Farm, Ely, Cambridgeshire

Westfield Farm lies approximately 1.5km southwest of the modern centre of the small city of Ely, Cambridgeshire (Figure 5.1). Archaeological evidence suggests activity and occupation occurring west of Ely during the Anglo-Saxon period, including a small contemporary settlement and cemetery at Westfield Farm.

The cemetery was first identified during archaeological evaluation works by Cambridge Archaeological Unit in early 2006 and was later excavated prior to housing development. The excavation uncovered 15 mostly east-west-oriented graves arranged around a central burial (Newman, 2007). The population demographic tended towards younger age-at-death ranges (Lucy, 2007 b). A full pathological report for this population has yet to be published (Natasha Dodwell, personal communication), hence the lack of disease prevalence rates in Table 5.6. The proximity of the cemetery to both freshwater Fen and salt marsh, and hence a variety of favourable mosquito breeding grounds, would likely have exposed the population to any mosquito-borne pathogens, such as *vivax* malaria.

5.3.3(v): Castledyke South, Barton-upon-Humber, North Lincolnshire

The small town of Barton-upon-Humber is located on the south bank of the River Humber in the county of North Lincolnshire, approximately five miles southeast of Hull (Figure 5.2). The town lies at the interface of a marshland belt associated with the Humber floodplain and the rising ground of the Lincolnshire Wolds (Rodwell et al., 2007). Historically, Barton occupied a relatively isolated position, due in part to separation from nearby Ermine Street by the marshy Ancholme River Valley to the west of the town. Archaeological evidence suggests that the first major phase of occupation at Barton occurred in the early Anglo-Saxon period, possibly signifying the nucleation of small localised settlements (Rodwell et al., 2007). The Castledyke South Anglo-Saxon cemetery, located on the southern periphery of modern Barton, represents the burial ground for this population.

Partial excavation of the cemetery, undertaken by the Archaeology Unit of Humberside County Council from 1975 to 1990, yielded the remains of 227 individuals. Stratigraphic and artefact dating suggested an intensive cemetery use-life of approximately two hundred years from the late 5th to early 8th centuries (Drinkall et al., 1998) . Skeletal preservation was generally good due to the chalk bedrock, although fragmentation was

frequently observed; 27% and 22% of the individuals could not be attributed a sex or age. Non-adults represented 23% of the interments (Boylston et al., 1998). Prevalence rates of enamel hypoplasia (9% TPR), and *cribra orbitalia* (16.6% CPR) are not exceptional for an Anglo-Saxon population (Boylston et al., 1998). Overall poor cranial preservation may, however, have contributed to an artificial reduction in prevalence of these pathologies. The nearby marshlands associated with the Humber tidal flats would have offered suitable *anopheline* breeding grounds in the Anglo-Saxon period. Malaria may well, therefore, have been a threat to pre-modern populations of the Humber Estuary environs.

5.3.3(vi): Baston, Lincolnshire

The small village of Baston is located in south Lincolnshire, close to the border with Cambridgeshire, approximately 60km south of Lincoln and 18km northwest of Peterborough (Figure 5.1). The village attests to its roots in the Roman period by its close proximity to the Carr Dyke and Ermine Street as it skirts the Lincolnshire Fen edge. First discovered in 1863, the Anglo-Saxon cemetery site lay relatively undisturbed until deep ploughing in the 1960s prompted recovery works by the Ministry of Public Buildings and Works. Excavations revealed a small 5th to 6th century mixed cremation and inhumation cemetery (Mayes et al., 1976).

Four inhumed individuals were recovered, each in generally good condition. Pathological analysis revealed enamel hypoplasia in two (50% CPR) individuals (Manchester, 1976). Despite the very limited number of individuals recovered at Baston, the scarcity of Anglo-Saxon burials directly associated with the south Lincolnshire silt Fens means that this small population should not be ignored.

5.3.3(vii): Cleatham, Kirton-in-Lindsey, Lincolnshire

Cleatham is located approximately 1.5km north west of the town of Kirton-in-Lindsey, North Lincolnshire (Figure 5.2). The site of the Anglo-Saxon cemetery lies on the edge of a limestone escarpment with the Trent River valley to the west and Ancholme River valley to the east. Prior to modern drainage schemes, the latter was characterised by marshlands, described by Balfour (1891:145) as “Cars, which were once wide swamps” associated with the river. Although 7km from the Ancholme itself, the Cleatham Anglo-

Saxon population would likely have been within range of *anopheline* breeding grounds, and hence, exposed to any mosquito-borne parasites.

Originally discovered in 1856, the cemetery was fully excavated between 1984 and 1989 due to an increasing threat of destruction through deep ploughing. Excavation yielded over 1200 cremation urns and 64 inhumations, making Cleatham one of the largest Anglo-Saxon cemeteries in England. Dating suggests that the cemetery was used throughout the early Anglo-Saxon period through to the later 7th century (Leahy, 2007). Since the soil conditions were acidic, preservation of the skeletons was highly dependent on the surrounding geology of each grave; burials which disturbed the limestone bedrock invariably displayed better preservation than those that did not. The Cleatham population exhibited a relatively high prevalence of *cribra orbitalia* (16.6% CPR).

Site	Cribra orbitalia CPR/TPR (%)	Enamel hypoplasia CPR/TPR (%)	Porotic hyperostosis CPR/TPR (%)
Baston	NR	50/NR	0/0
Castledyke	16.9/NR	NR/9	0/0
Cleatham	16.6/NR	NR	0/0
Edix Hill	10.1/NR	10.8/4	0/0
Haddenham	0/0	0/0	0/0
Littleport	29/71	45/NR	7/4
Westfield Farm	0*	6.6*	0/0
British average	8/25	19/NR	NR

Table 5.6: Non-specific pathology prevalence from selected Anglo-Saxon sites. NR = not reported. British averages taken from Roberts and Cox (2003). *full pathological report pending.

5.3.4: Medieval sites

5.3.4(i): Orchard Lane, Huntingdon

Prior to 10th and 11th century references to the localised Saxon and Danish settlements (Oakey and Spoerry, 1996), little is known concerning the early development of Huntingdon. The settlement had evidently entered a phase of prosperity which peaked by the 13th century, as demonstrated by the presence of hospitals, six monastic houses, and thirteen parish churches. This affluence lasted until the mid-14th century decline, which has been attributed in part to disease epidemics and national economic turmoil (Oakey and Spoerry,

1996). As previously mentioned, Huntingdon lies close to the south Cambridgeshire Fens and within the Great Ouse floodplain (Figure 5.1). Both of these environments would likely have provided suitable breeding grounds for *anopheline* mosquitoes.

Orchard Lane lies within the historic medieval core of Huntingdon, running parallel to the river approximately 100m to the south. The site in question is located to the immediate south east of Orchard Lane and was excavated in 1994 by the Archaeological Field Unit of Cambridgeshire County Council, following the discovery of human remains during archaeological evaluation. Excavation revealed part of an inhumation cemetery dating to between the 11th and 14th centuries. The cemetery was likely associated with one of the now-lost medieval churches of St Clement or St Laurence (Oakey and Spoerry, 1996). The remains of 24 individuals were excavated, comprising 18 adult and six immature individuals, and an adult sex ratio of nearly 1:2 in favour of males (Duhig, 1996). The population exhibited a 31.3% (CPR) prevalence of enamel hypoplasia and a very high prevalence of *cribra orbitalia* (50% CPR), suggesting "that life was particularly hard for this group, with food shortages, parasitism, infections, or any of these in combination" (Duhig, 1996:145).

Site	Cribra orbitalia CPR/TPR (%)	Enamel hypoplasia CPR/TPR (%)	Porotic hyperostosis CPR/TPR (%)
Orchard Lane	50/NR	31.3/NR	0
British average	10.8/NR	35/NR	NR

Table 5.7: Non-specific pathology prevalence from Orchard Lane. NR = not reported. British averages taken from Roberts and Cox (2003).

5.4: Selection of skeletal individuals for biomolecular analysis

Since it was not feasible to analyse every individual for biomolecular evidence of malaria, certain selection criteria needed to be in place in order to keep the sample size reasonable, while maximising the chances of malaria detection. Although temperate *vivax* malaria may not be as virulent as its tropical counterparts, the relapsing nature of the infection and lack of effective treatments would have added additional immunological pressures to exposed populations, particularly those already living in proximity to pestilential Fenland or marshy environments (Dobson, 1997). It seemed prudent, therefore, to initially target individuals who fell into one or more of three categories:

1. Those most at risk of contracting malaria;
2. Those who might suffer more severe symptoms of the disease, such as children and females of child-bearing age (Anstey et al., 2009; Price et al., 2007; Williams et al., 1997);
3. Those with skeletal changes potentially suggestive of past malaria infection (e.g., *cribra orbitalia*, porotic hyperostosis, and enamel hypoplasia).

Given the close proximity of the chosen sites and their inhabitants to likely *anopheline* breeding grounds, it was an unrealistic task to pinpoint individuals in the first category. There were, however, plenty of individuals who fitted into either the second or third categories.

Due to a lack of standardisation for age categories between skeletal reports, all samples selected for this study were assigned into categories (Table 5.8) based on Ubelaker (1989), Buikstra and Ubelaker (1994), and Scheuer et al., (2004). It should be stated here that individual sex was taken from pathological reports/inventories, rather than being assessed as part of this study.

Foetal/neonate	<1 month
Infant	1-12 months
Child	1-6 years
Juvenile	7-12 years
Adolescent	13-17 years
Very young adult	18-24 years
Young adult	25-34 years
Middle adult	35-49 years
Old adult	50+ years
Adult	18+

Table 5.8: Age categories assigned to samples.

Samples chosen for this study are displayed in Appendix 2, Tables A2.3 to A2.16. A sufficiently large quantity of samples was taken to cover the criteria outlined above. Further to this, it was anticipated that at least one sample per individual would be subjected to testing for anti-malaria antibodies. As discussed later in section 5.6.2, it was important to examine all samples for histological preservation, as this provided a baseline for selecting the most appropriate sample(s) from each individual for testing.

5.5: Selection of bone elements

Many archaeological biomolecular studies have utilised compact bone, since it offers higher resistance to diagenetic agents than trabecular bone (Brandt et al., 2002). However, it is logical to assume that serum proteins are most likely to concentrate in bone matrix with the greatest blood supply (i.e., trabecular, haematopoietic bone). Trabecular bone also has a higher hydroxyapatite to collagen ratio than cortical bone (Wang et al., 2005) – a potentially relevant consideration, given the proposed high affinity of non-collagenous proteins with bone mineral. Trabecular bone is, unfortunately, intrinsically more porous and potentially subject to increased diagenetic activity (Trueman et al., 2004). Thus, for this study, a balance had to be struck between using compact bone with a potentially well-protected matrix, and the more porous, serum-rich trabecular bone.

When selecting bone samples for immunological testing, a balance is also required between choosing a large enough section of bone from an element most likely to contain surviving target proteins, and avoiding gratuitous sampling and destruction of the finite skeletal resource. Since published protein extraction protocols have required between 75mg and 15g of bone powder per sample (e.g. Cappellini et al., 2012; Kolman et al., 1999), a conservative decision was made to sample no more than 10g of bone from each individual. This may seem excessive, but collection of this quantity of bone would allow for further confirmatory testing or methodological refinement when necessary. The removal of samples did not compromise future osteological analysis of the skeletal populations. For instance, any elements displaying pathological modification or diagnostic morphologies (e.g., rib ends) were not selected. Any untested human bone will be returned to the archive from which it was taken, along with a full report of research findings. The chosen sampling strategy abided by the BABAO (British Association of Biological Anthropology and Osteoarchaeology) codes of ethics and practice concerning destructive analysis of skeletal material (BABAO, 2007; 2010), and the guidelines supplied by the Advisory Panel on the Archaeology of Burials in England (2013) .

Ribs and phalanges were the first choice of elements for immunological testing (after Wiechmann et al., 1999), since their removal does not cause excessive destruction to the individual skeleton, or loss to the skeletal archive. Ribs were also ideal since they retain a

haematopoietic function throughout life and are, therefore, always rich in blood supply (Rodak et al., 2012) and extracellular blood serum proteins (including immunoglobulin G, or IgG – the biomolecule targeted in this study). Additionally, archaeological ribs are usually numerous in quantity and are often fractured (post-mortem) during burial, excavation, analysis, and curation. A 10g sample of rib was easily encountered for many individuals with no sawing of larger rib elements necessary.

Single phalanges, where present, were also chosen from each individual. Foot phalanges were a preferred target element, given their usual distance from the ribs in the grave. Although it may seem unwise to sample phalanges from adults since they, unlike ribs, do not retain haematopoietic marrow into adulthood, it was reasoned that the sampling of elements some distance apart should reduce the influence of diagenetic and taphonomic factors on protein survival. Critically, Wiechmann et al. (1999:384) found no significant differences "in terms of protein yield and quality according to sampling site" when testing archaeological human bone for the survival of non-collagenous proteins. In those skeletons without foot phalanges, hand phalanges were targeted. If no phalanges were present, cranial fragments were selected. It was possible to sample rib, phalanges, or cranial fragments from all target adult individuals. A slightly different strategy was required for many non-adult individuals: due the small size and often poor preservation of ribs and phalanges, particularly in those individuals under the age of five years, it was sometimes necessary to sample long bone fragments (e.g., sample HP117.2).

Targets selected for IgG detection (the early P1 protocols, see below) consisted of human bone and animal rib bone. Animal collagen was used as a control, as suggested by Brandt et al. (2002). The human bone consisted of unstratified, non-pathological, disarticulated fragments of adult and juvenile rib, phalanx, and cranium from the Hanging Ditch post-medieval site in Manchester (currently archived at Durham University). The animal rib fragments were disarticulated, unstratified archaeological bone from unspecified British sites dating to no earlier than the Roman period. These were curated at the Durham University Archaeology Department and obtained with permission of Professor Peter Rowley-Conwy. Collagen samples (Sigma-Aldrich®) were in the form of Type I, from bovine Achilles tendon. Each bone sample was histologically examined to characterise levels of preservation before being selected for extraction.

5.6: Sample preparation: bone cutting, histological analysis, and grinding

To help control for the effects of diagenesis and protein degradation in their target samples, some studies advocate preliminary histological analysis in the form of bone sectioning and subsequent microscopic analysis (e.g., Hanson and Buikstra, 1987; Schoeninger et al., 1989; Schmidt-Schultz and Schultz, 2007). Suitable fragments from bone cutting were set aside for histological analysis. Nitrile gloves were worn at all stages of the analysis in order to minimise external contamination.

5.6.1: Bone cutting

1. Bone fragments were gently washed in distilled water and allowed to completely dry.

2. Each fragment was then prepared for cutting by abrasion with a Dremel® 200 series drill and disposable cutting discs to carefully remove the external surface and any exposed surfaces (and hence surface contaminants), taking care to leave some cortical bone intact.

3. Small samples were then cut from each bone fragment incorporating, where possible, both cortical and trabecular bone. At this stage small fragments were set aside for histological analysis.

5.6.2: Histological analysis

Histological analysis though bone sectioning characterises bone preservation and helps to control for diagenetic influences. Only those samples displaying good preservation are then usually selected for biomolecular analysis. Conflicting research on collagen diagenesis (Hedges et al., 1995), however, demonstrates a lack of correlation between bone structure and surviving collagen content; the authors suggest that collagen degradation and loss may occur independently of microbial attack and histological destruction. Even less is known about the influences of diagenetic factors on the survival of non-collagenous proteins (NCPs, including IgG antibodies), although their strong adherence (at least compared to collagen) to hydroxyapatite during life may offer increased protection from degradation (Masters, 1987; Schmidt-Schultz and Schultz, 2004). The work of researchers such as Schmidt-Schultz and Schultz potentially supports the work of Hedges et al. (1995), in that samples demonstrating excellent histological preservation often displayed poor collagen

preservation while NCPs remained comparatively intact. Since this study proposes to extract and detect ancient immunoglobulins (biomolecules of naturally low concentration within blood serum), it seemed prudent to initially target samples with good histological preservation (although a variety of samples were ultimately analysed to test the preferential NCP survival hypothesis). Prior to sample cutting, each sample was subjectively assigned a category of gross macroscopic preservation based on basic recording criteria suggested by McKinley (2004). These criteria are presented in Table 5.9. Gross and histological preservation are compared in section 7.2.1.

Poor preservation	Fair preservation	Good preservation
Heavy surface erosion and/or fissuring	Moderate surface erosion and/or fissuring	Limited surface erosion and/or fissuring

Table 5.9: Basic criteria for assigning sample macroscopic preservation (based on McKinley, 2004).

Histological analysis provides a relatively quick, cost-effective method of reducing the chances of contamination and cross-reactivity from exogenous, diagenetically-introduced material. For this study all samples from each target individual were sectioned for histological analysis and the sample exhibiting the best preservation was selected for initial protein extraction protocols. The first step in preparing samples for histological analysis was to cut small pieces from each bone sample. To save time this was done during the cutting stage (detailed above in 5.6.1). Cut pieces usually measured no larger than 5 x 4mm, although it was important to ensure sufficient length to survive the grinding/polishing stages of sample preparation. The bone sections were then resin impregnated under vacuum and polished, following the steps adapted from Hedges et al. (1995) and Millard (2001):

1) In a well-ventilated area plastic moulds were coated with a releasing agent (e.g., silicone or anti-friction spray) and allowed to dry.

2) Bone samples were fixed into each mould, transverse side down, using superglue. A small plastic marker was also placed and sketches made to aid in orientation. It was possible to place up to 20 samples in each mould, dependent upon sample sizes.

3) Resin was then prepared, consisting of Epoxy resin and Epoxy hardener (West System®) in a 5:1 ratio. Once well mixed, the resin was immediately added to each mould. The moulds were then transferred to a vacuum desiccator.

4) The resins were subjected to cycles of evacuation until bubbles were removed and no longer formed under vacuum. This was achieved by repeatedly evacuating the desiccator for five to ten minutes, then allowing air into the chamber. When bubbles no longer formed, the samples were left under vacuum for one hour before being left to cure for up to 48 hours. Once completely hardened, the resin blocks were removed from the moulds.

5) Each resin block was then subjected to wet grinding using increasing grades of silicone carbide paper mounted on a rotating wheel. Paper grades were 120B, 400B, 800B, 1000B, and 2500B, each of which removed surface scratches from the previous grade. Blocks were cleaned ultrasonically between grades to prevent transfer of grit particles between papers. Light microscopy was employed to check for surface scratches before proceeding to the next paper grade.

6) Blocks were then polished using polishing cloths mounted to a rotating wheel. Polishing was achieved using an ethanol-based lubricant and decreasing grades of diamond paste (6µm, 3µm, and 1µm). Ultrasonic cleaning and scratch analysis by microscope was also performed between grades. At the end of the process, the resin surface should be free of most scratches, rendering the samples suitable for microscopic histological analysis.

Microscopic histological evaluation and scoring of each resinated sample was based upon the Oxford Histological Index (OHI) developed by Hedges et al. (1995) and modified by Millard (2001). The OHI is presented in Table 5.10, below, followed by micrographic examples of OHI values from this study (Figures 5.3-5.8). Very subjectively, OHI 0-1 may compare to ‘poor’ macroscopic preservation, 1-2 to ‘fair’, and 4-5 to ‘good’ (Table 5.9). However, as discussed in section 7.2.1, there is rarely a correlation between macroscopic and microscopic preservation.

Index	Approx. % of intact bone	Description
0	<5	No original features identifiable, other than Haversian canals
1	<15	Small areas of well-preserved bone present, or some lamellar structure preserved by patterns of destructive foci
2	<50	Clear lamellate structure preserved between destructive foci
3	>50	Clear preservation of some osteocyte lacunae
4	>85	Only minor amounts of destructive foci, otherwise generally well preserved
5	>95	Very well preserved, virtually indistinguishable from fresh bone

Table 5.10: The Oxford Histological Index, summarising diagenetic changes in bone (Hedges et al., 1995:203; Millard, 2001).

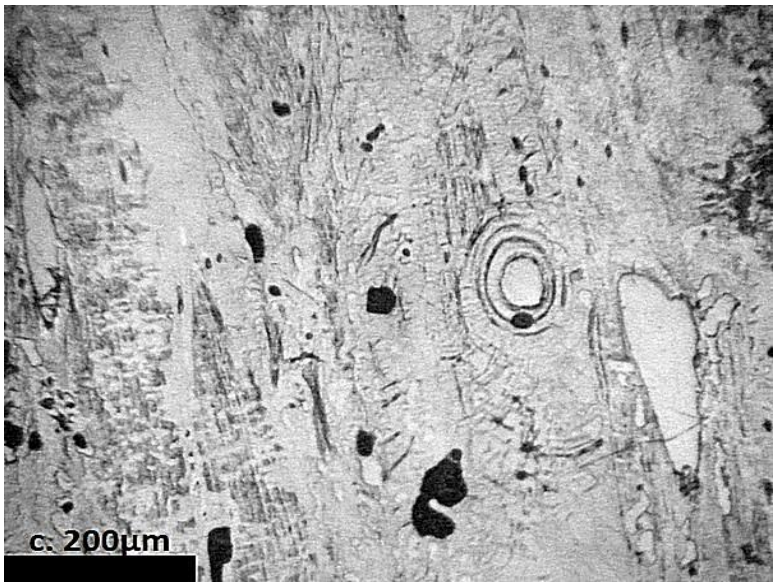

Figure 5.3: Micrograph displaying histology scoring 0 on the OHI. Cranium, sample LP5252.2. 100x magnification. Photograph by author.

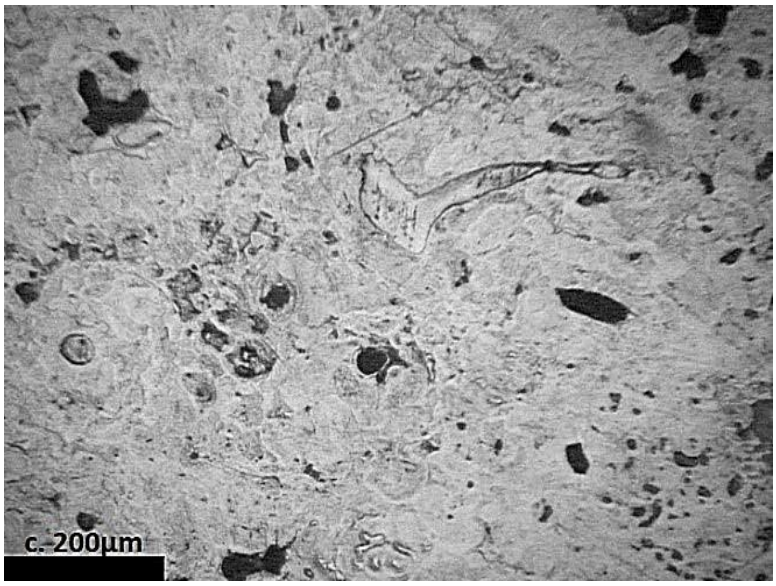

Figure 5.4: Micrograph displaying histology scoring 1 on the OHI. Cranium, sample HDAS3. 100x magnification. Photograph by author.

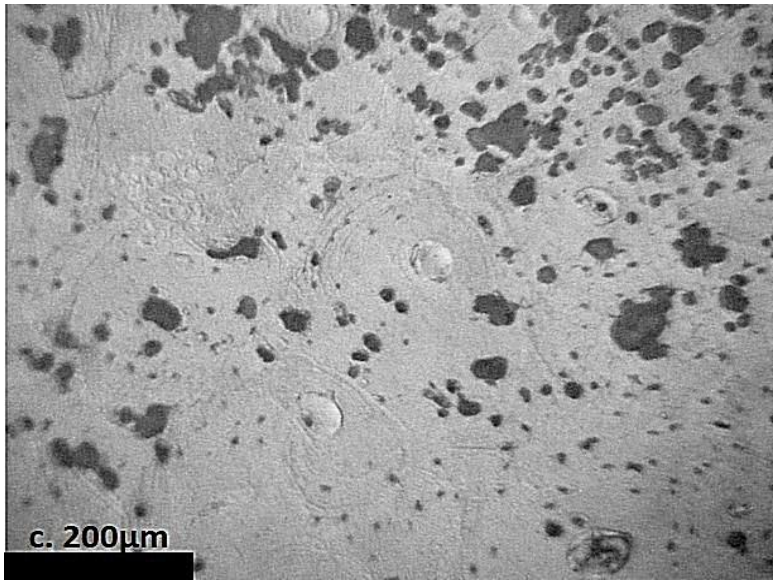

Figure 5.5: Micrograph displaying histology scoring 2 on the OHI. Rib, sample GM49.1. 100x magnification. Photograph by author.

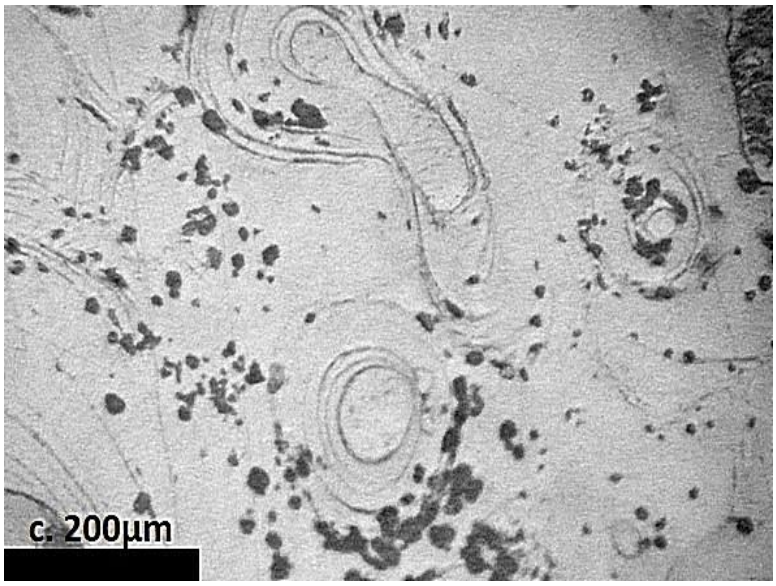

Figure 5.6: Micrograph displaying histology scoring 3 on the OHI. Metacarpal, sample HP117.2. 100x magnification. Photograph by author.

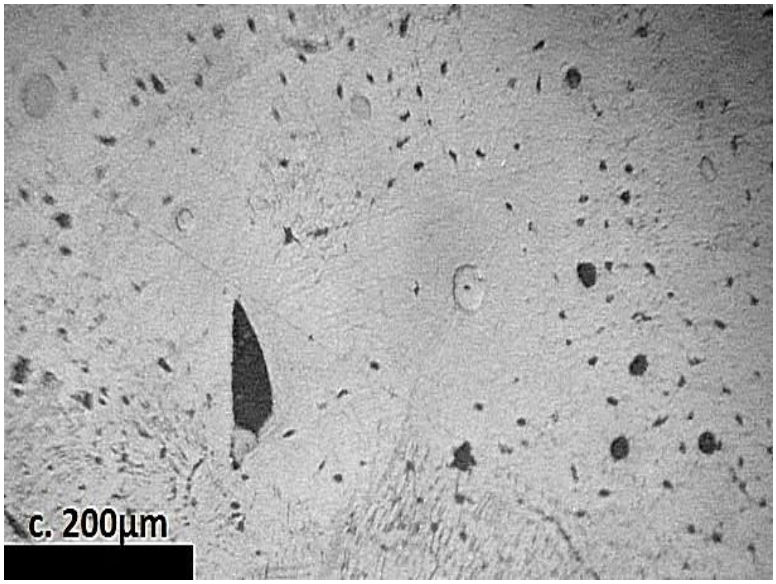

Figure 5.7: Micrograph displaying histology scoring 4 on the OHI. Rib, sample HPA3. 100x magnification. Photograph by author.

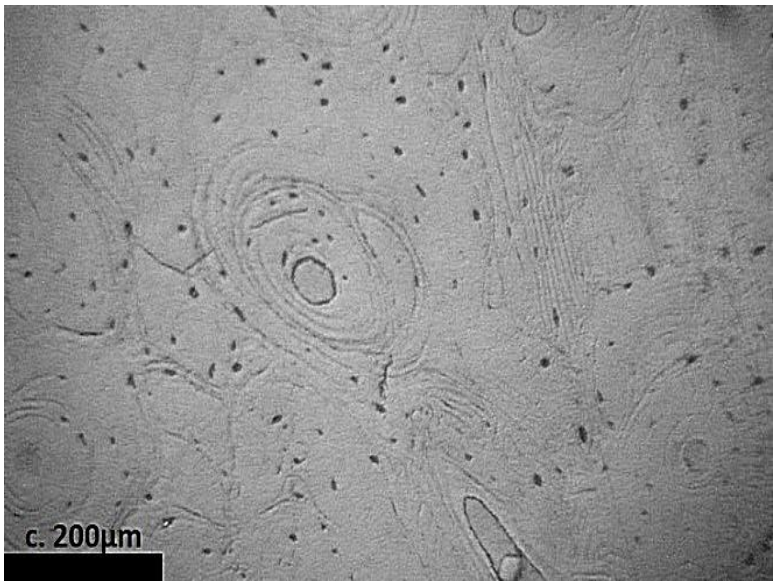

Figure 5.8: Micrograph displaying histology scoring 5 on the OHI. Hand phalanx, sample PW13.3. 100x magnification. Photograph by author.

5.6.3: Grinding

It was decided to grind the bone samples prior to analysis since the initial protein extraction protocol of choice (see section 5.7.1) followed this procedure. Prior to grinding, each sample was immersed in liquid nitrogen. This increases the brittleness of cell membranes, hence promoting release of proteins during grinding, while simultaneously decreasing the rate of protein degradation (Wu et al., 2009). After the nitrogen had dispersed (the sample remaining frozen), samples were subjected to a 20 second mechanical dismembration at 3000rpm using a Sartorius Mikro-Dismembrator S. Trial grinds of bone samples without prior liquid nitrogen immersion often required up to three minutes of dismembration to achieve adequate powdering. The use of liquid nitrogen ensured adequate, efficient sample powdering without the potentially uncontrolled, damaging temperature rises associated with extended mechanical grinding alone (Collins and Galley, 1998; Ericsson and Nister, 2011). Following grinding, samples were stored at room temperature until required for testing.

5.7: Protein and immunoglobulin G (IgG) extraction protocols

The vast range of published protein extraction techniques is indicative of a sub-discipline of biomolecular archaeology that is maturing in response to a growing

appreciation of the complexity and the challenges of extracting ancient, degraded proteins. While new extraction techniques continue to be published (e.g., Caputo et al., 2012), the choice of target protein strongly dictates the specific extraction protocol required (Cleland et al., 2012). With this in consideration, it should be stressed that the vast majority of published extraction protocols have been aimed at the retrieval of collagen for applications such as radiocarbon dating (e.g., Van Klinken et al., 1994; Minami et al., 2004), isotopic analyses (e.g., DeNiro and Weiner, 1988), and more recently, mass spectrometric peptide analysis (e.g., Buckley et al., 2008, 2009). Indeed, the recent synthesis by Cleland et al. (2012) displays a distinct lack of extractions aimed at non-collagenous proteins and, consequently, very few protocols have attempted to specifically extract immunoglobulins from archaeological bone.

Prior to attempting to detect human antibodies to malaria in archaeological samples, it was necessary to first select, adapt, or design a potential methodology that was capable of successfully and reliably extracting and purifying IgG antibodies. This precursory stage is critical, since a poorly functioning extraction protocol would inhibit later testing of the samples. Five published protein extraction protocols were attempted in this study, which, for ease of referencing, were labelled P1, P2, P3, P4, and P5. The first four protocols all required adaptation from the original published methodologies, as explained below.

5.7.1: P1: Extraction methodology adapted from Schmidt-Schultz and Schultz (2004)

It was initially decided to follow an extraction protocol published by Schmidt-Schultz and Schultz (2004) in which they reported successful extraction of IgGs from human archaeological bone and subsequent binding of these immunoreactive antibodies to antigens. The authors claim that their newly developed extraction method offers the best protein yield following solubilisation of archaeological bone matrix, and therefore a higher yield of NCPs (including IgGs). They argue that the high affinity of NCPs (particularly immunoglobulins) for hydroxyapatite means that target proteins are far more likely to be retained within the bone pellet matrix than released into the supernatant during extraction. They also suggest that sample dialysis and concentration (both commonly employed purification steps) of extracted material caused the loss of many NCPs. These suggestions may explain why earlier attempts had struggled to extract and detect IgGs: possible errors that influenced

Cattaneo et al. (1992), for instance, to declare that ancient IgGs represent a poor choice of target protein. Analysis of the bone pellet, rather than supernatant, is very rare amongst published protein extraction methodologies (see Cleland et al., 2012); the Schmidt-Schultz and Schultz research represents the only published protocol for extracting NCPs from the bone pellet itself.

It quickly became apparent that this extraction required some alteration from the published protocol for two main reasons. Firstly, the method specified the use of 2mM of the serine protease inhibitor aprotinin. This represents an enormous quantity of a very expensive reagent, far beyond the resources available for this study, and also clearly disproportionate to the other inhibitors used in the published protocol. It is likely that this requirement is erroneous (i.e., a 'typo'), although it is carried through to later published work (e.g., Schmidt-Schultz and Schultz, 2007). It was decided to omit the aprotinin due to the uncertainty over quantity required and, further to this, the protocol also included the serine protease inhibitors leupeptin and PMSF, which serve the same function as aprotinin (although the PMSF should ideally be solubilised in an alcohol-based solution, since it is water-insoluble). Secondly, the original protocol made no mention of the potential problems concomitant with the "abundant collagen" (Cleland et al., 2012:4) resulting from all ancient protein extractions from bone (Wiechmann et al., 1999). Ubiquitous degraded collagen has the potential to mask target proteins of smaller quantities (Wiechmann et al., 1999) and interfere with downstream analytical techniques, such as electrophoresis and mass spectrometry. Unfortunately, repeated requests to the authors for clarification on the aprotinin and collagen issues went unanswered.

Three attempts at protein extraction (P1.1, 1.2, and 1.3) were made using the adapted Schmidt-Schultz and Schultz (2004) protocol, each one driven by results (see section 6.4.1), and following evolving methodology from the previous attempts.

5.7.1(i): P1 samples

The bone samples selected for P1 extractions are presented in Table 5.11. The P1.1 samples represent histologically well preserved adult human and animal bone. Samples selected for extraction P1.2 represent two histologically well preserved adult human bone samples run in duplicate for consistency, and with the intention of choosing specific SDS-

PAGE gel bands to analyse by mass spectrometry (see section 5.7.3(iii)). Samples chosen for the third protein extraction (P1.3) represent a variety of bone types and preservation levels. This was performed mainly to test the suggestion by Masters (1987) that the high affinity of non-collagenous proteins for hydroxyapatite should protect the proteins from diagenetic factors that may result in poor histological preservation and the loss of collagen. A separate set of these samples were also treated with collagenase, as detailed below (section 5.7.2).

Sample	SDS#	Type	HI	Protocol
HDAR1	H1	Adult rib	4	1.1
HDAP2	H2	Adult phalanx	5	1.1
HDAP3	H3	Adult phalanx	5	1.1
HDAR4	H4	Adult rib	5	1.1
AN1	A1	Animal rib	5	1.1
AN2	A2	Animal rib	5	1.1
AN3	A3	Animal rib	5	1.1
AN7	A4	Animal rib	5	1.1
HDAP3	H3	Adult phalanx	5	1.2
HDAR4	H4	Adult rib	5	1.2
HDJS1		Juvenile cranium	2	1.3
HDAP5		Adult phalanx	5	1.3
HDAS1		Adult cranium	3	1.3
HDAP6		Adult phalanx	5	1.3
HDAR3		Adult rib	4	1.3
HDAP5		Adult phalanx	5	1.3
AN2		Animal rib	5	1.3

Table 5.11: Samples selected for P1 extractions. SDS# is the sample number assigned by Biological Sciences for SDS-PAGE electrophoresis. HI is histological preservation.

5.7.2: P1.1-1.3 protein extraction protocol

Protein extraction methodology remained consistent throughout the three P1 extractions:

1. 200mg of bone powder was extracted in 1ml of buffer A (4M guanidine HCL, 20mM NaH_2PO_4, 30mM Na_2HPO_4, and protease inhibitors 5mM benzamidine, 1mM PMSF, 50µM leupeptin, 10mM EDTA, pH 7.6), under constant agitation at 4°C for approximately 24 hours. Samples were then centrifuged for 30 minutes (10,000g) at 4°C and the supernatant discarded.

2. The pellet was then extracted in 1ml of buffer B (buffer A and 300mM EDTA, pH 7.6) at 4°C under constant agitation for approximately 24 hours.

3. Chelated calcium ions were removed by washing (centrifuged at 35,000g for 20 minutes at 4°C) three times with sterile molecular grade water.

4. The extracted bone pellet was lyophilised and stored at -20°C.

5. Approximately 20mg of lyophilised bone pellet was sonicated twice over ice for 7 seconds in 2.5ml of solubilisation buffer (20mM NaH_2PO_4, 30mM Na_2HPO_4, and protease inhibitors 1mM benzamidine, 10mM aminocaproic acid, 10mM EDTA, pH 7.0). According to Schmidt-Schultz and Schultz (2004), this procedure should liberate approximately 10µg of protein from the bone pellet.

In order to reduce the high quantity of collagen encountered in P1.1 and P1.2, which may mask target antibodies, a separate set of the same samples as used in P1.3 (Table 5.11) were treated with high purity type VII bacterial collagenase (Sigma-Aldrich®, 1335 units/mg) following solubilisation (after Tuross and Stathoplos, 1992; Ostrom et al., 2000). It should be noted that the solubilisation buffer used for samples intended for collagenase digestion did not contain EDTA, since this acts as a collagenase inhibitor (Worthington, 1993). The collagenase was prepared in a stock solution and then applied using the following steps:

1. 0.1mg of collagenase was dissolved in 10ml TESCA buffer solution (50mM TES and 0.36mM CaCl made up to 10ml with MQ water, pH 7.4) at 37°C. Unused stock solution was stored at -20°C.

2. 0.92ml stock solution was added to the 2.5ml solubilised sample, making a collagenase concentration of approximately 2.5 units to 1µg protein, or ~3.7 units/ml (following Hummelshoj et al., 2008).

3. Samples were then incubated under gentle agitation at 37°C for five hours, after which they were transferred to ice and immediately prepared for SDS-PAGE.

5.7.3: Identification of IgG antibodies from P1 extractions

In order to determine the presence of IgG antibodies in the extracted samples from the three P1 protocols, it was first necessary to identify and characterise the extracted proteins. The samples were thus subjected to a number of separate analytical techniques,

including one dimensional (1D) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, enzyme-linked immunosorbent assay (ELISA), and mass spectrometry.

5.7.3(i): P1 SDS-PAGE protocol

Working alongside the Durham University Biological Sciences department, selected samples were separated by 1D SDS-PAGE. Gels were produced for all three P1 extractions, including the collagenase treated samples in P1.3, using the following protocol:

1. Each sample was TCA (trichloroacetic acid) precipitated overnight at -20°C using an equal volume of 20% TCA to the sample.

2. The sample was then centrifuged for 15 minutes at 4°C and the supernatant removed.

3. Two acetone washes were performed on the supernatant by adding approximately 300µl of cold acetone to each sample and centrifuging at 4°C for 5 minutes, after which the supernatant was removed and the pellet allowed to air dry.

4. 200µl SDS sample buffer (63mM Tris HCl, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue, 5% DTT, pH 6.8) was added and the sample boiled for 5 minutes.

5. The supernatant was extracted and brought to neutral pH by adding sodium hydroxide (NaOH).

6. 20µl of each sample was run on pre-prepared polyacrylamide gels (10% resolving gel, 3% stacking gel) at 100V for approximately 10 minutes, and then 120V until dye reached the end of the gel.

7. Gels were stained overnight using Coomassie Brilliant Blue stain (40% methanol, 10% acetic acid, 0.025% Coomassie Brilliant Blue R-250), before being destained in 1% acetic acid solution.

8. Gels were scanned on a conventional flatbed scanner, before being silver stained following the protocol described in section 5.6.2.3.

5.7.3(ii): P1.3 Western blots

Western blot tests were performed by personnel in the Durham University Biological Sciences department on the two gels resulting from the P1.3 SDS-PAGE (collagenase and non-collagenase) protocol. In a Western blot test, the proteins from gels are transferred to a membrane, which can then be probed with an antibody to detect specific proteins of interest. These tests employed a commercially-available monoclonal mouse anti-human IgG antibody (Source Biosciences Lifesciences) to probe the membranes for the presence of human IgG heavy chains using the following protocol (supplied by Joanne Robson, Durham University Biological Sciences department):

Transfer:

1. Transfer apparatus consisting of sponges, 6x filter paper, and nitrocellulose membrane were soaked for a few minutes in 100ml transfer buffer (2.4g tris; 11.4g glycine) and 200ml ethanol. The gels were soaked separately in the same solution after careful removal of the stacking gels.

2. The transfer cassettes were opened with black panel flat on the bottom tray, which was filled with transfer buffer.

3. The transfer sandwiches were prepared by stacking the components in the flowing order: sponge, 3x filter paper, gel, nitrocellulose membrane, 3x filter paper, and sponge. The gel and membrane were placed on the cathode and anode sides of the cassette, respectively. The sandwiches were then firmly rolled over with a glass tube to remove air bubbles.

4. The transfer cassettes were clamped together and placed into an electrophoresis tank (BioRad®), before being submerged in transfer buffer. Ultrapure water was added to the outer chamber.

5. Blotting was achieved at 135V for one hour at 4°C.

Development:

1. The membranes were removed from the cassettes and placed in a small container of Ponceau S stain.

2. Membranes were stained with Ponceau S stain until weight markers became visible. Coomassie stain was then added to check successful protein transferral.

3. Membranes were rinsed in 100ml TBS-T (137mM sodium chloride; 2.7mM potassium chloride; 19mM tris; 0.5ml/l Tween 20), before being blocked overnight in 2% skimmed milk at 4°C. Blocking helps prevent nonspecific antibody/membrane binding.

4. The blocking solution was rinsed off with TBS-T and membranes incubated for one hour in a solution of monoclonal mouse anti-human IgG (heavy chain) antibody at a dilution of 1:1000 (dilution following Schmidt-Schultz and Schultz, 2004).

5. The membranes were then washed three times in TBS-T for 15 minutes, five minutes, and five minutes, before being incubated for one hour in a solution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Sigma-Aldrich®) at a dilution of 1:20000.

6. The membranes were then washed three times in TBS-T for 15 minutes, five minutes, and five minutes.

Detection:

1. Membranes were incubated in Supersignal West Pico Chemiluminescent substrate (Thermo Scientific®) for five minutes.

2. The membranes were then transferred to Saran wrap, covered in x-ray film and developed in a dark room. Results were recorded at exposures of 30 seconds, one minute, five minutes, and eight minutes.

5.7.3(iii): Human IgG Enzyme-linked immunosorbent assay (ELISA)

A commercially available human IgG capture ELISA kit (Molecular Innovations®, USA) was utilised in order to detect surviving IgG molecules in samples subjected to the adapted Schmidt-Schultz and Schultz protocol described above (section 5.7.2). This particular kit was chosen based on its ability to detect IgG quantities as low as 1ng/ml. Samples chosen for the ELISA represent a range of lyophilised samples from the three P1 extractions (Table 5.11). The supplied microtitre plate was pre-coated with affinity purified polyclonal anti-human IgG antibody, thus increasing the chances of detecting potentially degraded IgG molecules. Samples selected to test for the presence of human IgGs are shown in Table 5.13. It was necessary to test a variety of human bone in terms of age (adult or non-adult), element, and histological preservation. Controls in the form of final extraction buffer (Buffer C), bovine collagen types, and animal bone were essential.

The protocol used closely followed that supplied by the manufacturer (Molecular Innovations, 2012), with some minor alterations due to the nature of the samples being tested (e.g., testing in triplicate, rather than duplicate). The assay was performed at room temperature, with the plate being shaken on a microtitre plate shaker (150rpm) at each step of the assay:

1. A Tris-buffered saline (TBS) solution was made up consisting of 0.1M Tris and 0.15M NaCl (pH 7.4), followed by a blocking buffer consisting of 3% BSA (bovine serum albumin) in TBS.

2. The standard vial was then reconstituted with 1ml BSA blocking buffer to give a 500ng/ml solution. Since it was expected that the archaeological samples would contain significantly less IgG than fresh human serum (5-12mg/ml), standards were prepared using the dilutions shown in Table 5.12, and samples were not diluted.

IgG concentration (ng/ml)	Dilution
20	600µl (BB) + 400µl (from 50ng/ml)
10	500µl (BB) + 500µl (from 20ng/ml)
5	500µl (BB) + 500µl (from 10ng/ml)
2	600µl (BB) + 400µl (from 5ng/ml)
1	500µl (BB) + 500µl (from 2ng/ml)

Table 5.12: Standard dilutions chosen for use in the human IgG ELISA. (BB) = blocking buffer.

1. 100µl of each standard and sample was then added to the microtitre plate wells, with the well position of each being carefully recorded. The plate was then shaken at 150rpm for 30 minutes.

2. The washing buffer was diluted to a 1:10 concentration with deionised water, and the microtitre wells washed three times with 300µl of wash buffer. After each wash, excess wash was removed by gently tapping the plate over paper towels.

3. The peroxidise-conjugated antibody was then reconstituted by adding 10ml BSA blocking buffer and gently shaken to completely dissolve contents. 100µl of this was then added to all wells and the microtitre plate shaken at 150rpm for 30 minutes.

4. The wells were then washed with washing buffer as in step 2.

5. 100µl of tetramethylbenzidine (TMB) substrate solution was then added to all wells and the plate shaken at 150rpm for 10 minutes in darkness (TMB is light sensitive).

6. The reaction was quenched by adding 50µl of 1M H_2SO_4 (sulphuric acid) to each well.

7. Final absorbance values were read using a microtitre plate spectrophotometer (Perspective Biosystems Cytofluor® Multi-well plate reader) set at 450nm. Zero point was subtracted from all standards and unknowns to determine corrected absorbance (A_{450}).

Sample	Type	HI	Sample	Type	HI
Buffer C	Buffer control	-	HDAR4	Adult rib	5
Collagen	Bovine Type II	-	HDAS1	Adult cranium	3
Collagen	Bovine Type III	-	HDAR2	Adult rib	5
AN1	Animal rib	5	HDAR4	Adult rib	5
AN2	Animal rib	5	HDAR6	Adult rib	5
AN7	Animal rib	5	HDAP3	Adult phalanx	5
HDAR1	Adult rib	4	HDAP5	Adult phalanx	5
HDAR2	Adult rib	5	HDJS1	Juvenile cranium	2
HDAR3	Adult rib	4			

Table 5.13: Samples selected for the human IgG ELISA. HI is histological index.

5.7.3(iv): Matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry

Bands from the SDS-PAGE analysis of samples from P1.2 were chosen and excised based on molecular weights closely corresponding to IgG heavy chains and light chains (approximately 50kDa and 25kDa, respectively). Figures 6.41 and 6.42 display the gels and selected bands. Bands were excised immediately following visualisation and stored at -20°C before being analysed by laboratory personnel at the Durham University Biological Sciences department, first by MALDI-TOF mass spectrometry (MS), then by nLC-MS/MS (see section 5.7.3(v) for the latter).

MALDI-TOF mass spectrometry (MS) is a fast, powerful, and sensitive protein fingerprinting method. Put simply, in MALDI-TOF MS, gel band samples undergo tryptic digestion before being dissolved into a solvent matrix. The matrix is then dried into a solid phase, which is ablated by short laser pulses within the mass spectrometer. This turns the solid phase into an ion-containing gas phase. The ions are then accelerated and allowed to

drift towards a detector. Their drift speed, or 'time of flight' is directly proportional to their mass. These masses are the 'fingerprints', which are searched against databases of known protein spectra (de Hoffman and Stroobant, 2007; ProTech Inc., 2011)

Tryptic digestion of excised protein bands was performed using a ProGest robot (Genomic Solutions) programmed with the long trypsin digestion method as described immediately below. MALDI-TOF analyses were performed on a 4800 proteomic analyser (Applied Biosystems). All steps and analyses were carried out by laboratory personnel in the Biological Sciences Department of Durham University utilising the following protocols (Joanne Robson, personal communication, August 2013):

Tryptic digestion:

1. Excised protein bands were transferred to the wells of a 96 well microtitre plate. This plate is designed with microscopic holes at the bottom of the wells which allows for positive displacement of liquids during reagent changes on the robot.

2. Gel pieces were equilibrated in 50µl of 50Mm ammonium bicarbonate and proteins were reductively alkylated with 10mM DTT and 100mM iodoacetamide.

3. Bands were then de-stained and remaining gel plugs desiccated with acetonitrile.

4. Gel plugs were rehydrated with 50mM ammonium bicarbonate, containing 5% trypsin, and the proteins were digested for 12 hours at 37ºC.

5. Resulting peptides were extracted from the gel plugs with 2 x 25µl washes of 50% acetonitrile, 0.1% triflouroacetic acid (TFA).

6. Peptide extracts were lyophilised and then re-suspended in 10µl of 0.1% formic acid.

MALDI sample spotting:

7. A saturated matrix solution of α-cyano-4-hydroxy-cinnamic acid was prepared in 50% acetonitrile, 0.1% TFA, 10mM ammonium acetate.

8. For each sample 1 µl of matrix solution was spotted on the MALDI target immediately followed by 1µl of sample into the matrix spot and the sample/matrix droplet allowed to slowly air dry.

MALDI-TOF analyses:

9. Analyses were first performed on all of the target spots using automated data acquisition and processing under the control of Applied Biosystems 4000 series Explorer software (version 3.5) using reflector mode, a mass range of 700-4000 m/z, 1000 total laser shots per spectrum and a laser intensity of 3300v.

10. Following acquisition the TOF-MS spectra were noise corrected, peak de-isotoped and internally calibrated using the trypsin autolysis peaks 842.500 and 2211.100 m/z.

11. The eight most abundant precursor ions from each spectra were then selected by the software for fragmentation and MS-MS analyses using a 1kV CID fragmentation method collecting 4000 laser shots per spectra with a laser intensity of 3800 over the mass range.

Database searching and protein identification:

12. Peak lists of ion masses were generated by GPS Explorer software version 3.6 (Applied Biosystems) from the calibrated and de-isotoped MS and MS-MS spectra for each sample. Combined lists of MS-and MS-MS data were used for database searching with MASCOT version 2.2 (Matrix Science), against all entries in the NCBI database.

13. Database search parameters used were; digestion enzyme trypsin, single missed cleavage allowed, variable modifications of carboxymethyl cysteine and oxidised methionine, precursor mass tolerance of 50ppm and fragment ion tolerance of 0.2Da

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5.7.3(v): Nanospray liquid chromatography-mass spectrometry (nLC-MS/MS)

Nanospray liquid chromatography-mass spectrometry is an extremely powerful analytical tool which offers advantages over MALDI-TOF MS in terms of much greater sensitivity and reliability in protein identification. However, operationally, nLC-MS/MS is a much more complex task than MALDI-TOF MS. Data acquisition for the former takes also far longer due to the vast quantity of spectra generated (ProTech Inc., 2011). NLC-MS/MS has been demonstrated to be most useful for characterising whole proteomes from ancient bone samples (e.g., Cappellini et al., 2012).

Nanospray LC-MS/MS analysis was performed on the remaining tryptic digests of samples following the MALDI-TOF MS analysis outlined above in section 5.7.3(iv). All steps and analyses were carried out by laboratory personnel in the Biological Sciences

Department of Durham University utilising the following protocols (William Simon, personal communication, August 2013):

1. 15µl of each sample fraction of tryptic peptide digest (see section 5.7.3(iv) for digestion protocol) was analysed by nLC-MS/MS using a Dionex Ultimate 3000 nano-flow HPLC coupled to a hybrid quadrapole-TOF mass spectrometer (QStar Pulsar *i*, Applied Biosystems) fitted with a nanospray source (Protana) and a PicoTip silica emitter (New Objective).

2. Each sample was loaded and washed on a Zorbax 300SB-C18, 5mm, 5 x 0.3mm trap column (Agilent) and online chromatographic separation was achieved over 2 hours on a Zorbax 300SB-C18 capillary column (15cm x 3.5 x 75µm) with a linear gradient of 0-40% acetonitrile, 0.1% formic acid at a flow rate of 200nl/minute.

3. MS and MS-MS data were acquired using 1 second survey scan and 3 x 3 second product ion scans throughout the peptide elution. Only ions with 2^+ to 4^+ charge state and with TIC > 10 counts were selected for fragmentation.

Throughout the chromatographic run the mass spectrometer cycles every 10 seconds between a 1.0 second survey scan (MS peptide parent ion mass) and 3 x 3.0 second MS-MS scans (3 peptides fragmented). This gives intact mass data and fragment ion mass data for as many as possible of the peptides eluting into the instrument. These combined masses are used in the database searches to identify and characterise proteins present in the sample (William Simon, personal communication, August 2013).

Database searching and protein identification:

1. All MS/MS data files were processed using Paragon search algorithm of Protein Pilot Software 4.0 (Applied Biosystems, USA) and searched against the Swiss-Prot and/or TrEMBL databases. MS and MS/MS tolerances were set to 0.15 and 0.1Da respectively and cleavage sites were defined as lysine and arginine with a single missed cleavage.

5.7.4: P2: Extraction methodology adapted from Jiang et al. (2007)

The protocol developed by Jiang et al. (2007) uses an exhaustive four stage extraction method aimed at retrieving as many proteins as possible from fresh animal bone. The total protein yield from each step was measured by mass spectrometric analysis. Although not originally aimed at archaeological bone, the exhaustive nature of the

extractions may prove suitable for retrieving IgGs from such material for further analysis. The extraction steps utilised by Jiang et al. (2007) follow a more traditional formula than Schmidt-Schultz and Schultz (2004), in that supernatants were collected and analysed from each step, rather than being discarded in favour of the bone pellet until the final solubilisation stage.

For consistency, the same cutting/grinding steps were employed as with P1. Five sets of samples (P2.1, P2.2, P2.3, P2.4, and P2.5) were selected for this extraction protocol. As with the P1 extractions detailed above, the five P2 extractions follow evolving, results-driven methodologies; changes made in consecutive P2 extractions were designed to address problems encountered in each preceding extraction. Most of these problems arose due to difficulties of applying a protocol designed for fresh, modern bone samples to ancient, degraded samples.

In each P2 extraction (other than P2.1 – see section 5.7.4(i)), four samples were processed, as dictated by the immuno-affinity purification protocol subsequently chosen (see section 5.7.5 for details). Whereas Schmidt-Schultz and Schultz (2004) used a denaturant (guanidine-HCl) and chelator (EDTA) as the first extraction step, many other extraction methods (particularly collagen extractions) use hydrochloric acid (HCl) to initially demineralise the bone before denaturing and chelating. It is suggested that HCl demineralisation "induces 'swelling' of the collagen matrix and increases the ability of both collagen I and collagen-associated proteins [possible including cross-linked IgGs] to go into solution... [and] may allow for characterization of NCPs from ancient bone" (Cleland et al., 2012:4-5).

5.7.4(i): P2 samples

The samples chosen for the P2 extractions are show in Table 5.14. Two samples were chosen for P2.1 mainly to test the efficacy of the adapted Jiang et al. (2007) protocol and the functionality of the newly acquired thiophilic columns (see section 5.7.5). P2.2 consisted of samples displaying high histological preservation as well as a pure bovine collagen control sample. The control was necessary to rule out the presence of collagen following thiophilic adsorption chromatography (TAC) purification (see section 5.7.5). P2.3 samples were chosen based upon a range of histological preservations. As with P1.3, this

was performed to test the aforementioned suggestion by Masters (1987) concerning preferential NCP survival. The P2.4 and P2.5 human samples are from potentially malarious sites, thereby representing a range of periods (i.e., Roman and Anglo-Saxon) and histological preservations. An animal control sample was also included for P2.4.

Sample	Type	HI	Protocol
HDAP5	Adult phalanx	5	2.1
AN1	Adult rib	5	2.1
HDAP5	Adult phalanx	5	2.2
HDAR9	Adult rib	5	2.2
AN2	Animal rib	5	2.2
COLL	Bovine collagen	-	2.2
HDAR2	Adult rib	5	2.3
HDAP5	Adult phalanx	5	2.3
HDAR3	Adult rib	4	2.3
HDJS3	Juvenile cranium	1	2.3
WM2316.1	Adolescent rib	5	2.4
CD127.2	Very young adult cranium	5	2.4
LP3845.1	Adolescent rib	0	2.4
AN5	Animal rib	0	2.4
HP154.1	Young adult rib	5	2.5
LP3760.1	Juvenile rib	0	2.5
LP4585.3	Middle adult hand phalanx	2	2.5
OL1104.3	Young adult hand phalanx	5	2.5

Table 5.14: Samples selected for the P2 extractions adapted Jiang et al. (2007) protocol. HI is histological preservation.

Samples chosen for the P2 extractions were processed using an adapted Jiang et al. (2007) protocol. Adaptations were necessary due to the nature of the samples: Jiang and colleagues extracted protein from modern dog bones and therefore required additional steps not required with ancient samples (e.g., soft tissue removal). Additionally, Jiang and colleagues required the use of 1.2M and 6M HCl in the initial and final demineralisation stages, respectively. It has been suggested that 0.6M HCl is preferable for archaeological bone in order to reduce acid-hydrolysis of already degraded target proteins (Buckley et al., 2009; Cleland et al., 2012). Bone mineral (hydroxyapatite) solubility increases as pH decreases, from being mostly insoluble at pH 7.5, to very soluble below pH 6 (Lindsay, 2001). 0.6M HCl has a pH of 0.2, so should prove more than suitable for enhancing bone

mineral solubilisation. Although 1.2M and 6M HCl demineralisation may work well for the mass spectrometric peptide analysis intended by Jiang et al. (2007), these highly acidic conditions degrade proteins, rendering them unsuitable for the electrophoretic characterisation used later in this study. The P2 extractions, therefore, employed an initial 0.6M demineralisation stage throughout. The fourth stage in P2.1 was changed to a 0.6M final demineralisation (adapted from published 6M HCl concentration), but this was dropped after P2.1, since it resulted in no spectrophotometrically measurable protein release following thiophilic adsorption chromatography (see 5.7.5). Benzamidine and aminocaproic acid were chosen as protease inhibitors, since it was felt that they would inhibit a sufficiently wide range of proteolytic activity likely to be encountered in archaeological bone. Protease inhibitors are not required during HCl demineralisation (Collins and Galley, 1998), since proteases are unable to function under such conditions.

After each extraction stage, supernatants were reserved for thiophilic adsorption chromatography (TAC), which took place immediately following each extraction stage (see 5.7.5). Bone pellets were immediately utilised in the next extraction stage.

Stage 1:

1. 200mg of ground bone sample was incubated overnight at 4°C in 1ml 0.6M HCl, with occasional agitation.

Stage 2:

1. The bone pellet from extraction stage 1 was incubated for 24 hours at 4°C in 1ml 100mM Tris, 6M guanidine-HCl, 5mM benzamidine, and 10mM aminocaproic acid, pH 8, with occasional agitation.

Stage 3:

1. The bone pellet from extraction stage 2 was incubated for 24 hours at 4°C in 1ml 100mM Tris, 6M guanidine-HCl, 0.5M EDTA, 5mM benzamidine, and 10mM aminocaproic acid, pH 8, with occasional agitation. (It should be noted that in extractions P2.3, P2.4, and P2.5 samples were incubated for 48 hours at this stage, in an attempt to extract more protein).

Stage 4 (only utilised in P2.1):

1. The bone pellet from extraction stage 3 was incubated overnight in 1ml 0.6M HCl at 4°C.

2. Upon careful removal of the supernatant, the bone pellets were discarded.

5.7.5: Purification of IgG antibodies from P2 extractions using thiophilic adsorption chromatography (TAC)

Given the high quantity of expected proteins extracted from the phases of the adapted Jiang et al. (2007) technique, it was decided that an antibody purification approach would be necessary to separate the inevitably small quantity of IgGs from the overall protein yield. This is akin to the use of collagenase in the previous extraction technique. However, while collagenase reduces the quantity of collagen that may mask target IgG proteins, antibody purification should isolate target antibodies from samples, thus eliminating potentially interfering/masking proteins altogether.

Due to the generally low abundance of the target protein even in modern serum samples, antibody purification requires techniques that result in the highest purity yields. To achieve this, the most powerful purification techniques involve some type of immunoaffinity chromatography, in which the target antibodies are separated by interactions with a solid ligand (usually within a gel), based upon specific chemical or physical binding properties of the antibody (Thermo Scientific, 2010). Such techniques include the use of high affinity liquid chromatography (HPLC) over melon gel, or protein A, G, or L columns (all of which have a high affinity for IgGs). Kolman et al. (1999) successfully extracted IgGs from relatively recent archaeological bone and purified them using HPLC over protein A columns, prior to ELISA against syphilis antigens. Their testing of unpurified samples failed to produce positive antigen binding. There are, however, drawbacks to using protein columns, the most significant being the high cost, low sample throughput, the need for subsequent dialysis/cleaning steps, and potential damage to the antibodies during elution (Low et al., 2007; Thermo Scientific, 2010).

Thiophilic adsorption chromatography (TAC) was developed during the 1980s and has become well established as a cost-effective, gentle method of purifying IgG antibodies (Hardouin et al., 2007). TAC follows a similar process to most other protein column immunoaffinity chromatographic techniques in that target immunoglobulins are attracted to, or have a high affinity to, specific chemical ligands within a matrix. The chemical matrix structure in this case was named thiophilic gel, or T-gel (Hutchens and Porath, 1986), to which IgGs of all subclasses have a particularly high affinity. In anticipation of the likely degraded nature of the target proteins in archaeological bone and the extensive extraction

process of Jiang et al. (2007), it was thought prudent to attempt the gentlest IgG purification method available. Thiophilic purification operates at a near-neutral pH and is reliant on the presence of hydrophobic lyotrophic salt (e.g., potassium sulphate) as opposed to chaotropic salt (e.g., sodium chloride or ammonium sulphate) to promote IgG adsorption (Hardouin et al., 2007). The conditions serve to protect the antibodies from further degradation (Thermo Scientific, 2010) and also to remove many salts, which would normally require post-extraction desalting or dialysis; it is, therefore, considered a one-step process. It is a relatively low-cost alternative to other IgG purification methods in that the supplied resins can be regenerated and reused at least ten times before exhaustion (Thermo Fisher, 2010). The major disadvantages of thiophilic resins are the time required for sample processing and the low sample through-put.

Sample supernatants were subjected to thiophilic purification after each stage of the adapted Jiang et al. (2007) protocols, using a Pierce® Thiophilic Adsorption Kit (Thermo Scientific), which provided thiophilic columns and all necessary buffers and methodology:

1. 87mg of ACS Reagent Grade crystalline potassium sulphate per ml was added to each 1ml sample supernatant before gentle mixing. Samples were then centrifuged at 10,000g for 20 minutes.

2. Sample supernatants were then aspirated into separate 1.5ml tubes through 0.45µm filters (using 5ml syringe, filter, and 20 gauge needle) and brought to pH 8.

3. Thiophilic columns and buffers were equilibrated to room temperature. Columns were then uncapped and allowed to drain, followed by equilibration with 12ml of binding buffer (flow-through fractions being discarded).

4. Sample supernatants were then applied to the columns and allowed to completely enter the resin.

5. Columns were washed with consecutive 3ml volumes of binding buffer. The flow-through fractions were collected after each wash, with absorbance being monitored at 280nm against pure binding buffer using a CamSpec M330 spectrophotometer. This step determined when all non-bound material was removed from the column.

6. Bound proteins were then eluted from each column using as many elution buffer washes as was necessary, until no further protein was measurable at 280nm against pure elution buffer. Fractions containing protein from this step were

retained and frozen at -20°C, while those containing no measurable protein were discarded.

7. Each column was then regenerated with 12ml of 8M guanidine-HCl solution, before being washed with 20ml of ultrapure water.

8. 6ml of storage buffer was then added to the columns, which were capped when 3ml remained. Columns and buffers were then stored at 4°C.

It should be noted that the TAC protocol for the P2.3, 2.4, and 2.5 samples changed slightly in the type of 0.45µm filter used in sample preparation. The cellulose acetate filters used for the P2.1 and P2.2 samples were replaced with PVDF filters, which are demonstrated to significantly decrease the prevalence of IgG binding (Walsh and Coles, 1980).

5.7.6: Identification of IgG Antibodies from P2 Extractions and TAC Purification

Selected samples from the P2 extractions and subsequent TAC purifications were subjected to 1D SDS-PAGE and mass spectrometric analysis in order to identify and characterise any surviving IgGs. As with the Jiang et al.'s (2007) extraction protocols, changes in SDS-PAGE techniques were implemented as and when problems arose. Methodology was hampered by the dearth of published protocols aimed specifically at electrophoretic characterisation of ancient antibodies; this study was, therefore, required by trial and error to identify the most suitable SDS-PAGE techniques for the target proteins. Samples chosen for SDS-PAGE were generally those displaying the highest spectrophotometric absorbance values at 280nm following TAC. These should, theoretically, represent the samples with the highest concentrations of eluted IgGs.

5.7.6(i): SDS-PAGE (P2.1 and P2.2)

Working in cooperation with the Durham University Biological Sciences department, samples from P2.1 and P2.2 were separated by 1D SDS-PAGE (with a Bio-Rad Mini PROTEAN® II Electrophoresis Cell) using the following protocol, adapted from Laemmli (1970):

1. Each sample was TCA (trichloroacetic acid) precipitated overnight at 20°C using an equal volume 20% TCA to the sample.

2. The samples were then centrifuged for 15 minutes at 4°C and the supernatant carefully removed.

3. Two acetone washes were performed on the supernatants by adding approximately 300µl of cold acetone to each sample and centrifuging at 4°C for 5 minutes, after which the supernatant was removed and the pellet allowed to air dry.

4. Approximately 200µl SDS sample buffer (63mM Tris HCl, 10% Glycerol, 2% SDS, 5% DTT, 0.0025% Bromophenol Blue, pH 6.8) was added and the samples boiled for 5 minutes.

5. The supernatants were extracted and brought to neutral pH by adding sodium hydroxide (NaOH).

6. 20µl of each sample was run on pre-prepared polyacrylamide gels (10% resolving gel, 3% stacking gel) 100V for approximately 10 minutes, and then 120V until dyes reached the end of the gel. See Table 5.20 for the running buffer recipe.

7. Silver staining was achieved following the protocol described in 5.7.2.3.

5.7.6(ii): SDS-PAGE (P2.3, P2.4, and P2.5)

Following SDS-PAGE of P2.1 and P2.2 samples, it was felt necessary to run further trials to optimise the protocol for visualising target archaeological IgGs using equipment loaned from the Biological Sciences department. The acquisition of equipment and reagents in-house (within the Archaeology department of Durham University) meant that it was possible to run many trials using multiple variations of the SDS-PAGE protocol used in P2.1 and P2.2. Techniques presented here represent the most successful trials (from over 50 attempts) for samples from P2.3, P2.4, and P2.5, in terms of visualizing bands possibly representative of IgG. A notable difference in sample preparation for electrophoresis occurred with the P2.5 samples. These were precipitated with trichloroacetic acid (TCA) rather than with acetone (see below for protocol). Acetone was used to precipitate the earlier P2.3 and 2.4 samples, since TCA damages the light chain receptors, thereby reducing or destroying any remaining functionality of already potentially degraded ancient IgGs. Retaining functionality would be important should immunological testing for anti-malarial antibodies be attempted. However, acetone precipitation resulted in large phosphate pellets,

the phosphate being residual from the TAC elution buffer. This became a particular problem when more than 1ml of sample was prepared for electrophoresis in attempts to boost protein concentration: increased sample resulted in larger phosphate pellets, which made solubilisation in SDS sample buffer extremely difficult. TCA does not precipitate salts alongside protein, so was preferred to acetone for the P2.5 samples. Since desalting the samples would likely further reduce any ancient protein yield (Schmidt-Schultz and Schultz, 2004), it was decided to return to TCA precipitation. If IgGs were successfully visualised, an alternative, less destructive, yet inherently desalting precipitation method (e.g., chloroform-methanol) may have been considered for any downstream immunological analyses.

All in-house SDS-PAGE trials were conducted using a Bio-Rad Mini PROTEAN® II Electrophoresis Cell. An IgG positive control (Source Bioscience Lifesciences®) was also introduced at this stage in order to facilitate comparisons between gel lines and a positive sample. The addition of a positive control proved useful in determining the ideal gel concentrations, at least in terms of modern IgGs. The denaturing SDS-PAGE followed an adapted Laemmli (1970) methodology using gel concentrations appropriate for separating proteins below 100kDa in size, namely 10%, 12 %, or 15% resolving gels, and a 5% stacking gel (Nikolayenko et al. 2005). Tables 5.15-5.20 show recipes for these gels, along with the sample and running buffers. These are followed by the adapted Laemmli (1970) methodology employed in each of the extractions.

Reagent	Quantity
dH_20	4.1ml
3M Tris, pH 8.8	1.25ml
0.8% SDS	1.25ml
30% acrylamide	3.33ml
10% Ammonium persulphate (APS)	50µl
Tetramethylethylenediamine (TEMED)	20µl

Table 5.15: 10% resolving gel recipe for SDS-PAGE of P2.3, P2.4, and P2.5 samples. Makes 10ml.

Reagent	Quantity
dH_20	3.43ml
3M Tris, pH 8.8	1.25ml
0.8% SDS	1.25ml
30% acrylamide	4ml
10% Ammonium persulphate (APS)	50µl
Tetramethylethylenediamine (TEMED)	20µl

Table 5.16: 12% resolving gel recipe for SDS-PAGE of P2.3, P2.4, and P2.5 samples. Makes 10ml.

Reagent	Quantity
dH_20	2.43ml
3M Tris, pH 8.8	1.25ml
0.8% SDS	1.25ml
30% acrylamide	5ml
10% Ammonium persulphate (APS)	50µl
Tetramethylethylenediamine (TEMED)	20µl

Table 5.17: 15% resolving gel recipe for SDS-PAGE of P2.3, P2.4, and P2.5 samples. Makes 10ml.

Reagent	Quantity
dH_20	3.105ml
1M Tris, pH 6.8	625µl
30% acrylamide	600µl
0.8% SDS	625µl
Bromophenol blue	Trace
10% Ammonium persulphate (APS)	25µl
Tetramethylethylenediamine (TEMED)	20µl

Table 5.18: 5% Stacking gel recipe for SDS-PAGE of P2.3, P2.4, and P2.5 samples. Makes 5ml.

Reagent	Quantity
dH_20	2.4ml
0.5M Tris, pH 6.8	200µl
Sucrose	1g
Bicine	490mg
2-mercaptoethanol	250µl
SDS	150mg
Bromophenol blue	Trace

Table 5.19: Sample buffer recipe for SDS-PAGE of P2.3, P2.4, and P2.5 samples. Makes 3ml.

Reagent	Quantity
dH_20	776ml
1M Tris, pH 8.3	16ml
Bicine	2.64g
10% SDS	8ml

Table 5.20: Gel running buffer recipe for SDS-PAGE of all P2 samples. Makes 800ml.

1. 300µl of each P2.3, and P2.4 sample was precipitated at -20°C overnight in 1.2ml cold acetone. For P2.5 samples, 500µl of each sample was precipitated overnight in an equal volume 20% TCA at 4°C.

2. The P2.3 and P2.4 samples were then centrifuged for 15 minutes at 10,000g, after which the acetone was carefully removed and the remaining protein pellets allowed to air dry. For 2.5 samples, each sample was centrifuged for 15 minutes at 10,000g. TCA was then carefully removed and the remaining pellet washed twice in 300µl cold acetone, before centrifuging for 5 minutes at 10,000g. The supernatant was then removed and the pellet air dried for a maximum of 10 minutes.

3. A 10, 12, or 15% polyacrylamide resolving gel (Tables 5.15-5.17) was made and allowed to polymerise.

4. A 5% stacking gel (Table 5.18) was made and pipetted onto the polymerised resolving gel. The stacking gel was then left to polymerise with Teflon combs in place to create the sample wells.

5. Air dried sample pellets were resuspended in 200µl sample buffer (Table 5.19: recipe following Wiechmann et al. (1999) for SDS concentration and Page and Thorpe 2002)).

    a. For P2.3 samples, half of the samples were incubated at 55°C for 48 hours. This follows the suggestion by Wiechmann et al. (1999) that extended heating of archaeological samples in sample buffer promotes protein separation.

b. Remaining P2.3 samples were boiled for three minutes and allowed to return to room temperature. All P2.4 and 2.5 samples were boiled.

6. Polymerised gels were placed into running buffer (Table 5.20), before 10-20µl of each boiled and incubated sample was pipetted into the sample wells. 10µl of human IgG control sample and/or 3 µl of low range molecular weight marker and/or wide range molecular weight marker (Sigma-Aldrich®) were added to sample wells, respectively. Unused wells were filled with 10-20µl of sample buffer.

7. Gels were run at 100V for approximately 10 minutes, then 150V until samples reached the end of the gels.

5.7.6(iii): P2.3, P2.4, and P2.5 gel staining (Coomassie and silver)

Gels for P2.3 were visualised initially with Brilliant Blue G (Colloidal) Coomassie stain (Sigma-Aldrich®) following the supplied product protocol (see below). Colloidal Coomassie stain offers up to ten times the detection sensitivity over traditional Coomassie blue staining (Neuhoff et al., 1988). If further protein visualisation was deemed necessary, gels were then silver stained using a ProteoSilver™ Silver Stain Kit (Sigma-Aldrich®) and supplied protocol (see below). Both stain types are highly sensitive and compatible with downstream applications, such as mass spectrometric analyses.

Colloidal Coomassie Brilliant Blue G (Sigma-Aldrich®) staining:

The staining protocol used was as follows:

1. After gels had completed the SDS-PAGE run, they were fixed overnight in 100ml 40% ethanol, 10% acetic acid.

2. Gels were then washed twice for 10 minutes in 100ml dH_20.

3. Dye working solution was prepared by taking four parts dye stock solution and stirring in one part methanol. Gels were left covered overnight to stain.

4. Following staining, gels were washed in 1% acetic acid until the background became clear (wash solution was changed several times).

5. Gels were recorded using a conventional flatbed scanner. Protein bands of interest (i.e., those potentially signifying IgG heavy or light chains) were excised and stored at -20° for mass spectrometric analysis.

Silver staining using ProteoSilver™ Plus Silver Staining Kit (Sigma-Aldrich®):

The silver staining protocol was as follows. All steps were carried out with constant agitation:

1. Gels were fixed for 40 minutes in 100ml of a 50% ethanol, 10% acetic acid, 40% ultrapure H_20 solution.
2. Gels were then washed for 10 minutes in 100ml of a 30% ethanol solution.
3. The gels were washed for 10 minutes in 200ml ultrapure water.
4. The gels were then incubated for 10 minutes in 100ml of sensitiser solution (1ml sensitiser, 99ml ultrapure H_20).
5. Gels were washed twice for 10 minutes in 200ml ultrapure H_20.
6. The gels were then incubated for 10 minutes in 100ml silver solution (1ml silver, 99ml ultrapure H_20).
7. Gels were washed in 200ml ultrapure H_20 for one minute.
8. Gels were then developed in 100ml developer solution (5ml developer 1, 100µl developer 2, 95ml ultrapure H_20) until protein bands were sufficiently visualised.
9. 5ml of stop solution was added and gels incubated for 5 minutes to stop development.
10. Gels were then washed for 15 minutes in 200ml ultrapure water, and then stored in fresh ultrapure water.

5.7.7: P3: Adapted Schmidt-Schultz and Schultz protocol with TAC purification

Subsequent to running the P1 and P2 protocols, it was decided to prepare a further batch of samples (Table 5.21) using the adapted Schmidt-Schultz and Schultz methodology (P1), but this time collecting the supernatants from the separate extractions and subjecting them to TAC purification in order to remove excess collagen that may obscure IgGs. SDS-PAGE and gel staining were then performed on post-TAC eluted samples following the protocols described above. Selected gel bands (see section 6.4.2(iv)) were excised and subjected to nLC-MS/MS analysis following the protocol described in section 5.6.3(v). It should be stated here that the gels shown in sections 6.4.2(iii) and 6.4.2(iv) represent testing

of the P3 samples *prior* to TAC purification, since SDS-PAGE trials failed to produce visible bands following TAC. A Western blot was also performed on a selection of P3 samples (see section 5.7.10).

Sample	Type	HI
EH198.1	Very young adult rib	4
CD120.2	Very young adult hand phalanx	5
CD165.1	Child rib	5
OL1104.2	Young adult cranium	5

Table 5.21: Samples selected for P3 extraction. HI – histological preservation.

5.7.8: P4: Full Jiang et al. (2007) extraction and proteomic analysis

It was decided to run one further adapted Jiang et al. (2007) protocol with the intention of increasing protein yield from the TAC purification and preparing eluted samples from each extraction stage for mass spectrometric analysis. It was hoped that the increase in protein (and hence IgG) yield would result in better visualisation of target IgGs in colloidal Coomassie stained gels, and hence provide bands suitable for proteomic analysis. Furthermore, mass spectrometric analysis of eluted samples should identify at which extraction stage IgGs were being released, if at all. Whole sample analysis (i.e., pre-TAC) may provide a full protein proteome, which could reveal a vast range of other surviving ancient biomolecules, such as were encountered in recent ground-breaking proteomic research on Pleistocene mammoth bone (Cappellini et al. 2012). Unfortunately whole sample analysis proved prohibitively expensive and was therefore beyond the limitations of this study. Select eluted samples (Table 5.23) were, however, analysed by nLC-MS/MS.

The protocol used with the P4 samples (Table 5.22) follows the published Jiang et al. (2007) sample preparation technique more closely than that used with the P2 samples, since the main intention with the P4 samples was mass spectrometric analysis of eluted samples from each extraction stage. Other than omitting the degreasing steps required for fresh bone, the only deviations from the published protocol were the choice of protease inhibitors and the pH of solutions used in the second and third extraction stages. As previously, it was felt that aminocaproic acid and benzamidine would provide sufficient protease inhibition for the

ancient samples, rather than the cocktail of protease inhibitors required with fresh samples. The pH of hydrochloric acid in the first (demineralisation) stage was altered from 1.2M to 0.6M to reduce early hydrolysis and facilitate TAC binding. Finally, the pH of solutions was changed from 7.4 to 8 in preparation for the TAC following extractions, which requires the latter for the selective binding of only IgG. All four extraction stages and reagent concentrations (e.g., 6M HCl in the fourth extraction) were otherwise identical to the published protocol. The samples chosen represent a range of histological preservations, with EH156.3 (HI: 5), an exceptionally well preserved cranial section, most approximating the fresh cranial sample used by Jiang et al. (2007).

Supernatant fractions from the first three stages (approximately 3ml per sample) were reserved for TAC (see 5.7.5 for methodology) in order to compare quantity of bound 'IgGs' resulting from the P2 and P4 extractions. The fourth stage supernatants were not subjected to TAC, since the high concentration of HCl would likely completely hydrolise and degrade proteins to the peptide level. Although peptide-level IgGs would be suitable for proteomic characterisation, they would not be suitable for TAC purification or SDS-PAGE visualisation.

Sample	Type	HI
EH156.3	Young adult cranium	5
EHA4	Animal rib	4
CD112.3	Very young adult hand phalanx	2
CD84.2	Very young adult cranium	0

Table 5.22: Samples selected for P4 extraction. HI – histological preservation.

The following describes the protocol followed for extraction of P4 samples. After the first, second, and third stages, 250µl of each sample was reserved for nLC-MS/MS analysis (see 5.7.9).

Stage 1:

1. 1g of ground bone sample was split into five 200mg portions in separate micro tubes. Each was incubated overnight at 4°C in 1ml 0.6M HCl, with occasional agitation.

Stage 2:

1. The bone pellets from extraction stage 1 were incubated for 72 hours at 4°C in 1ml 100mM Tris, 6M guanidine-HCl, 5mM benzamidine, and 10mM aminocaproic acid, pH 8, with occasional agitation.

Stage 3:

1. The bone pellet from extraction stage 2 was incubated for 72 hours at 4°C in 1ml 100mM Tris, 6M guanidine-HCl, 0.5M EDTA, 5mM benzamidine, and 10mM aminocaproic acid, pH 8, with occasional agitation.

Stage 4:

1. The bone pellets from extraction stage 3 were incubated overnight in 1ml 6M HCl at 4°C.

2. Upon careful removal of the supernatant, the bone pellets were discarded. 250µl of supernatant from each sample was set aside for proteomic analysis (see 5.7.8), while the remaining supernatant was reserved at -20°C, should further analysis be required.

5.7.9: nLC-MS/MS analysis of post-TAC P3 and P4 samples

A small number of samples (Table 5.23) representing a range of preservations and protein concentrations were analysed by laboratory staff at the Durham University Biological Science Department using nLC-MS/MS for the presence of IgG-related peptides. Searches for matching peptides were made on the Swiss-Prot database and a translation of all human DNA sequences at EMBL (European Molecular Biology Laboratory) (Adrian Brown, personal communication, March 2014).

The two P3 samples were subjected to a longer separation gradient (three hours) with an exclusion list of 198 and 187 m/z values, respectively, in an attempt to exclude common keratin and collagen isoforms detected in previous runs (Adrian Brown, personal communication, April 2014). See section 6.4.5 for further details of this. The resulting peptides were searched against the human DNA sequences at EMBL (Adrian Brown, personal communication, April 2014).

Sample	Protocol	Extraction stage	Protein conc. (mg/ml)
CD120.2	P3	2	0.054
CD120.2	P3	3	0.084
CD84.2	P4	1	0.025
CD84.2	P4	2	0.08
CD84.2	P4	3	0.064
EH156.3	P4	1	0.05
EH156.3	P4	2	0.093
EH156.3	P4	3	0.044

Table 5.23: P3 and P4 samples analysed by nLC-MS/MS.

5.7.10: P2, P3, and P4 Western Blot

A number of samples from the later P2, P3 and P4 extractions (Table 5.23) that were analysed by SDS-PAGE were also selected for Western blot analysis following the protocol described below (which differs slightly from the test run previously). The same primary and secondary antibodies were employed in this testing, since it was anticipated that the much lower quantities of collagen expected in these samples would significantly reduce the cross-reactivity experienced in the P1 Western blot (see section 6.4.1(v)). The following samples were selected for testing based on their likelihood to contain IgG and/or to compare results of other analyses (e.g., proteomic) on the same samples.

Sample	HI	Protocol	Extraction Stage	Protein Concentration (mg/ml)
AN5	0	2.4	3rd	0.084
LP3845.1	0	2.4	3rd	0.088
OL1104.2	5	2.5	2nd	0.013
HP154.1	5	2.5	3rd	0.019
OL1104.2	5	3	3rd	0.021
OL1104.2	5	3	3rd	0.021
OL1104.2	5	3	Post-3rd HCl	0.021
AN5	0	3	1st	0.054
EH198.1	4	3	3rd	0.055
EH198.1	4	3	3rd	0.055
LP3845.1	0	3	1st	0.073
CD120.2	5	3	3rd	0.084
CD120.2	5	3	Post-3rd HCl	0.084
CD165.1	5	3	3rd	0.112
EH198.1	4	3	1st	0.117
HP156.3	5	4.1	3rd	0.044
HP156.3	5	4.1	1st	0.05
CD84.2	0	4.1	3rd	0.064
CD84.2	0	4.1	2nd	0.08
HP156.3	5	4.1	2nd	0.093

Table 5.24: P2, P3 and P4 samples selected for Western blot analysis. HI is histological preservation.

SDS-PAGE:

1. A gel was prepared consisting of 15% resolving and 5% stacking portions.

2. 10µl of each of the twenty samples and IgG positive control were loaded and the gel was run at 100v until samples reached the end of the gel. The use of a positive control negated the need for a molecular weight marker.

Transfer:

1. Transfer apparatus consisting sponges, 6x filter paper, and nitrocellulose membrane were soaked for a few minutes in 100ml transfer buffer (2.4g tris; 11.4g glycine) and 200ml ethanol. The gels were soaked separately in the same solution after careful removal of the stacking gels.

2. The transfer cassettes were opened and with black panel flat on the bottom tray, filled with transfer buffer.

3. The transfer sandwiches were prepared by stacking the components in the flowing order: sponge, 3x filter paper, gel, nitrocellulose membrane, 3x filter paper, and sponge. The gel and membrane were placed on the cathode and anode sides of the cassette, respectively. The sandwiches were then firmly rolled over with a glass tube to remove air bubbles.

4. The transfer cassettes were clamped together and placed into an electrophoresis tank (BioRad®), before being submerged in transfer buffer. Ultrapure water was added to the outer chamber.

5. Protein transfer (blotting) was achieved at 135V for two hours at 4°C.

Development:

1. Membranes were removed from the cassettes and placed in a small container of Ponceau S stain.

2. Membranes were stained with Ponceau S stain until lanes became visible.

3. Membranes were then rinsed in 100ml TBS-T (137mM sodium chloride; 2.7mM potassium chloride; 19mM tris; 0.5ml/l Tween 20), before being blocked overnight in 2% skimmed milk at 4°C. Blocking helps prevent nonspecific antibody/membrane binding.

4. The blocking solution was rinsed off with TBS-T and membranes incubated for 90 minutes in a solution of monoclonal mouse anti-human IgG (heavy chain) antibody at a dilution of 1:1000 (dilution following Schmidt-Schultz and Schultz 2004).

5. The membranes were then washed three times in TBS-T for 15 minutes, five minutes, and five minutes, before being incubated for one hour in a solution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Sigma-Aldrich®) at a dilution of 1:20000.

6. The membranes were then washed three times in TBS-T for 15 minutes, five minutes, and five minutes.

Detection:

1. Membranes were incubated in Supersignal West Pico Chemiluminescent substrate (Thermo Scientific®) for five minutes.

2. The membranes were then transferred to Saran wrap and covered in x-ray film and developed in a dark room and results recorded at exposures of one minute and five minutes.

5.7.11: P5: protein extraction following adapted Cappellini et al. (2012) protocol

Recent research (e.g., Cappellini et al. 2012) has highlighted the vast potential offered by high resolution proteomic methods in accessing previously unobtainable information concerning the survival of ancient proteins in mammalian (Pleistocene mammoth) bone. This included peptides associated with immunoglobulins. It was, therefore, considered prudent to attempt to utilise this methodology to attempt to extract IgGs from selected archaeological human bone samples. Samples chosen for this protocol (Table 5.25) represent a range of histological preservation and include animal bone samples for control purposes.

Sample	Type	HI
AN5	Animal rib	0
LP3760.1	Juvenile rib	0
EH133.1	Child rib	1
HP157.2	Adult rib	2
HP104.2	Child rib	3
CD107.1	Very young adult rib	4
CD120.2	Very young adult hand phalanx	5
OL1104.2	Young adult hand phalanx	5
HPAN5	Animal rib	5

Table 5.25: Samples selected for P5 extraction protocol. HI – histological preservation.

The published extraction protocol (Cappellini et al. 2012) was followed with an additional 0.6M HCl demineralisation stage. This was intended to further break down the remaining hydroxyapatite scaffold within the bone pellets following the ammonium bicarbonate extraction stage, thereby increasing the chances of releasing any retained, tightly mineral-bound non-collagenous proteins. This protocol was incompatible with TAC, since the extended 75°C incubation in the second extraction stage would completely denature any IgGs (Vermeer and Norde, 2000). SDS-PAGE was, however, run on each sample from each extraction stage in order to compare gel profiles to other protocols.

1. 75mg of ground bone sample was incubated overnight at 4°C in 0.5M EDTA, pH 8.01.

2. Samples were then centrifuged for 15 minutes at 17,000g. Supernatants were carefully collected and reserved.

3. Remaining bone pellets were vortexed in 800µl of 50mM ammonium bicarbonate, pH 7.4 and incubated at 75°C for 24 hours.

4. Samples were then centrifuged for 15 minutes at 17,000g. Supernatants were then collected and reserved.

5. Pellets were then incubated overnight at 4°C in 0.6M HCl, before being centrifuged for 15 minutes at 15,000g. Supernatants were collected and the pellets discarded.

5.8: Analysing histological preservation and protein yield

As previously mentioned (section 5.6.2), there exists a lack of consensus concerning bone diagenesis and the survival of collagen, the most abundant bone protein. Less is known about the survival of non-collagenous proteins, although their high affinity to hydroxyapatite may offer preferential protection (Masters, 1987). Histological analysis can characterise bone preservation, providing a simple method of controlling for contamination by exogenous, diagenetically-introduced materials. This research intends to investigate the relationship between histological preservation and quantity of protein yielded from the different extraction protocols described above. This may provide evidence of which extraction protocols and stages are most suitable for each histological preservation category and address the question of preferential survival of the non-collagenous protein IgG. In order to perform these analyses, the protein yields from each wash and elution stage of the protocols employing TAC was measured using a CamSpec M330 spectrophotometer (at 280nm). The resulting figures were then analysed to investigate relationships between histological preservation and protein yield.

5.9: Malaria EIA (ELISA)

A commercially available anti-malaria antibody EIA (ELISA) kit was run on post-TAC eluted samples from the P3, P4 protocols, and samples from the P5 extraction (Table 5.25) which remained after other analyses. The Malaria Total Antibody EIA (Lab 21)

provided all reagents alongside a 96 well plate coated with recombinant antigens capable detecting IgG, IgM, and IgA antibodies to all four species of human malaria. Controls tested included negative and positive controls supplied with the kit, the elution buffer from P3 and P4, and the three buffers from P5, along with ultrapure water. Animal bone controls were also tested. The samples represent a variety of histological preservation.

Sample	Type	HI	Protocol	Stage	Protein conc. (mg/ml)	Sample	Type	HI	Protocol	Stage	Protein conc. (mg/ml)
Negative	Control	-	-	-	-	EH156.3	Human	5	4	3	0.044
Positive	Control	-	-	-	-	HP157.2	Human	2	5	1	-
Elution buffer	Control	-	3 and 4	-	-	CD107.1	Human	4	5	1	-
0.5M EDTA	Control	-	5	-	-	AN5	Animal	0	5	1	-
50mM Ammonium bicarbonate	Control	-	5	-	-	OL1104.2	Human	5	5	1	-
0.6M HCl	Control	-	5	-	-	HPAN5	Animal	5	5	1	-
uH2O	Control	-	-	-	-	EH133.1	Human	1	5	1	-
EH198.1	Human	4	3	1	0.117	HP104.2	Human	3	5	1	-
CD165.1	Human	5	3	1	0.021	HP157.3	Human	2	5	2	-
CD120.1	Human	5	3	2	0.054	CD107.1	Human	4	5	2	-
EH198.1	Human	4	3	3	0.055	AN5	Animal	0	5	2	-
CD165.1	Human	5	3	3	0.112	OL1104.2	Human	5	5	2	-
CD120.1	Human	5	3	3	0.084	HPAN5	Animal	5	5	2	-
OL1104.2	Human	5	3	3	0.021	EH133.1	Human	1	5	2	-
EHA4	Animal	4	4	1	0.025	HP104.2	Human	3	5	2	-
CD84.2	Human	0	4	1	0.025	HP157.3	Human	2	5	3	-
CD112.3	Human	2	4	1	0.034	CD107.1	Human	4	5	3	-
EH156.3	Human	5	4	1	0.05	AN5	Animal	0	5	3	-
EHA4	Animal	4	4	2	0.037	OL1104.2	Human	5	5	3	-
CD84.2	Human	0	4	2	0.08	HPAN5	Animal	5	5	3	-
CD112.3	Human	2	4	2	0.067	EH133.1	Human	1	5	3	-
EH156.3	Human	5	4	2	0.093	HP104.2	Human	3	5	3	-
EHA4	Animal	4	4	3	0.04	HP157.3	Human	2	5	3	-
CD84.2	Human	0	4	3	0.064	CD107.1	Human	4	5	3	-

Table 5.26: P3, P4, and P5 samples tested by malaria ELISA. Protein concentration is taken from Appendix 1, Tables A1.1-A1.7. HI is histological preservation. Stage is extraction stage.

The malaria ELISA was run following the protocol supplied with the kit. All controls and samples were tested in duplicate (apart from the negative control, which was tested three times) to aid in the validation of the results:

1. All reagents and samples were brought to room temperature prior to use.
2. The wash buffer was diluted to 1:20 with distilled water.
3. 50µl of undiluted sample or control (in duplicate) was added to each well. The negative control was tested in triplicate. The plate was then shaken gently for 30 seconds to mix.
4. The plate was incubated (covered) at 37°C for 30 minutes.
5. The wells were then washed five times with wash buffer, allowing 30 seconds soak time between each wash cycle.
6. The conjugate was diluted 1:10 in conjugate buffer (50µl+ 500µl per 10 wells). 50µl of diluted conjugate was then added to each well.
7. The plate as then incubated (covered) at 37°C for 30 minutes. The wells were then washed five times with wash buffer, allowing 30 seconds soak time between each wash cycle.
8. 50µl of substrate/chromogen mixture was added to each well.
9. The plate was then incubated at room temperature for 30 minutes. As the substrate was photosensitive, the plate was protected from light during this incubation.
10. 50µl of stop solution was added to each well. The results were read at 450nm (A_{450}) using a BioTek® Synergy H4 Hybrid Plate Reader.
11. The cut-off value was calculated by taking the mean of the negative control values and adding 0.100.

5.10: Rapid testing using the CareStart™ Malaria Rapydtest®

Following the apparent success of Fornaciari et al. (2010) in extracting and detecting *P. falciparum*-specific antigens using commercially-available rapid tests, it was decided to subject a selection of samples (Table 5.27) to similar testing in the search for any surviving malaria antigens. Two rounds of testing were performed utilising the CareStart™ Malaria

Rapydtest® VOM (Apacor), a cassette-type rapid test capable of detecting *P. falciparum* histidine-rich protein-2 (PfHRP2) and non-*falciparum* lactate dehydrogenase. Analysis requires the introduction of 5µl of sample into the sample well, followed by 90µl of assay buffer to the buffer well. Results are read in 20 minutes and interpreted following the directions in Figure 5.9. Two separate batches of CareStart™ Malaria Rapydtests® were required for this analysis. In the second batch of the 'VOM' line was replaced with a '2', and the 'Pf' line was replaced with a '1' (see Figures 5.9 and 5.10 for examples). Both older and newer styles of test were identical in function. An example of functioning tests is given for reference purposes in Figure 5.10

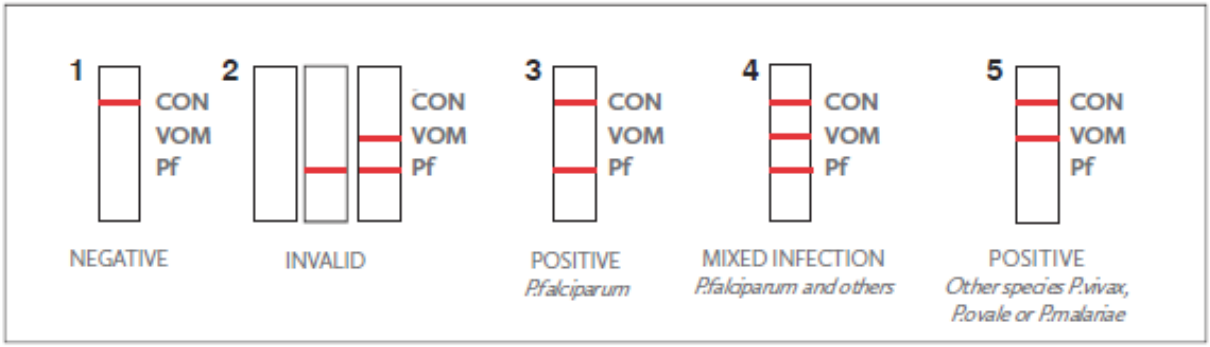

Figure 5.9: Interpretation of the CareStart™ Malaria Rapydtest® (Apacor 2013). In the newer style of cassette, 'VOM' is equivalent to '2', while 'Pf' is equivalent to '1'.

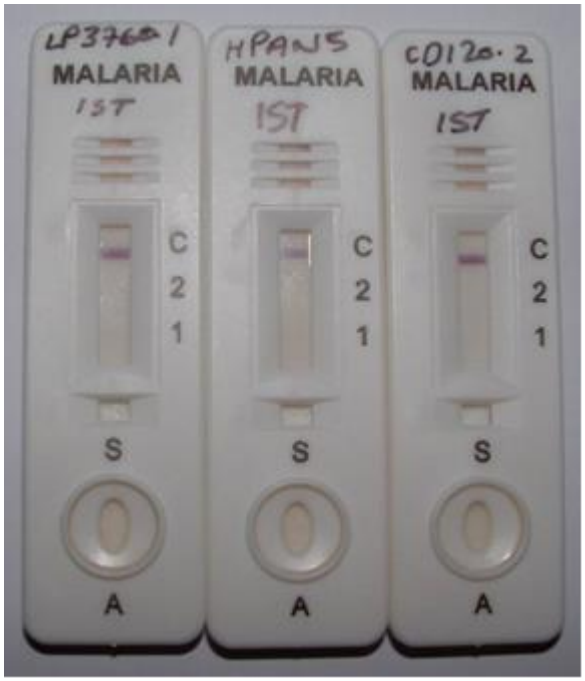

Figure 5.10: Functional CareStart™ Malaria Rapydtests®. Negative results shown (photograph by author).

The first round of testing was performed on samples (Table 5.27) following the extraction protocol of Fornaciari et al. (2010):

1. 50mg of ground bone sample was suspended in 200µl sterile PBS and subjected to four 10-second liquid nitrogen freeze thaw cycles.

2. The sample was then incubated for 24 hours at 4°C, before being incubated for 30 minutes at 37°C.

3. Following centrifugation for 10 minutes at 10,000 rpm, each sample supernatant was tested using the CareStart™ Malaria Rapydtest®.

4. Samples are considered positive by the manufacturer if the appropriate lines develop within 20 minutes of testing.

Sample	Type	HI
HP136.1	Adolescent rib	2
HP154.1	Young adult rib	5
HP227.2	Young child rib	2
WM2291.1	Child rib	0
CD120.2	Very young adult hand phalanx	5
CD165.1	Child rib	5
LP3760.1	Juvenile rib	0
LP3687.1	Young adult rib	1
LP3819D	Very young adult dentine	?
LP4116.1	Juvenile rib	0
LP4585.3	Middle adult hand phalanx	2
EH133.1	Child rib	1
EH198.1	Very young adult rib	4
EH584.1	Child rib	0
OL1100.2	Child cranium	1
OL1104.2	Young adult hand phalanx	5

Table 5.27: Samples tested using protein extraction protocol of Fornaciari et al. (2010). HI - histological preservation.

The second round of testing was analysed P2.5 samples (Table 5.28) after each the three adapted Jiang et al. (2007) extraction stages, along with collagen, and animal bone controls. These samples were analysed to see if detectable malaria antigens were released during each state of this exhaustive protein extraction protocol. Extracts from P2.5 samples (third extraction stage) following TAC were also subjected to rapid testing. Finally, a small number of samples (Table 5.28) following each of the three P5 extraction stages were selected for rapid testing.

Sample	Type	HI
HP154.1	Young adult rib	5
LP3760.1	Juvenile rib	0
LP4585.3	Middle adult hand phalanx	2
OL1104.3	Young adult hand phalanx	5
LP3819D	Very young adult dentine	?

Table 5.28: P2.5 samples subjected to rapid testing. HI – histological preservation.

Sample	Type	HI
LP3760.1	Juvenile rib	0
CD120.2	Very young adult hand phalanx	5
HPAN5	Animal rib	5

Table 5.29: P5 samples selected for rapid testing. HI – histological preservation.

5.11: Statistical analyses

Statistical testing was performed on data resulting from the palaeodemographic analysis of cemetery reports from Fen/non-Fen populations (sections 4.2.11 and 5.2) in order to investigate the hypothesis that the presence of *vivax* malaria would significantly contribute to increased mortality in Fenland environments, and that this increase would be reflected in age-at-death mortality ratios. It is this comparison which forms the null hypothesis that age at death is independent of population location, where location is the dependent nominal variable, and age-at-death category is the independent nominal variable. Testing of the hypothesis was achieved by comparing the observed and expected age-at-death frequencies for archaeological Fen and non-Fen cemetery populations using the chi-square (χ^2) test for independence, with an alpha-level of <0.05 and degrees of freedom (DF) dependent upon number of categories. Kolmogorov-Smirnov (K-S) testing was also performed to investigate the null hypothesis that there is no significant difference in age-at-death distributions between Fen and non-Fen populations. K-S testing was chosen due to the nonparametric nature of the data and small samples sizes involved.

Chi-square independence testing was also performed to investigate the null hypothesis that histological preservation of bone samples is independent of the age-at-death of the sampled individual. All chi-square analyses were performed using Microsoft Excel, while K-S analyses were performed using the PAST (Paleontological Statistics) software package (Hammer et al., 2001).

CHAPTER 6: RESULTS

This chapter presents the results of analyses described in Chapter 5 concerning the presence of *Plasmodium vivax* malaria in the past British Fenlands. First to be presented are the results of the analysis of published osteological reports aimed at identifying thalassaemia sequelae in archaeological populations from potentially malarious areas. Results of the demographic analyses of selected Fen and non-Fen cemetery populations are shown, followed by the histological testing of bone samples selected for biomolecular analysis. Outcomes of the five protocols (P1 to P5) aimed at extracting immunoglobulin G (IgG) from archaeological human bone are then described, followed by a brief investigation of the histological preservation and post-thiophilic adsorption chromatography (TAC) protein yields. Finally, the results of the malaria EIA and rapid testing are presented.

6.1: Identification of thalassaemia sequelae in skeletal reports

The results of the analysis of published osteological reports, each of which was examined in the light of the recently suggested skeletal sequelae of thalassaemia discussed in section 5.1, are presented here. The selected reports represent the sites outlined in section 5.3. The significance of the findings in terms of possible thalassaemia is discussed in section 7.1.

The identification of potentially unrecognised or misdiagnosed cases of thalassaemia in archaeological and palaeopathological reports is highly dependent upon the quality and detail of the original analysis of the skeletal material. In a number of cases, reports consisted of brief communications on the main pathologies encountered within populations, with very limited detail on individual skeletons (e.g., the *Durobrivae* and Baston reports). The limited pathological descriptions of skeletal individuals from these sites may be the result of a number of factors. Firstly, and as mentioned in section 4.2.8, the pressures of osteological analysis in a developer-funded setting may result in a short skeletal analysis included within a larger site report, with the intention of providing an in-depth report (often depending upon funding, which may not materialise) at a later date. This seems to have been the case for some sites in which the cemetery is not necessarily the primary focus of excavation, such as *Durobrivae* and The Parks, Godmanchester. In instances where the cemetery is the primary

focus, palaeopathological analyses are usually more thoroughly reported. It is these reports of larger cemetery sites that provided sufficient pathological detail at the individual level to be able to identify sequelae of interest. It is no surprise, therefore, that the majority of skeletons displaying pathologies consistent with possible thalassaemia (listed in Table 6.1) were encountered in these larger sites. These sites comprised The Hoplands, Castledyke South, and Highfield Farm, Littleport. It may also be the case that larger cemetery sites are usually analysed by highly experienced palaeopathologists, who are likely to recognise potential indicators of putative thalassaemia and perform additional diagnostic investigations, such as radiography (e.g., see Figure 6.2).

The level of detail of pathological analyses is, of course, dependent upon skeletal preservation. At certain sites, such as Watersmeet, Huntingdon and Westfield Farm, Ely, taphonomic processes and post-depositional disturbance resulted in fragmentation and poor survival of many inhumed individuals (Nicholson 2006; Lucy 2007a). Such preservation would hinder any diagnosis of thalassaemia, since many of the 'specific' sequelae highlighted in Table 5.1 affect elements that are inherently fragile (e.g., facial bones and ribs). Pathologies affecting the ribs (e.g., cortical erosion and multiple healed fractures) or that result in structural weakening of the bone may be difficult to distinguish in highly fragmented skeletal material.

Table 6.1 shows the possible thalassaemia sequelae identified and which sites yielded individuals presenting with one or more of these changes. Littleport skeleton 3760, an individual of approximately 8-12 years, was the only one to display more than one sequela (porotic hyperostosis and associated 'hair-on-end' appearance of the diploë). This individual was, therefore, subjected to further macroscopic and radiographic analysis (section 6.1.4). The majority of sequelae were encountered in Anglo-Saxon populations, which likely reflects the bias towards this time period in site selection.

Site		Period	Skeleton affected	Sequelae				
				Porotic hyperostosis	**Cranial diploë thickening**	**'Hair-on-end' appearance in cranial diploë**	**Scoliosis**	Multiple rib fractures
Cambridgeshire	The Parks, Godmanchester	Rom	37					X
	Edix Hill	A-S	198	?				
	Highfield Farm, Littleport	A-S	3479					X
			3745				X	
			3760	X		X		
			4095				X	
			4858				X	
Lincolnshire	Castledyke South, Barton-upon-Humber	A-S	15					X
			49					X
			73					X
			75					X
	The Hoplands, Sleaford	Rom	46				X	
			59					X
			63					X
			96				X	
			117					X
			128					X
			164					X
			168					X
			202					X
			211		X			

Table 6.1: Summary table of sites and skeletal individuals displaying possible thalassaemia sequelae. Those in bold are cited as being more specific for thalassaemia (after Hershkovitz et al., 1997; Ortner, 2003; Lagia et al., 2007; Lewis, 2010; Yochum and Rowe, 2005; Perisano et al. 2012). Rom – Roman, A-S – Anglo-Saxon.

6.1.1: Porotic hyperostosis

One skeleton (SK3760) from the Littleport site (Table 6.1) displayed cranial lesions consistent with porotic hyperostosis (Figure 6.1), with radiographic analysis confirming the characteristic 'hair-on-end' arrangement within the diploë (Figure 6.2). Skeleton 198 from Edix Hill (Table 6.1) also displayed cranial lesions suggestive of porotic hyperostosis, but

radiographic analysis conducted at the time of reporting proved inconclusive (Duhig 1998). Unfortunately, the burial catalogue was not sufficiently detailed to provide any further relevant pathological description for this individual, and the individual was unavailable for further study due to archive relocation. One prevalence of thickening of the cranial diploë was reported in the Hoplands population (Western, 2011), but no additional suggestive sequelae were reported in this individual.

Figure 6.1: Porotic hyperostosis of the left parietal bone from Littleport individual SK3760 (photograph reproduced with permission of G. Western).

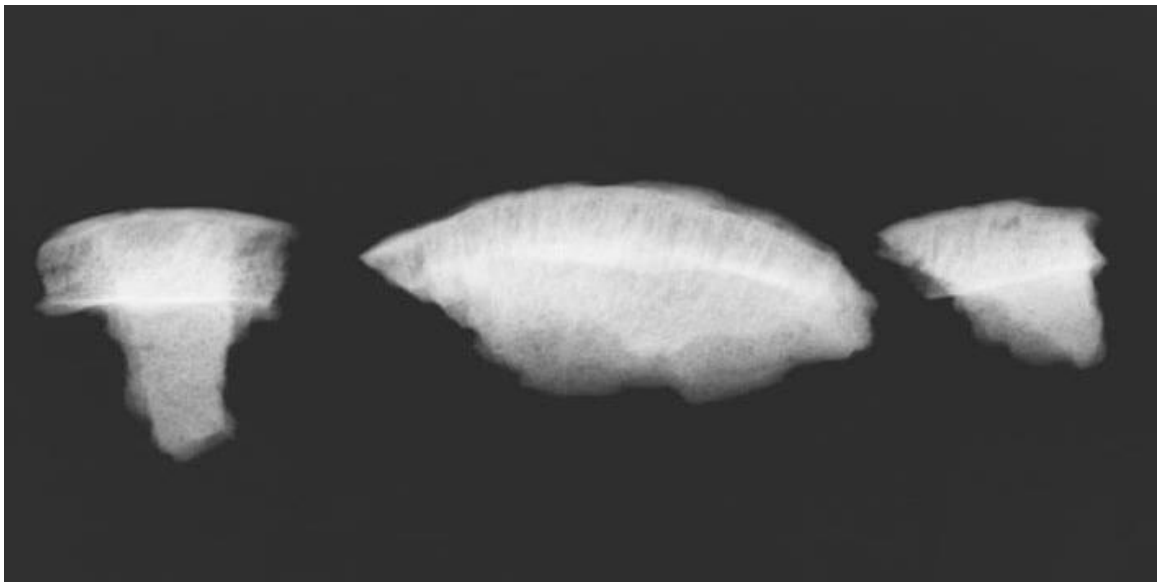

Figure 6.2: Littleport skeleton 3760 left parietal radiograph showing distinctive diploë 'hair-on-end' appearance (reproduced with permission of G. Western. Image taken by Mark Farmer on behalf of Reveal Imaging Ltd).

6.1.2: Multiple rib fractures

Multiple rib fractures in individuals were the most frequently encountered 'thalassaemia' sequela listed in Table 5.1. This is perhaps unsurprising, given that rib fractures are one of the most frequently reported traumatic lesions in the palaeopathological literature (Brickley, 2006). It has been argued that since rib fractures are so commonly observed, they are "rarely included in studies investigating trauma in past societies" (Brickley 2006:62). This may explain why most of the reports selected for this study failed to provide details of rib fractures affecting individual skeletons, instead offering population-wide prevalence rates.

6.1.3: Scoliosis

Scoliosis was reported in three individuals from Littleport and two from the Hoplands, Sleaford site (Table 6.1). Idiopathic scoliosis has a reported prevalence rate of 5.2% in modern populations (Konieczny et al., 2013). Up to 40% of thalassaemic individuals develop non-idiopathic scoliosis (Taher et al., 2006), but the morphological difference between the two is very difficult to identify in archaeological skeletons, particularly if the vertebrae are poorly preserved. A differential diagnosis of thalassaemic scoliosis would rely on the presence of supportive sequelae within individuals. As preservation and careful analysis of each element are key to diagnosis, the condition may sometime be under-represented in archaeological populations.

6.1.4: Further analysis of Littleport skeleton 3760

In response to the combination of skeletal sequelae suggestive of possible thalassaemia (Table 6.1), Littleport individual 3760 was acquired for further macroscopic and radiographic analysis. The latter was based on the macroscopic appearance of the extant elements, and with reference to the diagnostic radiography performed by Lewis (2010) on the Poundbury sub-adults. The skeleton was very fragmentary (less than 25% complete), which precluded observation of a number of criteria listed in Table 5.1 (e.g., no facial bones and very few vertebrae). Extant elements showed no macroscopically observable changes suggestive of thalassaemia. However, a selection of the fragmentary ribs was radiographed in order to compare to Lewis' observed 'rib-within-a-rib' pathology. No evidence of this

was observed (Figure 6.3). None of the ribs showed macroscopic evidence of fractures, calluses, or cortical erosion beyond that usually associated with post-depositional damage.

Figure 6.3: Radiograph of rib fragments from Littleport skeleton 3760 (radiograph by author).

6.2: Demographic analysis of Fen and non-Fen cemetery populations

The following presents the results from the demographic analysis of cemetery populations from Fen/non-Fen sites, as discussed in section 5.2. The age categories are defined in Table 6.2.

Foetal/neonate	<1 month
Infant	1-12 months
Child	1-6 years
Juvenile	7-12 years
Adolescent	13-17 years
Very young adult	18-24 years
Young adult	25-34 years
Middle adult	35-49 years
Old adult	50+ years
Adult	18+

Table 6.2: Age category definition for demographic analysis.

6.2.1: The sites and their individuals

The Fen and non-Fen sites selected for this demographic study and their respective number of individuals (separated into the age categories shown in Table 6.2) are presented in summary Tables 6.3 and 6.4. Full tables (A2.1 and A2.2) for these data are shown in Appendix 2.

	< 1 month	**1-12 months**	**1-6 years**	**7-12 years**	**13-17 years**	**18-24 years**	**25-34 years**	**35-49 years**	**50+**	**Adult 18+**	**Total**
						M/F	**M/F**	**M/F**	**M/F**	**M/F**	
Roman	4	3	15	18	9	2/5	17/17	18/17	18/18	5/7	173
Anglo-Saxon	22	138	202	158	94	84/117	182/179	144/115	189/131	172/142	2069
Medieval	25	128	136	126	63	28/38	80/62	43/16	84/44	120/177	1170
Total	51	269	353	302	166	114/160	279/258	205/148	291/193	297/326	3412

Table 6.3: Numbers of individuals from combined Fen sites. M/F – Males/Females.

	< 1 month	**1-12 months**	**1-6 years**	**7-12 years**	**13-17 years**	**18-24 years**	**25-34 years**	**35-49 years**	**50+**	**Adult 18+**	**Total**
						M/F	**M/F**	**M/F**	**M/F**	**M/F**	
Roman	89	32	67	50	17	20/27	59/48	71/49	53/36	45/41	704
Anglo-Saxon	26	66	140	111	100	101/122	138/120	89/92	89/42	76/73	1385
Medieval	51	61	157	71	42	44/30	41/27	36/33	91/50	86/39	859
Total	166	159	364	232	159	165/179	238/195	196/174	233/128	207/153	2948

Table 6.4: Numbers of individuals from combined non-Fen sites. M/F – Males/Females.

6.2.2: Mortality, survivorship, and probability of death

The data presented in Tables 6.3 and 6.4 were used to construct period-specific and total Fen and non-Fen mortality profiles, survivorship curves, and probability of death curves. The aim was to investigate differences in cemetery demographics between locations in order to address the question of whether such differences might support the presence of *vivax* malaria in the Fens in antiquity. Where appropriate, chi square (χ^2) tests of independence were conducted for total Fen/non-Fen samples to test the null hypothesis that age-at-death is independent of Fen/non-Fen location. Kolmogorov-Smirnov (K-S) tests were also applied to investigate differences in Fen/non-Fen populations in terms of age-at-death distributions. Table 6.5 summarises the results of these tests. The demographics are first addressed by period, then by overall picture of total Fen/non-Fen mortality, survivorship, and probability of death. Given the vast quantity of information presented from these data, the most useful and potentially enlightening trends are emphasised here (in terms of how they may relate to health and the presence of putative *vivax* malaria). These are then discussed in section 7.1.2, 7.1.3, and 7.1.4. The limitations of such palaeodemographic analyses are addressed in section 7.1.5.

Mortality profiles (in the form of population percentages) for each period are presented for the total excavated skeletal populations in all age categories. The aged adults are then separated by sex in order investigate possible differential mortality based on sex. Survivorship (l_x) and probability of death (q_x) curves were calculated following the construction of basic life tables (after Chamberlain, 2006) based upon the raw data presented in Tables 6.3 and 6.4. Both types of analysis require a zero start point, so they must assume that all individuals are alive at birth. Survivorship curves also assume a constant decline in numbers between 50 and 100 years, since the final age category is 50+ and it is highly unlikely that a significant quantity of individuals in antiquity would live beyond 100. Both types also assume that populations are relatively stable in terms of fertility and mortality rates, a situation more likely to be encountered in pre-modern populations as instability "associated with demographic transitions appear to be a recent historical phenomenon" (Chamberlain, 2006:27).

6.2.2(i): The Roman period

Figure 6.4 suggests that overall age-at-death in the Roman period was significantly affected by location (χ^2 $p<0.01$), although the significance is lost when comparing aged adults (Figure 6.5). K-S testing showed significance ($p<0.01$) for total population and aged adults (Figures 6.4 and 6.5). K-S testing was also performed omitting the <1 age category, since the Roman Fen sample is severely lacking in individuals of this age. Significance ($p<0.01$) remained the same, however. It is important to note the low Fen sample numbers due to the lack of excavated Roman Fen cemetery sites (Table 6.3), and the subsequent disparity between Fen and non-Fen sample numbers. The impact of very low numbers of Fen infants (<1 year old) can be been seen in Figures 6.6 and 6.7 (survivorship) and 6.8 (probability of death), which assume that low numbers equates to low mortality. The overall disparity in population numbers may indeed account for the observed difference in survivorship between Fen and non-Fen (Figures 6.6, 6.7, and 6.9). Roman adult male and female mortality, survivorship, and probability of death are shown in Figures 6.5, 6.7, and 6.7).

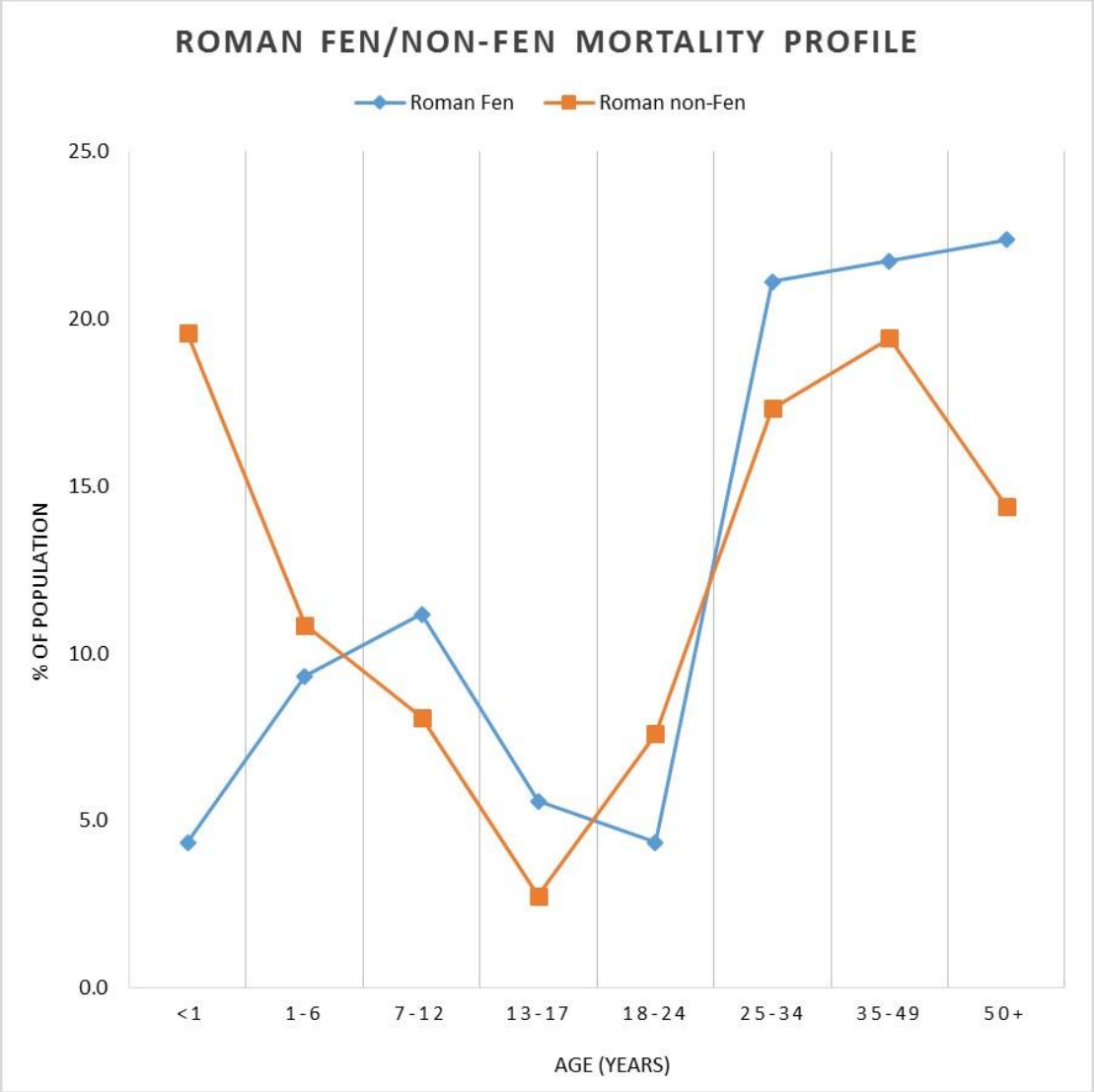

Figure 6.4: Roman Fen/non-Fen mortality profile. χ^2 p= <0.01. K-S p = <0.01. Omitting <1 category: K-S p = <0.01. *n* – 161 Fen, 618 non-Fen.

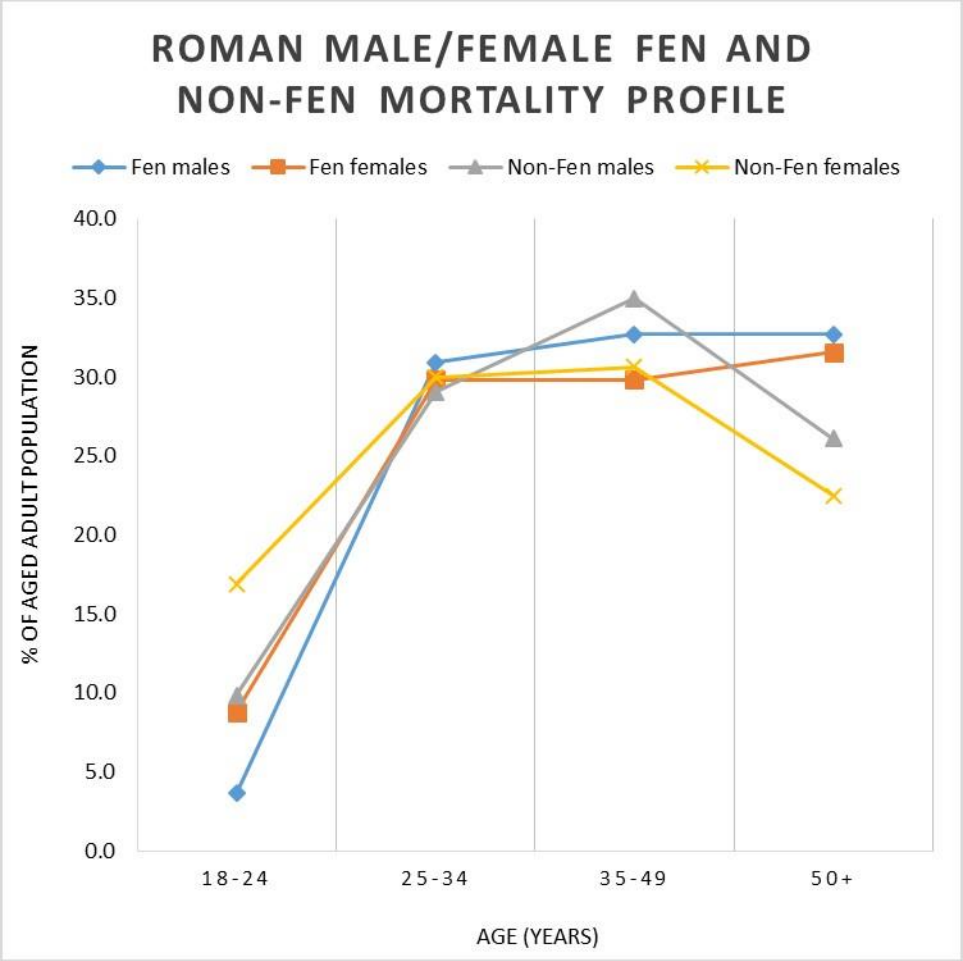

Figure 6.5: Roman male and female Fen and non-Fen mortality profile. χ^2 p = 0.07. *n* – 55 Fen males, 203 non-Fen males – K-S p = 0.01; 57 Fen females, 160 non-Fen females – K-S p = 0.01.

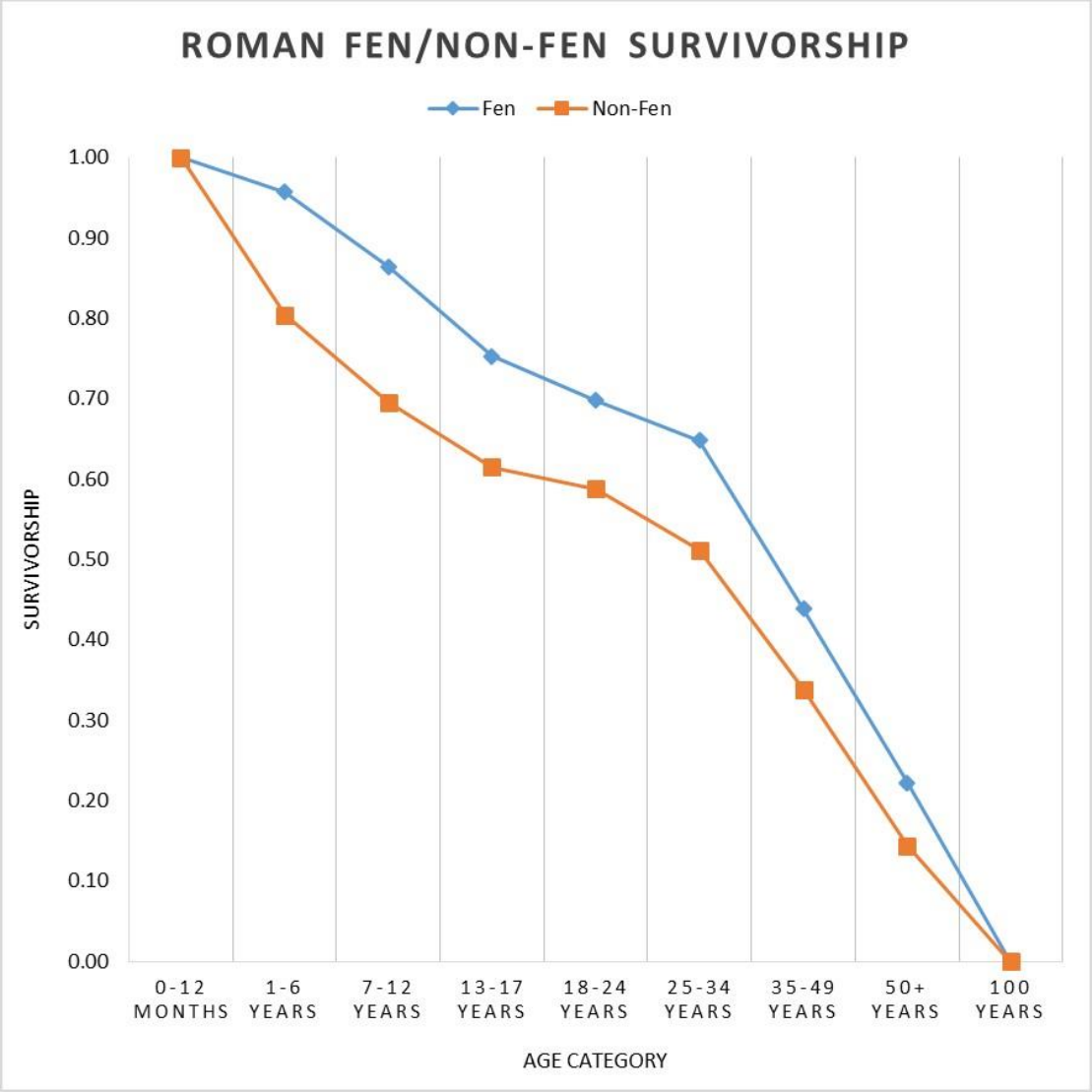

Figure 6.6: Roman Fen and non-Fen survivorship (l_x). *n* – 161 Fen, 618 non-Fen.

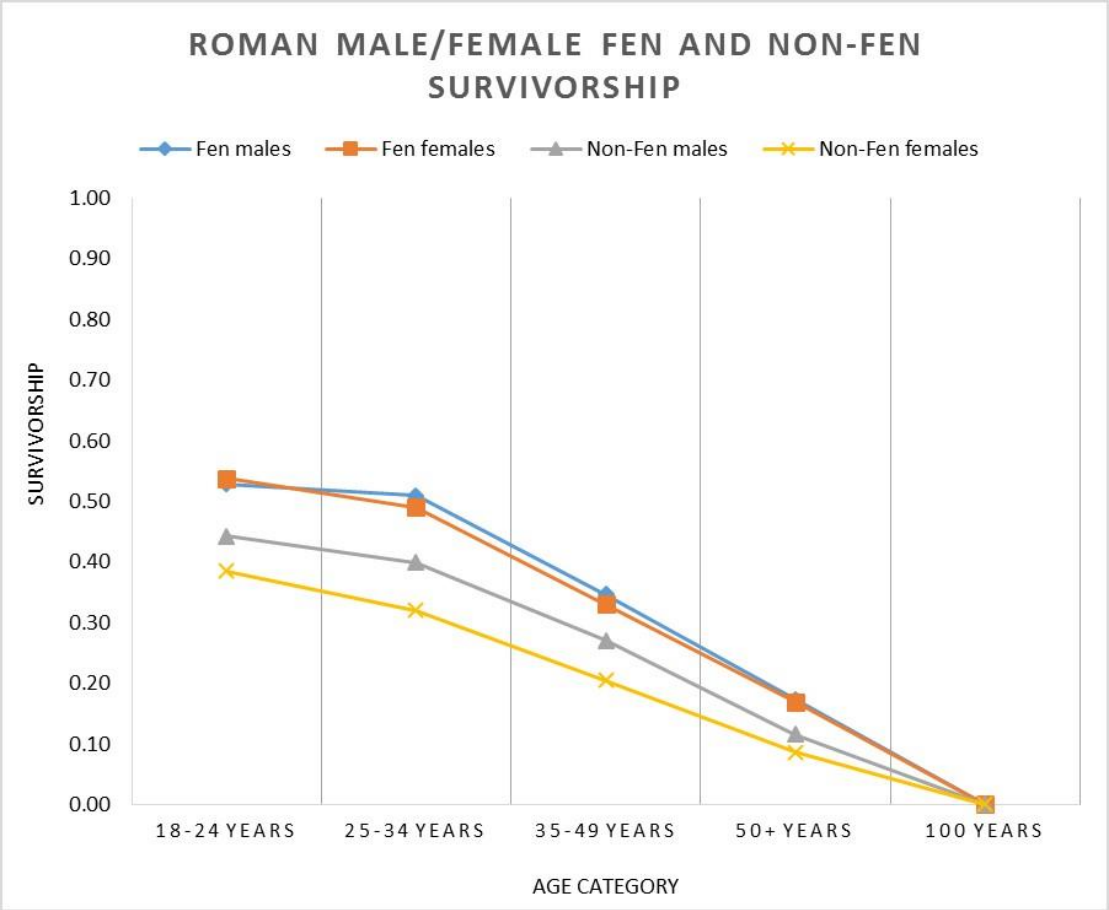

Figure 6.7: Roman male and female Fen and non-Fen survivorship (l_x). *n* – 55 Fen males, 203 non-Fen males; 57 Fen females, 160 non-Fen females.

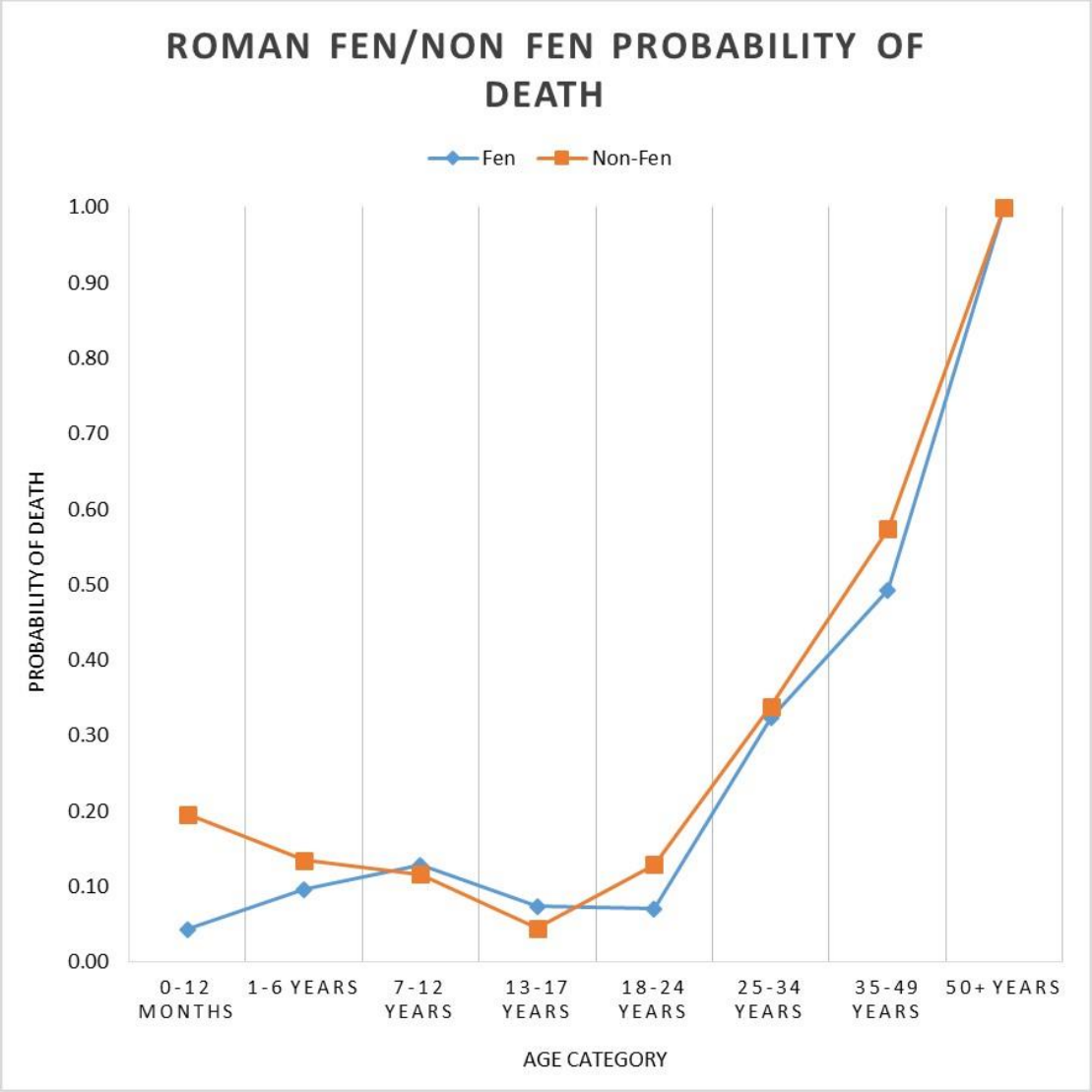

Figure 6.8: Roman Fen and non-Fen probability of death (q_x). n – 162 Fen, 618 non-Fen.

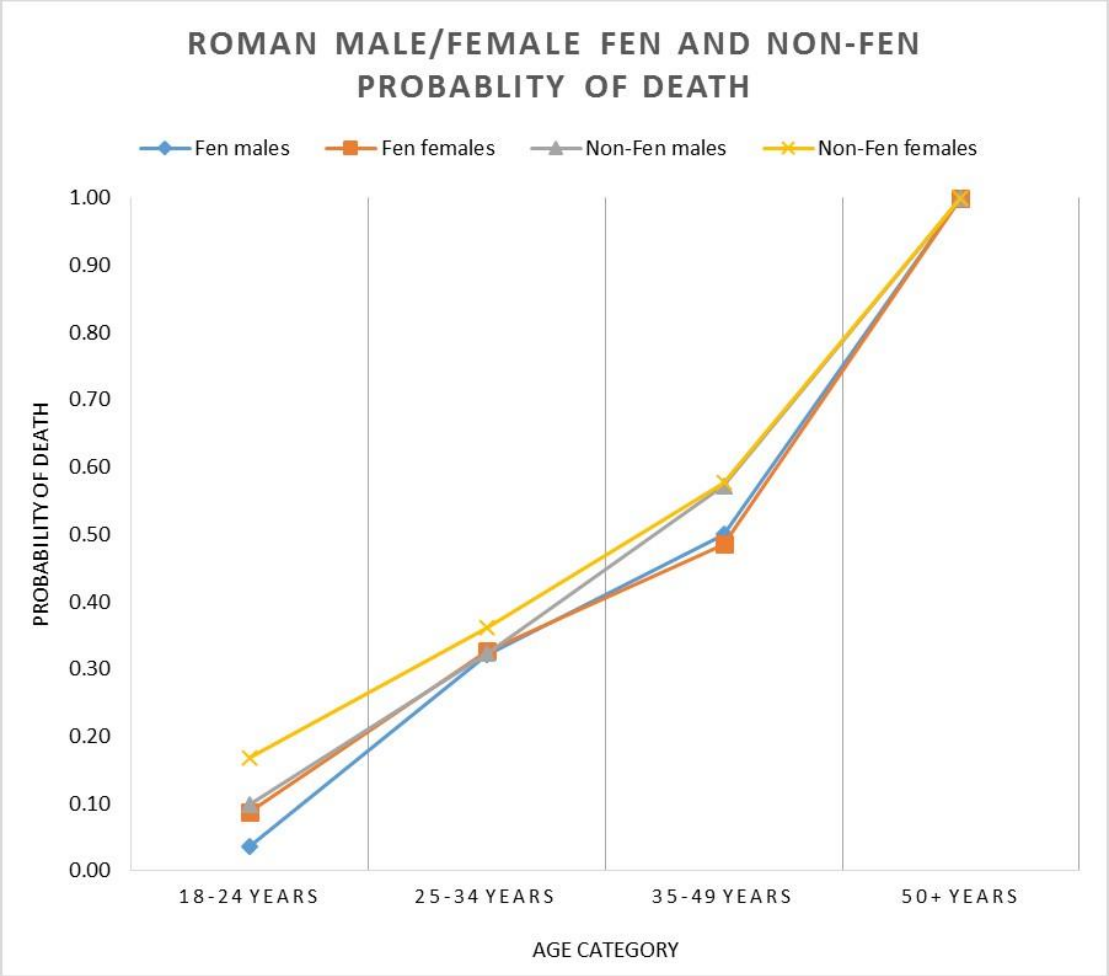

Figure 6.9: Roman male and female Fen and non-Fen probability of death (q_x). *n* – 55 Fen males, 203 non-Fen males; 57 Fen females, 160 non-Fen females.

6.2.2(ii): The Anglo-Saxon period

The Anglo-Saxon period is represented by the largest numbers of both Fen and non-Fen individuals (1755 and 1236 aged individuals, respectively). As with the Roman period, there is a correlation (χ^2, $p < 0.01$) between location and age-at-death (Figure 6.10). Conversely, no significance in terms of age-at-death distribution (K-S, $p = 0.42$) is observed for the Anglo-Saxon period. The relatively low numbers in the <1 year category reflects a general dearth of infant burials in the Anglo-Saxon period (discussed in Section 7.1.5), rather than being an artefact of overall low numbers such as was encountered in the Fen Roman sample. This period-specific trend can clearly be seen in Figures 6.10, 6.11, and 6.15, particularly when compared to the corresponding medieval data (Figure 6.17, 6.18,

6.23, and 6.24). Note the relatively high mortality rates for non-Fen females aged 18-24, compared to their Fen counterparts (Figure 6.12). It is interesting to note that while non-Fen females seem to have been at particular risk of premature death (as further evidenced in Figures 6.14 and 6.16), Fen males display the opposite trend.

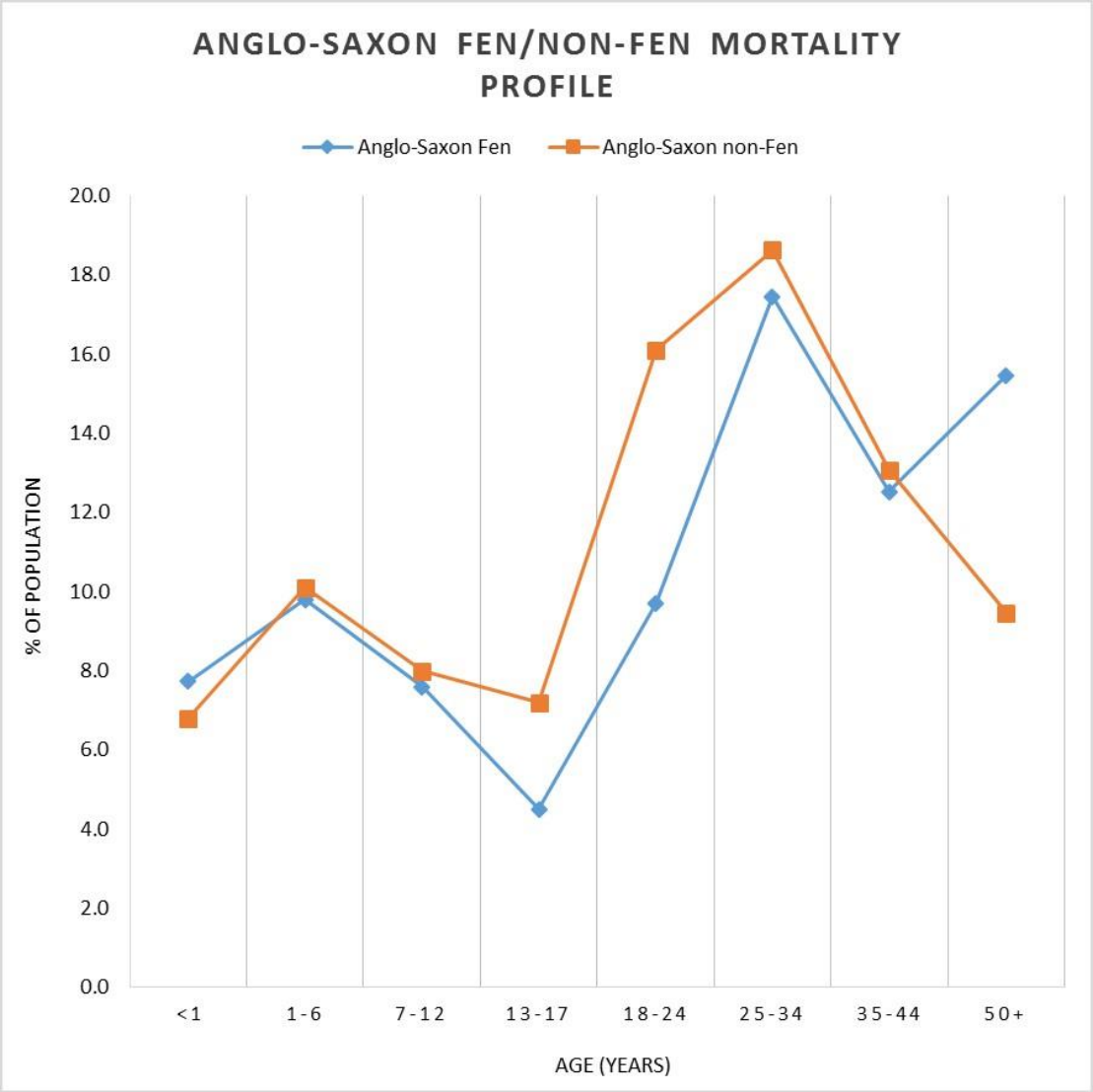

Figure 6.10: Total Anglo-Saxon Fen and non-Fen mortality profile. χ^2, p <0.01. K-S, p = 0.42. *n* = 1755 Fen, 1236 non-Fen.

Figure 6.11 compares Fen and non-Fen mortality in the Anglo-Saxon period when the St. Peter's Barton-upon-Humber sample is removed from the Fen population set (the total Fen population is included in the graph for comparison purposes). Since this sample accounts for 56% of the Anglo-Saxon aged Fen population, it is important to investigate any

skewing effect that this site has on the overall picture of Anglo-Saxon Fen mortality. The significance of location remains the same (χ^2 p <0.01) with the St. Peter's sample removed, although the K-S testing showed no significant difference (K-S p = 0.93) in age-at-death distribution between Fen (St. Peter's sample removed) and non-Fen sites. A main point of interest here is a trend towards a larger numbers of adults (particularly in the 35-49 year category) in the St. Peter's sample, perhaps suggesting that more people at this site lived into older age, or that younger individuals are less well represented at this cemetery. These data are discussed further in Section 7.1.3.

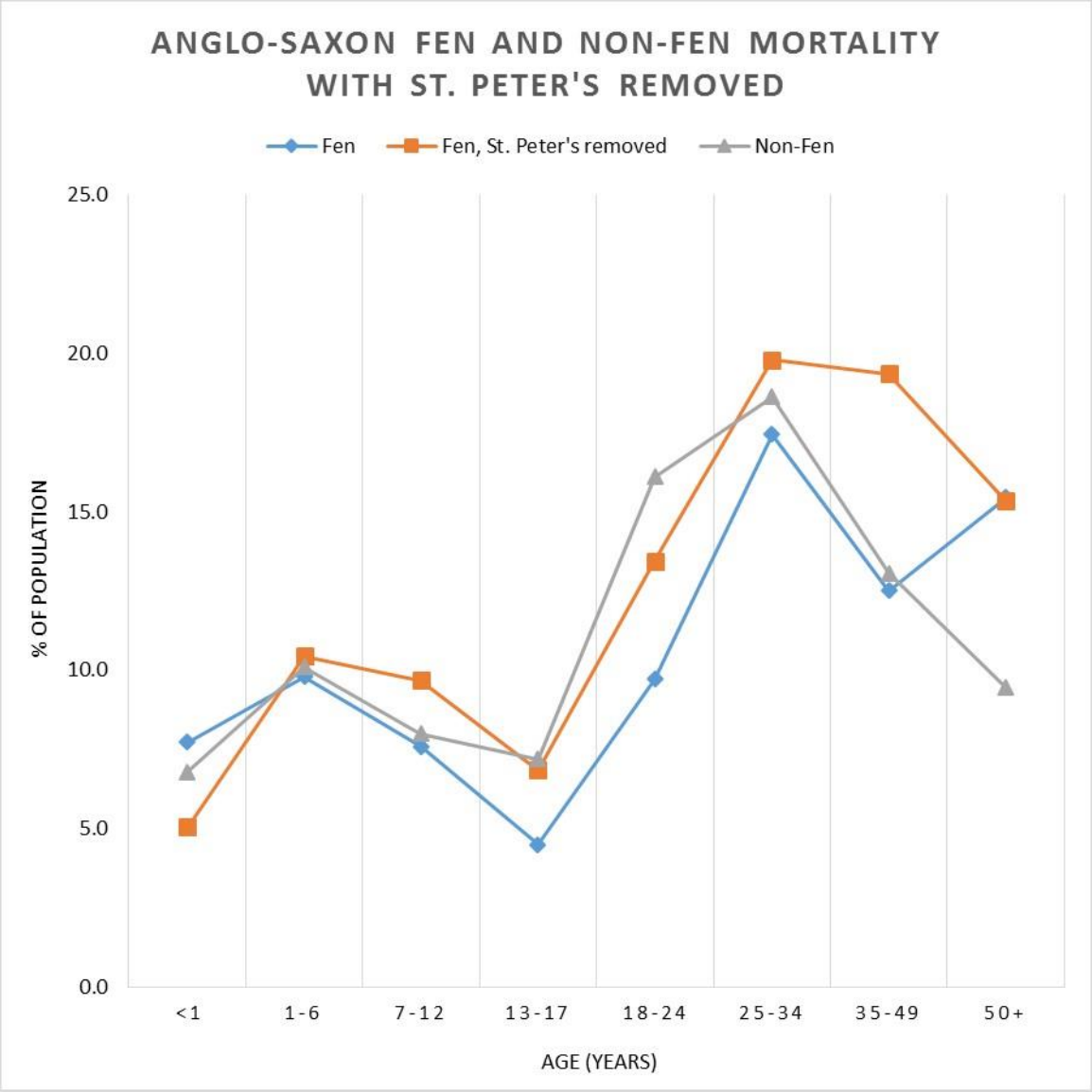

Figure 6.11: Anglo-Saxon Fen/non-Fen mortality profiles with St. Peter's sample removed. χ^2, p <0.01. K-S, p = 0.93. *n* – 1328 Fen, 1385 non-Fen.

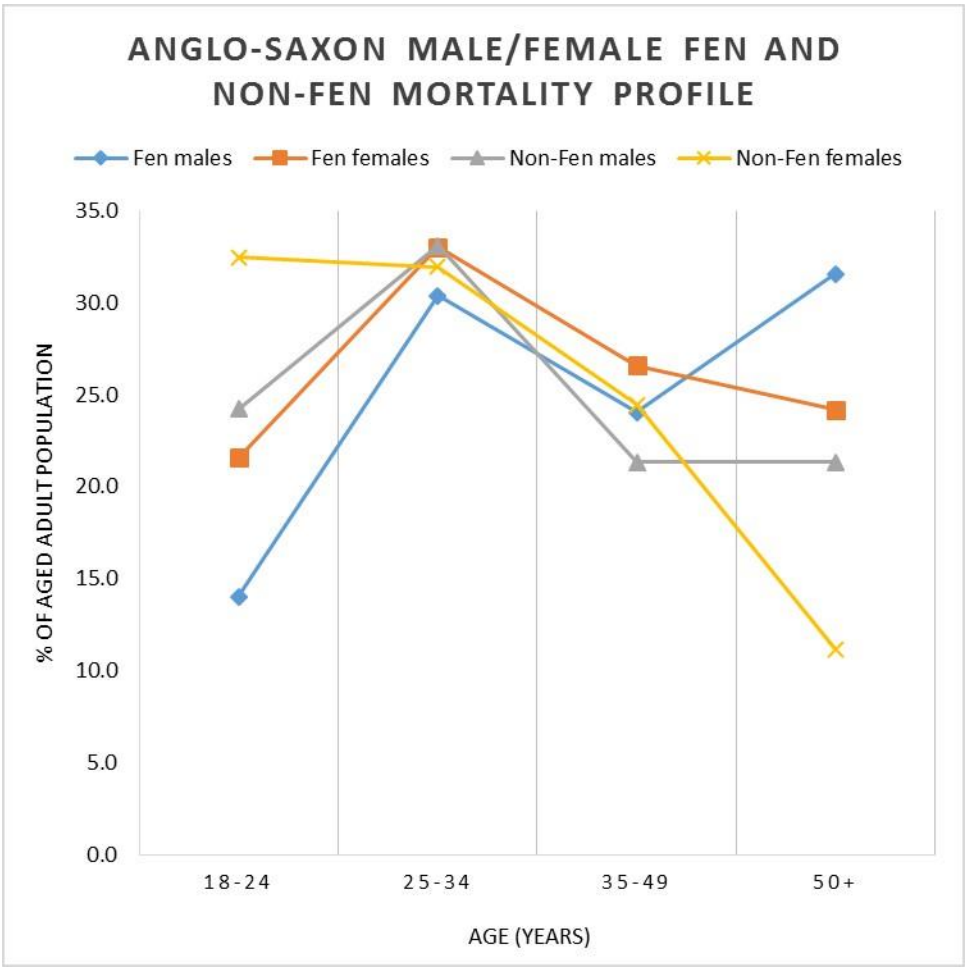

Figure 6.12: Anglo-Saxon male and female Fen and non-Fen mortality profile. χ^2, p <0.01. *n* = 599 Fen males, 417 non-Fen males – K-S, p = 0.11; 542 Fen females, 376 non-Fen females – K-S, p = 0.53.

Figure 6.13: Anglo-Saxon Fen and non-Fen survivorship (l_x). *n* – 1755 Fen, 1236 non-Fen.

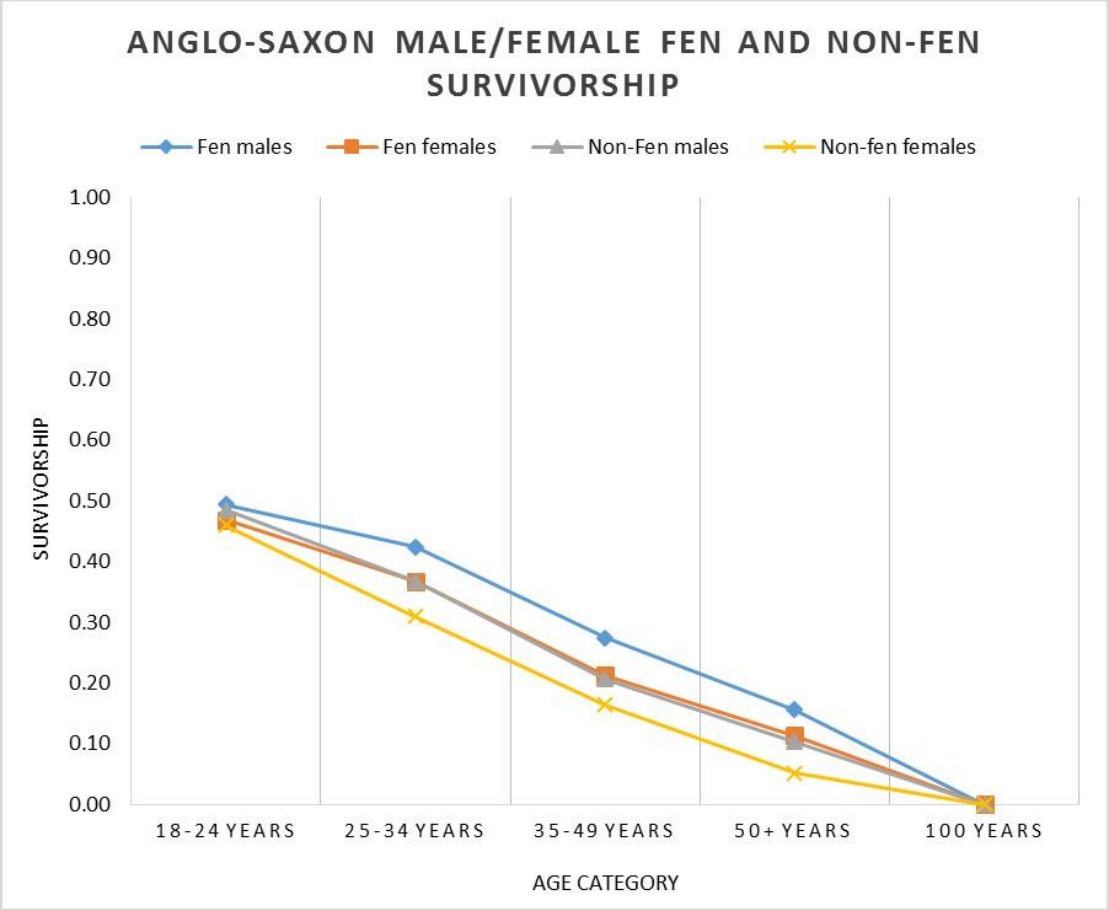

Figure 6.14: Anglo-Saxon male and female Fen and non-Fen survivorship (l_x). *n* – 599 Fen males, 417 non-Fen males; 542 Fen females, 376 non-Fen females.

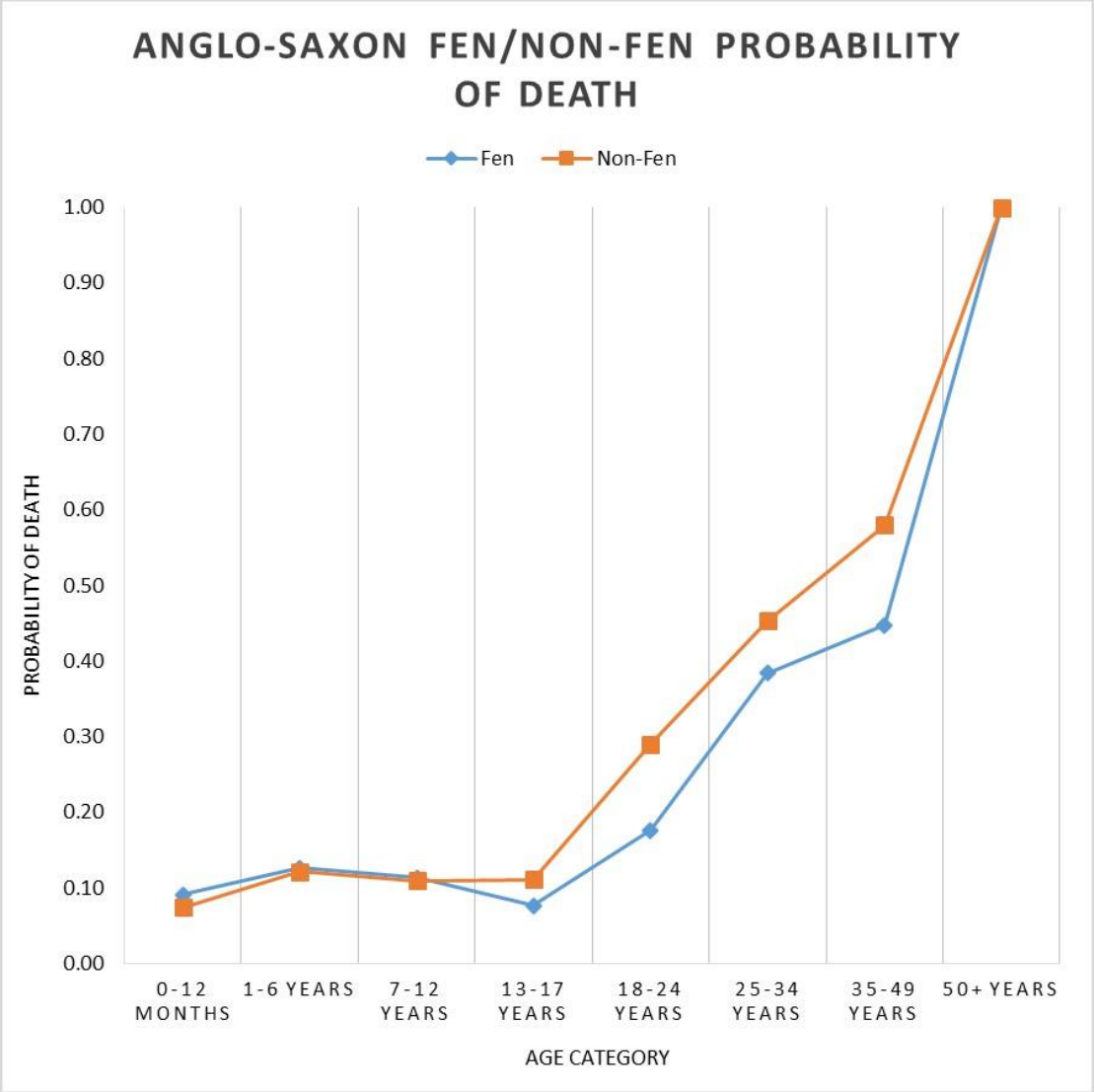

Figure 6.15: Anglo-Saxon probability of death (q_x). *n* – 1755 Fen, 1236 non-Fen.

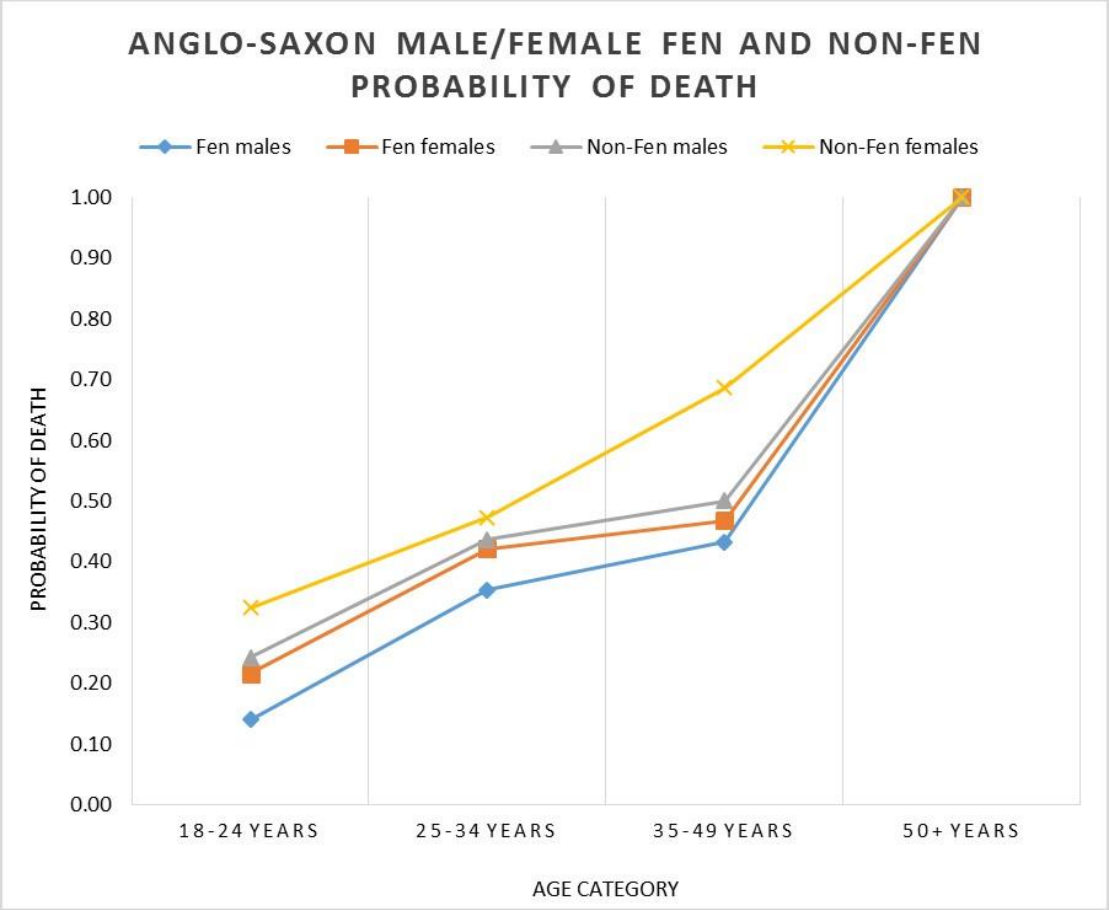

Figure 6.16: Anglo-Saxon male and female Fen and non-Fen probability of death (q_x). *n* – 599 Fen males, 417 non-Fen males; 542 Fen females, 376 non-Fen females.

6.2.2(iii): The medieval period

The Fen and non-Fen medieval samples are dominated by two large cemetery populations: St. Peter's (Barton-upon-Humber) and Wharram Percy account for 73% and 75% of the medieval Fen and non-Fen samples, respectively. It was important to investigate any skewing effect that the two largest sample might have on the tests. Consequently, the same statistical tests were run after removing these sites. Chi-square suggests location to be a significant ($p<0.01$) factor in age-at-death, before (Figure 6.17) and after (Figure 6.18) removal of these sites. Prior to removal, K-S testing shows no significance ($p=0.52$) in age-at-death distribution between Fen and non-Fen. However, this reverses upon removal of the two largest sites, with significance emerging ($p<0.01$).

Figure 6.17 shows high infant (<1 year old) mortality at both St. Peter's and Wharram Percy, with a subsequent reduction in the number of people surviving to

adulthood. These figures drop quite dramatically when the sites are removed (Figure 6.18). The peak in childhood mortality at Wharram Percy is followed by a subsequent reduction in the number of people surviving to adulthood. This skewing effect is evident in Figures 6.17, 6.19, and 6.23, which show lower-than-expected percentages of aged adults. The non-Fen mortality profile following the removal of the Wharram Percy sample may be more representative of a 'normal' medieval non-Fen profile, particularly as concerns the effects of high childhood mortality on subsequent age category representation.

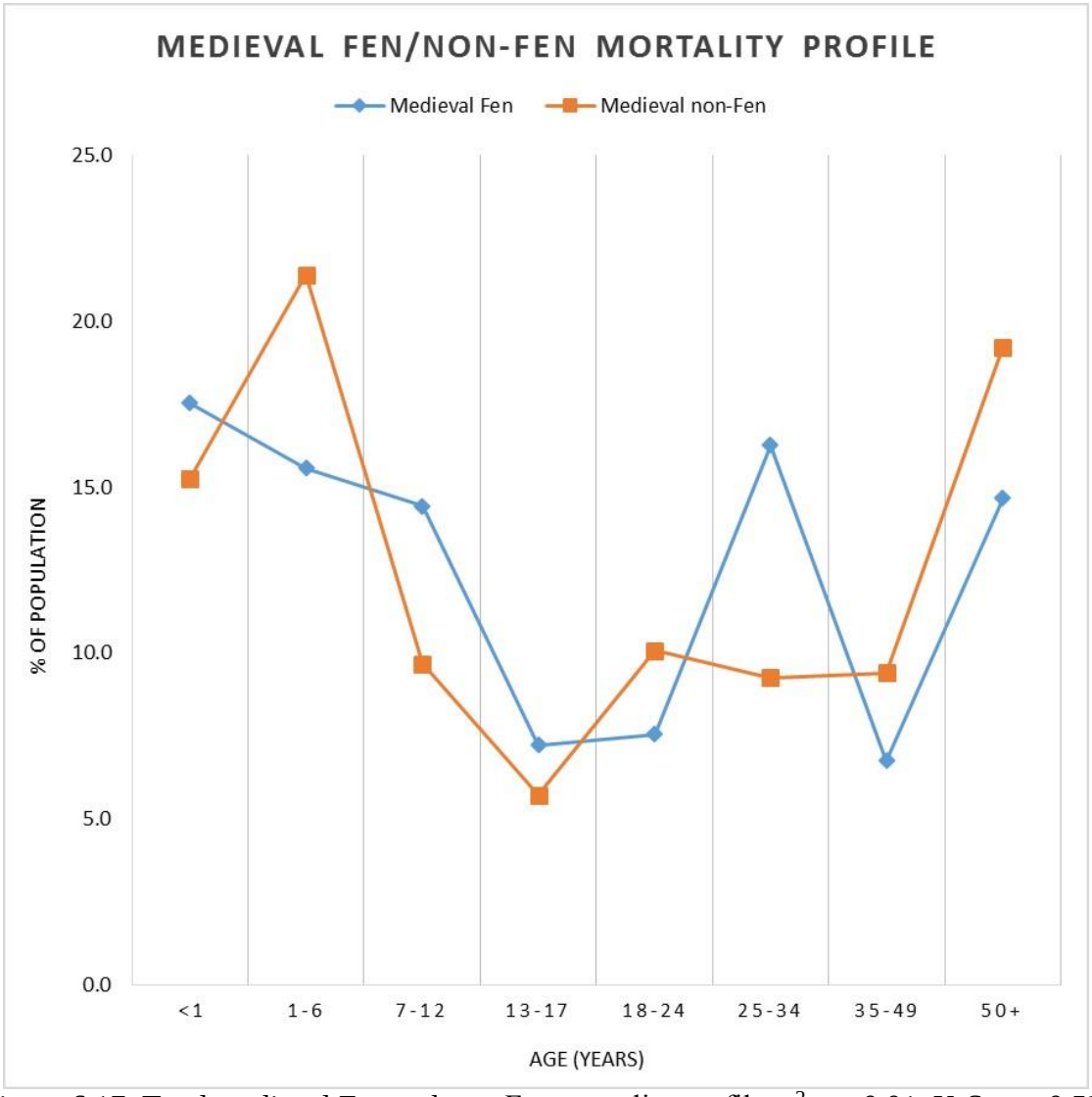

Figure 6.17: Total medieval Fen and non-Fen mortality profile. χ^2, p <0.01. K-S, p = 0.52. *n* = 873 Fen, 834 non-Fen.

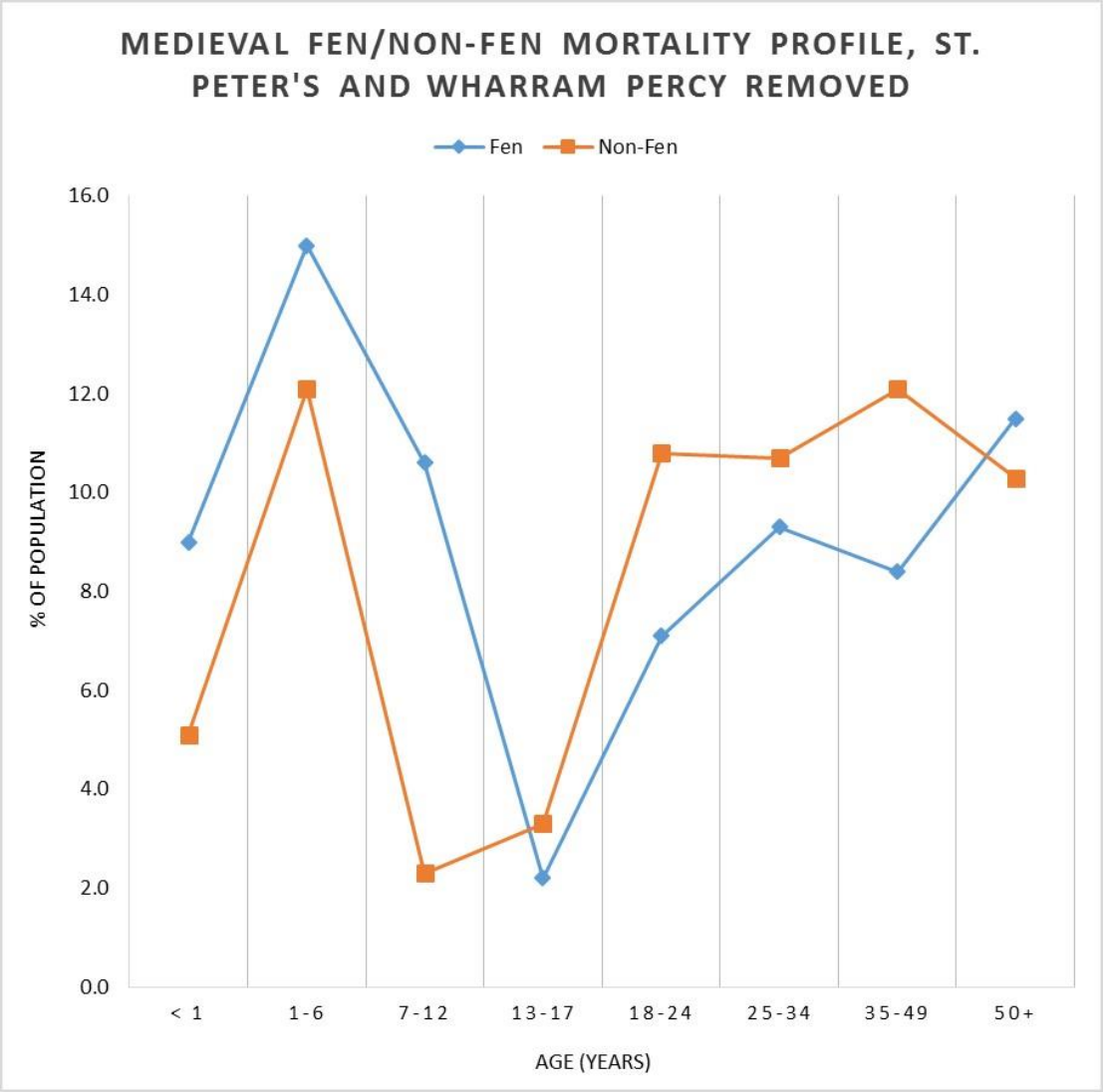

Figure 6.18: Medieval Fen and non-Fen mortality profile with St. Peter's and Wharram Percy samples removed. χ^2, p <0.01. K-S, p <0.01. *n* = 321 Fen, 214 non-Fen.

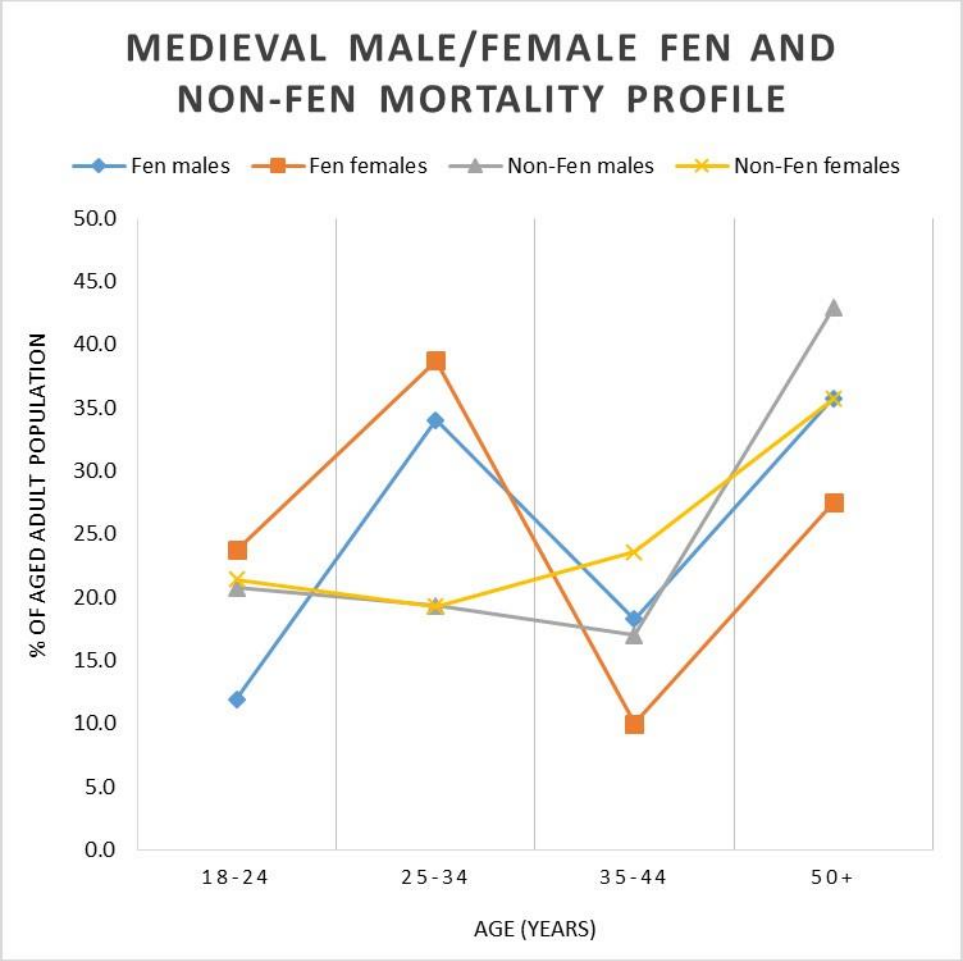

Figure 6.19: Medieval male and female Fen and non-Fen mortality profile. χ^2, p <0.01. K-S, p = 0.53. *n* = 235 Fen males, 212 non-Fen males – K-S, p = 0.10; 160 Fen females, 140 non-Fen females – K-S, p = 0.53.

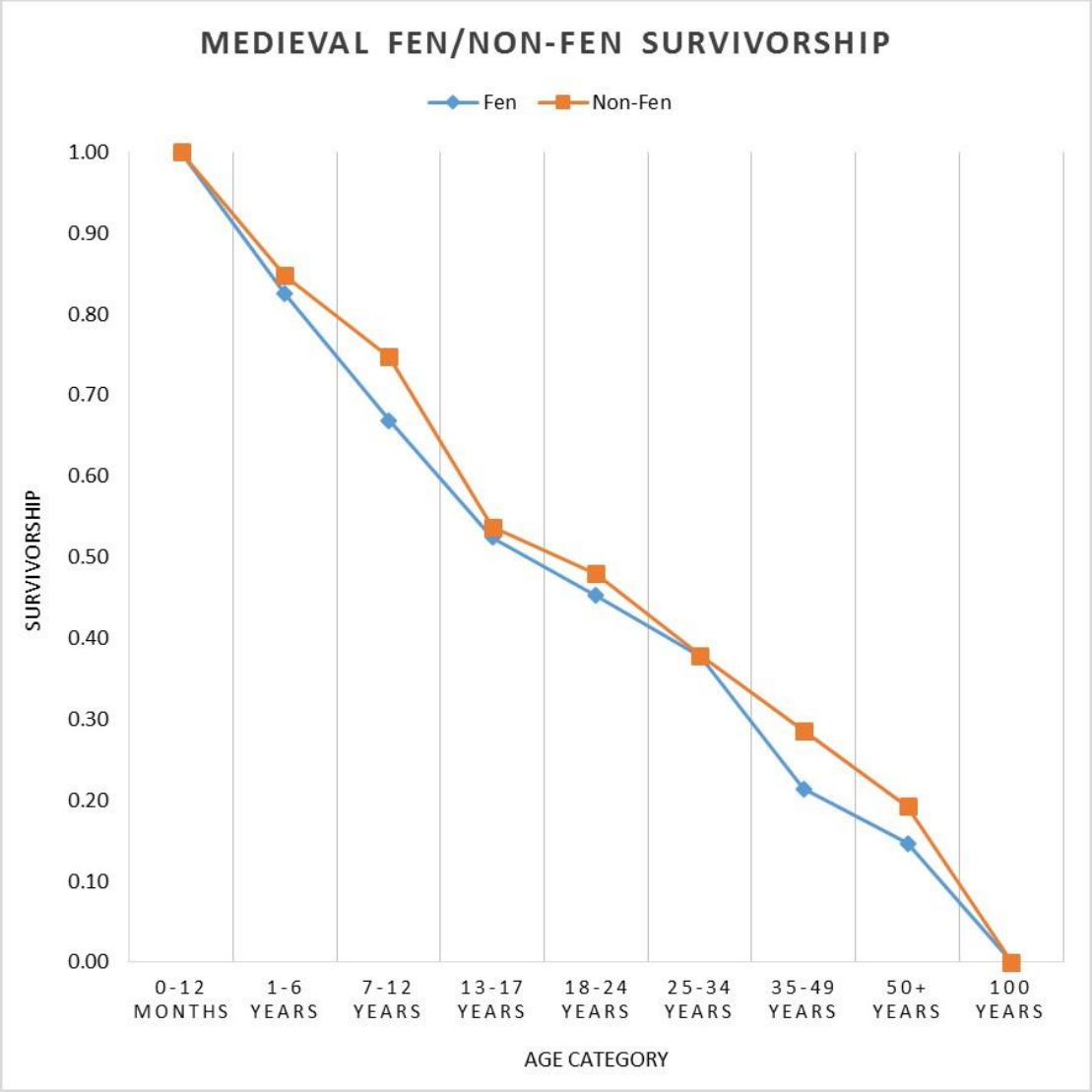

Figure 6.20: Total medieval Fen and non-Fen survivorship (l_x). n = 873 Fen, 734 non-Fen.

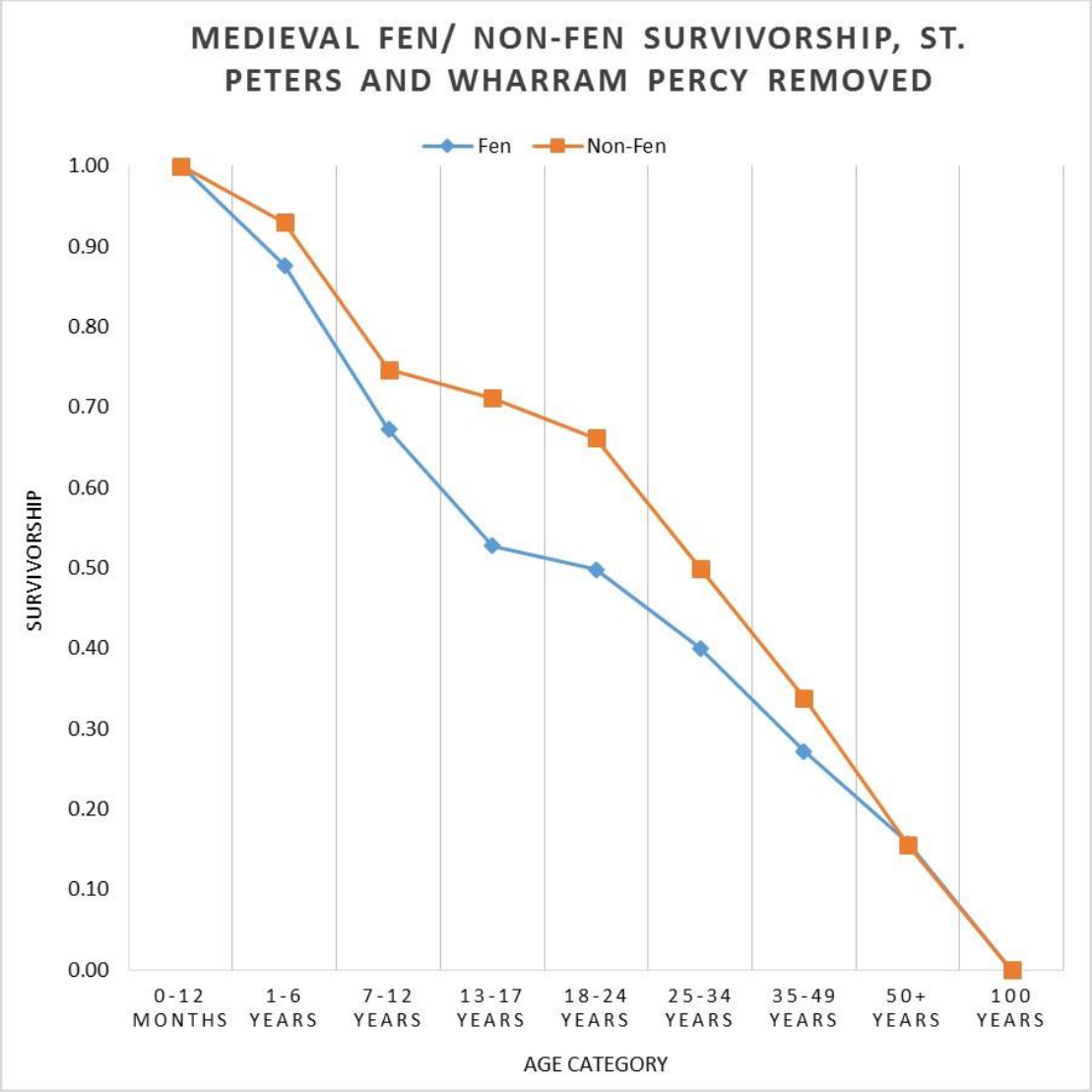

Figure 6.21: Medieval Fen and non-Fen survivorship (l_x) with St. Peter's and Wharram Percy samples removed. n = 235 Fen, 142 non-Fen.

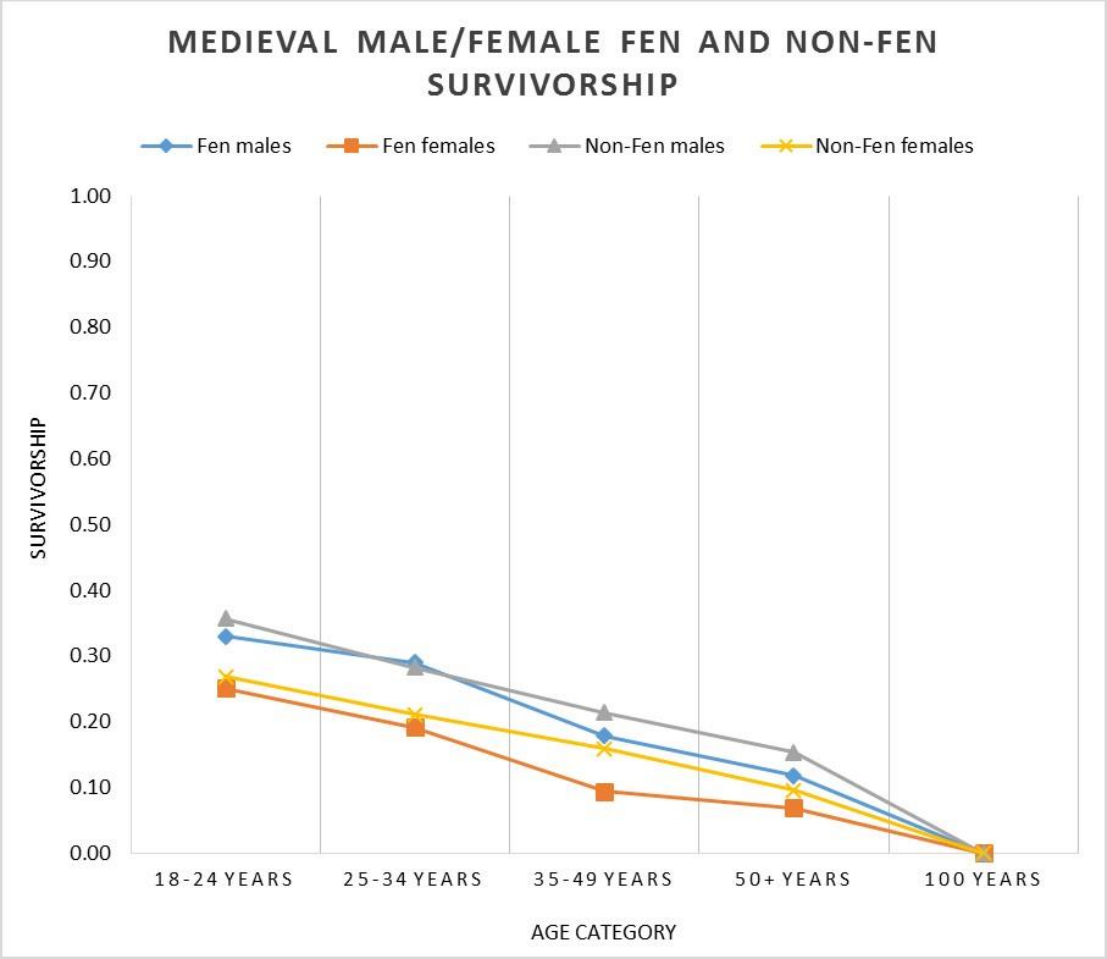

Figure 6.22: Medieval male and female Fen and non-Fen survivorship (l_x). n = 235 Fen males, 212 non-Fen males; 160 Fen females, 140 non-Fen females.

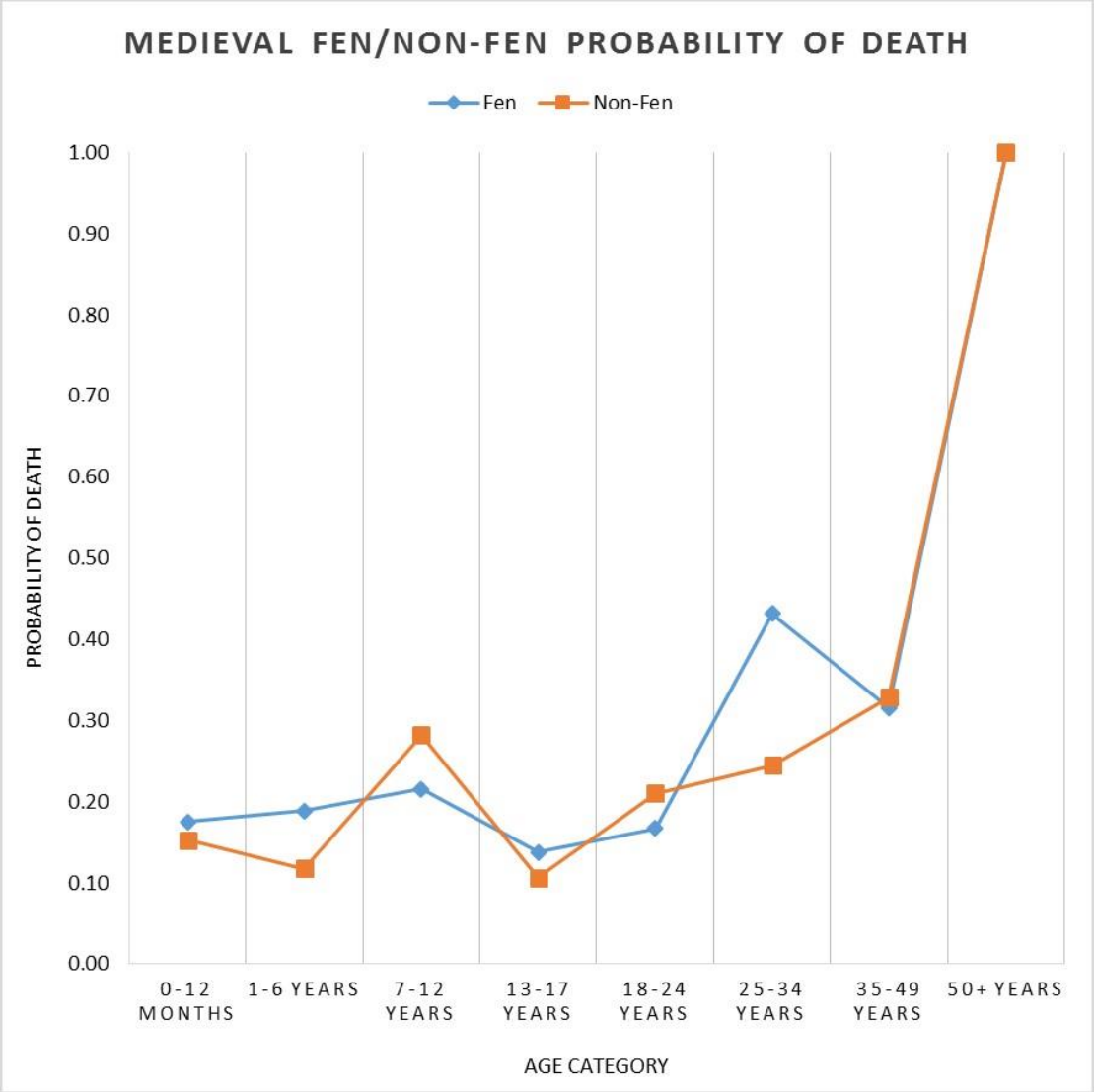

Figure 6.23: Total medieval Fen and non-Fen probability of death (q_x). n = 873 Fen, 734 non-Fen.

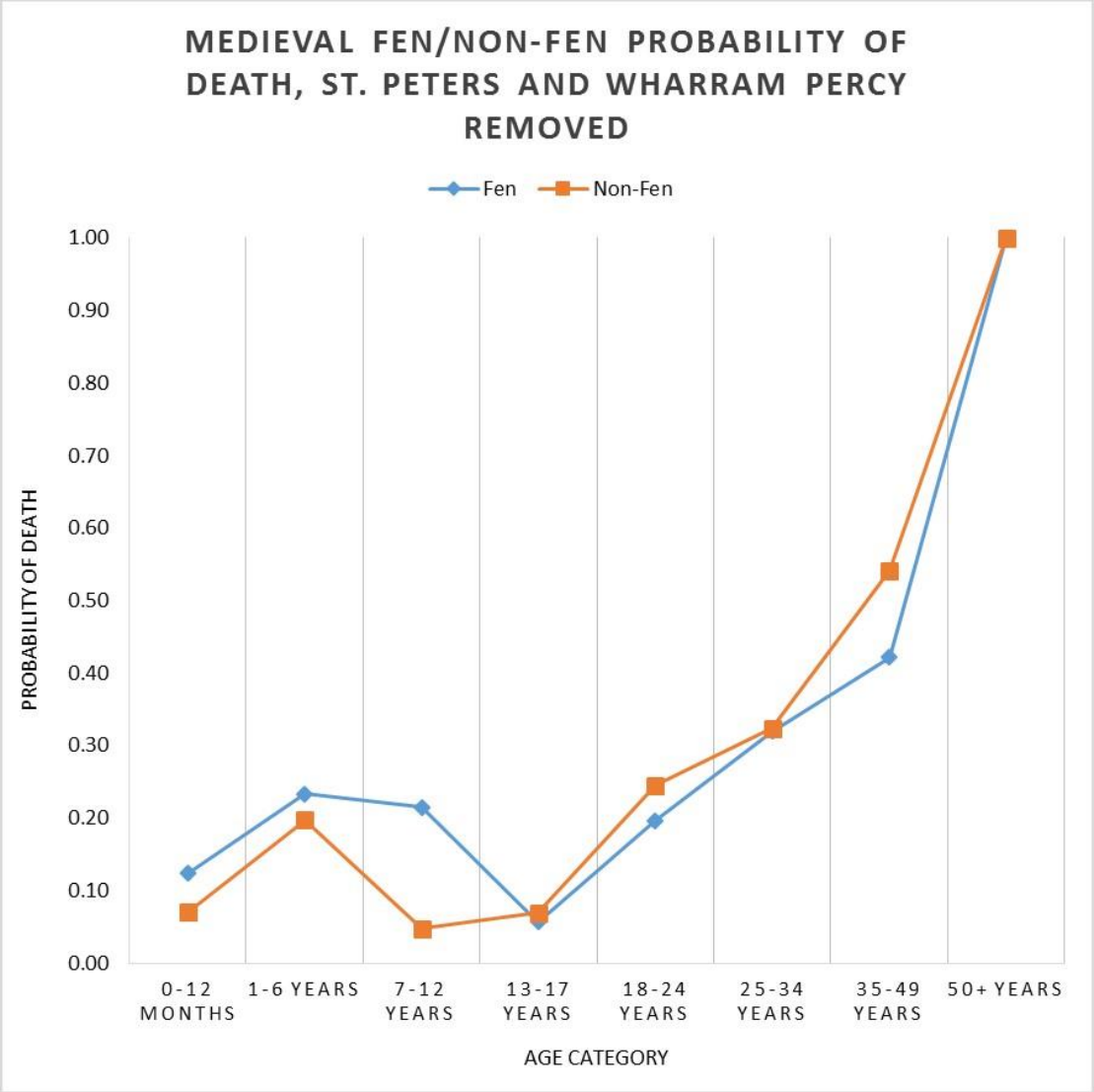

Figure 6.24: Medieval Fen and non-Fen probability of death (q_x) with St. Peter's and Wharram Percy samples removed. n = 235 Fen, 142 non-Fen.

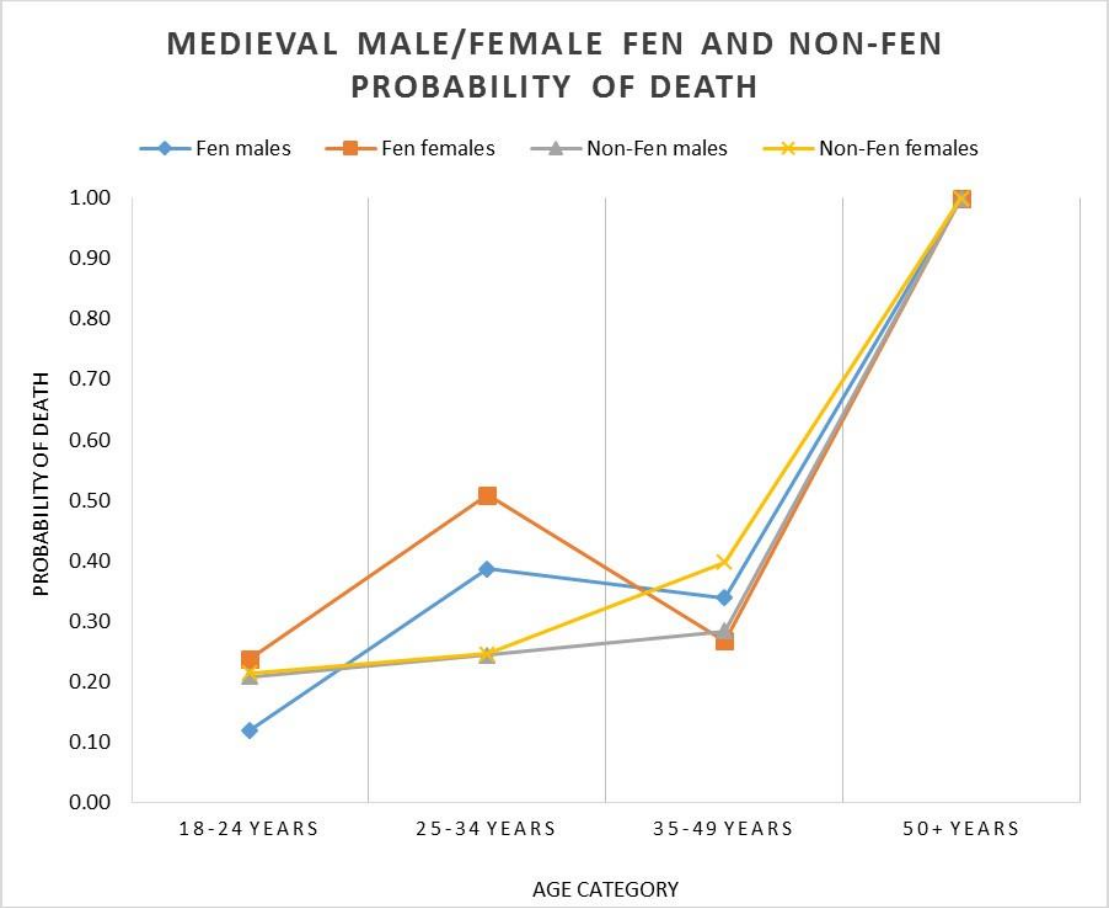

Figure 6.25: Medieval male and female Fen and non-Fen probability of death (q_x). n = 235 Fen males, 212 non-Fen males; 160 Fen females, 140 non-Fen females.

6.2.2(iv): Total demographics

Figures 6.26-6.31 present the mortality, survivorship, and probability of death data for all periods combined. This is followed by Table 6.5, which summarises the results of statistical testing for all periods. Testing of total mortality data (Figure 6.26) shows that while location has a significant impact upon age-at-death (χ^2 p <0.01), there is no significant difference between Fen and non-Fen age-at-death distributions (K-S p = 0.89). In terms of general observed trends in mortality, infant mortality (<1 year old) is lower than expected, but this may be an artefact of low Roman and Anglo-Saxon skeletal numbers (addressed in Section 7.1.5), or the small interval of age category in comparison to subsequent categories. Figure 6.26 shows that the lowest mortality occurred in the adolescent age category (13- 17 years), and peaked in the young adult category (25-34 years). Figures 6.27 and 6.29 suggest that Fen and non-Fen males tended to live longer than their female counterparts, although

the adult age-at-death distribution does not display a significant difference (Figure 6.27). The poor outlook for non-Fen Roman and Anglo-Saxon females (Figures 6.9 and 6.16) is reflected in the survivorship (Figures 6.28 and 6.29) and probability of death (Figures 6.30 and 6.31) charts.

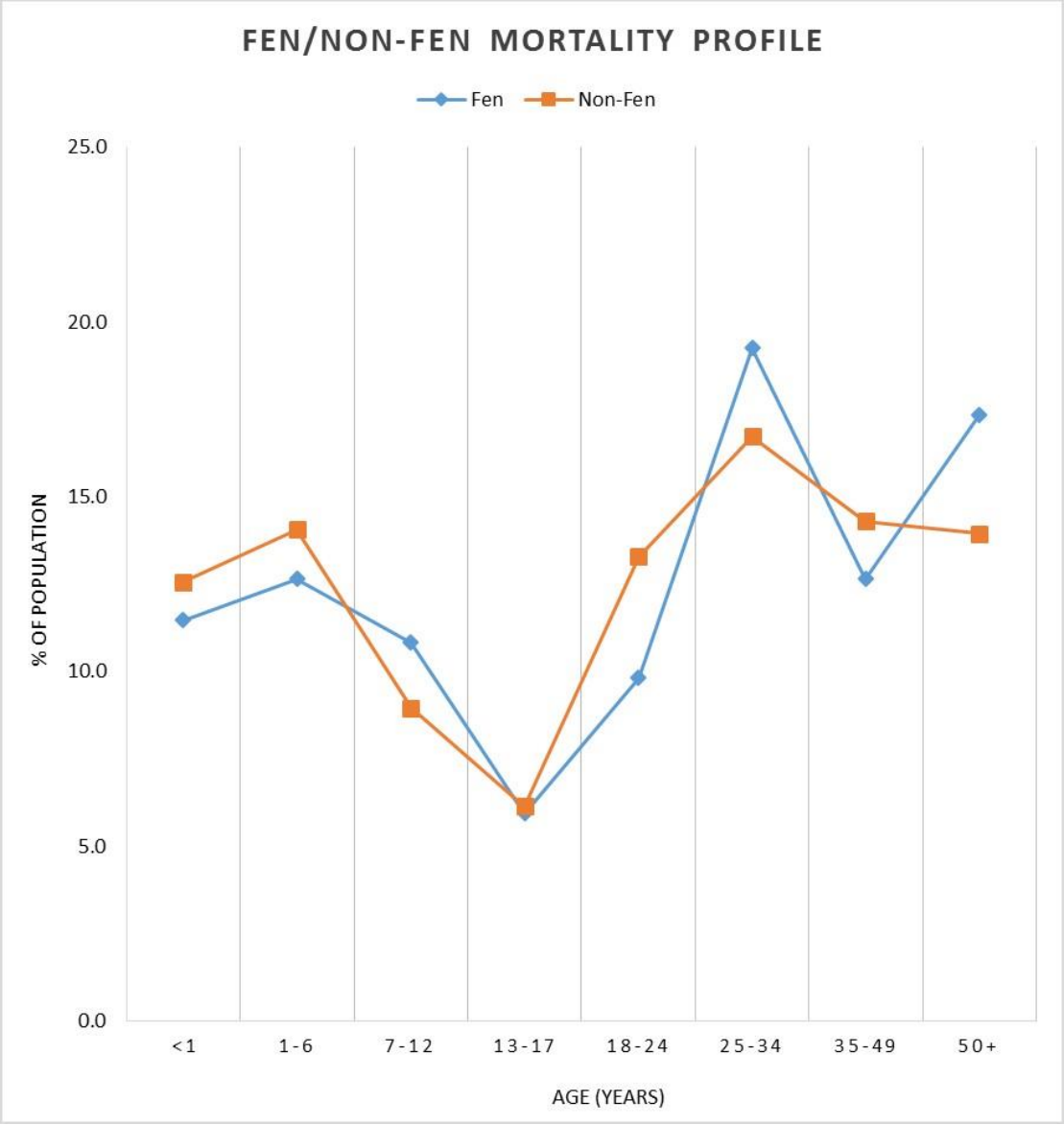

Figure 6.26: Total Fen and non-Fen populations by percentage of buried individuals in each age category. χ^2, p <0.01. K-S, p = 0.93. Omitting <1 category: K-S, p = 0.89. *n* = 2789 Fen, 2588 non-Fen.

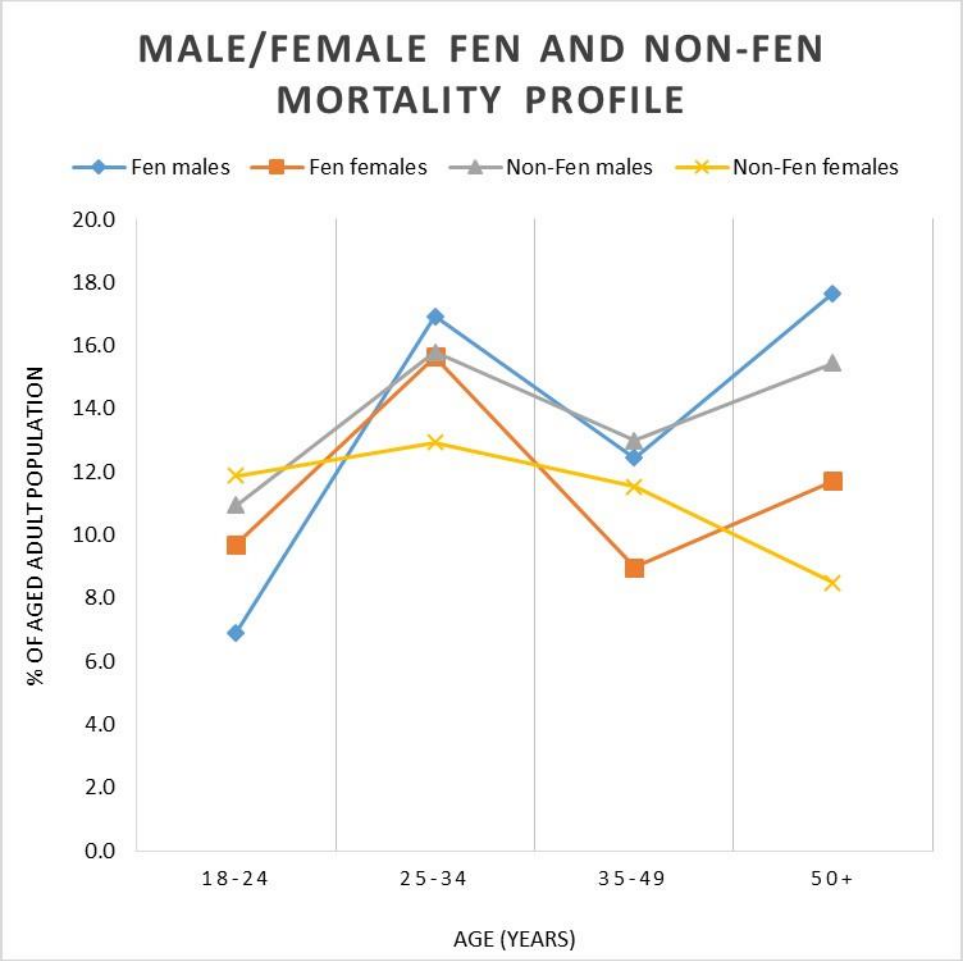

Figure 6.27: Total male and female Fen and non-Fen mortality profile. χ^2, p <0.01. *n* = 889 Fen males, 832 non-Fen males - K-S, p = 0.53; 759 Fen females, 676 non-Fen females – K-S, p = 0.10.

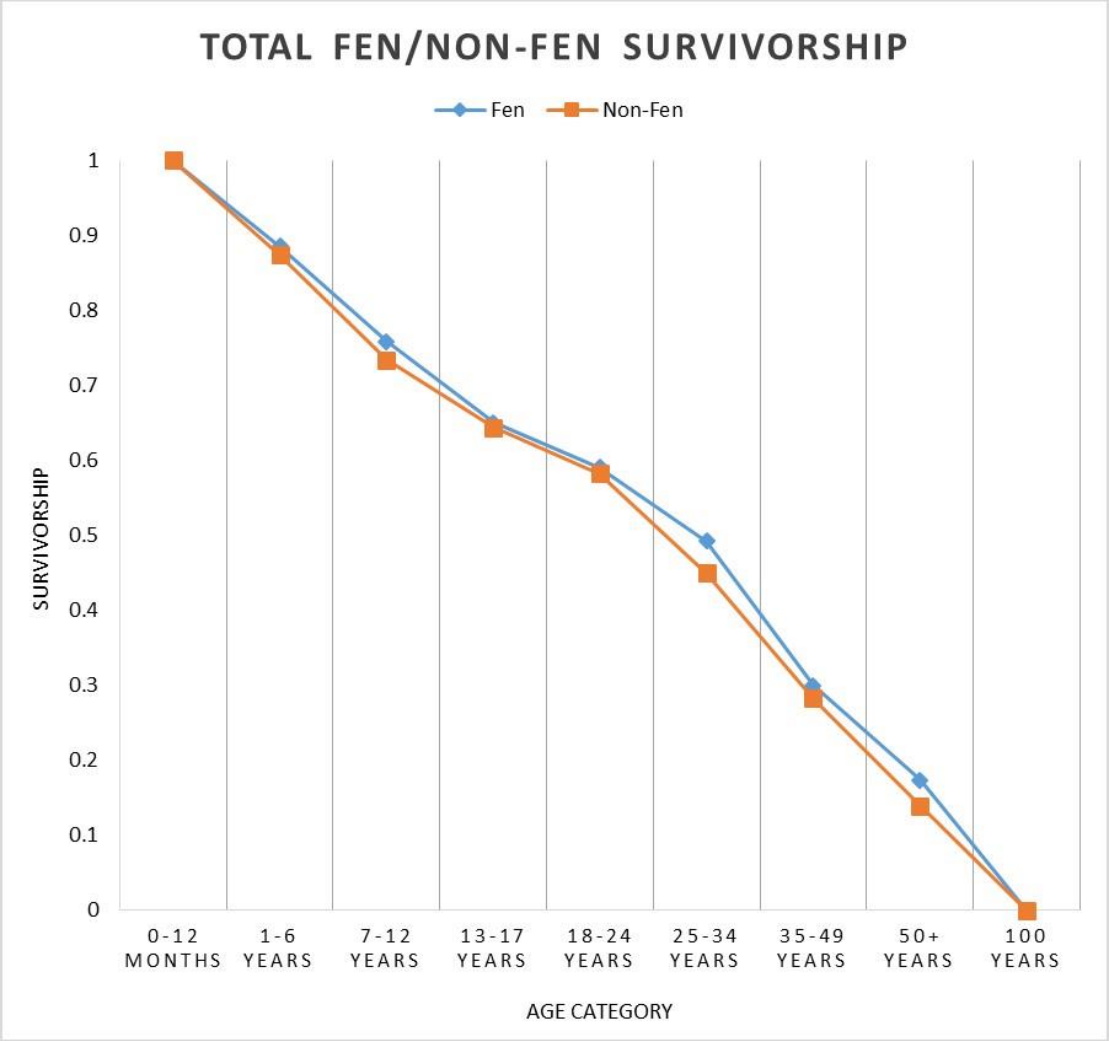

Figure 6.28: Total Fen and non-Fen survivorship (l_x). n = 2789 Fen, 2588 non-Fen.

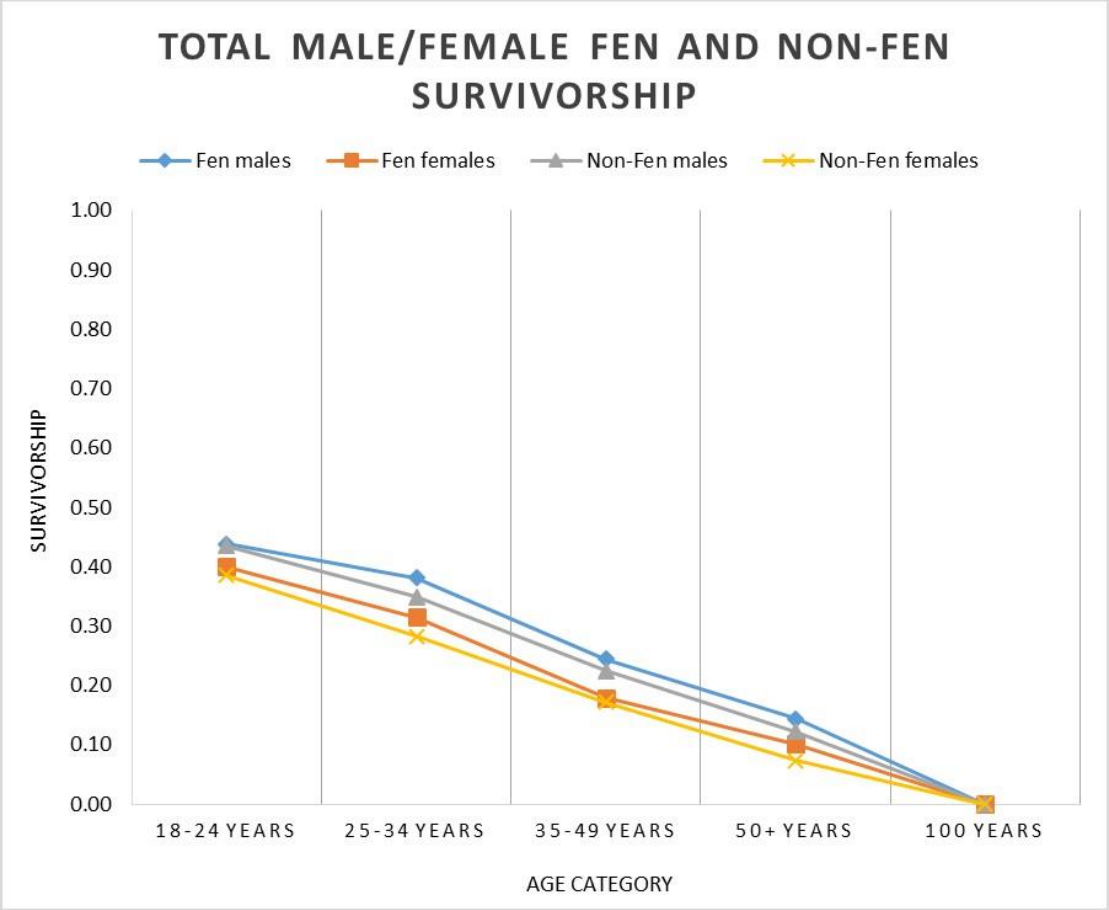

Figure 6.29: Total male and female Fen and non-Fen survivorship (l_x). n = 1648 Fen males, 832 non-Fen males; 1508 Fen females, 676 non-Fen females.

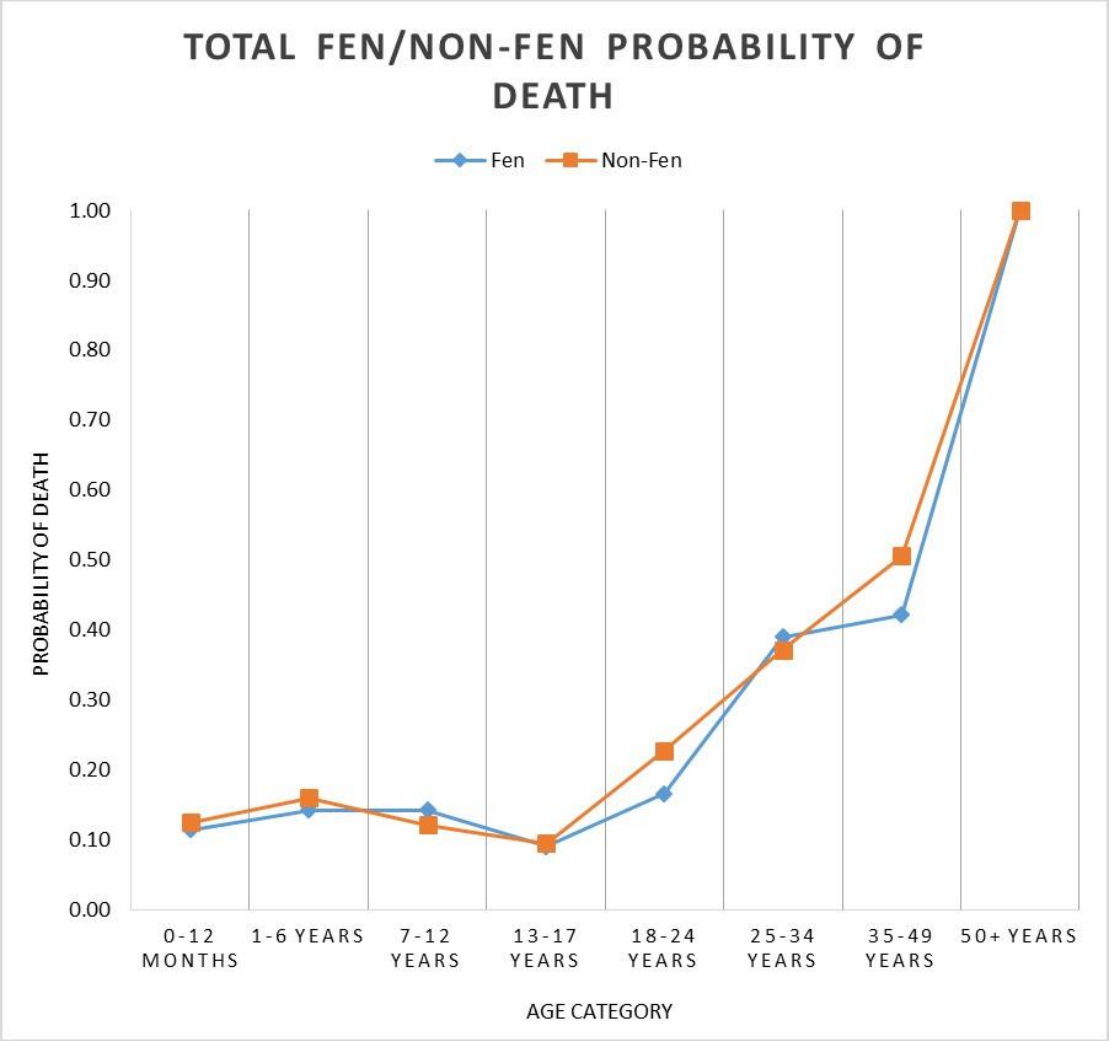

Figure 6.30: Total Fen and non-Fen probability of death (q_x). n = 2789 Fen, 2588 non-Fen.

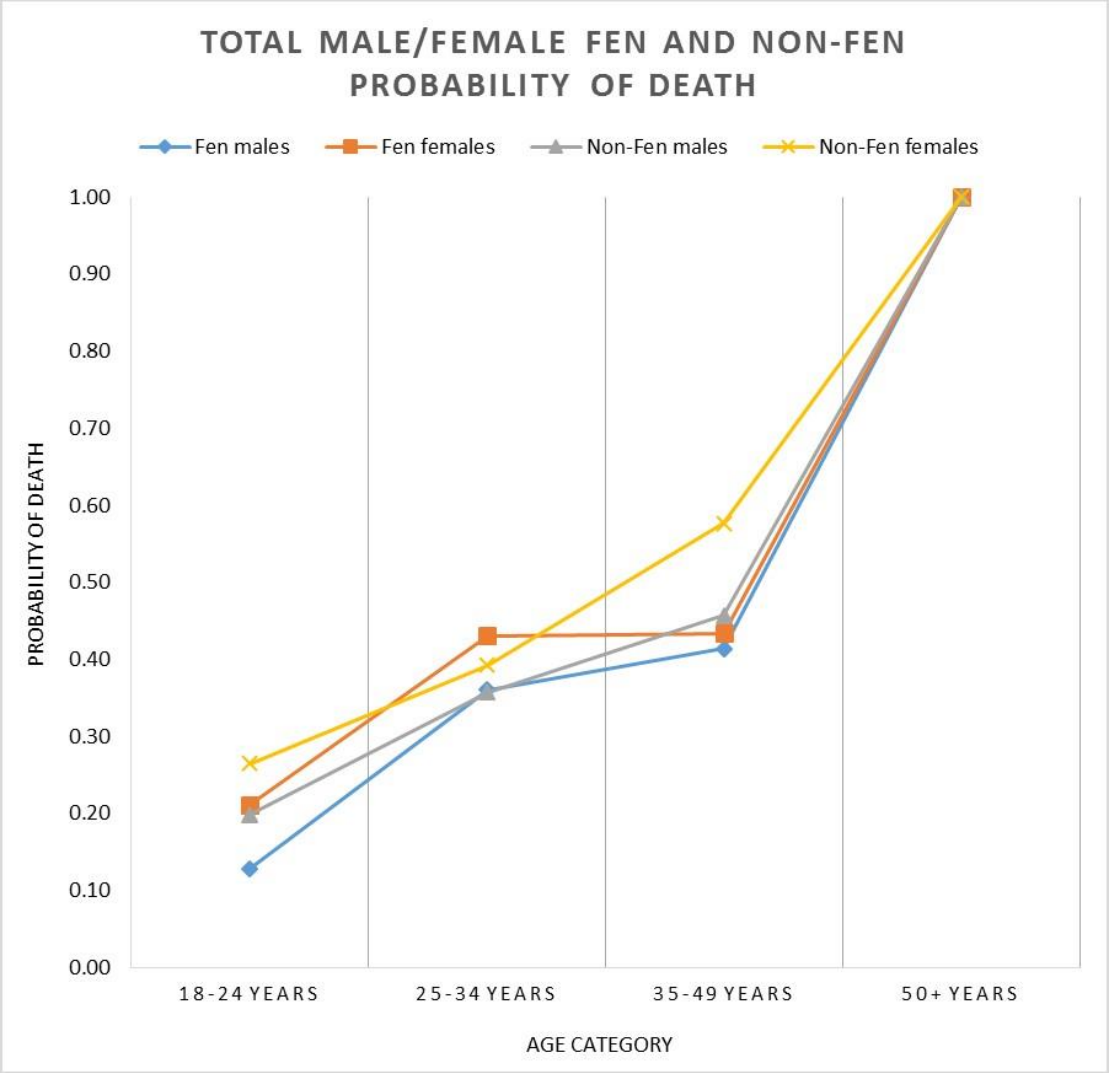

Figure 6.31: Total male and female Fen and non-Fen probability of death (q_x). n = 1648 Fen males, 832 non-Fen males; 1508 Fen females, 676 non-Fen females.

Fen/non-Fen mortality data	Figure	Chi-square (a = 0.05)	Kolmogorov-Smirnov (a = 0.05)
		Reject H_0?	Reject H_0?
Roman total	6.4	Yes	Yes
Roman males	6.5	No	Yes
Roman females	6.5	No	Yes
Anglo-Saxon total	6.10	Yes	No
Anglo-Saxon total with St. Peter's removed	6.11	Yes	No
Anglo-Saxon males	6.12	Yes	No
Anglo-Saxon females	6.12	Yes	No
Medieval total	6.17	Yes	No
Medieval total with St. Peter's and Wharram Percy removed	6.18	Yes	Yes
Medieval males	6.19	Yes	No
Medieval females	6.19	Yes	No
Total	6.26	Yes	No
Total males	6.27	Yes	No
Total females	6.27	Yes	No

Table 6.5: Summary of statistical test results on mortality profiles. H_0 = null hypothesis.

6.3: Histological analysis

Summary tables for histological analysis results are presented firstly for the control samples (Table 6.6) and then by period and site in Tables 6.8-6.22. Table 6.7 presents a key to the age category abbreviations employed in Tables 6.8-6.22. Sample percentages in each histological index (HI) category are included in each table to provide a summary of overall element preservation at each site. Summary tables for all Roman (Table 6.13) and Anglo-Saxon (Table 6.21) data are also included for ease of comparison. The raw data for all summary tables are presented in Appendix 2 (Tables A2.3 to A2.16).

HI	0	1	2	3	4	5
Type						
Adult rib	0	0	0	1	3	4
Adult cranium	2	1	1	1	0	0
Adult phalanx	0	0	1	1	0	3
Sub-adult rib	0	1	2	0	0	0
Sub-adult cranium	0	2	0	0	0	0
Animal rib	1	0	0	1	2	4

Table 6.6: Control samples in each HI category, by element type.

Title	Abbreviation	Age
Child	CH	1-6 years
Juvenile	JU	7-12 years
Adolescent	AD	13-17 years
Very young adult	VYA	18-24 years
Young adult	YA	25-34 years
Middle adult	MA	35-49 years
Old adult	OA	50+ years
Adult	A	18+ years

Table 6.7: Key to age category abbreviations used in summary Tables 6.8-6.20.

6.3.1: Roman sites

Tables 6.8 to 6.12 display the types of element selected from each age category, and the histological preservation of selected samples from the Roman sites. The larger sample sets (e.g., The Parks, Godmanchester, The Hoplands, and Watersmeet) likely offer the best representations of Roman Fen histological preservation. These sites (Tables 6.9, 6.10, and 6.12) show similar patterns in preservation levels, with the majority of samples scoring below 2 on the HI. This is confirmed by summary Table 6.13.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Rib	0	0	2	0	0	0
AD	Rib	0	0	1	0	0	0
AD	Cranium	1	0	0	0	0	0
VYA	Rib	0	0	0	1	1	0
MA	Rib	0	0	0	1	0	0
	Cranium	0	0	1	0	0	0
-	Animal rib	1	0	1	0	0	0
	Total	2	0	5	2	1	0
	% of Total	20	0	50	20	10	0

Table 6.8: Samples from the *Durobrivae* site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Rib	1	1	0	0	0	0
	Cranium	2	0	0	0	0	0
JU	Rib	3	1	4	0	0	0
	Cranium	0	0	1	0	0	0
	Hand phalanx	3	1	0	0	0	0
AD	Cranium	0	0	0	0	1	0
VYA	Rib	3	0	0	1	0	0
	Cranium	3	1	0	0	0	0
	Hand phalanx	1	0	0	0	0	1
-	Animal rib	2	1	1	0	0	0
	Total	18	5	6	1	1	1
	% of Total	56	16	19	3	3	3

Table 6.9: Samples from The Parks, Godmanchester site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Rib	2	0	1	1	1	0
AD	Rib	1	1	1	0	0	0
	Foot phalanx	0	0	0	0	1	0
	Hand phalanx	0	3	0	0	1	0
VYA	Rib	1	0	0	0	0	0
	Foot phalanx	0	1	0	0	0	0
	Hand phalanx	0	1	0	0	0	0
YA	Rib	3	0	1	0	0	1
	Foot phalanx	2	0	0	0	0	1
	Hand phalanx	0	0	0	0	0	2
-	Animal rib	1	2	0	1	0	5
	Total	10	8	3	2	3	9
	% of Total	29	23	9	6	9	26

Table 6.10: Samples from The Hoplands site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
YA	Rib	0	0	0	1	1	0
	Hand phalanx	0	0	0	0	0	1
	Total	0	0	0	1	1	1
	% of Total	0	0	0	33	33	33

Table 6.11: Samples from the Prickwillow Road, Ely site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Rib	2	2	0	0	0	0
JU	Rib	1	0	0	1	0	0
	Cranium	0	0	1	0	0	0
	Foot phalanx	1	0	0	0	0	0
AD	Rib	0	0	1	0	0	0
	Foot phalanx	0	0	0	0	0	1
YA	Rib	2	0	0	2	0	0
	Cranium	1	0	0	0	0	0
	Hand phalanx	1	0	0	0	0	2
	Animal rib	2	0	1	0	0	0
	Total	11	2	3	3	1	3
	% of Total	48	9	13	13	4	13

Table 6.12: Samples from the Watersmeet, Huntingdon site in each category, by age and element type.

HI	0	1	2	3	4	5
Type						
Rib	20	5	11	8	4	1
Cranium	7	1	3	0	1	0
Foot phalanx	3	1	0	0	1	2
Hand phalanx	5	5	0	0	1	6
Total	35	12	14	8	7	9
% of total	41	14	16	9	8	11

Table 6.13: Summary of element histological preservation from Roman sites (human samples).

6.3.2: Anglo-Saxon sites

Tables 6.14 to 6.20 show the element types selected from each age category, and the histological preservation of selected samples from the Anglo-Saxon sites. As with the Roman sites, the trend in preservation is towards the lower HI categories, although there is greater variation between sites. For example, Castledyke South (Table 6.15) and Edix Hill (Table 6.17) display a greater proportion of well-preserved samples levels than Cleatham (Table 6.16) and Littleport (Table 6.19), which are generally poorly preserved. However, as

seen with the Roman samples, overall preservation (Table 6.21) is heavily weighted towards the lower HI categories.

	HI	0	1	2	3	4	5
Age	**Type**						
VYA	Rib	0	1	0	0	0	0
	Hand phalanx	0	0	1	0	0	0
	Animal rib	1	2	0	0	0	0
	Total	1	3	1	0	0	0
	% of Total	20	60	20	0	0	0

Table 6.14: Samples from the Baston site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Rib	2	0	1	1	0	1
	Cranium	2	1	0	1	1	0
JU	Rib	1	2	0	1	1	0
	Cranium	2	2	0	1	0	0
	Foot phalanx	0	0	1	0	0	0
AD	Rib	0	0	1	1	0	0
	Cranium	0	0	0	1	1	0
	Hand phalanx	0	0	0	0	0	1
VYA	Rib	2	5	3	0	2	0
	Cranium	3	2	0	1	1	1
	Foot phalanx	2	1	0	0	0	0
	Hand phalanx	0	0	1	0	0	1
-	Animal rib	4	0	1	0	0	0
	Total	18	13	8	7	6	4
	% of Total	32	23	14	13	11	7

Table 6.15: Samples from the Castledyke South, Barton-upon-Humber site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Cranium	0	0	0	1	0	0
JU	Cranium	1	0	0	0	0	0
AD	Rib	3	1	0	0	0	0
	Cranium	1	3	0	0	0	0
	Hand phalanx	2	1	0	0	0	0
YA	Rib	2	1	0	0	1	0
	Cranium	3	1	0	0	0	0
	Total	12	7	0	1	1	0
	% of Total	57	33	0	5	5	0

Table 6.16: Samples from the Cleatham site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Rib	1	1	0	0	0	0
	Cranium	1	0	1	0	0	0
JU	Rib	0	0	0	2	0	0
	Cranium	1	0	0	0	1	0
	Hand phalanx	0	1	0	0	0	0
AD	Cranium	0	0	1	0	0	0
	Hand phalanx	0	0	0	1	0	0
VYA	Rib	2	0	0	0	1	0
	Cranium	0	0	1	1	0	0
	Foot phalanx	0	2	0	0	1	0
	Hand phalanx	0	0	0	0	1	0
YA	Rib	1	0	0	1	0	0
	Cranium	0	0	0	0	0	1
	Foot phalanx	1	0	0	0	0	0
	Hand phalanx	0	0	1	0	0	0
	Animal rib	1	0	1	0	0	0
	Total	8	4	5	5	4	1
	% of Total	30	15	19	19	15	4

Table 6.17: Samples from the Edix Hill site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
?	Rib	0	0	0	0	1	0
	Cranium	1	0	0	0	0	0
	Total	1	0	0	0	1	0
	% of Total	50	0	0	0	50	0

Table 6.18: Samples from the Haddenham site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Rib	3	0	0	0	0	0
	Cranium	3	1	1	0	0	0
AD	Rib	5	1	1	0	0	0
	Cranium	4	2	1	0	0	0
	Foot phalanx	1	0	2	0	0	0
JU	Rib	5	1	0	0	0	0
	Cranium	5	0	0	0	0	0
	Foot phalanx	0	1	0	0	0	0
	Hand phalanx	0	1	0	0	0	0
YA	Rib	3	3	0	0	0	0
	Cranium	1	3	0	0	0	0
	Foot phalanx	1	0	3	0	0	0
	Hand phalanx	1	0	0	1	0	0
MA	Rib	1	1	1	0	0	0
	Cranium	2	1	0	0	0	0
	Foot phalanx	0	0	0	1	0	0
	Hand phalanx	1	0	1	0	0	0
A	Rib	1	0	0	0	0	0
	Cranium	0	0	1	0	0	0
	Hand phalanx	1	0	0	0	0	0
	Animal rib	0	0	0	2	0	0
	Total	38	15	11	4	0	0
	% of Total	56	22	16	6	0	0

Table 6.19: Samples from the Littleport site in each HI category, by age and element type.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Rib	2	1	0	0	0	0
JU	Rib	1	1	0	0	0	0
AD	Rib	0	1	0	0	0	0
	Foot phalanx	0	0	2	0	0	0
YA	Rib	0	0	1	0	0	0
	Hand phalanx	0	1	0	0	0	0
	Total	3	4	3	0	0	0
	% of Total	30	40	30	0	0	0

Table 6.20: Samples from the Westfield Farm, Ely site in each HI category, by age and element type.

HI	0	1	2	3	4	5
Type						
Rib	35	20	8	6	6	1
Cranium	30	16	6	6	4	2
Foot phalanx	5	4	8	1	1	0
Hand phalanx	5	4	4	2	1	2
Total	75	44	26	15	12	5
% of total	42	25	15	8	7	3

Table 6.21: Summary of element histological preservation from Anglo-Saxon sites (human samples).

6.3.3: Medieval site

Table 6.22 shows results of the histological analysis of the Orchard Lane, Huntingdon. The pattern of preservation is similar to that seen in the earlier Roman and Anglo-Saxon periods, with poorer preserved samples dominating.

	HI	0	1	2	3	4	5
Age	**Type**						
CH	Rib	1	1	0	0	0	0
	Cranium	0	2	0	0	0	0
JU	Rib	0	0	0	0	1	0
	Cranium	0	1	0	0	0	0
	Hand phalanx	0	1	0	0	0	1
AD	Rib	1	0	0	0	0	0
	Hand phalanx	0	0	0	0	1	0
VYA	Rib	2	0	0	0	0	0
	Cranium	2	0	0	0	0	0
	Hand phalanx	0	1	0	0	0	0
YA	Rib	0	0	1	0	0	0
	Cranium	0	1	0	0	0	0
	Hand phalanx	0	0	0	0	0	1
	Animal rib	0	0	2	0	0	0
	Total	6	7	3	0	2	2
	% of Total	30	35	15	0	10	10

Table 6.22: Samples from the Orchard Lane, Huntingdon site in each HI category, by age and element type.

6.3.4: Summary analysis

Figure 6.32 demonstrates the trend towards poor preservation as observed in the summary tables presented above. Table 6.23 and Figure 6.33 suggest differential preservation between element types, with ribs and cranial samples tending towards poorer microscopic preservation. This is discussed further in Section 7.2.

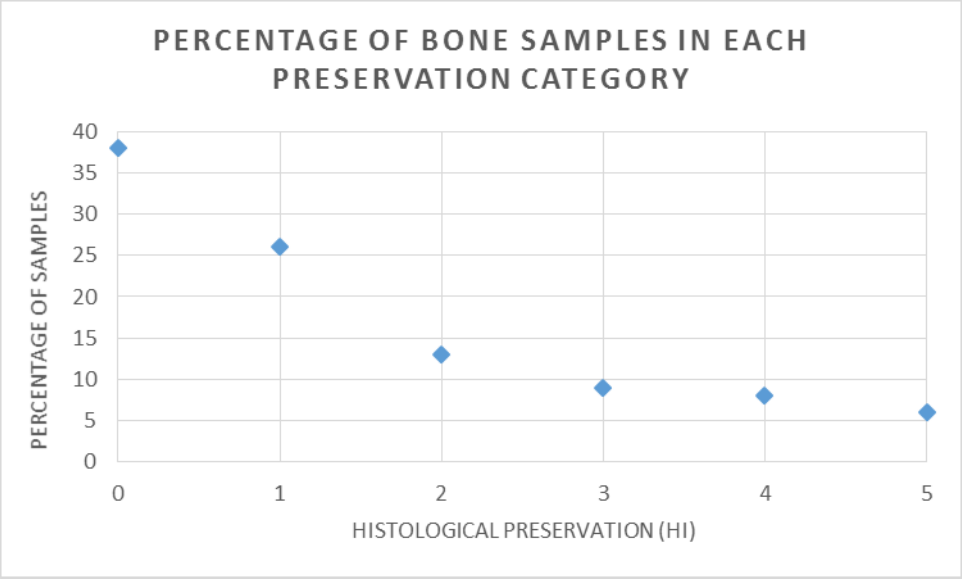

Figure 6.32: Percentage of bone samples in each category of histological preservation.

HI	Rib	Cranium	Hand Phalanx	Foot Phalanx
0	54	50	28	28
1	18	24	23	25
2	6	10	13	32
3	12	6	8	4
4	8	8	8	4
5	2	2	20	7

Table 6.23: Percentages of bone types in each HI category.

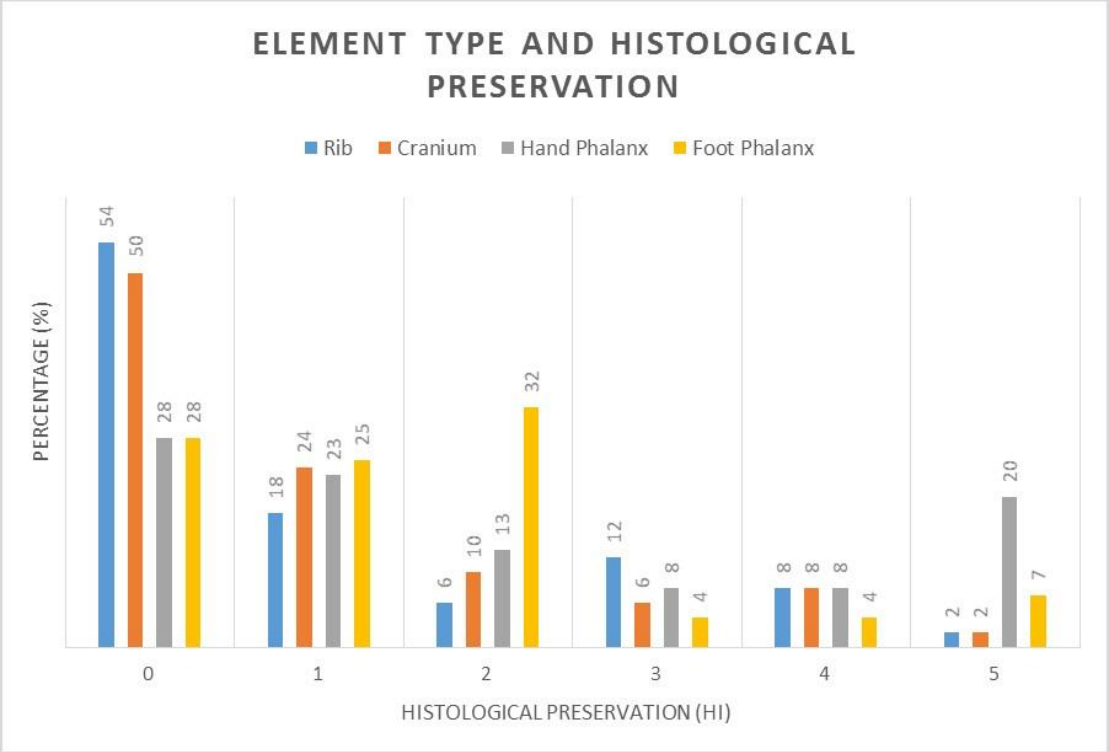

Figure 6.33: Percentage of bone elements in each HI category.

Figure 6.34 compares overall histological preservation between the Roman and Anglo-Saxon sites. The medieval site was omitted here due to limited sample size. The χ^2 test ($p<0.01$) rejects the null hypothesis that histological preservation is independent of period.

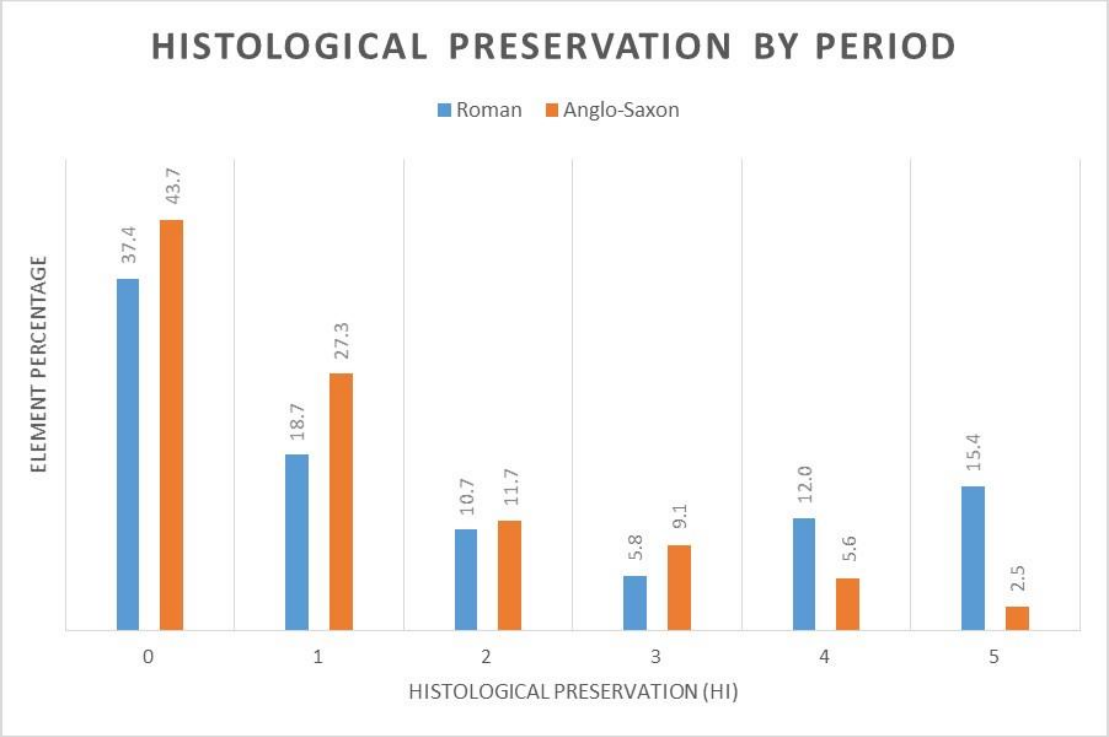

Figure 6.34: Element percentage in each HI category by period. χ^2, $p < 0.01$.

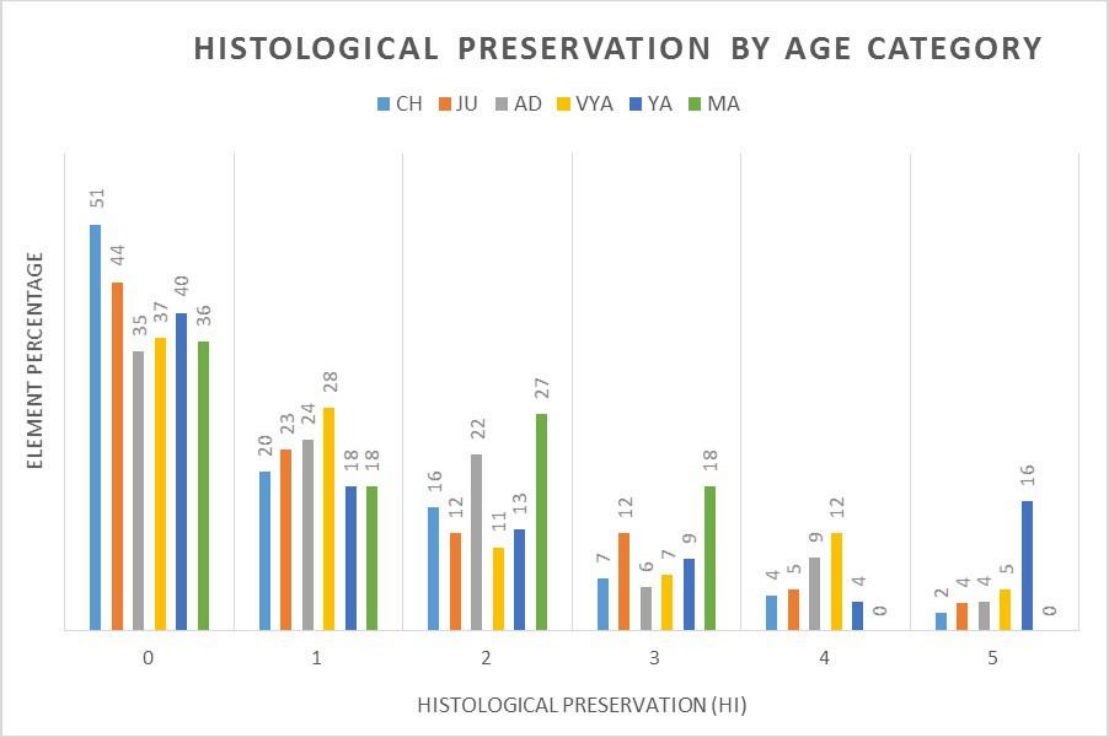

Figure 6.35: Element percentage in each HI category by age group (see Table 6.7 for category abbreviations). χ^2 , p = 0.19.

Figure 6.35 displays histological preservation levels in each age category (abbreviations defined in Table 6.7). The χ^2 test ($p<0.19$) suggests that age-at-death is independent of histological preservation.

6.4: Protein and immunoglobulin G (IgG) extraction protocols

This section presents the results from the five protein extraction, purification, and characterisation protocols (P1-P5) detailed in section 5.7. For SDS-PAGE analyses, only the most 'successful' gels in terms of their potential for IgG identification are presented here. Schmidt-Schultz and Schultz (2004) identified likely archaeological IgG heavy chain bands at around 60kDa molecular weight bands in very well preserved samples (Figures 6.36 and 6.37). It was, therefore, expected that IgG heavy chain bands in the samples chosen here would approximate similar weights, and it was assumed that light chains would appear as bands at around 25kDa.

6.4.1: P1: Extraction methodology adapted from Schmidt-Schultz and Schultz (2004)

6.4.1(i): P1.1 SDS-PAGE gels

Figures 6.38, 6.39, and 6.40 show the Coomassie and silver stained gels for samples resulting from the P1.1 and P1.2 extractions. Samples chosen for these SDS-PAGE tests (see Table 5.11 for explanation of sample abbreviations) were all well preserved, in order to compare results to those achieved by Schmidt-Schultz and Schultz (2004) in Figures 6.36 and 6.37. Inserted arrows in Figures 6.38-6.40 illustrate the approximate molecular weight of IgG heavy chains (c. 60kDa).

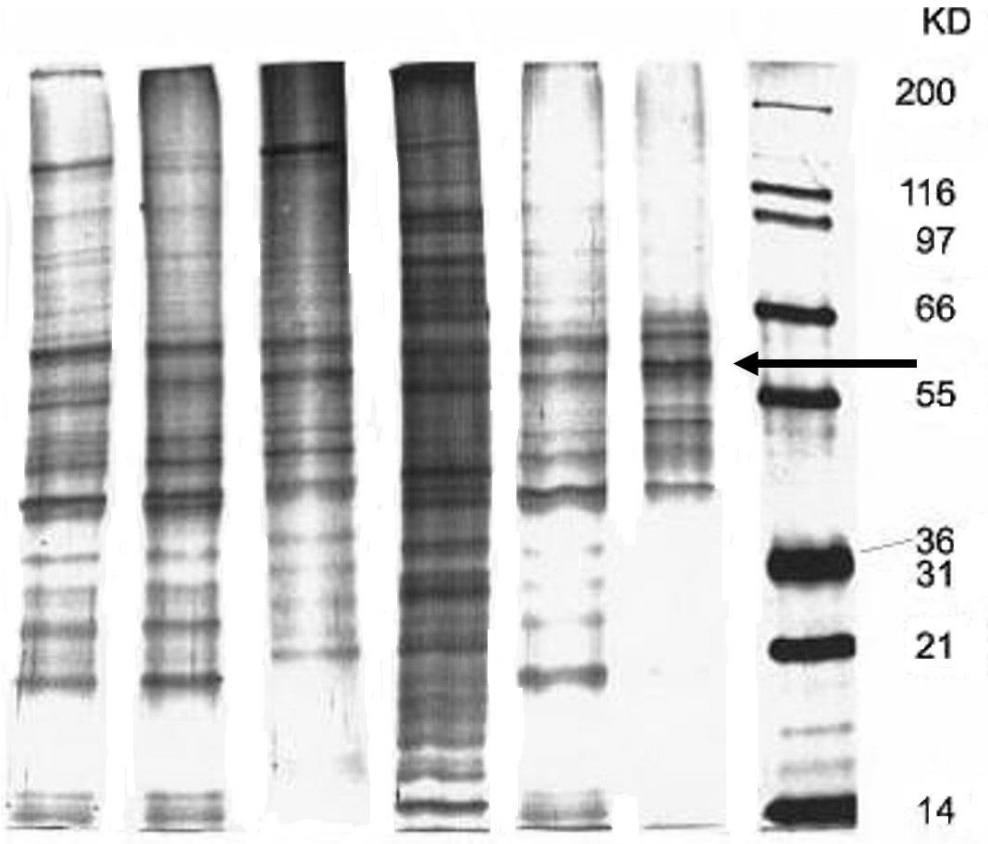

Figure 6.36: Silver stained SDS-PAGE gel showing six ancient samples with molecular weight ladder on the right. KD = molecular weight. Arrow indicates expected IgG heavy chain bands, as shown in Figure 6.37 (adapted from Schmidt-Schultz and Schultz 2004:33).

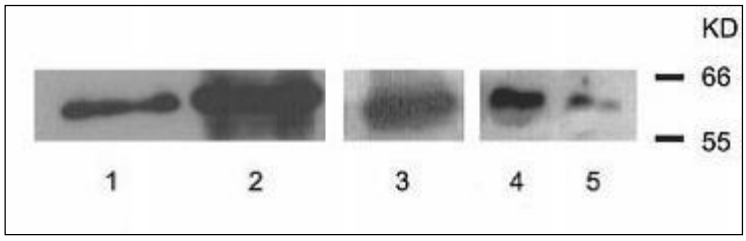

Figure 6.37: Schmidt-Schultz and Schultz (2004:34) confirmation of IgG heavy chains at approximately 60kDa by Western blot.

Examination of the silver stained gel (Figure 6.39) shows considerable smearing of proteins above approximately 37kDa, resulting in very poor band resolution when compared to Schmidt-Schultz and Schultz's ancient samples (Figure 6.36). Excessive smearing of ancient samples on SDS-PAGE gels often results from degraded proteins and is discussed in section 7.3. The poor resolution of Coomassie bands (Figure 6.38) and silver stained bands (Figure 6.39) around 60kDa (top arrows) made selection and excision of potential IgG heavy chain bands impossible, although Figure 6.39 displays possible bands corresponding to IgG light chains (c. 25kDa).

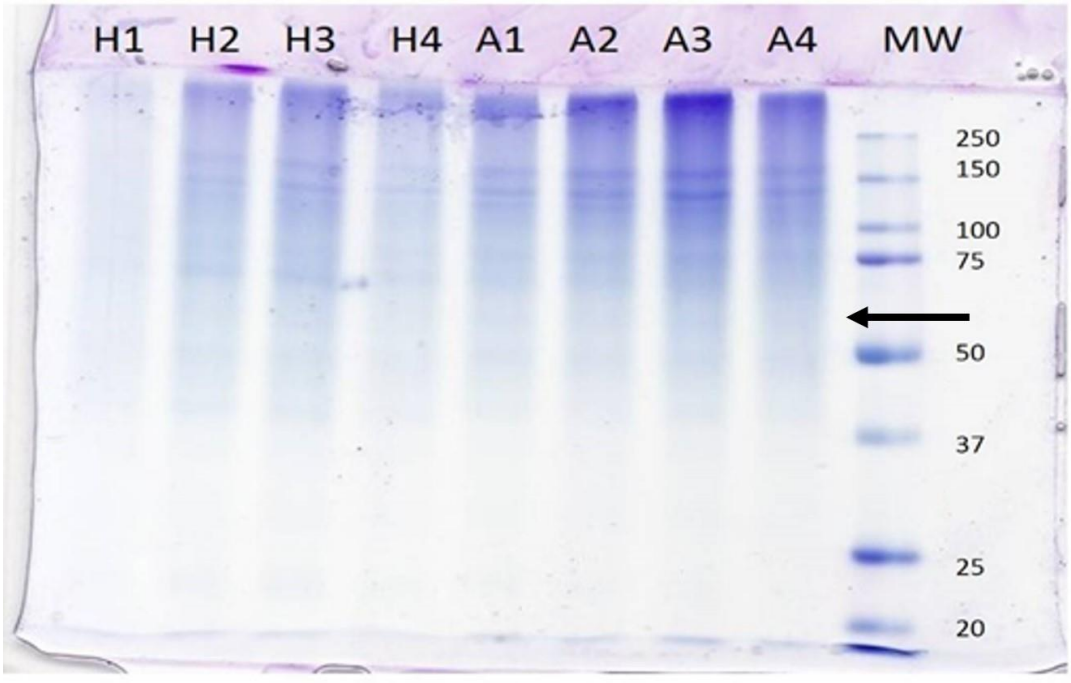

Figure 6.38: P1.1 Coomassie stained SDS-PAGE gel. MW is molecular weight marker (kDa). H1 – HDAR1; H2 – HDAP2; H3 – HDAP3; H4 – HDAR4; A1 – AN1; A2 – AN2; A3 – AN3; A4 – AN7 (see Table 5.11). Gel/samples prepared as in section 5.7.3(i). Arrow indicates expected bands for IgG heavy chains.

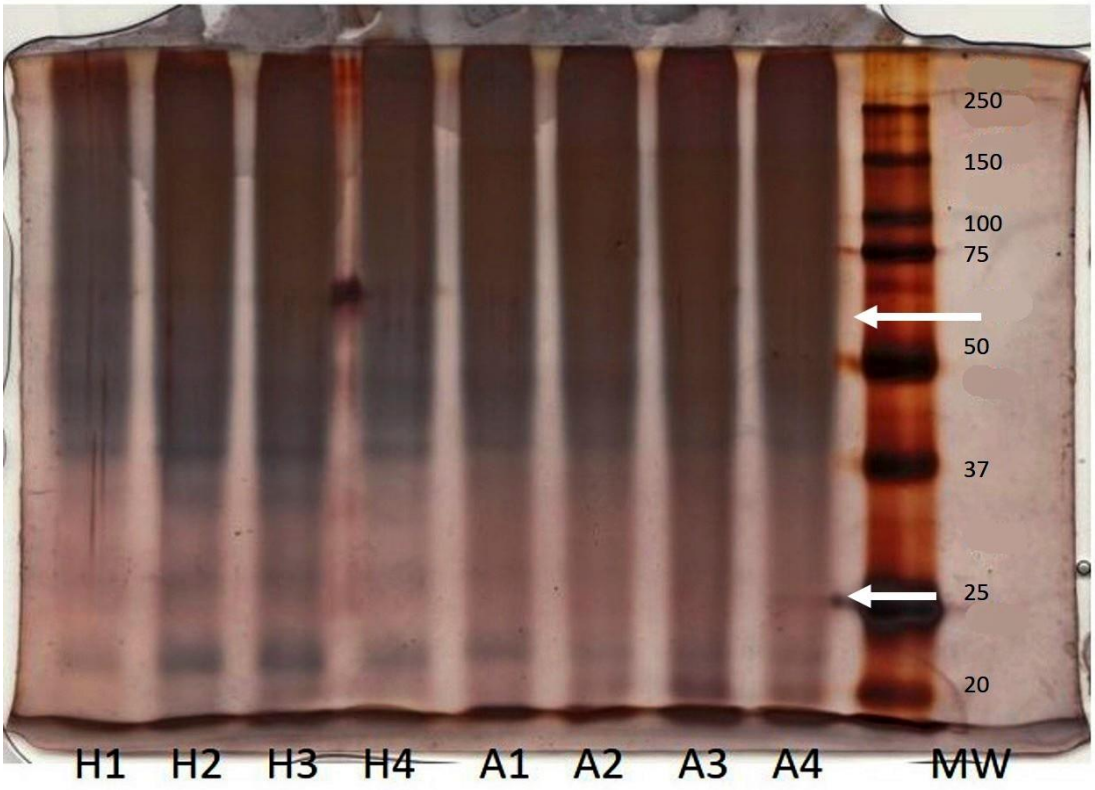

Figure 6.39: P1.1 silver stained SDS-PAGE gel. MW is molecular weight marker (kDa). H1 – HDAR1; H2 – HDAP2; H3 – HDAP3; H4 – ADAR4; A1 – AN1; A2 – AN2; A3 – AN3; A4 – AN7 (see Table 5.10). Gel/samples prepared as in section 5.7.3(i). Top arrow indicates expected bands for IgG heavy chain; bottom arrow indicates expected bands for IgG light chains.

6.4.1(ii): P1.2 SDS-PAGE gels

A further set of samples (Table 5.11) were tested in an attempt to reduce the excessive smearing encountered in the P1.1 gels. Figure 6.40 shows slight improvement in band clarity in the Coomassie stained gel, with possible IgG heavy chain bands present (see arrow). The silver stained gel, however, demonstrated the same obscuring smear above approximately 30kDa, although clarity improved below this, with potential IgG light chain bands visible (Figure 6.40, bottom right arrow).

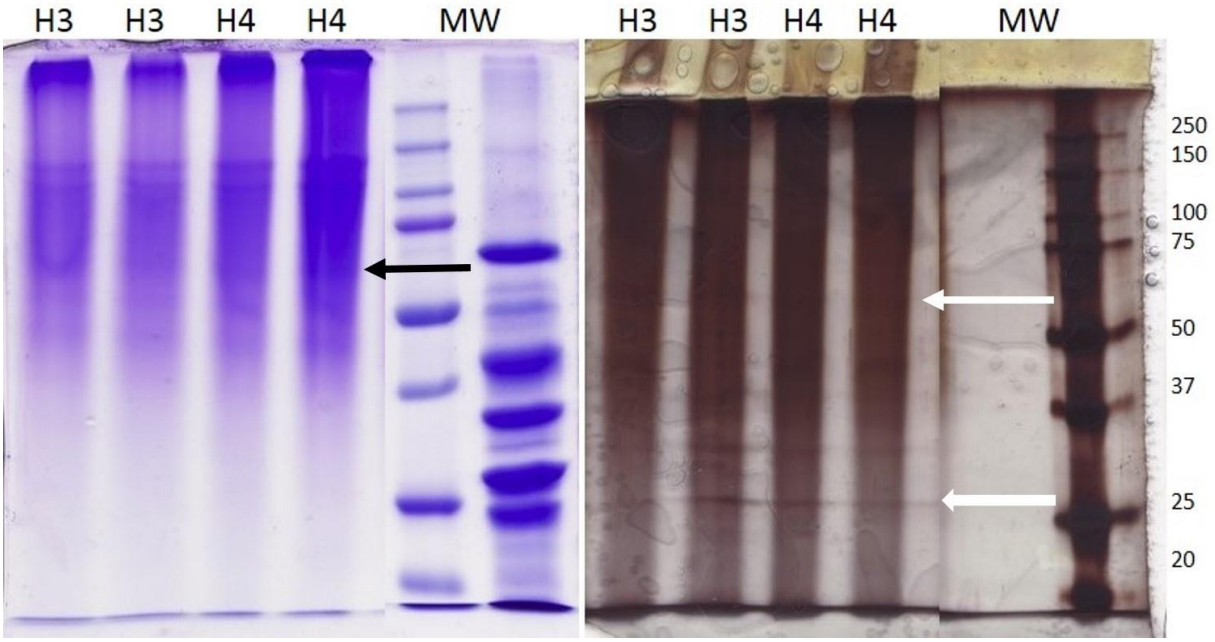

Figure 6.40: P1.2 SDS-PAGE gels following Coomassie (left) and silver (right) staining. MW is molecular weight marker (kDa). H3 – HDAP3; H4 – HDAR4 (see Table 5.11). Gels/samples prepared as in sections 5.7.3(i). Top arrows indicate expected bands for IgG heavy chains; bottom white arrow indicates expected bands for IgG light chains.

6.4.1(iii): P1.2 SDS-PAGE gel bands selected for proteomic analysis, and proteomic results

The slightly improved Coomassie stained band clarity in Figure 6.41 and the potential silver stained light chain bands in Figure 6.42 allowed for selection of promising bands for proteomic analysis. The chosen bands were assigned numbers by the Biological Sciences personnel (bands 2, 5, and 7 from gel in Figure 6.41, and 9, 10, and 11 from gel in Figure 6.42). Those chosen in Figure 6.41 (2, 5, and 7) approximate the IgG heavy chain bands identified by Schmidt-Schultz and Schultz (2004). It was assumed that IgG light chains might present around 25kDa, given the evidently well-preserved heavy chains identified by Schmidt-Schultz and Schultz. Since they did not attempt to identify light chains, three clear bands (bands 9, 10, and 11) around and slightly above 25kDa were excised for proteomic analysis (Figure 6.42).

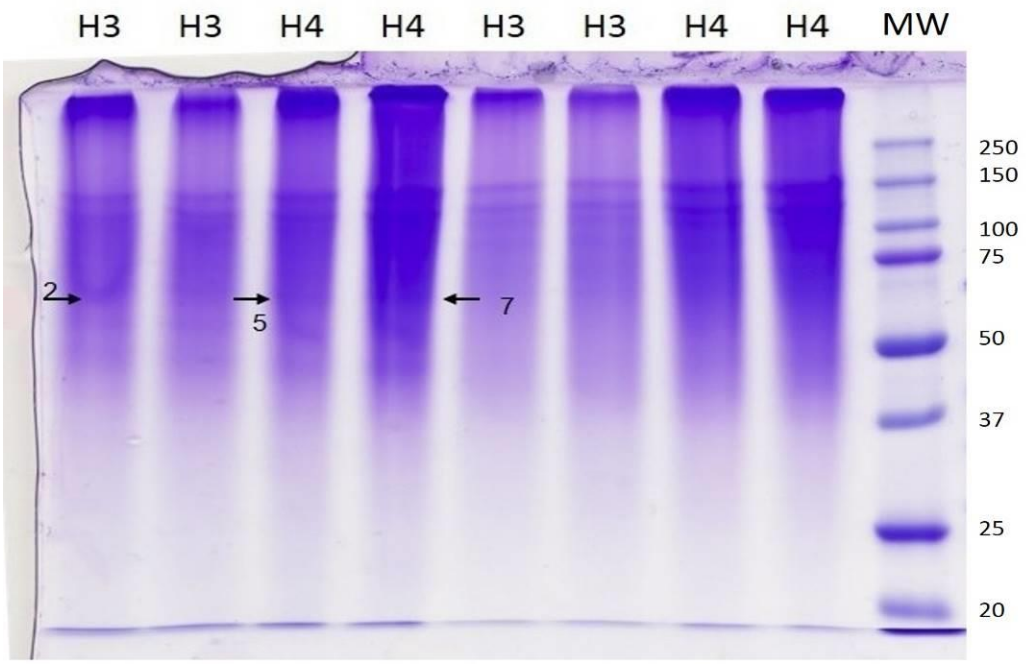

Figure 6.41: P1.2 Coomassie stained SDS-PAGE gel bands (2, 5, and 7) excised for proteomic analysis. H3 – HDAP3; H4 – HDAR4 (see Table 5.11).

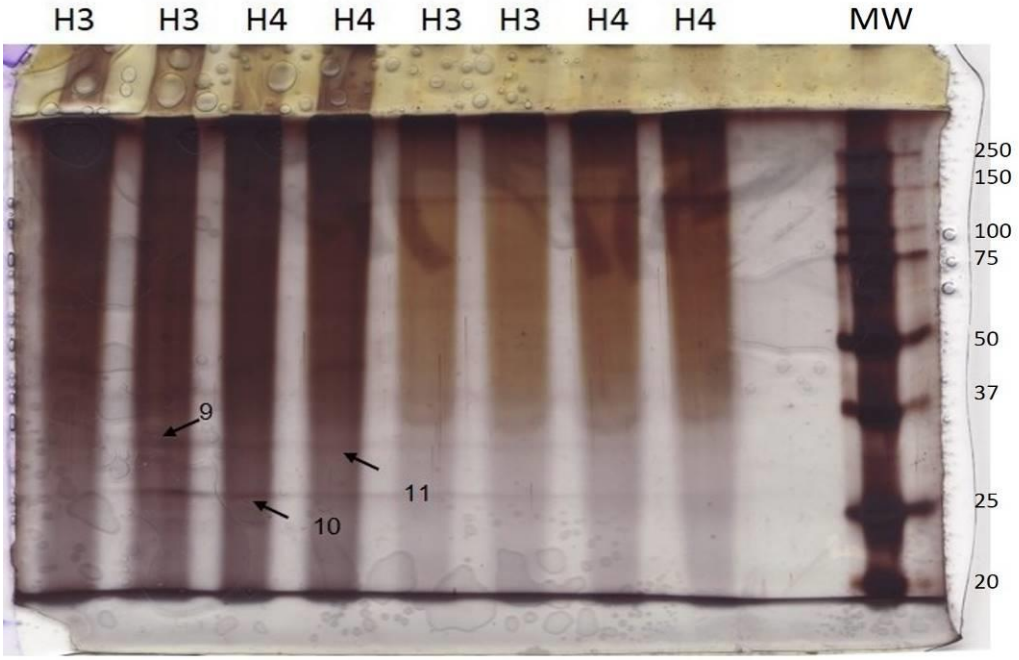

Figure 6.42: P1.2 silver stained SDS-PAGE gel bands (9, 10, and 11) excised for proteomic analysis. H3 – HDAP3; H4 – HDAR4 (see Table 5.11).

MALDI analysis results tested negative for any protein. Laboratory personnel were unsure of the reasons behind this failure (Joanne Robson, personal communication,

September 2012). The samples were subsequently analysed by nanospray LC-MS/MS, the results of which are presented in Table 6.24. No human proteins beyond collagen (other than exogenous contaminating keratin) were detected when matched against the Swiss-Prot database.

Gel band	Name	Sequence Coverage (%)	Peptides (95%)
2	Pro alpha 1 collagen	38.3	81
5	Pro alpha 1 collagen	47.8	67
7	Pro alpha 1 collagen	42	64
9	Alpha type 2 collagen	33	30
10	Alpha 1 type 1 collagen	29.4	29
11	Pro alpha 1 collagen	24.4	26

Table 6.24: Results of P1.2 nLC-MS/MS analysis, showing most prevalent proteins and sequence coverage. Swiss-Prot database. Gel bands as in Figures 6.41 and 6.42.

6.4.1(iv): P1.3 SDS-PAGE gels

Analysis of the P1.2 SDS-PAGE gels and proteomic results demonstrated the requirement for the removal of smearing collagen, which may mask non-collagenous proteins (NCPs) of lower abundance, including IgG. Selected samples (Table 5.11) were tested in duplicate, with one batch (Figure 6.44) treated with collagenase prior to SDS-PAGE, and one batch (Figure 6.43) left untreated. This allowed for direct comparison between gels and assessment of the efficacy of the collagenase treatment. As can be seen, the collagenase treatment was partially successful in reducing smearing for some samples (compare AP3, AR6, and AN4), yet unsuccessful for others (compare AR3, AP5, and AN2).

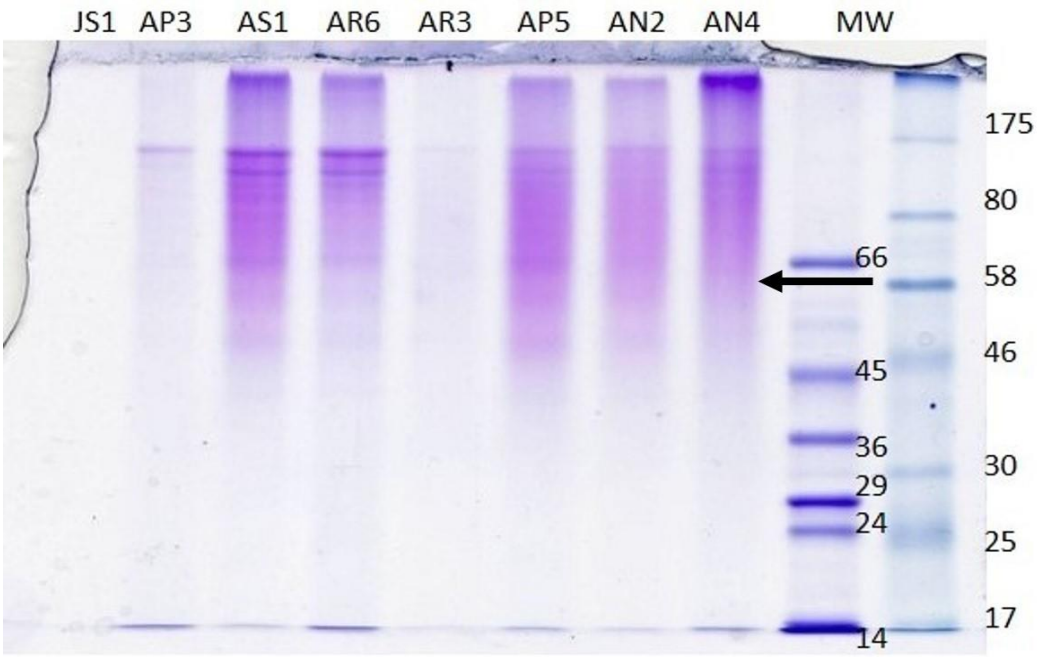

Figure 6.43: P1.3 Coomassie stained SDS-PAGE gel, non-collagenase treated. Samples as in Table 5.11. MW is molecular weight (kDa). Gel/samples prepared as in 5.7.3(i). Arrow indicates expected bands for IgG heavy chains.

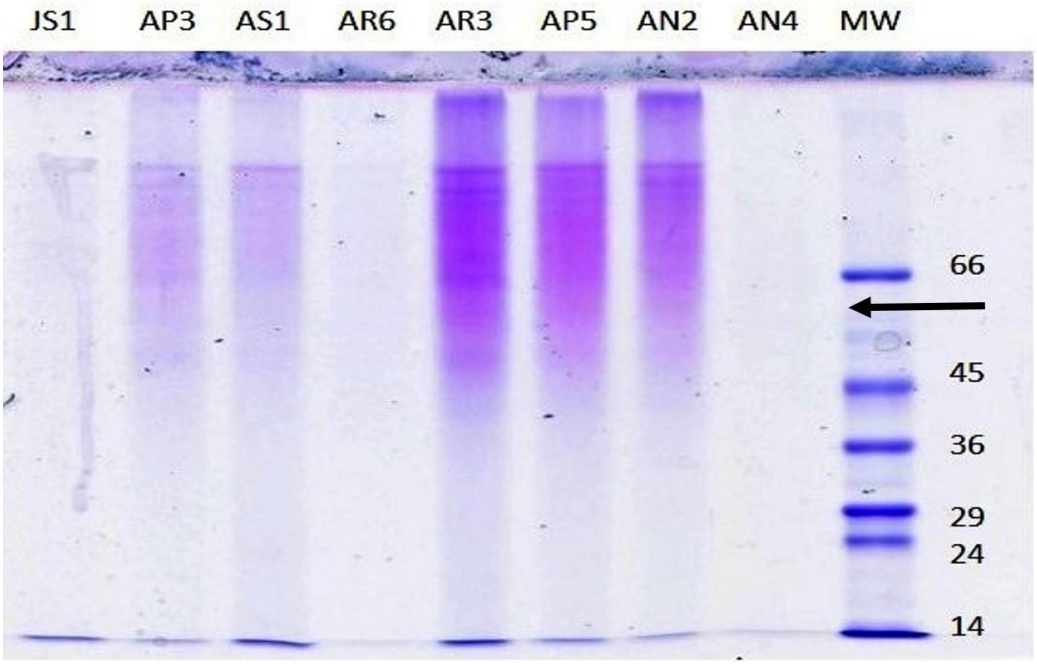

Figure 6.44: P1.3 Coomassie stained SDS-PAGE gel following collagenase treatment. Samples as in Table 5.11. MW is molecular weight (kDa). Gel/samples prepared as in 5.7.3(i) and 5.7.2. Arrow indicates expected bands for IgG heavy chains.

6.4.1(v): Western blot

In an attempt to directly detect the presence of IgG heavy chains, the P1.3 collagenase and non-collagenase treated gels (Figures 6.43 and 6.44) were subjected to a Western blot analysis (see section 5.7.3(ii)). This was performed by probing transferred protein bands with a monoclonal mouse anti-human IgG (Fc chain) antibody and secondary anti-mouse IgG conjugated antibody. Both gels were exposed for 10, 30, 60 seconds, and eight minutes following transfer of proteins. A comparison of the membranes (Figures 6.45-6.51) to Schmidt-Schultz and Schultz's (2004) positive Western blot results (Figure 6.37), fails to show any correlation with their 'positive' for IgG heavy chains at approximately 60kDA. The appearance of bands above the expected molecular weight range for IgG heavy chains (see Figure 6.46 for an example of this) should be treated with caution, as they likely represent non-specific cross reactivity. Bands below 60kDa may, however, represent degraded IgG Fc chains (see Figure 6.46 for an example, indicated by the white arrow).

Figures 6.45, 6.46, and 6.47 show the non-collagenase treated membranes at 10, 30, and 60 second exposures, respectively. The left side of this membrane was damaged during the protocol; the lane representing JS1 is, therefore, not present here. Cross-reactivity is shown at all exposure stages, becoming increasingly evident over time. The eight minute exposure of the non-collagenase treated membrane is not shown, due to over-exposure and subsequent loss of clarity. A possible positive for degraded IgG heavy chain can be seen on Figures 6.46 and 6.47 (marked by a white arrow).

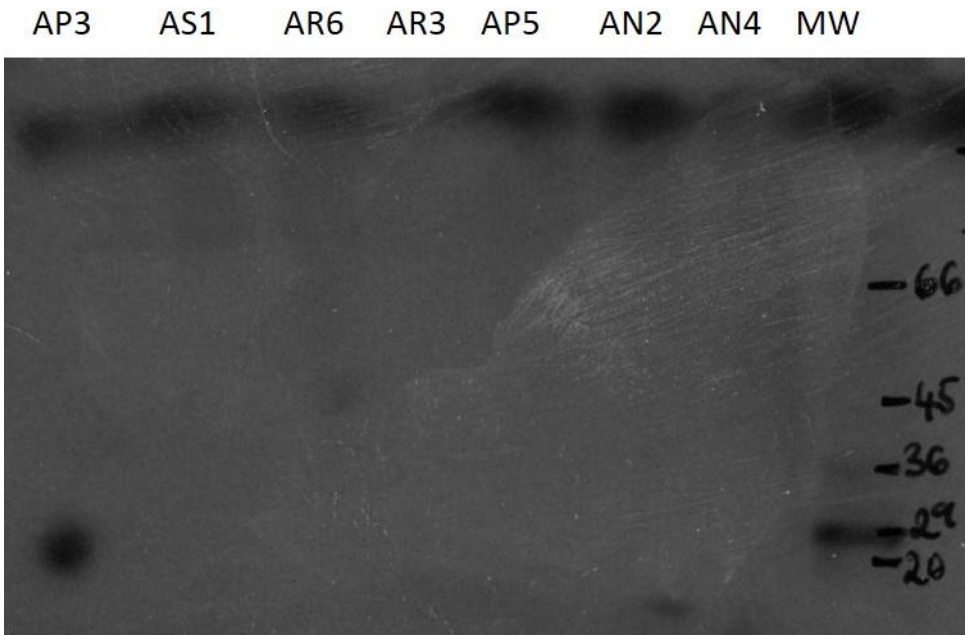

Figure 6.45: Western blot of non-collagenase-treated P1.3 SDS-PAGE gel, exposed for 10 seconds. MW – molecular weight markers.

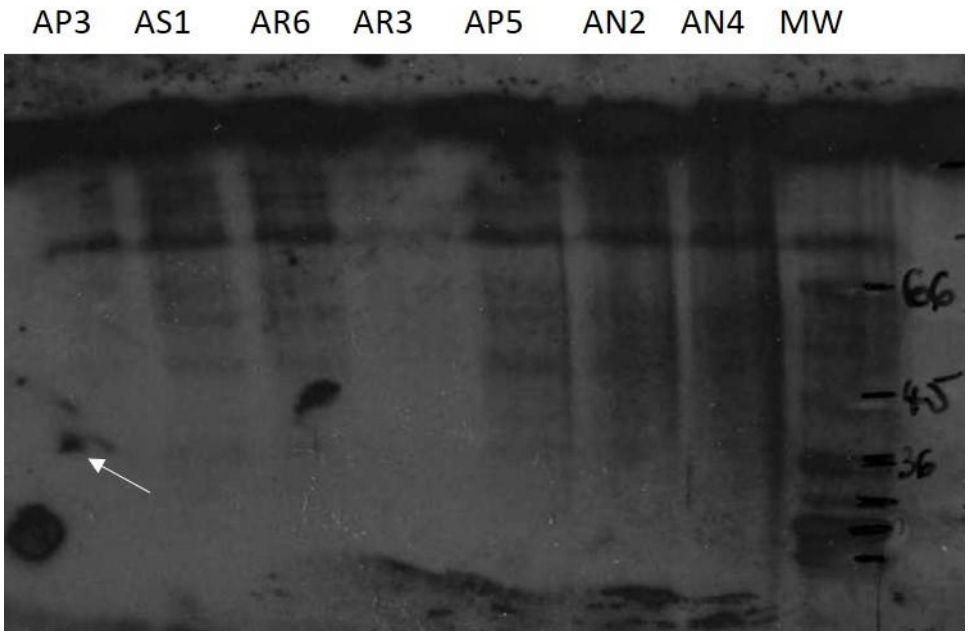

Figure 6.46: Western blot of non-collagenase-treated P1.3 SDS-PAGE gel, exposed for 30 seconds. Arrow indicates possible positive for human IgG. MW – molecular weight markers.

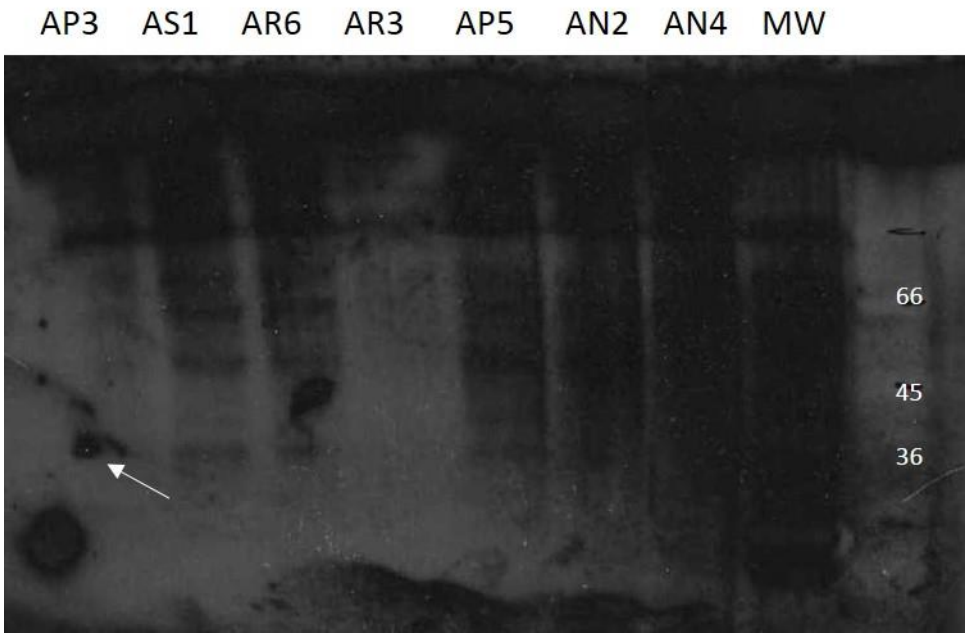

Figure 6.47: Western blot of non-collagenase-treated P1.3 SDS-PAGE gel, exposed for 60 seconds. Arrow indicates possible positive for human IgG. MW – molecular weight markers.

Figures 6.48, 6.49, and 6.50 display the collagenase-treated membrane at 10 seconds, 30 seconds, 60 seconds, and eight minute exposure times, respectively. Far less cross-reactivity is observed here. The possible IgG heavy chain positive is marked by white arrows. This band is discussed in section 7.3.1(ii), as is the appearance of a band in Figure 6.50 adjacent to the 29kDa molecular weight (MW) marker. Very faint bands at approximately 25kDa (Figure 6.50) for samples AR6, AR3, and AP5 may tentatively suggest the presence of IgG light chains. Their appearance, however, would be the result of cross-reactivity, given the heavy-chain specificity of the primary antibody and anti-mouse IgG specificity of the secondary antibody.

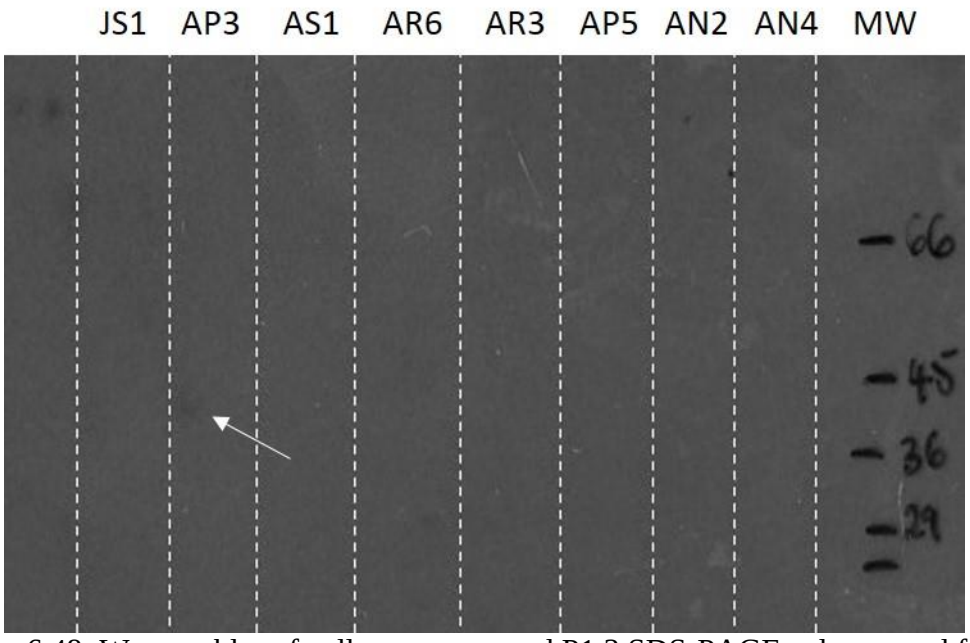

Figure 6.48: Western blot of collagenase-treated P1.3 SDS-PAGE gel, exposed for 10 seconds. Arrow indicates possible positive for human IgG. MW – molecular weight markers.

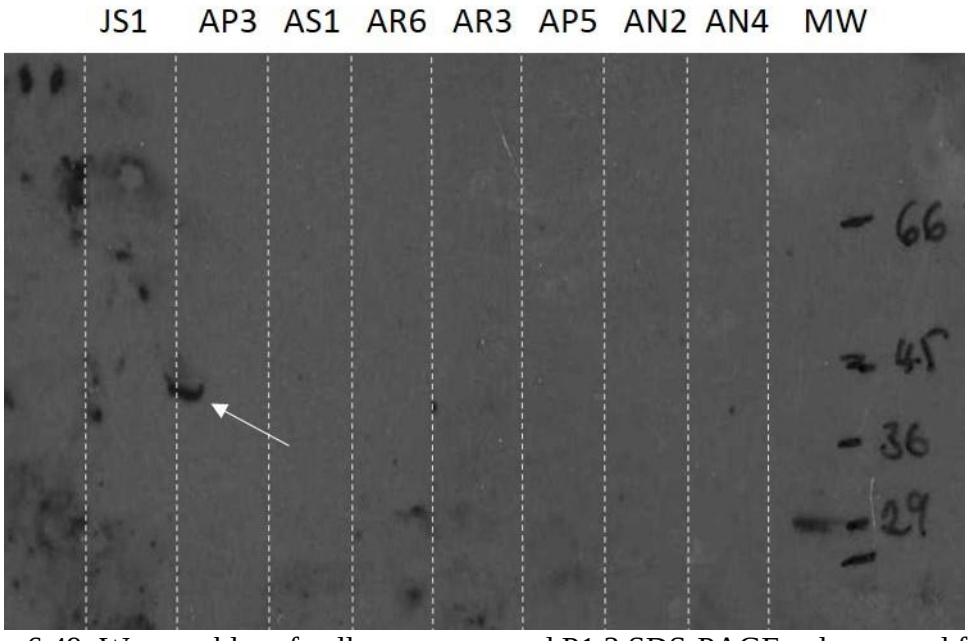

Figure 6.49: Western blot of collagenase-treated P1.3 SDS-PAGE gel, exposed for 30 seconds. Arrow indicates possible positive for human IgG. MW – molecular weight markers.

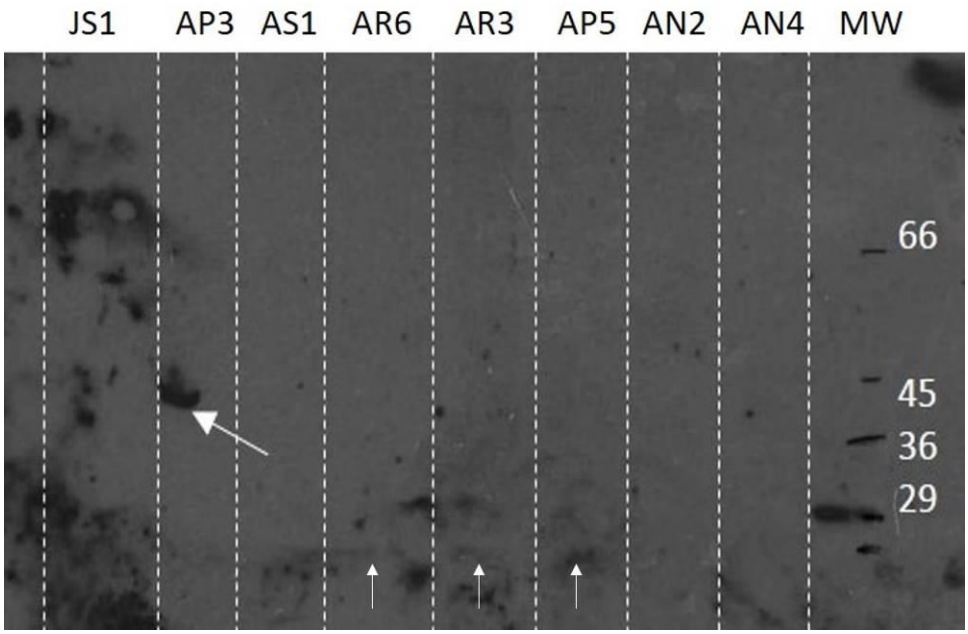

Figure 6.50: Western blot of collagenase-treated P1.3 SDS-PAGE gel, exposed for 60 seconds. Arrows indicates possible positive for human IgG. MW – molecular weight markers.

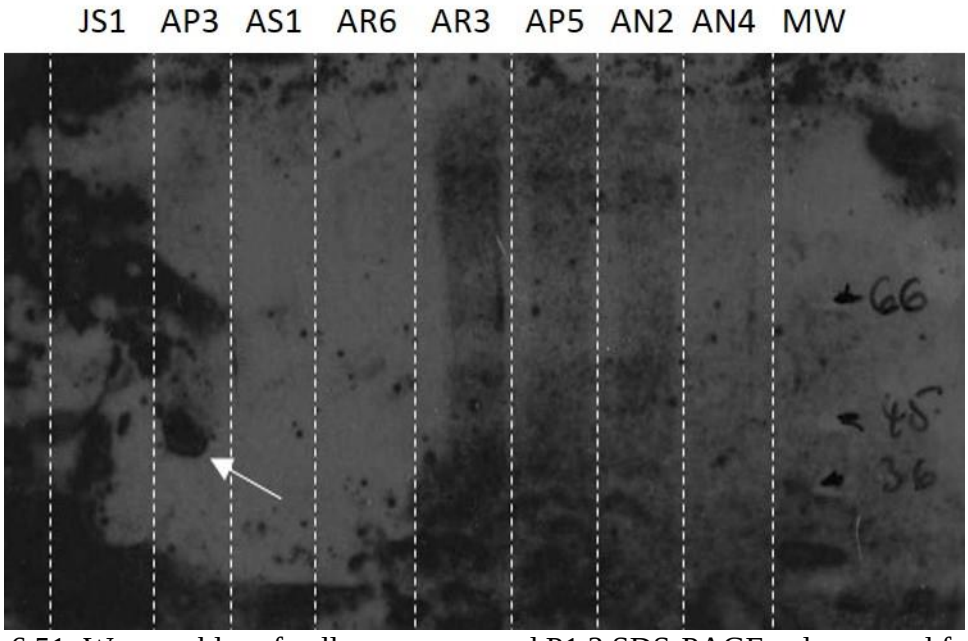

Figure 6.51: Western blot of collagenase-treated P1.3 SDS-PAGE gel, exposed for eight minutes. Arrow indicates possible positive for human IgG. MW – molecular weight markers.

6.4.1(vi): P1 human IgG ELISA

Two types of enzyme-linked immunosorbent assays (ELISAs) were employed as part of this study. The first was a human IgG capture ELISA that analysed samples resulting from the P1.1 extraction protocol alongside a variety of standards and controls (Table 6.25). These controls included bovine collagen, animal bone, and buffers. Table 6.25 suggests that IgGs were detected at extremely low concentrations, with the highest concentration in sample HDAR3 (4.79ng/ml). Note that many of the results fall close to the ELISA detection limit of 1ng/ml. These results are discussed in section 7.3.1(iii). The test functioned correctly, as evidenced by the standards.

Sample	Type	IgG quantity (ng/ml)	Sample	Type	IgG quantity (ng/ml)
Standard (1ng/ml)	Standard	1	AN7	Animal rib	3.43
Standard (2ng/ml)	Standard	2	HDAR1	Adult rib	2.07
Standard (5ng/ml)	Standard	5	HDAR2	Adult rib	4.47
Standard (10ng/ml)	Standard	10	HDAR3	Adult rib	4.79
Standard (20ng/ml)	Standard	20	HDAR4	Adult rib	2.18
Buffer C	Buffer control	2.8	HDAS1	Adult cranium	1.97
Collagen	Bovine Type I	0.61	HDAR6	Adult rib	1.76
Collagen	Bovine Type III	3.53	HDAP3	Adult phalanx	1.66
AN1	Animal rib	1.97	HDAP5	Adult phalanx	-0.22
AN2	Animal rib	2.5	HDJS1	Juvenile cranium	0.82

Table 6.25: Results from P1 human IgG ELISA described in section 5.7.3(iii).

6.4.2: P2 extraction methodology adapted from Jiang et al. (2007)

This section presents the results of the P2 extractions following the adapted Jiang et al. (2007) protocol outlined in section 5.7.4. Samples selected for the P2 extractions are shown in Table 5.14. Results of thiophilic adsorption chromatography (TAC) purifications for all P4 extractions are show in summary Tables 6.26 to 6.29. Each table displays the total spectrophotometric absorbance values at 280nm for each sample at each extraction stage, which equates to the approximated mg/ml concentration of the tested sample. Full results tables for these TAC tests are shown in Appendix 1. The SDS-PAGE gels corresponding to

each P2 extraction are displayed immediately following the appropriate table. Samples with higher protein concentrations (post-TAC) were usually selected for SDS-PAGE analysis, since it was assumed that these were the most likely to result in clearer IgG bands.

6.4.2(i): P2.1 thiophilic adsorption results and SDS-PAGE gels

Table 6.26 displays the TAC results from the P2.1 extractions. Total resin-retained eluted protein concentrations (mg/ml) from the TAC resins for each sample at each extraction stage (stages described in section 5.7.4(i)) are shown. Note the extremely low totals, with the highest total (HDAP5 stage 1) approximating only 38µg/ml, and the absence of retained protein from the fourth extraction stage.

Sample	HI	Stage 1 (mg/ml)	Stage 2 (mg/ml)	Stage 3 (mg/ml)	Stage 4 (mg/ml)
HDAP5	5	0.038	0	0.025	0
AN1	5	0.004	0.004	0.007	0

Table 6.26: Summary of P2.1 TAC showing total protein (mg/ml) eluted from each extraction stage. HI – histological index. Samples described in Table 5.14.

Figure 6.52 shows the SDS-PAGE gel (sliver stained) with selected samples from the P2.1 TAC. The white arrow indicates the expected molecular weight of extracted IgG heavy chains. The gel shows a cluster of bands for most samples between approximately 45 and 66kDa. No further analytical action (e.g., excision for proteomics) was taken on this gel due to the extremely low protein concentrations.

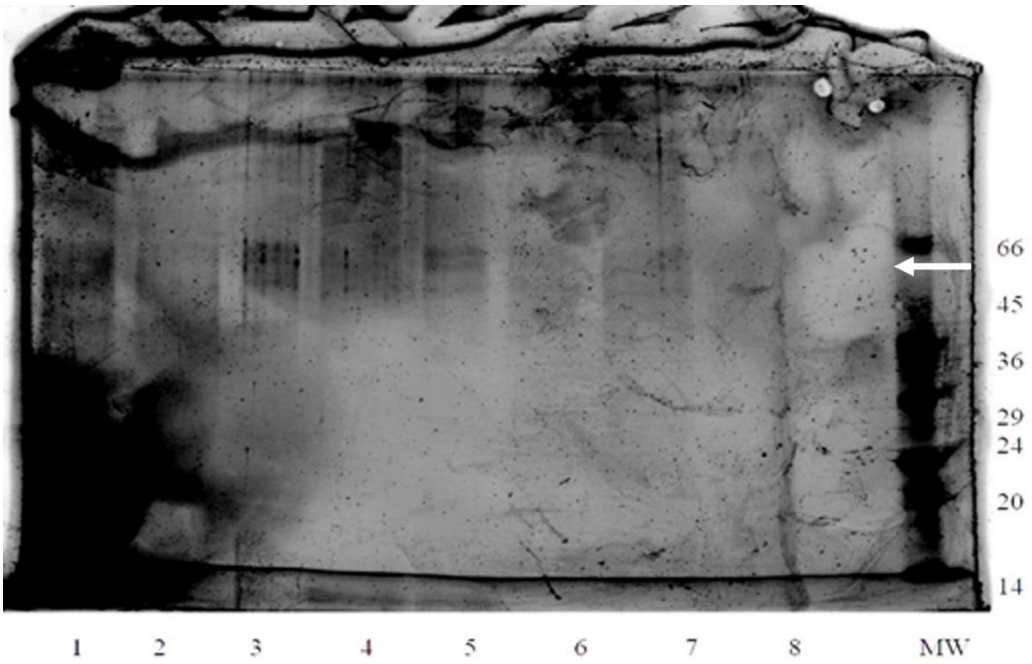

Figure 6.52: P2.1 SDS-PAGE silver stained gel. 1, 2, and 3 – HDAP5, 1st stage; 4, 5, and 6 – HDAP5, 3rd stage; 7 – AN1, 1st stage; 8 – AN1, 3rd stage. See Table 5.14 for sample descriptions. MW is molecular weight (kDa). Gel/samples prepared as in sections 5.7.4(i) and 5.7.3(i). Arrow indicates expected bands for IgG heavy chains.

6.4.2(ii): P2.2 thiophilic adsorption results and SDS-PAGE gels

In an attempt to increase protein yield, a wider range of samples were extracted and subjected to TAC (Table 6.27) and SDS-PAGE (Figure 6.53). Once again, however, silver staining was required due to the low protein concentrations resulting from the TAC. Bands were not, therefore, excised for further analysis. Note the retention and elution of protein from the collagen control sample (Table 6.27), and appearance of lanes around 50-60kDa on the corresponding gel lanes (7 and 8). This demonstrates non-specific binding of collagen by the TAC resins; matching bands for the other samples (lanes 1-6) are suggestive of similar collagen binding in these archaeological samples.

Sample	HI	Stage 1 (mg/ml)	Stage 2 (mg/ml)	Stage 3 (mg/ml)
HDAR9	5	0	0.103	0.027
HDAP5	5	0.137	0.085	0.022
HDAN2	5	0.006	0.072	0.009
Collagen	-	0.003	0.062	0

Table 6.27: Summary of P2.2 TAC showing total protein (mg/ml) eluted from each extraction stage. HI – histological index. Samples described in Table 5.14.

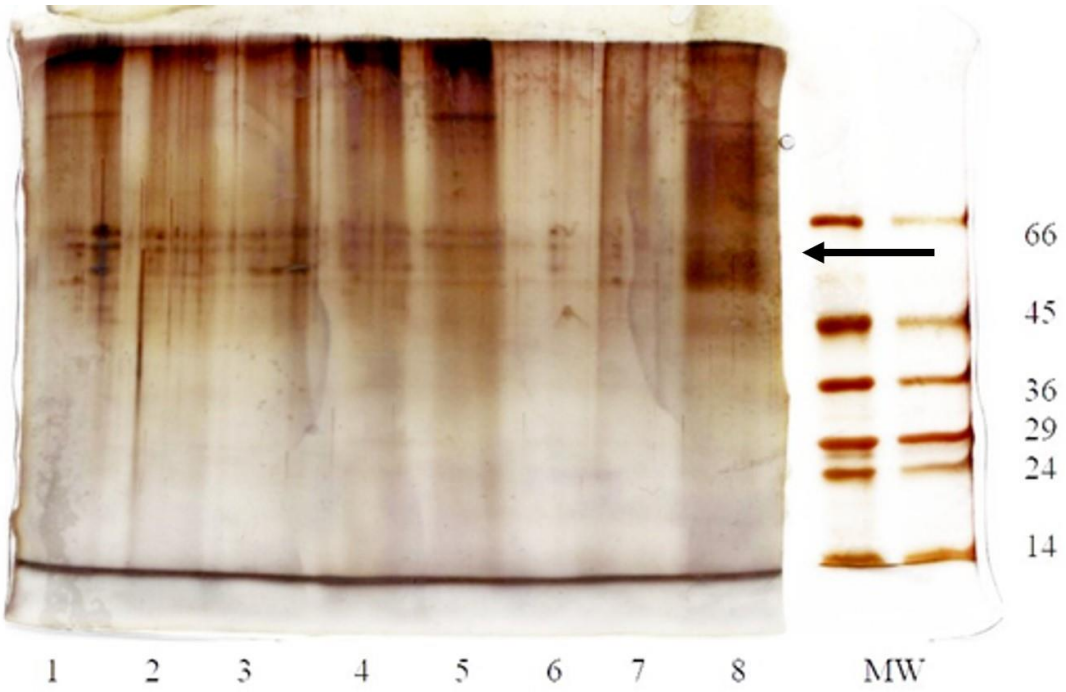

Figure 6.53: P2.2 silver stained SDS-PAGE gel. MW is molecular weight marker (kDa). 1 - HDAP3, 2nd stage; 2 - HDAP9, 2nd stage; 3 – AN2, 2nd stage; 4 – HDAR9, 3rd stage; 5 – HDAP3, 3rd stage; 6 – AN2, 1st stage; 7 – Collagen, 1st stage; 8 – Collagen, 2nd stage. See Table 5.14 for sample descriptions. Gel/samples prepared as in sections 5.7.4(i) and 5.7.3(i). Arrow indicates expected bands for IgG heavy chains.

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6.4.2(iii): P2.3 thiophilic adsorption results and SDS-PAGE gels

Results of the TAC test for P2.3 extraction samples are shown in Table 6.28. Sample HDJS3 was included here to investigate the effect of poor histological preservation (HI) on protein yield (discussed later in section 6.5) and TAC retention. Table 6.28 suggests that HI

had little impact on the concentration of proteins eluted from the TAC resins, which remained very low, regardless of sample preservation.

Sample	HI	Stage 1 (mg/ml)	Stage 2 (mg/ml)	Stage 3 (mg/ml)
HDAP5	5	0.1	0	0.041
HDAR2	5	0.001	0.005	0.026
HDAR3	4	0.004	0.018	0.026
HDJS3	1	0.04	0.003	0.055

Table 6.28: Summary of P2.3 TAC showing total protein (mg/ml) eluted from each extraction stage. HI – histological index. Samples described in Table 5.14.

The SDS-PAGE gel for selected P2.3 samples is shown in Figure 6.54. A positive fresh IgG control was included at this stage in order to help identify potential ancient IgG bands. The gel shows the positive heavy chains at approximately 50kDa (lower than Schmidt-Schultz and Schultz's ancient Fc chains at 60kDa, interestingly) and light chains very faintly at approximately 25kDa. A comparison of the extended heating (advocated by Wiechmann et al., 1999) and traditional short boil steps during sample preparation was attempted with three P2.3 post-TAC samples. It can be seen that the latter approach resulted in more intense bands. Potential matches to the positive heavy chains can be seen in all samples, particularly the boiled versions.

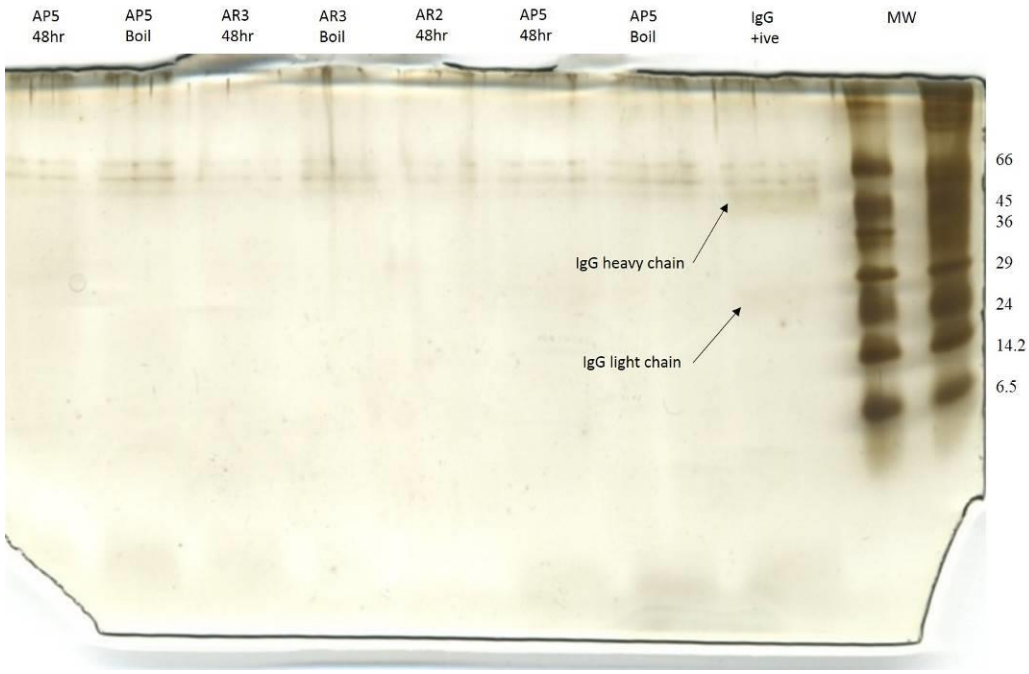

Figure 6.54: P2.3 SDS-PAGE silver stained gel. MW is molecular weight (kDa), with positive control. Samples described in Table 5.14.

6.4.2(iv): P2.3 gel bands selected for proteomic analysis, and proteomic results

Selected bands potentially matching the IgG positive control were excised for proteomic analysis (Figure 6.55). Bands from the strongest non-boiled sample (AP5 48hr) and its boiled counterpart (AP5) were tested to compare results, and IgG positive control bands were selected to test the efficacy of proteomic analysis of silver stained bands.

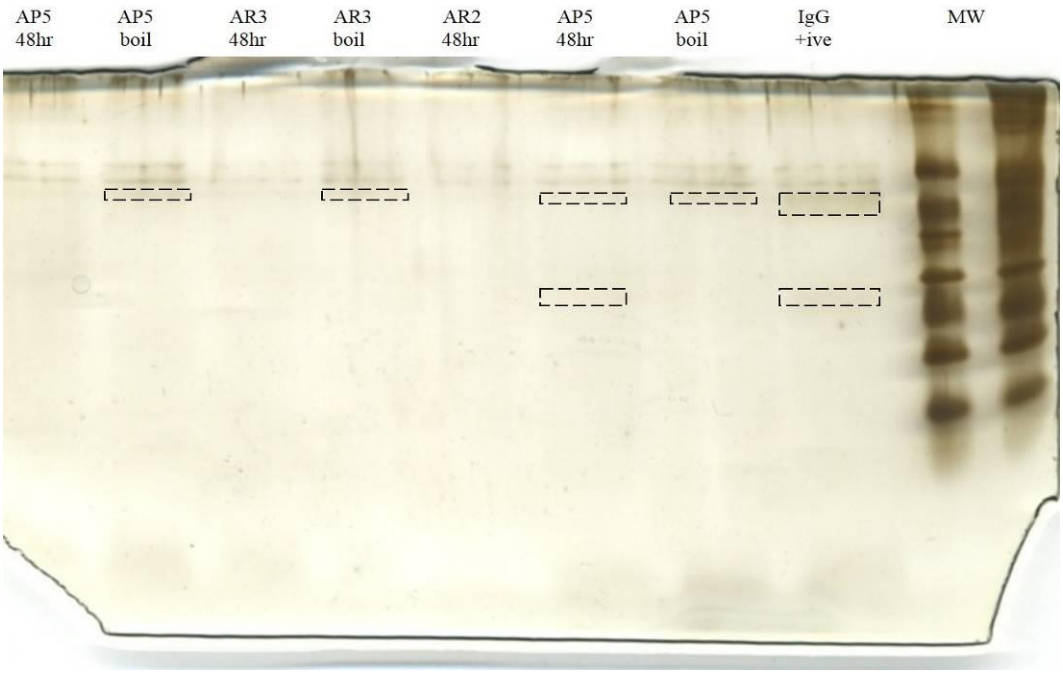

Figure 6.55: P2.3 gel bands excised for proteomic analysis.

Unfortunately neither MALDI nor nLC-MS/MS analysis yielded reportable results for analyses of the excised gel bands, including the IgG positive controls. This was likely in part due to the extremely low protein quantity being visualised in post-Coomassie silver staining and potential interference with residual acrylamide (William Simon, personal communication, July 2013).

6.4.2(v): P2.4 thiophilic adsorption results and SDS-PAGE gels

Table 6.29 shows that the P2.4 samples resulted in noticeably higher TAC elution concentrations than in previous samples. An absence of protein from the stage 2 extractions is likely representative of human error, rather than a problem with the extraction or TAC methodology. This increase in protein concentration allowed for band visualisation by colloidal Coomassie staining (Figure 6.56), thereby increasing the chance of successful proteomic analysis of selected bands (Figure 6.57).

Sample	HI	Stage 1 mg/ml)	Stage 2 (mg/ml)	Stage 3 (mg/ml)
WM2316.1	5	0.083	0	0.022
CD127.2	5	0.077	0	0.01
LP3845.1	0	0.073	0	0.088
AN5	0	0.054	0	0.084

Table 6.29: Summary of P2.4 TAC showing total protein (mg/ml) eluted from each extraction stage. HI – histological index. Samples described in Table 5.14.

Figure 6.56 shows faint bands potentially matching the IgG positive control heavy chains at around 50kDa (see arrow) for most of the ancient samples (lanes 1-7). The gel also shows the effectiveness of acetone precipitation on fresh IgGs (compare lanes 8 and 9).

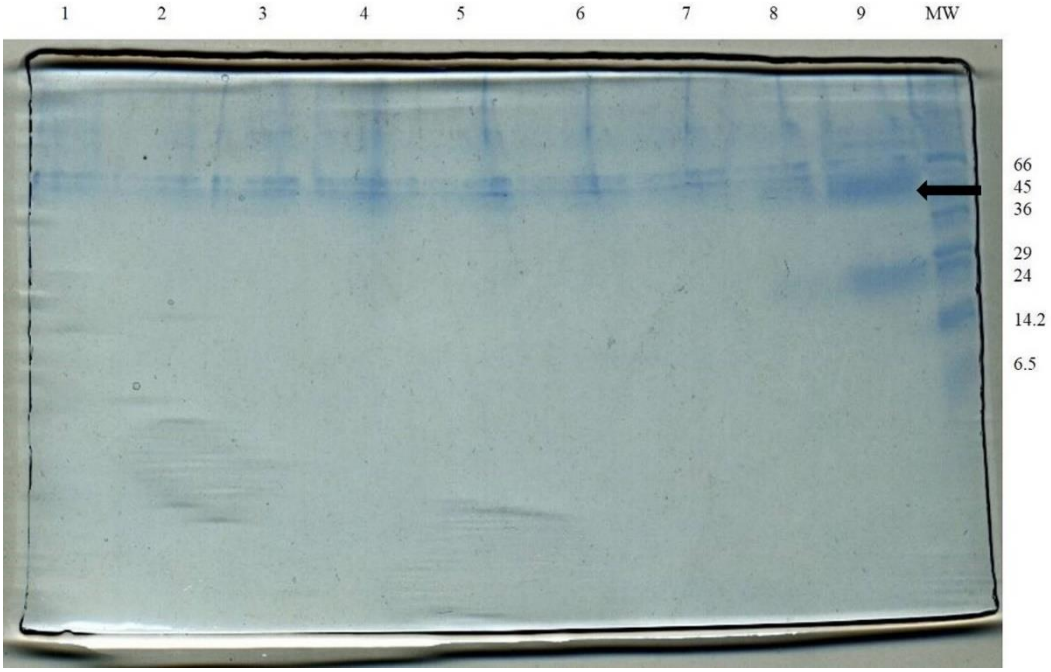

Figure 6.56: P2.4 SDS-PAGE. Colloidal Coomassie stained gel. 1 – WM2316.1, 1st stage; 2 – CD127.2, 1st stage; 3 – LP3845.1, 1st stage; 4 – LP3845.1, 3rd stage; 5 – AN5, 1st stage; 6 – AN5, 3rd stage; 7 – HDAR3 (from P2.3); 8 – IgG positive control (not precipitated); 9 – IgG positive control (acetone precipitated); MW – Molecular weight marker (kDa). Samples described in Table 5.14. Gel/samples prepared as in section 5.7.6(ii). Arrow indicates expected bands for IgG heavy chains.

6.4.2(vi): P2.4 gel bands selected for proteomic analysis, and proteomic results

Bands of potential interest matching the IgG positive control (including the control itself) were excised for proteomic analysis (Figure 6.57).

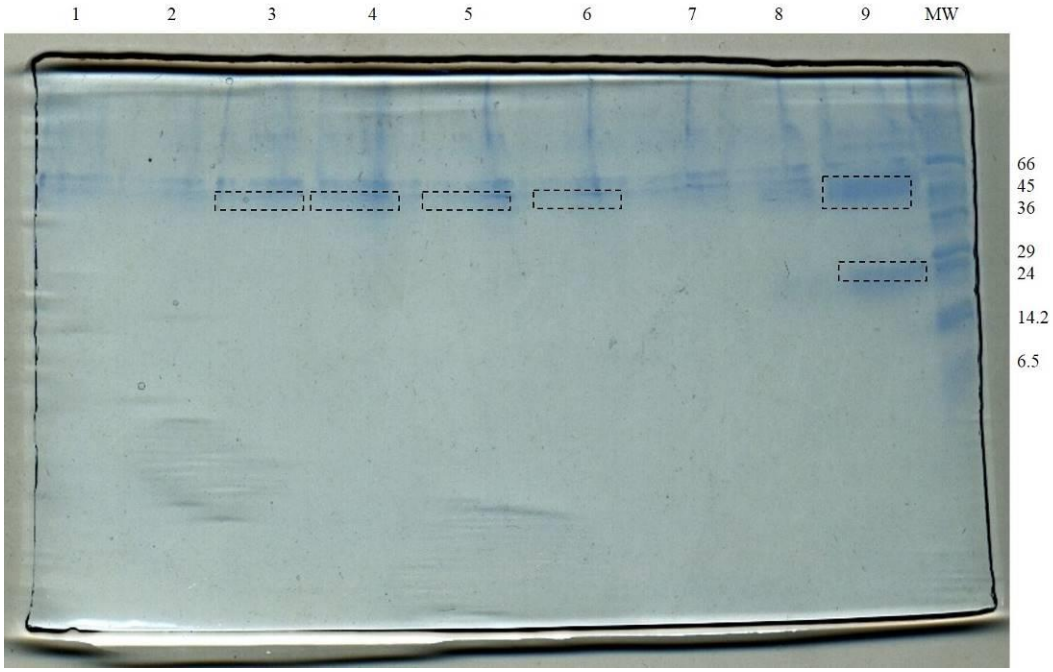

Figure 6.57: P2.4 gel bands excised for proteomic analysis, including the IgG positive control (band 9).

The results of proteomic analysis of the selected bands are presented in Tables 6.30 (MALDI) and 6.31 (nanospray LC-MS/MS). Both analyses showed only contaminating exogenous keratin for the ancient samples (LP3845 and AN5), but successfully identified the positive controls. Best matches to databases of sequenced proteins (NCBI for MALDI and Swiss-Prot for nLC-MS/MS) are shown. Put simply, protein score (Table 6.30) is the number of successful matches for each identified protein, and sequence coverage (Table 6.31) is the percentage of a peptide sequence that match a known the sequence of a known protein.

Sample	Best Match	Protein Score
LP3845.1, 1st stage	Keratin 5 [Homo sapiens]	52
LP3845.1, 3rd stage	No result	No result
AN5, 1st stage	Predicted: similar to keratin 10 [Pan troglodytes]	86
AN5, 3rd stage	No result	No result
IgG heavy chain, positive control	Immunoglobulin gamma 2 heavy chain constant region [Homo sapiens]	107
IgG light chain, positive control	Chain A, crystal structure Fab fragment of monoclonal IgG [Homo sapiens]	86

Table 6.30: Results from MALDI analyses of excised P2.4 gel bands. NCBI database.

Sample	Name	Sequence Coverage (%)
LP3845.1, 1st stage	Keratin type 1, cytoskeletal 9 [Homo sapiens]	6.3
LP3845.1, 3rd stage	Keratin type I, cytoskeletal 10 [Homo sapiens]	16.4
	Keratin type I, cytoskeletal 9 [Homo sapiens]	7.5
	Keratin type 2, cytoskeletal 1 [Homo sapiens]	9
AN5, 1st stage	Keratin type I, cytoskeletal 10 [Homo sapiens]	16.4
	Keratin type 2, cytoskeletal 1 [Canis familiaris]	6.8
	Keratin type I, cytoskeletal 9 [Homo sapiens]	7.4
AN5, 3rd stage	No result	No result
IgG heavy chain, positive control	Ig gamma-3 chain C region [Homo sapiens]	19.9
	Keratin type I, cytoskeletal 10 [Homo sapiens]	8.9
	Keratin type I, cytoskeletal 9 [Homo sapiens]	9.3
	Keratin type II, cytoskeletal 2 [Homo sapiens]	8.6
	Ig alpha-1 chain C region [Gorilla gorilla]	7.9
	Ig gamma-2 chain C region [Homo sapiens]	22.4
	Ig gamma-1 chain C region [Homo sapiens]	22.7
	Ig heavy chain V-I region [Homo sapiens]	15.4
	Ig heavy chain V-III region [Homo sapiens]	16.7
	Keratin type II, cytoskeletal 1 [Canis familiaris]	11.3
IgG light chain, positive control	Ig kappa chain C region [Homo sapiens]	35.8
	Ig kappa chain V-II region [Homo sapiens]	25.6
	Ig gamma-1 chain C region [Homo sapiens]	11.2
	Ig lambda chain V-I region [Homo sapiens]	19.2
	Ig lambda chain V-I region [Homo sapiens]	20.2
	Ig kappa chain V region [Oryctolagus cuniculus]	14.8
	Ig lambda chain V-I region [Homo sapiens]	16.5
	Ig kappa chain V-III region [Homo sapiens]	23.5
	Ig lambda chain V-II region [Homo sapiens]	11.7

Table 6.31: Results of nLC-MS/MS analyses of excised P2.4 gel bands. Swiss-Prot database. Peptide numbers were not supplied.

6.4.2(vii): P2.5 thiophilic adsorption results and SDS-PAGE gels

Table 6.32 shows the total eluted protein concentrations (mg/ml) from TAC testing of the P2.5 samples described in Table 5.14, and Figure 6.58 displays the corresponding colloidal Coomassie stained SDS-PAGE gel. As mentioned in section 5.7.6(ii), these samples were precipitated in TCA, rather than acetone, since the latter resulted in large phosphate pellets which were difficult to solubilise in SDS sample buffer. No bands clearly matching the IgG control are present in Figure 6.58, although possible heavy chain matches are observable for all samples in the silver stained version (Figure 6.59).

Sample	HI	Stage 1 (mg/ml)	Stage 2 (mg/ml)	Stage 3 (mg/ml)
HP154.1	5	0.023	0.053	0.025
LP3760.1	0	0.007	0.01	0.016
LP4585.3	2	0.007	0	0.006
OL1104.2	5	0	0.013	0.015

Table 6.32: Summary of P2.5 TAC showing total protein (mg/ml) eluted from each extraction stage. HI – histological index. Samples described in Table 5.14.

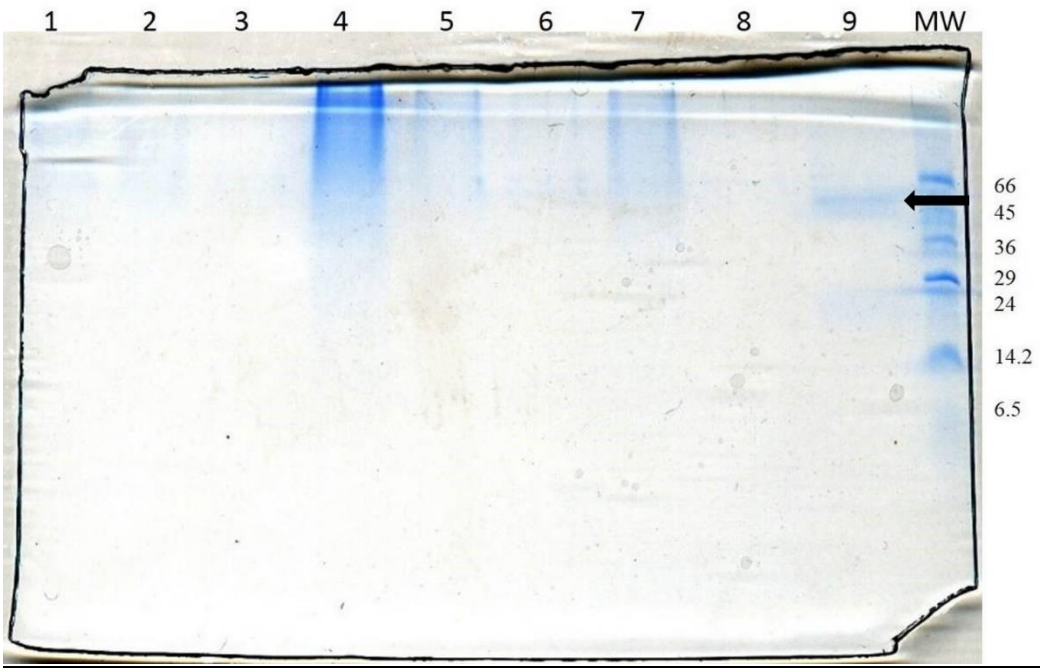

Figure 6.58: P2.5 SDS-PAGE. Colloidal Coomassie stained gel. 1 – HP154.1, 1st stage; 2 – HP154.1, 2nd stage; 3 – OL1104.2, 2nd stage; 4 – HP154.1, 3rd stage; 5 – LP4585.3, 3rd stage; 6 – 3819D, 3rd stage; 7 – OL1104.2, 3rd stage; 8 – Blank sample buffer; 9 – IgG positive control; 10 – Molecular weight marker (kDa). Samples described in Table 5.14. Gel/samples prepared as in section 5.7.6(ii). Arrow indicates expected bands for IgG heavy chains.

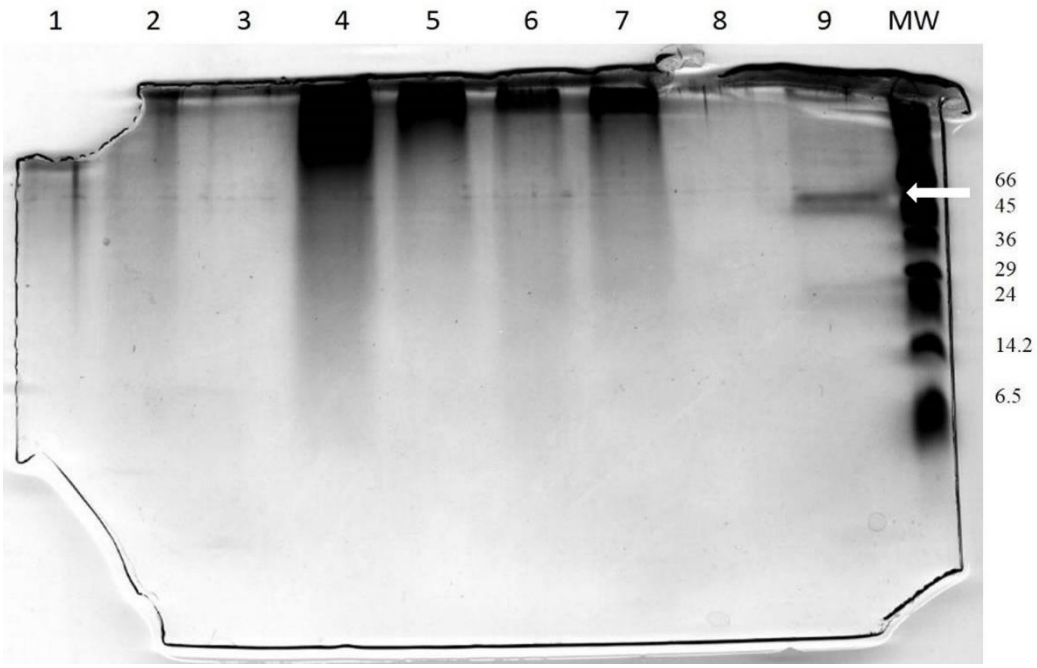

Figure 6.59: P2.5 SDS-PAGE. Silver stained gel. 1 – HP154.1, 1st stage; 2 – HP154.1, 2nd stage; 3 – OL1104.2, 2nd stage; 4 – HP154.1, 3rd stage; 5 – LP4585.3, 3rd stage; 6 – 3819D, 3rd stage; 7 – OL1104.2, 3rd stage; 8 – Blank sample buffer; 9 – IgG positive control; 10 – Molecular weight marker (kDa). Samples described in Table 5.14. Gel/samples prepared as in section 5.7.6(ii). Arrow indicates expected bands for IgG heavy chains.

6.4.2(viii): P2.5 gel bands selected for proteomic analysis, and proteomic results

Figure 6.60 displays the possible heavy chain bands from the P2.5 gel (Figure 6.59) selected for proteomic analysis. Unfortunately, no protein was detected in either MALDI or nLC-MS/MS analyses of the seven gel bands.

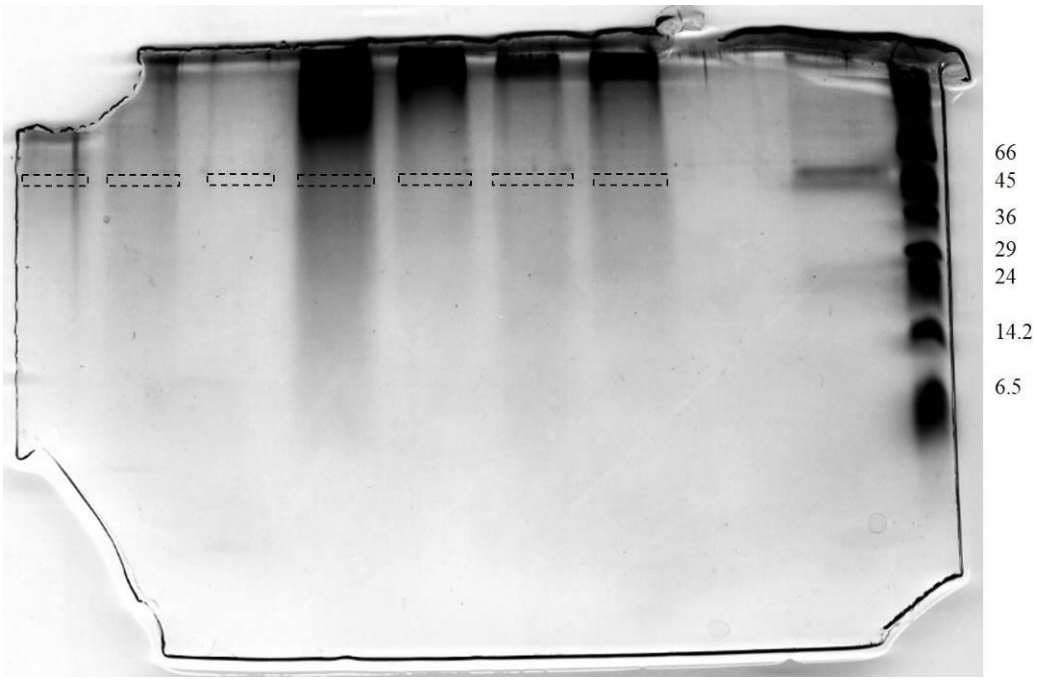

Figure 6.60: P2.5 gel bands excised for proteomic analysis. Samples as in Figure 6.59.

6.4.3: P3 extraction methodology adapted from Schmidt-Schultz and Schultz (2004), followed by thiophilic adsorption

The four well-preserved samples described in Table 5.21 were subjected to the adapted P1 extraction methodology followed by TAC. This was performed to remove degraded collagen which caused intense smearing of many P1 SDS-PAGE lanes, potentially masking lower abundance IgGs.

6.4.3(i): P3 thiophilic adsorption results and SDS-PAGE gel

Table 6.33 displays the total protein concentration (mg/ml) from each extraction stage for the selected samples. Although still relatively low concentrations, they compare favourably to most of the P2 samples subjected to TAC.

Sample	HI	Stage 1 (mg/ml)	Stage 2 (mg/ml)	Stage 3 (mg/ml)
EH198.1	4	0.117	0.105	0.055
CD120.2	5	0.055	0.054	0.084
CD165.1	5	0.021	0.103	0.112
OL1104.2	5	0.024	0.024	0.021

Table 6.33: Summary of P3 TAC showing total protein (mg/ml) eluted from each extraction stage. HI – histological index. Samples described in Table 5.21.

Repeated SDS-PAGE analyses of these samples post-TAC unfortunately resulted in no Coomassie-stained bands suggestive of IgG. Consequently, extracted samples that had not been subjected to TAC were instead analysed by SDS-PAGE. The resulting gel is shown in Figure 6.61. As expected, the sample lanes show significant smearing, since collagen was not removed prior to testing.

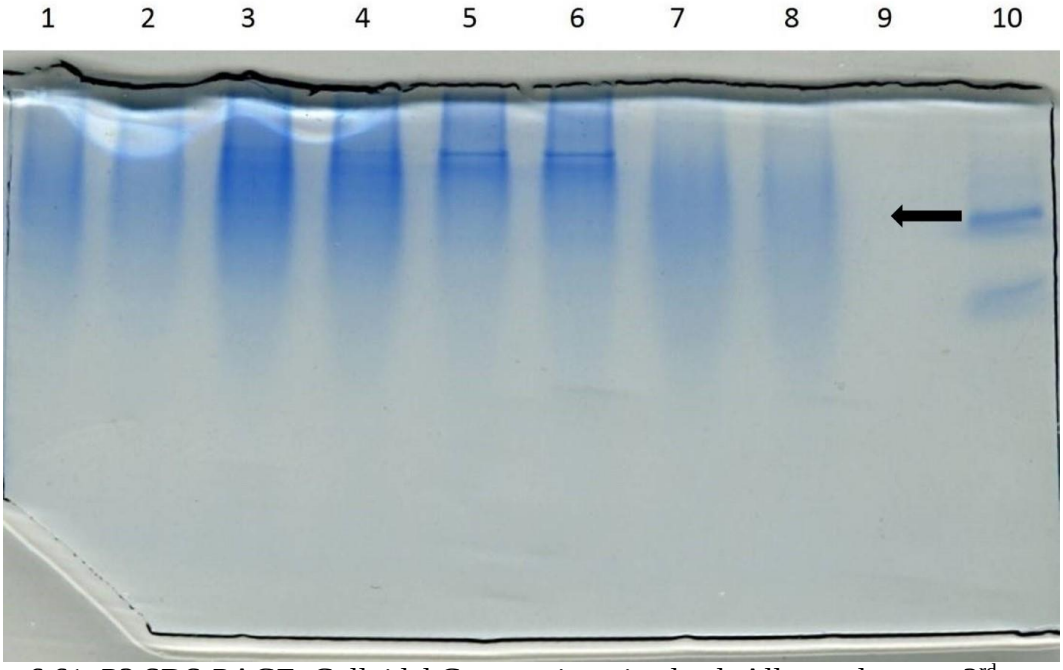

Figure 6.61: P3 SDS-PAGE. Colloidal Coomassie stained gel. All samples post 3rd stage. 1 – EH198.1; 2 – EH198.1; 3 – CD120.2; 4 – CD120.2; 5- OL1104.2; 6 – OL1104.2; 7 – CD165.1; 8 – CD165.1; 9 – Blank sample buffer; 10 – IgG positive control. Samples described in Table 5.14. Arrow indicates expected bands for IgG heavy chains.

6.4.3(ii): P3 gel bands selected for proteomic analysis, and proteomic results

Faint bands potentially representing IgG Fc chains were excised from the P3 gel (Figure 6.62) for nLC-MS/MS analysis. Given the nature of the samples (not subjected to

TAC), it is unsurprising that collagen was the dominant protein identified in the bands (Table 6.34), followed by exogenous keratin. No endogenous NCPs were identified.

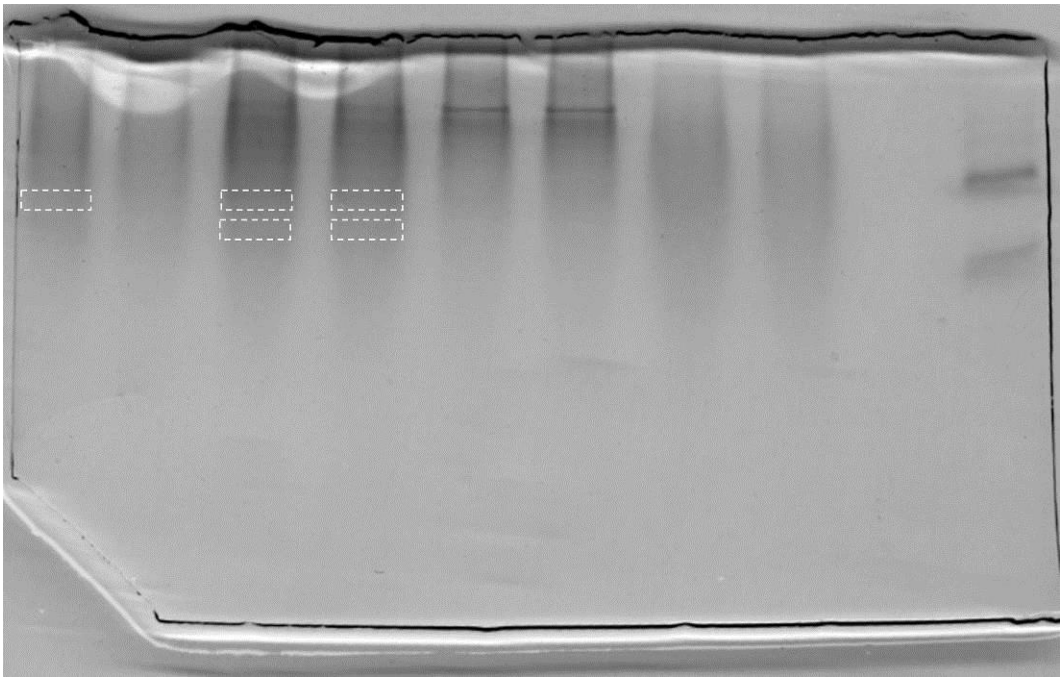

Figure 6.62: P3 gel bands excised for proteomic analysis. Samples as in Figure 6.61.

Sample	Name	Sequence Coverage (%)
EH198.1	Collagen alpha-2(I) chain [Homo sapiens]	34.5
	Collagen alpha-1(I) chain [Homo sapiens]	29.1
	Keratin type II, cytoskeletal 1 [Homo sapiens]	27.8
	Keratin, type I, cytoskeletal 14 [Homo sapiens]	35.6
	Keratin type I, cytoskeletal 9 [Homo sapiens]	22.5
	Keratin type II, cytoskeletal 6C [Homo sapiens]	32.6
	Keratin type I, cytoskeletal 10 [Homo sapiens]	11.8
	Keratin type I, cytoskeletal 16 [Homo sapiens]	31.1
	Keratin type II, cytoskeletal 5 [Homo sapiens]	15.8
CD120.2 lane 3 upper	Collagen alpha-1(I) chain [Homo sapiens]	48.4
	Collagen alpha-2(I) chain [Homo sapiens]	55.9
	Keratin type II, cytoskeletal 1 [Homo sapiens]	10.7
	Collagen alpha-2(I) chain [Rattus norvegicus]	44.5
CD120.2 lane 4 upper	Collagen alpha-1(I) chain [Homo sapiens]	62.6
	Collagen alpha-2(I) chain [Homo sapiens]	72.8
	Keratin 1 [Homo sapiens]	15.1
	Collagen type I, alpha 1 [Homo sapiens]	62.5
CD120.2 lane 3 lower	Collagen alpha-1(I) chain [Homo sapiens]	51
	Collagen alpha-2(I) chain [Homo sapiens]	57.8
	Keratin type II, cytoskeletal 1 [Homo sapiens]	12.4
	Collagen alpha-2(I) chain [Canis familiaris]	41.8
CD120.2 lane 4 lower	Collagen alpha-2(I) chain [Homo sapiens]	32.7
	Keratin type II, cytoskeletal 1 [Homo sapiens]	23.4
	Keratin, type I cytoskeletal 10 OS=Homo sapiens	11.8
	Keratin type I, cytoskeletal 9 [Homo sapiens]	16.7
	Keratin type II, cytoskeletal 2 [Homo sapiens]	13

Table 6.34: Results of nLC-MS/MS analysis of excised P3 gel bands. Swiss-Prot database.

6.4.4: P4 full Jiang et al. (2007) extraction, SDS-PAGE, and proteomic analysis

A further batch of samples (Table 5.22) were selected for extraction in an attempt to increase protein yield following an adapted Jiang et al. (2007) protocol similar to that employed for the P2 samples (see section 5.7.8 for details). These samples represented a range of preservation levels.

6.4.4(i): P4 thiophilic adsorption results

Despite the comparatively adequate protein concentrations shown in Table 6.35, repeated attempts at SDS-PAGE analysis failed to provide any Coomassie stained bands suggestive of IgG.

Sample	HI	Stage 1 (mg/ml)	Stage 2 (mg/ml)	Stage 3 (mg/ml)
CD84.2	0	0.025	0.08	0.064
CD112.3	2	0.034	0.067	0.059
EHA4	4	0.025	0.037	0.04
EH156.3	5	0.05	0.093	0.044

Table 6.35: Summary of P4 TAC showing total protein (mg/ml) eluted from each extraction stage. HI – histological index. Samples described in Table 5.21.

6.4.5: nLC-MS/MS analysis of P3 and P4 samples

This section presents the results of nLC-MS/MS analysis of selected P3 and P4 post-TAC samples (section 5.7.8; Table 5.23) in Tables 6.36 to 6.46. Peptide matches were made by searching the Swiss-Prot and TrEMBL (human genome) databases. Only proteins for which more than one peptide scored over 95% confidence are included in the tables. The number of successfully identified peptides is shown in the Peptides (95%) column. Sequence coverage represents the percentage of each unique protein sequence identified. For full analyses, see Appendix 3. The extracted NCPs of interest (namely the endogenous NCPs and soil-associated, infiltrating proteins) are discussed in sections 7.3.3(ii) and 7.3.3(iii).

Tables 6.36 and 6.37 show pre- and post-exclusion analysis for sample CD120.2 (2nd extraction stage). As explained in section 5.7.8, the post-exclusion samples were subjected to a longer separation gradient with lists excluding the collagen and keratin sequences encountered in the pre-exclusion analysis. The aim of this was to instruct the mass spectrometer to ‘ignore’ these high-abundance proteins to reveal lower abundance non-collagenous, non-contaminating proteins. Tables 6.36 and 6.37 suggest that this process was partially successful for this sample in that all collagen was excluded, and two previously masked NCPs (terminal uridylyltransferase 4; Ankyrin repeat and SOCS box protein 18) were detected post-exclusion.

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
CD120.2 2nd stage	5	Collagen alpha-2(I) chain [Homo sapiens]	58.9	24
		Collagen, type I, alpha 1 [Homo sapiens]	51.3	19
		Keratin, type I cytoskeletal 9 [Homo sapiens]	26.6	6
		Keratin 1 [Homo sapiens]	25	5
		Keratin, type I cytoskeletal 10 [Homo sapiens]	32	3
		Keratin, type II cytoskeletal 2 [Homo sapiens]	20.5	2
		Dermcidin [Homo sapiens]	29.1	2

Table 6.36: P3 sample CD120.2, 2nd extraction stage, nLC-MS/MS analysis, pre-exclusion. TrEMBL database.

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
CD120.2 2nd stage	5	Keratin, type I cytoskeletal 10 [Homo sapiens]	25.9	3
		Keratin, type II cytoskeletal 2 [Homo sapiens]	24.4	2
		Dermcidin [Homo sapiens]	25.5	1
		Terminal uridylyltransferase 4 [Homo sapiens]	8.7	1

Table 6.37: P3 sample CD120.2, 2nd extraction stage, nLC-MS/MS analysis, post-exclusion. TrEMBL database. Bold entries are endogenous non-collagenous proteins.

Tables 6.38 and 6.39 show results of analysis of P3 sample CD120.2 (3rd extraction stage), pre- and post-exclusion, respectively. Once again, endogenous NCPs (shown in bold) were revealed following exclusion.

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
CD120.2 3rd stage	5	Collagen alpha-2(I) chain [Homo sapiens]	25.8	15
		Keratin 1 [Homo sapiens]	25.49	12
		Keratin, type I cytoskeletal 9 [Homo sapiens]	19.21	10
		Collagen, type I, alpha 1 [Homo sapiens]	14.53	7
		Keratin, type II cytoskeletal 2 [Homo sapiens]	18.74	8
		Keratin, type I cytoskeletal 10 [Homo sapiens]	13.25	6
		Keratin, type I cytoskeletal 15 [Homo sapiens]	4.97	2
		Keratin 1 [Homo sapiens]	25.49	12
		Dermcidin [Homo sapiens]	2	1

Table 6.38: P3 sample CD120.2, 3rd extraction stage, nLC-MS/MS analysis, pre-exclusion. TrEMBL database.

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
CD120.2 3rd stage	5	Keratin, type I cytoskeletal 10 [Homo sapiens]	31.5	7
		Keratin, type I cytoskeletal 9 [Homo sapiens]	21.3	3
		Keratin 1 [Homo sapiens]	15.4	2
		Collagen, type I, alpha 1 [Homo sapiens]	28.5	2
		Keratin, type II cytoskeletal 2 [Homo sapiens]	22.4	1
		Terminal uridylyltransferase 4 [Homo sapiens]	6.5	1
		Dermcidin [Homo sapiens]	10	1
		Ankyrin repeat and SOCS box protein 18 [Homo sapiens]	9.2	1

Table 6.40: P3 sample CD120.2, 3rd extraction stage, nLC-MS/MS analysis, post-exclusion. TrEMBL database. Bold entries are endogenous non-collagenous proteins.

Two post-TAC P4 samples displaying different histological preservation levels were analysed for comparative protein data. These samples were not subjected to nLC-MS/MS analysis with exclusion lists due to the extended time required to create such lists. However, the apparent success of this step with the P3 samples suggests that exclusion should be considered for future analyses.

Tables 6.41, 6.42, and 6.43 display the proteins detected in sample CD84.2 (HI: 0) from the first, second, and third extraction stages, respectively. The first extraction stage (Table 6.41) revealed collagen, exogenous contaminating proteins, and two bacterial proteins likely introduced into the bone by soil infiltration (environmental contamination).

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
CD84.2 1st stage	0	Collagen alpha-1(I) chain [Homo sapiens]	72.4	105
		Collagen alpha-1(I) chain [Homo sapiens]	66.3	89
		Collagen, type I, alpha 1 [Homo sapiens]	69.9	71
		Collagen alpha-2(I) chain [Homo sapiens]	77.1	63
		Collagen alpha-2(I) chain [Homo sapiens]	70.3	62
		Keratin, type I cytoskeletal 9 [Homo sapiens]	31.1	11
		Keratin, type I cytoskeletal 9 [Homo sapiens]	29.5	10
		Keratin 1 [Homo sapiens]	30.4	10
		Keratin, type I cytoskeletal 10 [Homo sapiens]	34.2	5
		Keratin, type I cytoskeletal 10 [Homo sapiens]	23.5	5
		Keratin, type II cytoskeletal 2 [Homo sapiens]	14.6	2
		Keratin, type II cytoskeletal 2 [Homo sapiens]	8.1	2
		Dermcidin [Homo sapiens]	27.3	1
		MUC19 variant 12 [Homo sapiens]	2.6	1
		L-glutamine:scyllo-inososе aminotransferase [Streptomyces griseus]	13.2	1
		Probable tRNA sulfurtransferase [Lactobacillus casei]	10.6	1

Table 6.41: P4 sample CD84.2, 1st extraction stage, nLC-MS/MS analysis. Swiss-Prot and TrEMBL databases.

Analysis of the second extraction stage of sample CD84.2 (Table 6.42) revealed a number of endogenous NCPs, shown in bold.

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
CD84.2 2nd stage	0	Collagen alpha-2(I) chain [Homo sapiens]	79.8	95
		Collagen alpha-1(I) chain [Homo sapiens]	75.9	93
		Collagen alpha-1(I) chain [Homo sapiens]	61.7	87
		Collagen alpha-2(I) chain [Homo sapiens]	74.2	86
		Collagen, type I, alpha 1 [Homo sapiens]	74	67
		Protein S100-A7 [Homo sapiens]	30.7	2
		Keratin, type II cytoskeletal 1 [Homo sapiens]	5.9	2
		Protein S100-A7 [Homo sapiens]	30.7	2
		Keratin 1 [Homo sapiens]	22	2
		Vitronectin [Homo sapiens]	4.2	1
		Cystatin-A [Homo sapiens]	28.6	1
		Protein pelota homolog [Methanocaldococcus jannaschii]	9.2	1
		Chondroadherin [Homo sapiens]	20.3	1
		Vitronectin [Homo sapiens]	7.9	1
		Pigment epithelium-derived factor [Homo sapiens]	9.8	1
		Cystatin-A protein [Homo sapiens]	38.8	1
		Keratin, type I cytoskeletal 10 [Homo sapiens]	14.2	1

Table 6.42: P4 sample CD84.2, 2nd extraction stage, nLC-MS/MS analysis. Swiss-Prot and TrEMBL databases. Bold entries are endogenous non-collagenous proteins.

Table 6.43 shows the proteins detected in sample CD84.2, third extraction stage. The endogenous NCPs are again shown in bold. Note that the detected NCPs from both second and third extraction stages are represented by only one peptide.

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
CD84.2 3rd stage	0	Collagen alpha-1(I) chain [Homo sapiens]	70.4	97
		Collagen alpha-1(I) chain [Homo sapiens]	66	93
		Collagen alpha-2(I) chain [Homo sapiens]	76.2	73
		Collagen alpha-2(I) chain [Homo sapiens]	72.5	72
		Collagen, type I, alpha 1 [Homo sapiens]	67.6	65
		Keratin, type II cytoskeletal 1 [Homo sapiens]	35.7	23
		Keratin 1 [Homo sapiens]	39.3	23
		Keratin, type I cytoskeletal 9 [Homo sapiens]	34.8	14
		Keratin, type I cytoskeletal 9 [Homo sapiens]	34.3	13
		Keratin, type I cytoskeletal 10 [Homo sapiens]	28.3	13
		Keratin, type I cytoskeletal 10 [Homo sapiens]	35.8	13
		Keratin, type II cytoskeletal 2 [Homo sapiens]	35.5	12
		Keratin, type II cytoskeletal 2 [Homo sapiens]	37.2	12
		Keratin, type II cytoskeletal 6B [Homo sapiens]	20.9	6
		Keratin, type II cytoskeletal 6B [Homo sapiens]	30.5	5
		Keratin, type I cytoskeletal [Homo sapiens]	18.6	3
		Keratin, type II cytoskeletal 5 [Homo sapiens]	22.2	3
		Keratin, type I cytoskeletal 14 [Homo sapiens]	18.4	2
		Chondroadherin [Homo sapiens]	14.8	2
		Vitronectin [Homo sapiens]	8.2	1
		Hornerin [Homo sapiens]	6.7	1
		Pigment epithelium-derived factor [Homo sapiens]	10.3	1
		Dermcidin [Homo sapiens]	10	1
		Biglycan [Homo sapiens]	4.6	1
		Prothrombin [Homo sapiens]	12.9	1
		Vitronectin [Homo sapiens]	12.3	1
		Biglycan preproprotein variant (Fragment) [Homo sapiens]	9.8	1
		Hornerin [Homo sapiens]	12.2	1
		Pigment epithelium-derived factor [Homo sapiens]	12.2	1
		Dermcidin [Homo sapiens]	17.3	1

Table 6.43: P4 sample CD84.2, 3rd extraction stage, nLC-MS/MS analysis. Swiss-Prot and TrEMBL databases. Bold entries are endogenous non-collagenous proteins.

Tables 6.44, 6.45, and 6.46 display the results of nLC-MS/MS analysis of post-TAC P4 sample EH156.3 (HI: 5). Analysis of the first extraction stage revealed one endogenous

NCP (protein argonaute-4), which is represented by a single peptide (Table 6.44), along with a number of soil-associated infiltrating bacterial proteins. *Cronobacter sakazakii* is a pathogenic bacteria, the unusual presence of which is discussed further in section 7.3.3(iii).

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
EH156.3 1st stage	5	Collagen alpha-1(I) chain [Homo sapiens]	77.7	148
		Collagen alpha-1(I) chain [Homo sapiens]	73.8	129
		Collagen alpha-2(I) chain [Homo sapiens]	83.4	117
		Collagen alpha-2(I) chain [Homo sapiens]	77.8	97
		Collagen, type I, alpha 1 [Homo sapiens]	76.9	93
		cDNA FLJ56576, highly similar to Collagen alpha-2(I) chain [Homo sapiens]	94.1	14
		Collagen alpha-1(II) chain [Homo sapiens]	79.1	9
		Collagen alpha-6(IV) chain [Homo sapiens]	79.2	1
		Collagen, type XI, alpha 1 [Homo sapiens]	56	1
		Keratin 1 [Homo sapiens]	17.2	1
		Protein argonaute-4 [Homo sapiens]	7.9	1
		Keratin, type II cytoskeletal 1 [Homo sapiens]	5.7	1
		Collagen alpha-1(XXVIII) chain [Homo sapiens]	34	1
		HTH-type transcriptional regulator MalT [Cronobacter sakazakii]	3.6	1
		Dihydroxy-acid dehydratase [Methanosarcina barkeri]	8.5	1
		Diflavin flavoprotein A 1 [Synechocystis sp.]	6.3	1
		DNA polymerase catalytic subunit [Equine herpesvirus 1]	6.1	1

Table 6.44: P4 sample EH156.3, 1st extraction stage, nLC-MS/MS analysis. Swiss-Prot and TrEMBL databases.

Table 6.45 shows the range of proteins identified in the second P4 extraction stage of sample EH156.3. Although collagen once again dominates, two endogenous NCPs were detected. The two non-human proteins, representing highly exotic bacterial species *Trichodesmium erythraeum* and *Halorubrum lacusprofundi*, were identified through the Swiss-Prot database. Their 'presence' is discussed in section 7.3.3(iii).

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
EH156.3 2nd stage	5	Collagen alpha-2(I) chain [Homo sapiens]	81.7	146
		Collagen alpha-1(I) chain [Homo sapiens]	77.3	143
		Collagen alpha-1(I) chain [Homo sapiens]	72.3	123
		Collagen alpha-2(I) chain [Homo sapiens]	78.8	122
		Collagen, type I, alpha 1 [Homo sapiens]	76.5	96
		Collagen alpha-1(II) chain [Homo sapiens]	76.3	6
		Collagen alpha-1(II) chain [Homo sapiens]	65.4	5
		Keratin, type II cytoskeletal 1 [Homo sapiens]	8.01	4
		Keratin 1 [Homo sapiens]	20	4
		Keratin, type I cytoskeletal 9 [Homo sapiens]	7.2	1
		Imidazoleglycerol-phosphate dehydratase [Trichodesmium erythraeum]	5.2	1
		30S ribosomal protein S3Ae [Halorubrum lacusprofundi]	7.9	1
		Protein AHNAK2 [Homo sapiens]	11.1	1
		Keratin, type I cytoskeletal 9 [Homo sapiens]	14.1	1
		cDNA, FLJ94754, highly similar to Homo sapiens potassium inwardly-rectifying channel, subfamily J, member 2 (KCNJ2), mRNA [Homo sapiens]	11.2	1

Table 6.45: P4 sample EH156.3, 2nd extraction stage, nLC-MS/MS analysis. Swiss-Prot and TrEMBL databases.

The largest quantity of endogenous NCPs was detected in the third extraction of sample EH156.3 (Table 6.46). This sample was also the only one to contain NCPs represented by multiple peptides

Sample	HI	Name	Sequence Coverage (%)	Peptides (95%)
EH156.3 3rd stage	5	Collagen alpha-1(I) chain [Homo sapiens]	76.4	117
		Collagen alpha-1(I) chain [Homo sapiens]	68.4	104
		Collagen alpha-2(I) chain [Homo sapiens]	79.5	100
		Collagen alpha-2(I) chain [Homo sapiens]	78.3	94
		Collagen, type I, alpha 1 [Homo sapiens]	73.1	78
		Keratin, type II cytoskeletal 1 [Homo sapiens]	16.6	7
		Keratin 1 [Homo sapiens]	20.5	7
		Pigment epithelium-derived factor [Homo sapiens]	18.7	6
		Pigment epithelium-derived factor [Homo sapiens]	24.6	6
		Collagen alpha-1(II) chain [Homo sapiens]	74.8	6
		Collagen alpha-1(II) chain [Homo sapiens]	6.77	5

		Chondroadherin [Homo sapiens]	19.8	4
		Biglycan preproprotein variant (Fragment) [Homo sapiens]	24.7	4
		Matrix Gla protein [Homo sapiens]	36.9	4
		Biglycan [Homo sapiens]	6.12	3
		Vitronectin [Homo sapiens]	6	3
		Keratin, type II cytoskeletal 6B [Homo sapiens]	5.7	3
		Vitronectin [Homo sapiens]	21.5	3
		Keratin, type II cytoskeletal 6B [Homo sapiens]	21.3	3
		Keratin, type I cytoskeletal 10 [Homo sapiens]	4.92	2
		Keratin, type I cytoskeletal 10 [Homo sapiens]	12.2	2
		Chondroadherin [Homo sapiens]	28.7	2
		Prothrombin [Homo sapiens]	17.8	2
		cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein [Homo sapiens]	18.5	2
		Keratin, type I cytoskeletal 9 [Homo sapiens]	9.6	1
		Alpha-2-HS-glycoprotein [Homo sapiens]	14.4	1
		Keratin, type II cytoskeletal 2 [Homo sapiens]	11.9	1
		Osteomodulin [Homo sapiens]	4.3	1
		Lumican [Homo sapiens]	1.7	1
		Dermatopontin [Homo sapiens]	9.5	1
		Keratin, type I cytoskeletal 9 [Homo sapiens]	20.7	1
		cDNA, FLJ93532, highly similar to osteomodulin, mRNA [Homo sapiens]	8.8	1
		Collagen alpha-2(V) chain [Homo sapiens]	61.5	1
		Keratin, type II cytoskeletal 2 epidermal [Homo sapiens]	18.5	1
		Lumican variant (Fragment) [Homo sapiens]	5.6	1
		Keratin, type I cytoskeletal 17 [Homo sapiens]	20.8	1

Table 6.46: P4 sample EH156.3, 3rd extraction stage, nLC-MS/MS analysis. Swiss-Prot and TrEMBL databases. Bold entries are endogenous non-collagenous proteins.

6.4.5(i): TAC-retained collagen

Nanospray LC-MS/MS analysis of the P3 (CD120.2, HI: 5) and P4 post-TAC eluted samples CD84.2 (HI: 0) and EH156.3 (HI: 5) offers insights into the different extant isoforms of collagen non-specifically retained on the thiophilic resins (see sections 7.3.3(ii) and 7.3.3(iii) for details of retained non-human proteins and human NCPs). Tables 6.47-6.49 present a summary of the collagen isoforms identified in the three post-TAC samples, along with their sequence coverage and number of peptides confidently identified. It should be

recalled that the collagen has non-specifically bound to the thiophilic resin in very low concentrations, and may not be representative of the entire collagen content of the pre-TAC samples (e.g., for sample CD84.2 3rd stage extraction, the post-TAC protein concentration was 0.064mg/ml, compared to 5.61mg/ml pre-TAC). Note the large differences in collagen isoform diversity, average sequence coverage, and identified peptides between the P3 (pre-exclusion) and P4 samples.

Stage	Protein	Sequence Coverage (%)	Peptides (95%)
2 pre	Collagen alpha-2(I) chain	58.9	24
	Collagen, type I, alpha 1	51.3	19
	Average	**55.1**	**22**
3 pre	Collagen alpha-2(I) chain	25.8	15
	Collagen, type I, alpha 1	14.5	7
	Average	**20.2**	**11**
3 post	Collagen, type I, alpha 1	28.5	2

Table 6.47: Collagen from post-TAC P3 sample CD120.2, 2nd and 3rd extraction stages. Pre – pre exclusion; post – post exclusion.

Stage	Protein	Sequence Coverage (%)	Peptides (95%)
1	Collagen alpha-2(I) chain	77.1	63
	Collagen alpha-1(I) chain	72.4	105
	Collagen, type I, alpha 1	69.9	71
	Average	**73.1**	**80**
2	Collagen alpha-2(I) chain	79.8	95
	Collagen alpha-1(I) chain	75.9	93
	Collagen, type I, alpha 1	74	67
	Average	**76.6**	**85**
3	Collagen alpha-2(I) chain	76.2	73
	Collagen alpha-1(I) chain	70.4	97
	Collagen, type I, alpha 1	67.6	65
	Average	**71.4**	**78**

Table 6.48: Collagen from P4 post-TAC sample CD84.2 (HI: 0). Stage – extraction stage.

Stage	Protein	Sequence Coverage (%)	Peptides (95%)
1	Collagen alpha-2(I) chain	83.4	117
	Collagen alpha-1(I) chain	77.7	148
	Collagen, type I, alpha 1	76.9	93
	Collagen alpha-1(II) chain	79.1	9
	Collagen alpha-6(IV) chain	79.2	1
	Collagen, type XI, alpha 1	56	1
	Average	**75.4**	**62**
2	Collagen alpha-2(I) chain	81.7	146
	Collagen alpha-1(I) chain	77.3	143
	Collagen, type I, alpha 1	76.5	96
	Collagen alpha-1(II) chain	76.3	6
	Average	**78.0**	**98**
3	Collagen alpha-2(I) chain	79.5	100
	Collagen alpha-1(I) chain	76.4	117
	Collagen, type I, alpha 1	73.1	78
	Collagen alpha-1(II) chain	74.8	6
	Collagen alpha-2(V) chain	61.5	1
	Average	**73.1**	**60**

Table 6.49: Collagen isoforms from P4 post-TAC sample EH156.3 (HI: 5). Stage – extraction stage.

6.4.6: P2, P3, and P4 Western blot

The Western blot test run on selected P2, P3, and P4 samples following the protocol described in section 5.7.9 failed to produce any positive results, even for the positive IgG control; exposures at one and five minutes showed only background 'noise', and are therefore not presented here. Possible reasons for this failure are discussed in section 7.35.

6.4.7: P5: protein extraction following adapted Cappellini et al. (2012) protocol and SDS-PAGE gels

The results (SDS-PAGE gels) of samples extracted (Table 5.25) following the P5 adapted Cappellini et al. (2012) outlined in section 5.7.10 are presented in this section. The IgG positive control was also included in these gels (lane 10). Figures 6.63, 6.64, and 6.65 represent SDS-PAGE analysis of the three extraction stages. No clear bands suggestive of IgG were identified by colloidal Coomassie staining and smearing is evident on all gels. A possible match to IgG heavy chain can be seen in Figure 6.63, lane 9. However, the

extended development period (shown by yellowing of the gel) suggests extremely low protein concentration, and consequently the band was not excised for proteomic analysis.

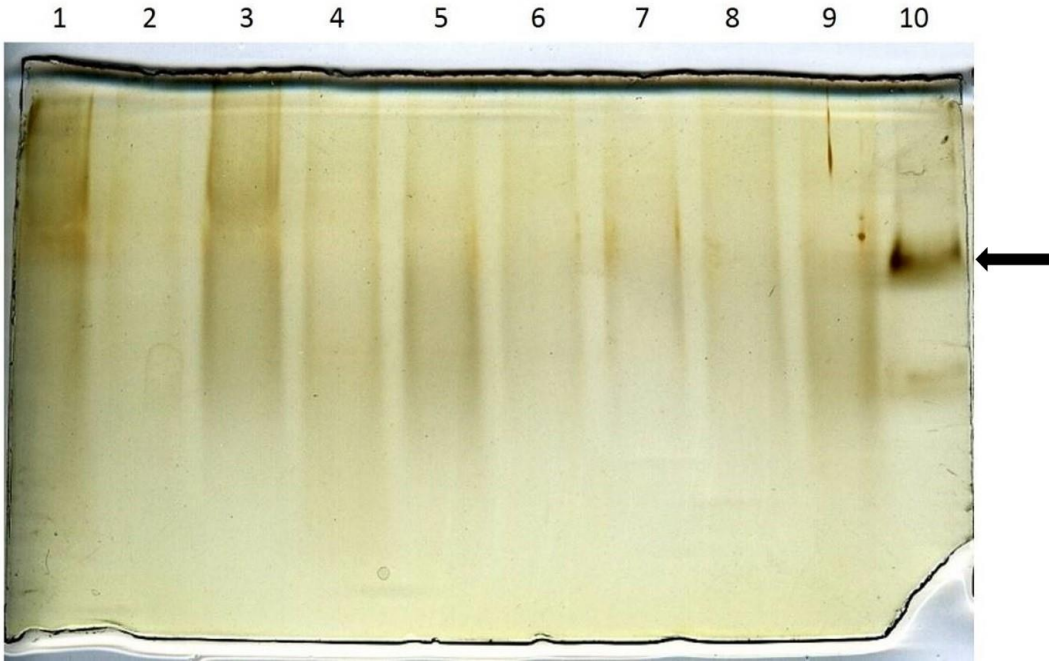

Figure 6.63: P5 first extraction stage SDS-PAGE silver stained gel. 1 – AN5; 2 – LP3760.1; 3 - EH133.1; 4 – HP157.3; 5 – HP104.2; 6 – CD107.1; 7 – CD120.2; 8 – OL1104.2; 9 – HPAN5; 10 – IgG positive control. Samples described in Table 5.25. Arrow indicates expected bands for IgG heavy chains.

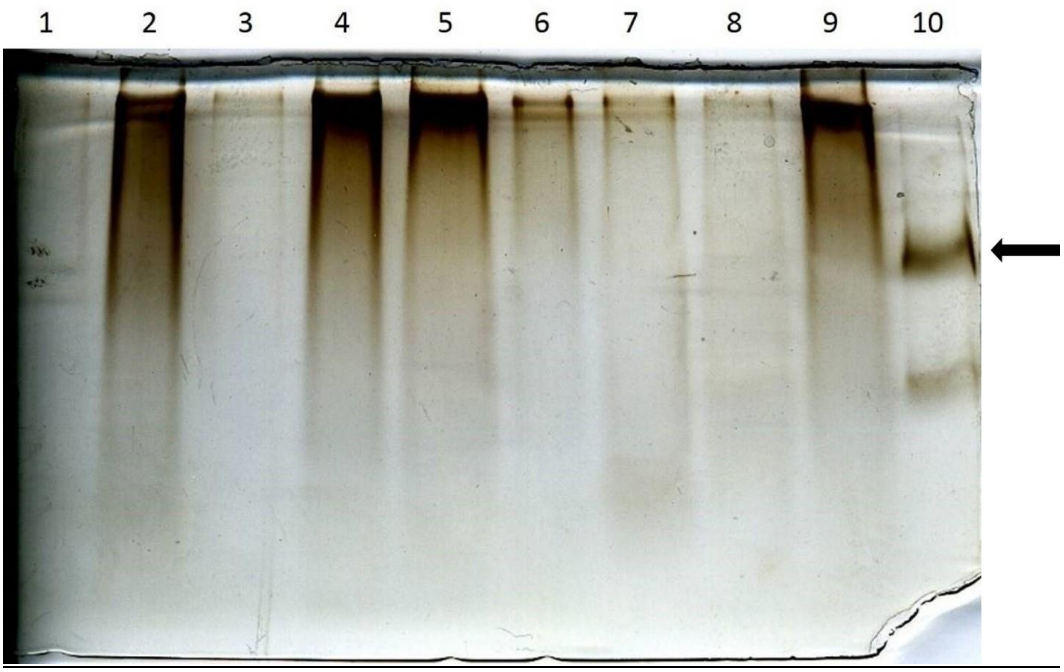

Figure 6.64: P5 second extraction stage SDS-PAGE silver stained gel. 1 – AN5; 2 – LP3760.1; 3 – EH133.1; 4 – HP157.3; 5 – HP104.2; 6 – CD107.1; 7 – CD120.2; 8 – OL1104.2; 9 – HPAN5; 10 – IgG positive control. Samples described in Table 5.25. Arrow indicates expected bands for IgG heavy chains.

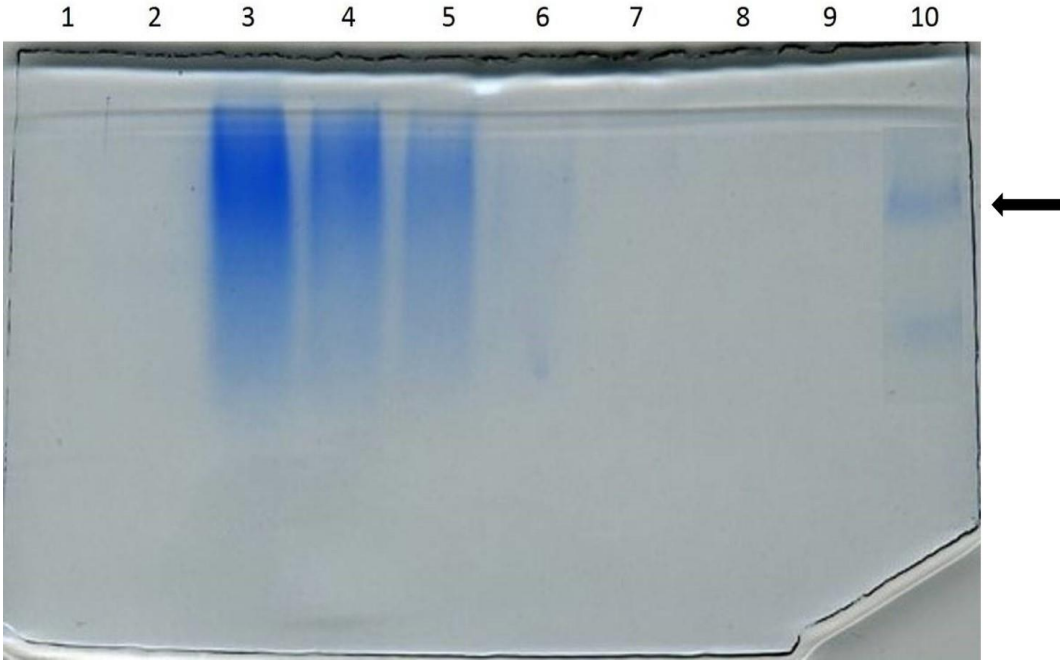

Figure 6.65: P5 third extraction stage SDS-PAGE colloidal Coomassie stained gel. 1 – AN5; 2 – LP3760.1; 3 – EH133.1; 4 – HP157.3; 5 – HP104.2; 6 – CD107.1; 7 – CD120.2; 8 – OL1104.2; 9 – HPAN5; 10 – IgG positive control. Samples described in Table 5.25. Arrow indicates expected bands for IgG heavy chains.

6.5: Histological preservation and protein yield

Protocols P2 and P4 provided protein yields from thiophilic adsorption chromatography for a range of histological preservations, while P3 (Table 6.50) concentrated on well preserved (HI 4 and 5) samples only. P2 and P4 cannot be directly compared to P3, since the extraction stages differ methodologically. Figures 6.66 and 6.67 show average P2 protein yields and the dependence of protein yield on histological preservation.

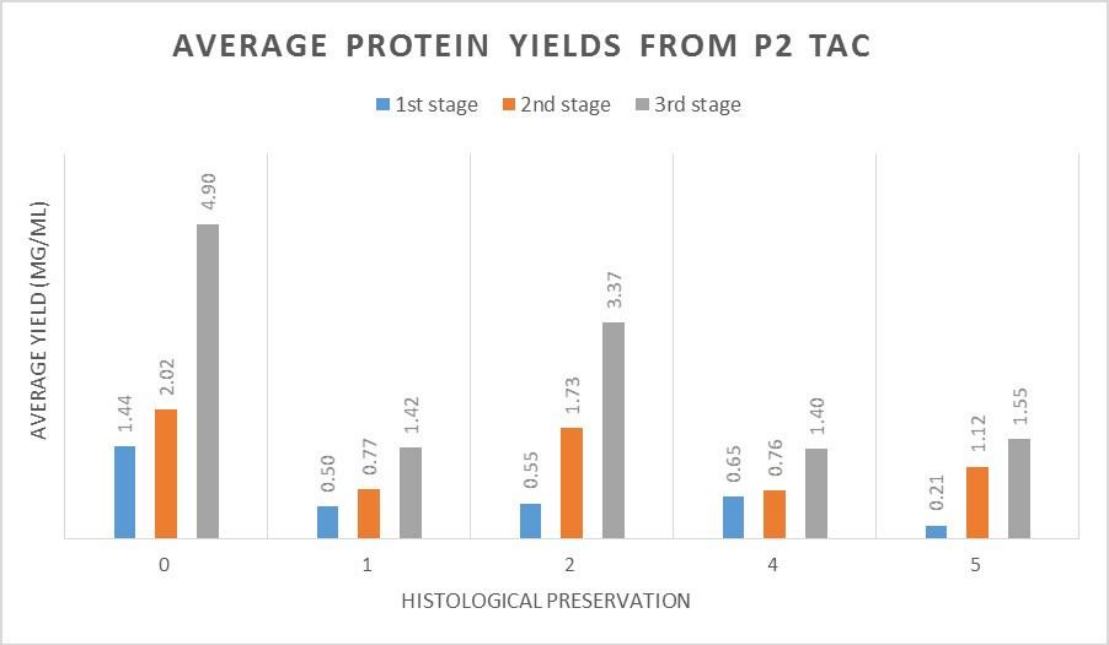

Figure 6.66: Average protein yields from each P2 extraction stage following TAC.

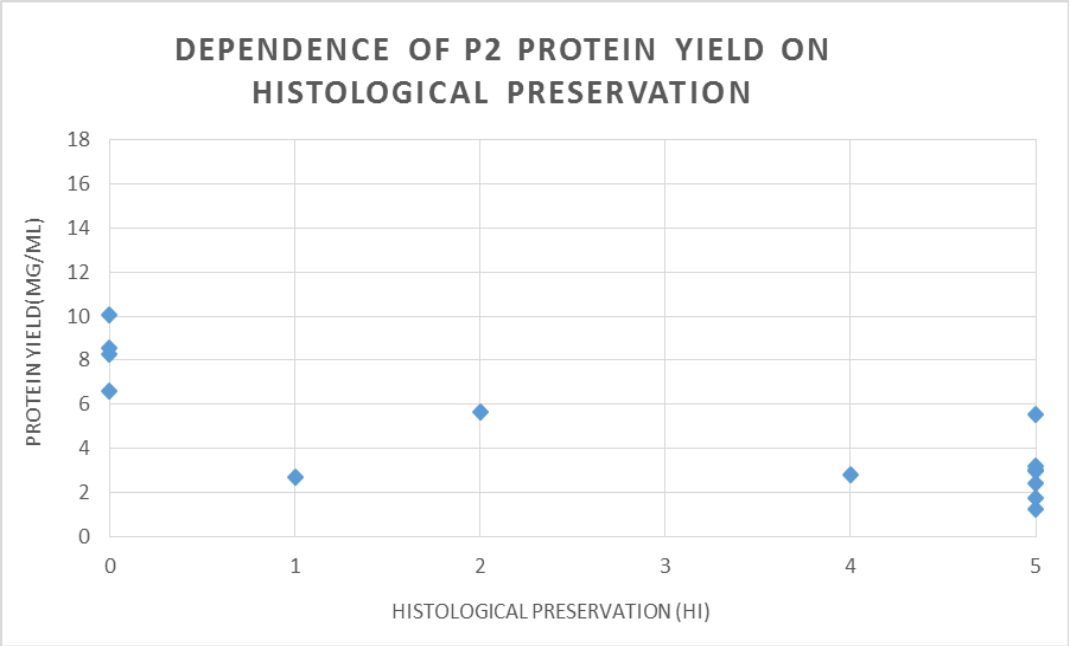

Figure 6.67: P2 TAC bulk protein yield (mg/ml) and histological preservation.

Figure 6.68 suggests that the P2 TAC elution yield was less dependent upon histological preservation than the bulk protein yield seen in Figure 6.67. A similar pattern to this is seen in the P4 bulk and elution yields (Figures 6.70 and 6.71).

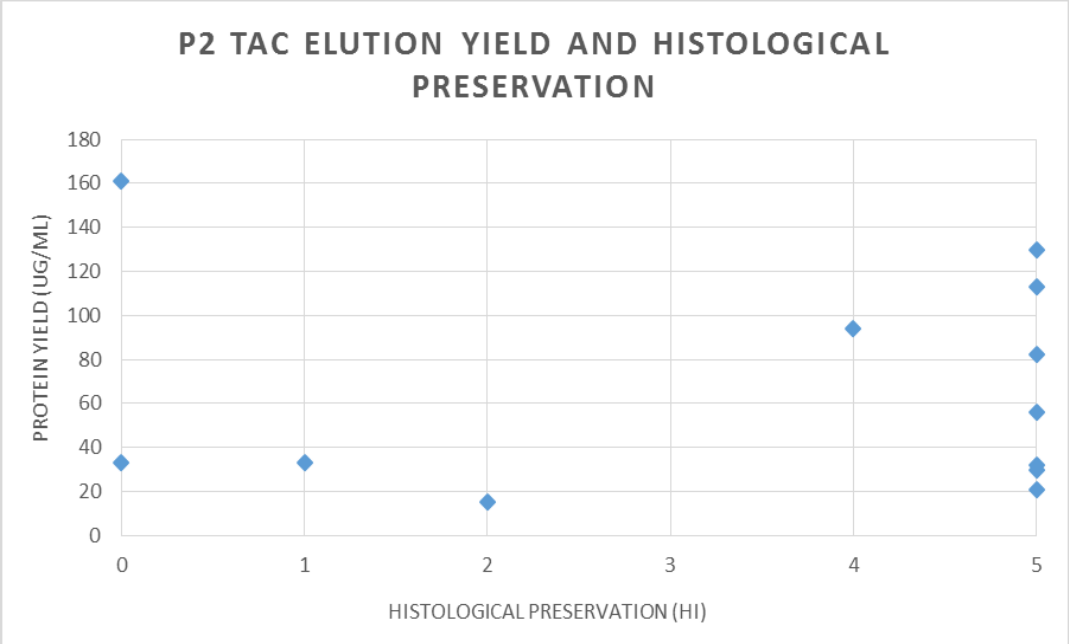

Figure 6.68: P2 TAC elution yield (µg/ml) and histological preservation.

Tables 6.50 and 6.51 display the total and average P3 TAC yield by extraction stage and histological preservation, respectively.

Sample	EH198.1	CD120.2	CD165.1	OL1104.2
Type	Rib	Hand phalanx	Rib	Cranium
HI	4	5	5	5
Total protein yield (mg/ml)	4.55	6.71	5.17	2.34

Table 6.50: Total post-TAC protein yield for P3 samples. HI – histological preservation.

	HI: 4	**HI: 5**
1st stage	1.713	1.300
2nd stage	1.818	1.995
3rd stage	1.014	1.445

Table 6.51: Average P3 extraction stage TAC protein yields (mg/ml) and histological preservation.

Figure 6.69 shows the total bulk protein yields from the four P4 samples of varying histological preservation. Note the general decrease in yield as preservation levels increase. This dependence upon histological integrity is shown in Figure 6.70, although as previously stated, the eluted yield (Figure 6.71) does not follow this pattern.

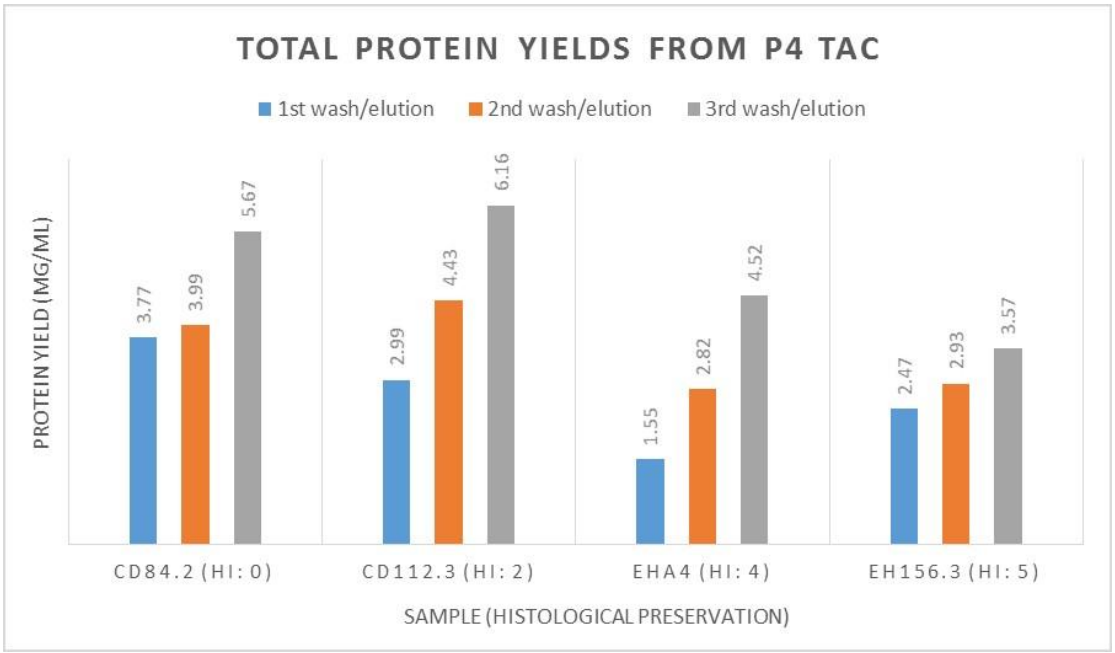

Figure 6.69: Total protein yields (mg/ml) from P4 TAC.

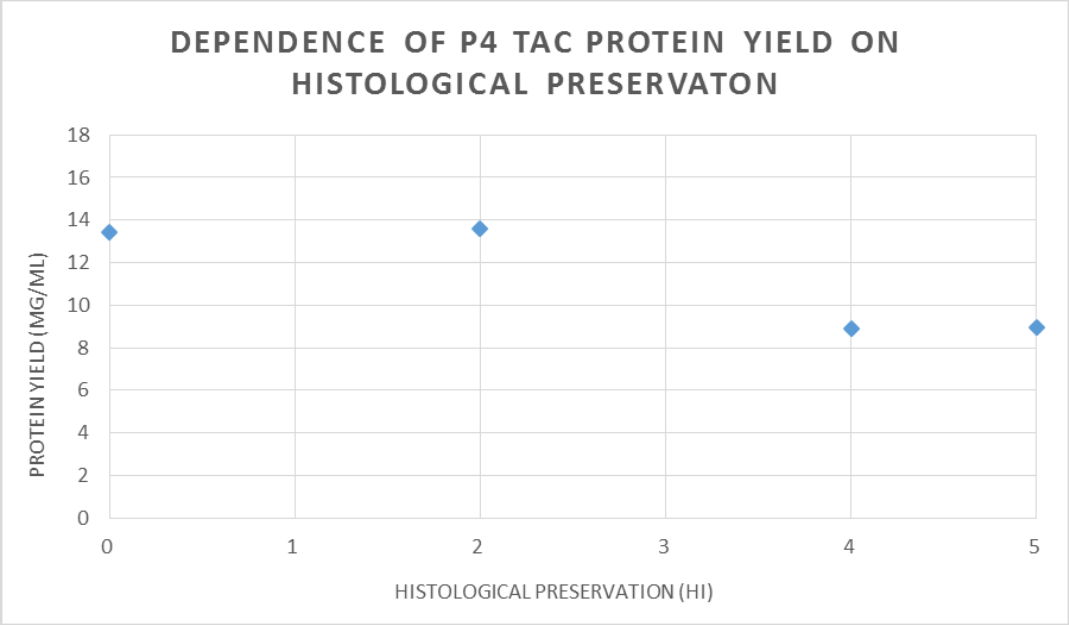

Figure 6.70: P4 TAC bulk protein yield (mg/ml) and histological preservation.

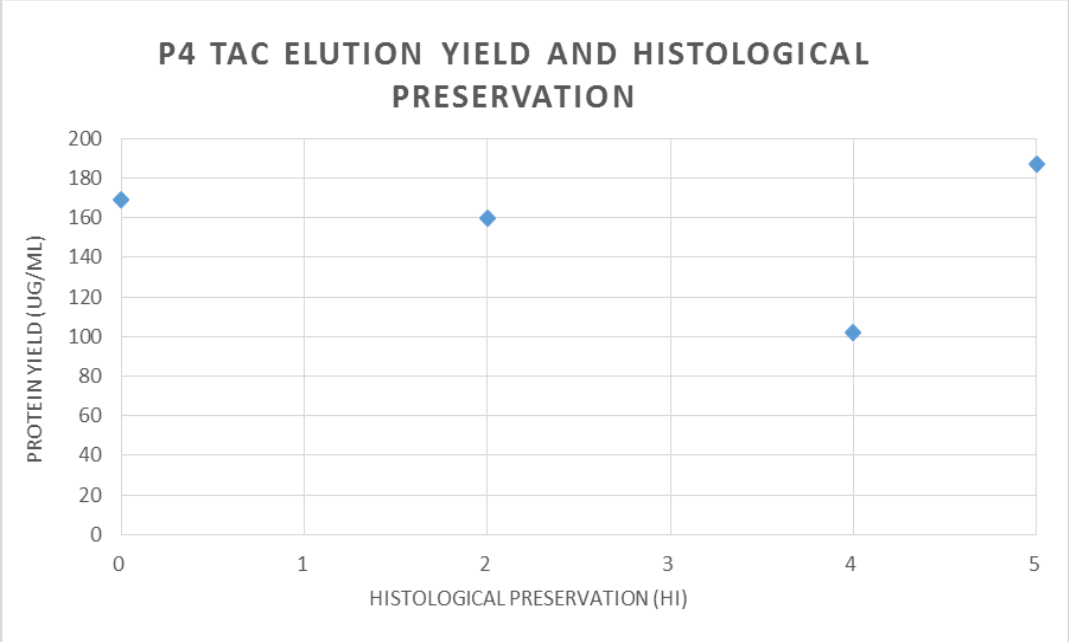

Figure 6.71: P4 TAC elution yield (µg/ml) and histological preservation.

Figures 6.72 and 6.73 display the average P2 and P4 TAC protein yields and their relationship to histological preservation, while Table 6.52 shows the total post-TAC protein yields for the P2 and P4 samples. The figures and table confirm the general trend towards better preservation and lower protein yields.

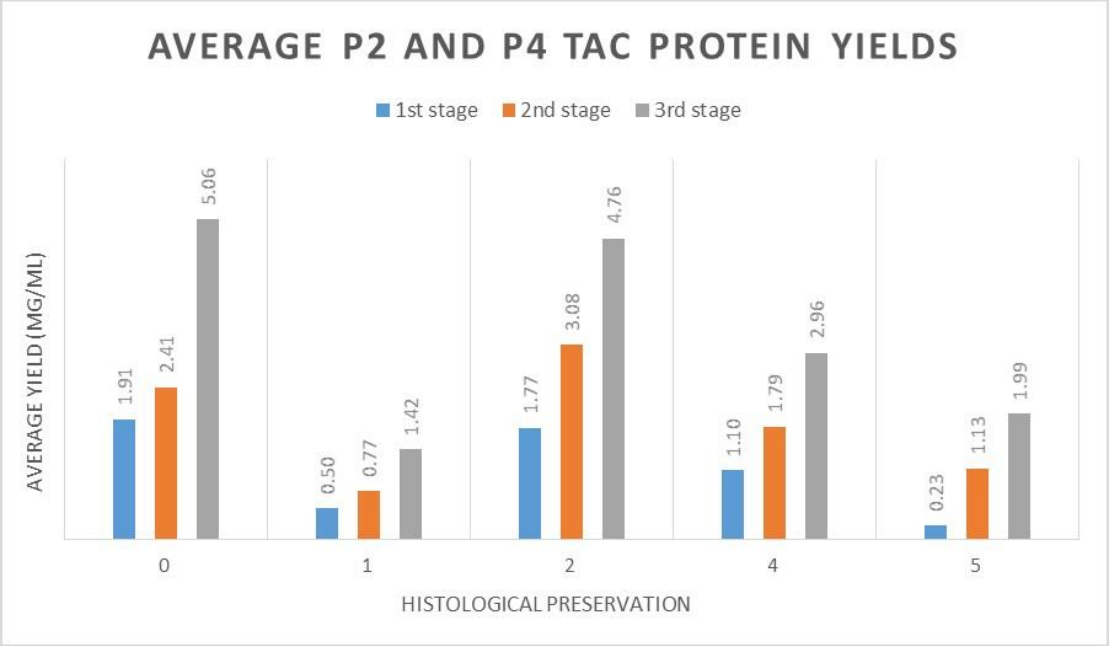

Figure 6.72: Histological preservation and average TAC protein yields (mg/ml) from each extraction stage (P2 and P4 combined).

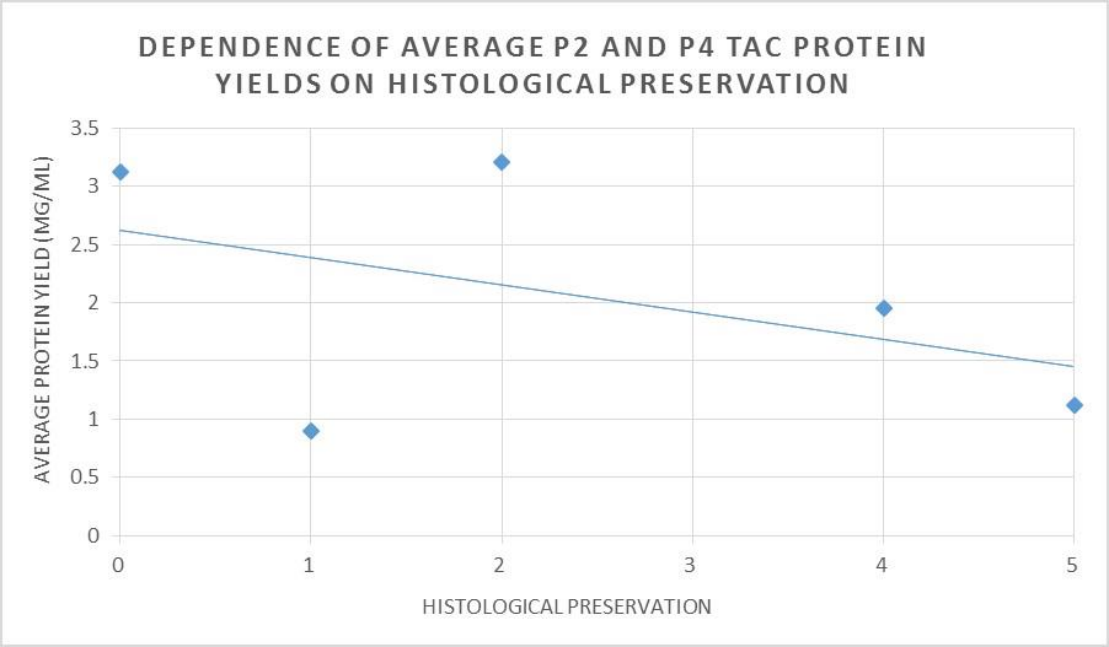

Figure 6.73: Average P2 and P4 TAC bulk protein yields (combined) and histological preservation.

Sample	Type	Period	HI	Total protein yield (mg/ml)
CD84.2	Cranium	A-S	0	12.44
LP3760.1	Rib	A-S	0	8.27
HDAN5	Rib	PM	0	8.57
HDJS4	Cranium	PM	1	2.70
CD112.3	Hand phalanx	A-S	2	13.58
LP4585.3	Hand phalanx	A-S	2	5.65
EHA4	Rib	A-S	4	8.89
HDAR3	Rib	PM	4	2.80
EH156.3	Cranium	A-S	5	8.97
OL1104.2	Hand phalanx	M	5	1.71
HP154.1	Rib	R	5	3.01
CD127.2	Cranium	A-S	5	3.21
WM2316.1	Rib	R	5	1.21
HDAR2	Rib	PM	5	2.43
HDAR3	Rib	PM	5	2.96
HDAP5	Hand phalanx	PM	5	5.53

Table 6.52: Total post-TAC protein yield for P2 and P4 samples. HI – histological preservation; R- Roman; A-S – Anglo-Saxon; M – medieval; PM – post medieval.

Tables 6.53 and 6.54 show average protein yields and histological preservation by element type for P2/P4 and P3, respectively. Note the usually high yield from the cranial sample (HI: 5) in Table 6.53.

	HI: 0	HI: 1	HI: 2	HI: 4	HI: 5
Rib	9.14	-	-	2.80	2.40
Cranium	13.44	2.70	-	-	8.97
Hand Phalanx	-	-	9.62	-	3.62

Table 6.53: Average TAC protein yields (mg/ml) from P2 and P4 extractions by histological preservation (HI) and element type.

	HI: 4	HI: 5
Rib	4.55	5.17
Hand Phalanx	-	4.52

Table 6.54: Average TAC protein yields (mg/ml) from P3 extractions by histological preservation (HI) and element type.

The following Tables (6.55 and 6.56) show average TAC elution yields for the P2/P4 and P3 extractions. Note the higher P3 elution yields and general lack of correlation between

yield and preservation in the P2 and P4 samples. However, Table 6.55 shows that the average P2/P4 elution yield percentages of the total protein yields are higher in the better preserved samples. This is not necessarily the case for the P3 samples (Table 6.56).

	HI: 0	HI: 1	HI: 2	HI: 4	HI: 5
Rib	97	-	-	94	89
Cranium	169	33	-	-	122
Hand Phalanx	-	-	88	-	26

Table 6.55: Average TAC elution yields (µg/ml) from P2 and P4 extractions by histological preservation (HI) and element type.

	HI: 4	HI: 5
Rib	277	236
Hand Phalanx	-	131

Table 6.56: Average TAC elution yields (µg/ml) from P3 extractions by histological preservation (HI) and element type.

	HI: 0	HI: 1	HI: 2	HI: 4	HI: 5
Rib	1.0	-	-	3.4	4.1
Cranium	1.3	1.2	-	-	2.1
Hand Phalanx	-	-	0.7	-	1.1

Table 6.57: Average TAC elution yield percentages of total protein for P2 and P4 extractions, by histological preservation (HI) and element type.

	HI: 4	HI: 5
Rib	6.1	4.6
Hand Phalanx	-	2.9

Table 6.58: Average TAC elution yield percentages of total protein for P3 extractions, by histological preservation (HI) and element type.

Finally, Table 6.59 shows average total TAC protein yields (mg/ml), average eluted yields (µg/ml), and average eluted percentage of total yield, by element type. The data suggests that although rib samples on average yielded the least total protein, they displayed the highest average eluted percentage. Note that this is likely due to the inclusion of the P3 ribs samples (see Table 6.58) in the data.

	Total Yield (mg/ml)	Eluted Yield (µg/ml)	Elution % of Total
Rib	4.56	152	3.9
Cranium	7.08	111	1.6
Hand Phalanx	5.57	94	1.9

Table 6.59: Average total TAC protein yields (mg/ml), average eluted yields (µg/ml), and average eluted percentage of total yield, by element type.

6.6: Malaria EIA (ELISA)

The results of the anti-malaria antibody EIA (ELISA) described in section 5.9 are presented here (Table 6.60). Samples and controls with an A_{450} value less than the cut-off (0.382) are considered negative, while samples just below the cut-off should be interpreted with caution. The samples and controls were run in duplicate, with the average absorbance at A_{450} reported. The negative control was read three times, with average absorbance measuring 0.282. The cut off value was calculated by adding 0.100 to the average negative control absorbance, as directed by the manufacturer. Note the false positive (in duplicate) recorded for the animal sample HPAN5.

Sample	Type	HI	Protocol	Stage	Protein conc.	Read 1	Read 2	Average Abs
Positive	Control	-	-	-	-	**2.639**	**2.530**	**2.585**
Elution buffer	Control	-	3 and 4	-	-	0.257	0.194	0.226
0.5M EDTA	Control	-	5	-	-	0.201	0.279	0.240
50mM ammonium bicarbonate	Control	-	5	-	-	0.218	0.248	0.233
0.6M HCl	Control	-	5	-	-	0.267	0.387	0.327
uH20	Control	-	-		-	0.217	0.402	0.310
EH198.1	Human	4	3	1	0.117	0.343	0.213	0.278
CD165.1	Human	5	3	1	0.021	0.232	0.268	0.250
CD120.1	Human	5	3	2	0.054	0.185	0.219	0.202
EH198.1	Human	4	3	3	0.055	0.255	0.268	0.262
CD165.1	Human	5	3	3	0.112	0.358	**0.570**	**0.464**
CD120.1	Human	5	3	3	0.084	0.173	0.192	0.183
OL1104.2	Human	5	3	3	0.021	0.185	0.187	0.186
EHA4	Animal	0	4	1	0.025	0.314	0.235	0.275
CD84.2	Human	0	4	1	0.025	0.224	0.346	0.285
CD112.3	Human	2	4	1	0.034	0.372	0.263	0.318
EH156.3	Human	5	4	1	0.05	0.231	0.220	0.226
EHA4	Animal	4	4	2	0.037	0.180	0.172	0.176
CD84.2	Human	0	4	2	0.08	0.186	0.188	0.187
CD112.3	Human	2	4	2	0.067	0.208	0.208	0.208
EH156.3	Human	5	4	2	0.093	0.181	0.192	0.187

EHA4	Animal	4	4	3	0.04	0.228	0.175	0.202
CD84.2	Human	0	4	3	0.064	0.179	0.161	0.170
CD112.3	Human	2	4	3	0.059	0.202	**2.080**	**1.141**
EH156.3	Human	5	4	3	0.044	**0.839**	0.169	**0.504**
HP157.2	Human	2	5	1	-	0.148	0.099	0.124
CD107.1	Human	4	5	1	-	0.119	0.212	0.166
AN5	Animal	0	5	1	-	0.207	0.200	0.204
OL1104.2	Human	5	5	1	-	0.193	0.190	0.192
HPAN5	Animal	5	5	1	-	0.202	1.562	0.882
EH133.1	Human	1	5	1	-	**0.396**	0.319	0.358
HP104.2	Human	3	5	1	-	0.183	0.195	0.189
HP157.2	Human	2	5	2	-	0.217	0.226	0.222
CD107.1	Human	4	5	2	-	0.099	0.157	0.128
AN5	Animal	0	5	2	-	0.433	0.201	0.317
OL1104.2	Human	5	5	2	-	0.252	**1.067**	**0.660**
HPAN5	Animal	5	5	2	-	**0.696**	**0.521**	**0.609**
EH133.1	Human	1	5	2	-	0.316	0.347	0.332
HP104.2	Human	3	5	2	-	0.290	0.265	0.278
HP157.2	Human	2	5	3	-	0.243	0.360	0.302
CD107.1	Human	4	5	3	-	0.311	0.337	0.324
AN5	Animal	0	5	3	-	0.274	0.378	0.326
OL1104.2	Human	5	5	3	-	0.332	0.266	0.299
HPAN5	Animal	5	5	3	-	0.244	0.337	0.291
EH133.1	Human	1	5	3	-	0.281	0.205	0.243
HP104.2	Human	3	5	3	-	0.262	0.232	0.247

Table 6.60: Results of malaria ELISA. HI is histological preservation. Stage is extraction stage. Protein concentration in mg/ml. Abs is absorbance. Cut of value is 0.382. Positive samples when read at A_{450} in bold.

6.7: Rapid testing using the CareStart™ Malaria Rapydtest®

As a part of the suite of analyses attempting to detect malaria-associated biomolecules in archaeological human, selected samples (Tables 5.27, 5.28, and 5.29) were subjected to rapid testing using the CareStart™ Malaria Rapydtest® (Apacor). This cassette-type RDT is capable of detecting malaria parasite antigens in the form of *P. falciparum* histidine-rich protein-2 (PfHRP2) and the non-*falciparum* enzyme lactate dehydrogenase in clinically-derived serum samples. One set of samples were tested following the published Fornaciari et al. (2010) protocol, while others tested represent samples from the P2.5 and P5 extractions. Figures for all tested samples are presented in Appendix 2 (Figures A2.1 to 2.14).

All samples subjected to rapid testing, regardless of protocol followed, registered as having worked correctly through appearance of the control line. Samples tested using the Fornaciari et al. (2010) protocol (Table 5.27) were the most likely to show true positives, rather than cross-reactivity potentially associated with the collagen control (see Figure A2.11) or any chemical reagents used in the P2.5 and P5 extractions. Unfortunately, no positives were identified from the samples tested following the Fornaciari et al. (2010) protocol. Neither were positives registered for any of the P5 samples at any of the three extraction stages. Although neither the Fornaciari et al. (2010) protocol samples, nor the P5 samples produced positive results, a number of the P2.5 tests did so (Table 6.61). These 'positives' are discussed in section 7.6.

	HI	Stage 1	Stage 2	Stage 3	Appendix 2 figure reference
HP154.1	5	VOM	-	-	A2.7
LP4585.3	2	VOM/Pf	VOM/Pf	-	A2.7; A2.8
LP3760.1	0	VOM/Pf	-	-	A2.7; A2.8
LP3819D	?	VOM	VOM	-	A2.7
Collagen	-	-	VOM	-	A2.11

Table 6.61: P2.5 rapid test positives. VOM – *P. vivax*, *P. malariae*, or *P. ovale*; VOM/Pf – mixed infection. HI – histological preservation. LP3819D – dentine sample.

This chapter has presented the results of the numerous lines of enquiry employed in the search for direct and indirect evidence of *Plasmodium vivax* in past British populations. The following chapter will critically interpret the data presented here, with reference to the limitations of each methodology chosen, and will attempt to identify possible research avenues for future investigations of past malaria through human remains analysis.

CHAPTER 7: DISCUSSION

This chapter discusses the evidence acquired during this study concerning the presence of putative *P. vivax* malaria in British antiquity. This research has attempted to identify indirect and direct evidence of the disease through analysis of palaeopathological reports, demographic burial profiles, and endogenous (antibody) and exogenous (antigenic) ancient biomolecules associated with malaria infection.

7.1 Indirect evidence for *P. vivax* malaria

Indirect evidence for ancient *Plasmodium vivax* in the Lincolnshire and Cambridgeshire Fens was sought through examination of published archaeological and pathological reports in attempts to identify previously unrecognised skeletal sequelae of thalassaemia, following the recent publication of new diagnostic criteria (Lewis, 2010). A palaeodemographic analysis of cemetery populations was also undertaken in order to identify possible differences in mortality rates based on Fen/non-Fen site location.

7.1.1: Thalassaemia in the past British Fens

As discussed in section 4.2.8, the establishment and consolidation of thalassaemia would require the amalgamation of a very particular set of circumstances in the presence of a strong selective pressure. The development of skeletal sequelae of thalassaemia would rely upon either a spontaneous point mutation, a deletion of non-coding parts of the genes responsible for α or β globin chain synthesis (Min-Oo and Gros, 2005), or the introduction of such genes through inheritance from an exogenous carrier into the population. Although any of these scenarios is possible, external introduction of the deleterious genes into the gene pool by heterozygous carriers is probably more likely to occur, since individuals affected by point mutations or deletions would need to survive and reproduce in order to pass on the defective genes; the presence of fellow carriers in the population for the transmission of genes to the subsequent generation would also be required. The influx of new genes with the Roman legions, for instance, may have introduced thalassaemia in to the gene pool of Poundbury Camp, Dorset, resulting in the first possible skeletal sequelae of thalassaemia to be encountered in the British archaeological record (Lewis, 2010).

For thalassaemia to become widespread within a past population, the newly introduced alleles would need to persist and become 'balanced'. This is a state in which the frequency of affected alleles is equilibrated due to the increased fitness of the heterozygote over the homozygote. Since thalassaemia minor and, to a lesser extent, intermedia sufferers are more likely to survive to reproduce, the affected alleles can persist within a gene pool and become equilibrated by the process of natural selection (Carter and Mendis, 2002). Even if *P. vivax* were exerting a strong enough selection pressure to drive the development of this particular haemoglobinopathy in Fenland populations, there may not have been sufficient time from the introduction of the required alleles to achieve polymorphic balancing and regular skeletal manifestation within the Roman and early Anglo-Saxon cemetery populations evaluated in this study (if it can be assumed that both malaria and thalassaemia were introduced into the British Isles during the former period). Futuer physical re-evaluation of a large skeletal sample from a Fen/marshland cemetery with a long use-life (e.g., potentially St. Peter's Church, Barton-upon-Humber) may be the best strategy in identifying the development of thalassaemia over time.

7.1.1(i): The archaeological record

As outlined in section 4.2.9, the most likely type of thalassaemia to be encountered in the archaeological record is the heterozygous form known as thalassaemia intermedia (Ortner, 2003). The disease is idiosyncratic in its expression, with a range of clinical outcomes depending upon severity. Since symptoms generally worsen with age (Taher et al., 2006), it could be expected that older individuals would display multiple skeletal sequelae, should they survive into adulthood without treatment. Survivability into adulthood is, of course, dependent upon the severity of symptoms. Lewis (2010) identified three possible cases of thalassaemia intermedia in older Poundbury children, although it remains uncertain as to whether their deaths were directly influenced by their condition. Had her reanalysis included the adults, she may well have observed an increased prevalence of skeletal changes suggestive of thalassaemia intermedia.

Regardless of the age of individuals, a differential diagnosis of thalassaemia would require the presence of at least two skeletal changes, if not more. Unfortunately, no individuals from the selected site reports in this study displayed more than one sequela that

might be indicative of thalassaemia. What follows is a discussion of the different sequelae listed in Table 5.1 and identified in Table 6.1 that were encountered in the reports. It should be recalled here that only individuals displaying two or more skeletal changes suggestive of thalassaemia were considered for further investigation, and that most of the Cambridgeshire samples were unavailable for further analysis due to archive relocation.

7.1.1(ii): Porotic hyperostosis, multiple rib fractures, and scoliosis

The Littleport skeleton displaying radiographically-confirmed porotic hyperostosis (section 6.11) represents the only positively identified case of this condition in any site selected for this study. Due to the rarity of the condition in British archaeological populations and its geographical association with potentially endemic malaria, a thorough macroscopic analysis of the surviving skeletal elements was performed with the intention of identifying other pathological changes that might be indicative of thalassaemia. This was accompanied by radiographic analysis of ribs in order to identify any of Lewis' (2010) diagnostic rib-within-a-rib features. Unfortunately, no further sequelae of interest were identified. Analysis was, however, highly restricted due to poor overall preservation and lack of many elements prone to developing thalassaemic sequelae.

Multiple rib fractures were the most commonly reported pathology suggestive of thalassaemia. Multiple pathological bone fractures in thalassaemic patients usually result from cortical thinning and medullary expansion (Ahmad et al., 2011). Such fractures are more frequently observed in homozygous thalassaemics; it is rarer to encounter them with thalassaemia intermedia (Haidar et al., 2010), but the fact that they do occasionally appear warranted their inclusion as a suggestive sequela. In suspected archaeological thalassaemia cases, radiography of ribs may be used to identify the 'rib-within-rib' feature encountered by Lewis (2010) at Poundbury. This would, of course, be contingent upon adequate preservation of ribs. No individuals in this study who exhibited multiple rib fractures displayed other sequelae suggestive of thalassaemia. They were not, therefore, targeted for further analyses.

The Hoplands site displays a 13% crude prevalence rate (CPR) of rib fractures, with seven individuals exhibiting multiple fractures (Western, 2011). This is considerably higher than any other study site, as well as the Romano-British average CPR of 2.9% (Roberts and

Cox, 2003), although it is more comparable to the 9.4% CPR seen at the Roman cemetery at Jesus Lane, Cambridge (Alexander et al., 2004; Western, 2011). Unfortunately, the absence of other supportive 'thalassaemia' sequelae in any of the Hoplands individuals displaying multiple rib fractures precludes possible diagnoses of genetic anaemia. The same pattern is evident at the Castledyke South site, with four individuals (2% CPR) displaying multiple rib fractures (Bolyston et al., 1998) with no further supportive skeletal changes reported. The elevated Hoplands figure may represent more rigorous reporting of rib fractures on the individual level, or better rib preservation at this site. Without further physical skeletal analysis, both of these scenarios currently offer more likely explanations than thalassaemia.

A further possible cause of multiple rib fractures can see seen at the Castledyke site, where two out of the four individuals were aged over 45 years. The fractures in these individuals may be associated with osteoporosis (Ortner, 2003). Osteoporotic rib fractures in modern European populations occur commonly in both men and women, with an estimated prevalence of over 3% (Ismail et al., 2006), yet it can be difficult to diagnose in skeletal material due to the challenge of distinguishing between pathological bone loss and post-mortem deterioration (Boylston et al., 1998). It is, of course, possible that multiple healed rib fractures are simply evidence of a series of traumatic events over an extended life period.

Taher et al. (2006) report that up to 40% of individuals affected by thalassaemia intermedia develop scoliosis. Research on the relationship between the two has demonstrated that the development of the condition in thalassaemics follows a different trajectory than in idiopathic scoliosis, reflecting the haematological abnormalities characteristic of thalassaemia. It would be difficult to be specific about the precise aetiology of scoliosis in archaeological cases, although thalassaemic scoliosis is often associated with retarded skeletal maturation (Korovessis et al., 1996; Papanastasiou et al., 2002). A differential diagnosis of thalassaemic scoliosis would rely on supportive sequelae within individuals. It is potentially interesting that scoliosis is recorded at the Littleport (three individuals, 8.8% TPR/4.9% CPR) and The Hoplands (2 individuals, 5.9% TPR) sites, both of which are situated in areas of the Fenland that may have experienced continuous malaria endemicity (as discussed in section 4.2.6).

Idiopathic scoliosis has a true prevalence rate (TPR) of up to 5.2% in modern adolescents, exhibiting a female to male ratio to up to 3:1, which increases with advancing

age (Konieczny et al., 2013). Post-medieval scoliosis prevalence (CPR) is a suggested 0.34% (Roberts and Cox, 2003). An explanation for this low prevalence may lie in the palaeopathological diagnosis of the condition, which benefits from a well preserved vertebral column. If preservation allows, each vertebrae should be carefully examined for abnormal morphology, since the conformation of the vertebral column is difficult to accurately reconstruct in dry bone (Ortner, 2003). As preservation and careful analysis of each element are key to diagnosis, the condition may sometimes be under-represented in archaeological populations. The Littleport site yielded three adult scoliotic individuals (8.8% TPR/4.9% CPR), two females and one male. Assuming that past populations exhibited similar patterns to modern, it could *very* tentatively be suggested that the Littleport prevalence rate may be suggestive of non-idiopathic scoliosis, despite an original differential diagnosis of developmental/idiopathic aetiologies (Western, 2007). Once again, the individuals affected by scoliosis at both The Hoplands and Littleport sites displayed no other sequelae suggestive of thalassaemia.

7.1.1(iii): Summary

The analysis of skeletal reports from the Fen sites revealed scant evidence for thalassaemia in these populations. There are a number of possible reasons for this:

1. Malaria was not present in the Fenlands during the periods under investigation;
2. Malaria was present, but any selective pressure was insufficient to drive the development of thalassaemia. For instance, fluctuating endemicity or transmission may not have stimulated the development of the condition;
3. Thalassaemia was present, but most sufferers (i.e., individuals with thalassaemia major or minor) would be undetectable in the archaeological record.

The first point forms the basis of this entire study, and is the reason for investigating thalassaemia, population demographics, and biomolecular malaria markers. The further possible reasons were explored through report analysis and, where necessary, supplementary physical examination of the individuals of interest. Skeletal evidence for thalassaemia in the past British Fens provides a far from conclusive answer to the presence of this disease. It may not be coincidental that some populations most likely to have experienced extended

malaria endemicity exhibited higher frequencies of putative thalassaemia sequelae, yet the lack of multiple indicators within individuals precludes any convincing differential diagnoses. A more in-depth investigation would require a full re-examination of archived skeletal populations, searching specifically for the presence of suggestive sequelae, rather than relying on skeletal reports which may be insufficiently detailed. This type of analysis would be akin to that performed by Lewis (2010) on the Poundbury non-adult skeletons, and supported by radiography of suspected cases.

The likelihood of encountering evidence of thalassaemia in skeletal material from British antiquity was very low from the outset. Many of the skeletal changes listed in Table 5.1 either affect elements which are inherently fragile and at high risk of post-mortem fragmentation, or affect the bone in such a manner as to increase its susceptibility to diagenesis and damage. Elevated neonate and infant mortality resulting from thalassaemia major within a population is difficult to assess, given the generally poor representation of this age group in Fenland cemeteries (see section 7.1.5 for a discussion of this bias). Methodological improvements in ancient DNA analysis may provide confirmatory evidence of thalassaemic genotypes in ancient Fenland skeletal material. Such research has recently been undertaken on Bronze Age Minoan (Cretan) populations (Hughey et al., 2012), which successfully identified genetic markers of heterozygous beta thalassaemia in one ancient individual. This showed a similar average frequency to modern Cretans. This type of investigation builds upon previous attempts to identify archaeological thalassaemia (e.g., Yang, 1997), and suggests that next generation aDNA sequencing techniques may prove extremely useful in the identification of ancient thalassaemia. New aDNA technology may yet prove extremely useful in identifying polymorphic traits suggestive of malaria resistance in archaeological populations which elicit no direct osseous response. These may include protective conditions, such as the Duffy-negative blood system and G6PD deficiency recently documented in populations exposed to endemic temperate *vivax* malaria (Sina, 2002; Louicharoen et al., 2009).

Although this small analysis of putative thalassaemia skeletal sequelae cannot support the presence of malaria in the ancient Fens, it does not, of course, prove that the polymorphism was *not* present. Highly tentative evidence from sites such as Littleport and The Hoplands may be suggestive of the disease, but secondary analysis of sequelae through

published reports remains insufficient for diagnosis at this time. *Vivax* malaria may have been present and indeed endemic in the past Fenlands, but may not have driven the specific development of thalassaemia as a protective mechanism. The emergence of thalassaemia tends to be associated with *falciparum*-endemic areas and it may be the case that *vivax* malaria (particularly temperate strains) exerts insufficient selective pressure. Conversely, it may reflect the lack of research on 'benign' *P. vivax* addressed in section 3.5. Research on the evolution of population-specific protective haemoglobinopathies has tended to concentrate on *P. falciparum,* with far less known concerning the interaction of *P. vivax* and genetic resistance (Louicharoen et al., 2009). The situation is complicated by the presence of both *falciparum* and *vivax* malaria in many areas where emerging protective polymorphisms have been recorded, such as Southeast Asia.

It may be the case that Defoe's (1722) reported 'seasoning' of male marshland inhabitants may be reflective of an acquired, rather than genetic, immunity to *vivax* malaria. As discussed in the next section, demographic analysis of Fen and non-Fen populations may suggest that Fen populations were indeed 'seasoned' to their environments. The malaria hypothesis suggests that populations exposed to long-term endemic malaria are the most likely to develop genetic resistance to the disease, rather than populations exposed intermittently. The lack of evidence for thalassaemia in past Fenland populations may support acquired immunity as the dominant form of resistance. It may also suggest that *vivax* malaria transmission was discontinuous or, if endemic, lacked the selective pressure required to drive the development of protective polymorphisms. This assumes, of course, that conditions such as G6PD deficiency were not present. As mentioned previously, technological developments may soon allow for detection of this polymorphism in archaeological contexts.

This study of the palaeopathological evidence has not been able to confirm the presence of thalassaemia in the past British Fenlands, yet it has added to a limited corpus of knowledge concerning Fen health. It also offers a base from which to expand research into any acquired immunity of haemoglobinopathies that may have developed in response to *vivax* malaria in past Fenland populations.

7.1.2: Cemetery population demography

Before discussing the results of the small Fen/non-Fen palaeodemographic study outlined in section 5.2, it is important to recall the type of mortality profile that might be expected in a population affected by *P. vivax*. As discussed in sections 4.2.10 and 4.2.11, the groups most at risk of developing severe illness and potentially dying from *P. vivax* (likely combined with comorbidity) are the very young and pregnant women (Nosten et al., 2004). In terms of age categories for this study, these would include neonates (0-1 month old), infants (1-12 months), young children (1-6 years), and adolescent to young adult females (13-34 years). Thirty four years is, of course, is an arbitrary cut-off point. It assumes that the majority of pregnancies would occur prior to 35 years of age and that the reduced number of pregnancies occurring in middle adulthood (35-49 years) are unlikely to affect the overall mortality profile of a population. If *vivax* malaria were a significant contributor to mortality in past Fen/marshland communities it should, theoretically, be reflected in elevated numbers of cemetery burials in comparison to non-Fen cemeteries, over and above those observed in non-Fen populations. General trends in mortality, survivorship, and force of mortality (also known as probability of death) are discussed, followed by an analysis of how these trends coalesce to form overall patterns of Fen and non-Fen demography. Consideration of the extent to which these trends inform us about the presence of *vivax* malaria in the Fens will be given throughout the interpretation. Finally, the limitations of such demographic analyses are addressed.

7.1.2(i): Fen/non-Fen mortality, survivorship (l_x), and probability of death (q_x)

When examining the mortality profiles for the Roman period, it is important to note the low Fen sample numbers due to the lack of excavated Roman Fen cemetery sites (Table 6.3). This is especially noticeable for the <1 year old categories and may be representative of a particular Roman burial custom in which "infants rarely received proper burial" (Sallares et al., 2004:319), instead being interred within settlements rather than in designated, organised cemeteries outside of settlement boundaries (Toynbee, 1971; Scott, 1990). The majority of non-Fen Roman individuals in the <1 year categories came from the Ancaster and Baldock sites (See Appendix 2, Table A2.2). The former was a small Roman town with likely communal burial areas, in which many of the infants were buried in groups (Cox,

1989). This suggests the presence of organised burial plots for infants, rather than ad-hoc burial within a settlement or domicile, such as was observed at the Rudston Villa site (Stead, 1980; Bayley 1980a). The disproportionate number of <1 year olds at Baldock may be suggestive of differential burial practice, or biased cemetery sampling. The non-Fen <1 year old mortality profile (Figure 6.4) may be more representative of a typical rural Roman profile, since the bulk of these individuals originated from two such cemeteries. The generally low Fen and non-Fen infant numbers may suggest that rural Roman infant burial was indeed less organised, with the mortality profile (Figure 6.4) perhaps representative of rural infant burial practices, regardless of Fen/non-Fen location.

The higher prevalence of Fen adults (Figures 6.4 and 6.5), particularly in the 50+ year category may indicate that more of these groups survived to an older age, thus potentially supporting higher pre-adult mortality in the non-Fen Roman locations. The survivorship data (Figures 6.6 and 6.7) support this hypothesis. However, as mentioned in section 6.2.2(i), the difference in sample numbers between Fen and non-Fen could potentially skew the data and subsequent interpretation for the Roman period. Chi-square testing ($p<0.01$) shows location to be a significant influence on age-at-death, while the Roman period is the only one for which the K-S null hypothesis can be rejected, with a significant difference ($p <0.01$) in total age-at-death (Figure 6.4) and adult age-at-death (Figure 6.5) mortality distributions by location. The K-S test perhaps offers a more reliable indicator of distribution than χ^2 here, since the former is more suited to analysing the small Fen samples size. The selected Fen Roman cemeteries are all relatively small, the largest (The Parks, Godmanchester) yielding only 52 aged individuals, whereas non-Fen Ancaster yielded over 300.

Figure 6.5 shows that non-Fen females make up the highest proportion of burials in the 18-24 year age category, with corresponding poor survivorship (Figure 6.7) and highest probability of death (Figure 6.9). The subsequent proportional fall in non-Fen females in the 50+ category suggests that this group was particularly at risk during the Roman period, a trend that continued into the Anglo-Saxon period. That the Fen populations show better survivorship and lower probability of death may be indicative of populations adapting to high-stress environments, with the survivors living longer than their non-Fen contemporaries. This theory is discussed further in section 7.14.

Demographic analysis of the Anglo-Saxon data suggests that this period was particularly difficult for the non-Fen groups, with females experiencing the highest probability of death and lowest survivorship (Figures 6.14 and 6.16). Given the post-medieval reports of elevated female immigrant deaths in marshland environments discussed in section 3.6.3, it might be expected that the number of very young adult and young adult female Fen burials would exceed that of non-Fen burials in the same age categories. Demographic analysis, however, may tentatively suggest the opposite. The very young adult female category (ages 18-24), for instance, exhibited higher non-Fen burial probability of death for the Roman and Anglo-Saxon periods, with the latter showing the greatest differences by location (Figures 6.9 and 6.16). Of course, even if the 18th century reports were entirely accurate, such a phenomenon may not be reflected in the burial record.

It is difficult to pinpoint exact reasons for this pattern, and there are likely to be multiple factors influencing the data. For instance, ongoing research (Ellen Kendall, personal communication, March 2014) tentatively suggests that Anglo-Saxon Fen-associated populations, including those at Littleport, may have adopted a practice of extended breastfeeding as an adaptive strategy to the harsh environment. This biocultural behaviour has been observed in developing countries, where poor health and growth in childhood influenced the extension of breastfeeding (e.g., Marquis et al., 1997; Simonden et al., 2001). If this practice was widespread in the Anglo-Saxon Fens, it may help to explain the overall improved Fen survivorship. Unfortunately, the lack of individuals in the <1 year old categories confounds comparisons of Fen/non-Fen infant survivorship. However, since more Fen individuals seem to have survived for longer, it may be suggestive of lower infant mortality. It is also possible that an adapted breastfeeding and weaning practice reduced the probability of death for Fen females compared to their non-Fen counterparts, given that extended breastfeeding reduces fertility and promotes natural birth spacing through lactational amenorrhoea (Tommaselli et al., 2000). Since increased frequency of pregnancy and birth would have directly correlated with maternal morbidity and mortality (Lewis, 1998; Norton, 2005), it follows that a practice which reduced this frequency may have reduced mortality. At this point and without further research, this conclusion must remain conjectural, yet is highlights a possible line of evidence that may illuminate our understanding of Fen health and cultural practices in the undocumented past.

The difficulties of traversing the "wide wilderness" of "vast fens and swamps" (Felix, quoted in Darby, 1940:8-9) would have meant near isolation for many Anglo-Saxon Fen island populations, such as Ely, which Bede recorded as only being accessible by boat in the 8th century (Darby, 1940). Whether such isolation served as a protective measure against the external pressures of conflict and disease is open to question. It is possible, for instance, that reduced mobility and migration may have led to Defoe's 'seasoning' mentioned in section 3.6.3, potentially producing a baseline population-wide resistance to local immunological threats. The rich, year-round resource availability in the Fens may also have ensured an adequate nutritional status, although this may have been offset by increased parasitism, such as was suggested for the Littleport and earlier Hoplands samples (Western, 2007; 2010).

Analysis following removal of the St. Peter's Anglo-Saxon individuals (Figure 6.11), which make up 56% of the total Anglo-Saxon Fen sample set, suggests a lower probability of death prior to 35 years at the St. Peter's site. This may be evidenced in the increased number of buried 25-49 year olds when including the St. Peter's individuals. Consideration of the large St. Peter's population and its influence on the Anglo-Saxon and medieval Fen demographic patterns introduces a potential problem of differential burial customs. The St. Peter's data set represents the only Fen sample associated with a church. Although many of the earliest burials may pre-date the extant early 11th century church, it is possible that the 10th century Christian cemetery was associated with a precursory chapel on the site (Rodwell and Atkins, 2011). The influence of the Church on later Anglo-Saxon burial customs is difficult to ascertain (Geake, 2003). It is unknown, for instance, whether Anglo-Saxon burial in direct association with the church was reserved for certain members of the local community (as was common in later medieval intra-mural church burials), or whether all who died locally were buried here. Certainly, the large numbers of infants at St. Peter's compared to other Fen sites suggests that the unique location allowed a focal point for burial and subsequent preservation of an age group often under-represented in the Anglo-Saxon period. This may, therefore, represent a more normalised pattern of infant mortality than is observed in most other Anglo-Saxon sites. In terms of tracing the presence of *Plasmodium vivax* in the Roman and Anglo-Saxon Fens though palaeodemographic analysis, there are no

obvious signs of its existence, although mortality and survivorship data suggest a picture of adaptation to the environment

An indirect indicator of putative *P. vivax* presence in the medieval Fens may be suggested by comparing data derived from infant burials at the St. Peter's Church and Wharram Percy sites, data which potentially suggests the effects of intrauterine growth restriction (IUGR) at the Fen site. As mentioned in section 4.2.10, a causative link has been suggested between malaria and up to 70% of clinically observed cases of IUGR (Steketee et al., 2001). *P. vivax* infection during pregnancy is a well-established, though poorly understood cause of IUGR and low birth-weight (Nostern et al., 1999; Rodriguez-Morales et al., 2006; Desai et al., 2007). An estimated 18 million babies of low birth-weight are born every year as a result of either pre-term birth or IUGR, the latter being responsible for approximately 2% of neonatal deaths (Lawn et al., 2005). Given the modern statistics, we should perhaps expect to observe IUGR in archaeological populations exposed to malaria.

The average femoral (diaphyseal) lengths of one year olds were 126mm at Wharram Percy (Mays, 1999) and approximately 75mm at St. Peter's (Waldron, 2007). The St. Peter's population exhibits evidence for adequate nutrition and an "unremarkable" disease load (Waldron, 2007:129), whereas health and nutrition at Wharram Percy was suggested to approximate that of poor 19th century urban communities (Mays, 2007). The relatively high child mortality rate observed at Wharram Percy (and evidenced in Figures 6.17 and 6.18) is likely a direct indicator of the poor nutritional status and high disease load of the population as a whole (Mays, 2007). The average 12 month old femoral length at Wharram Percy is comparable to other British archaeological populations (Mays, 2007), suggesting that low birth weight/size was not a major problem at this site. Since malnutrition in developing countries has been shown to influence the development of IUGR in up to 24% of pregnancies (Villar et al., 1986), one may expect to observe a higher prevalence of 'small' infants in a chronically under-nourished population. This is, however, not the case at Wharram Percy. There seems to be another as yet unidentified factor that influenced the high prevalence of small babies at St. Peter's. The strong correlation of *vivax* malaria infection with the development of IUGR provides a *possible* explanation for the increased prevalence of small infants at St. Peter's. It should, however, be acknowledged that IUGR

can be caused by numerous factors beyond *vivax* malaria and malnutrition, such as infectious disease, diabetes, and maternal high blood pressure.

The comparison of infant size, as determined by average femoral (diaphyseal) length, at Wharram Percy and St. Peter's Church may offer tentative support for the presence of *P. vivax*-associated IUGR at the latter site. The latter population certainly exhibits a large proportion of smaller-than-expected children (Waldron, 2007), a pattern that may reflect the norm in modern endemic *vivax* malaria areas (Nosten et al., 1999). Alternately, the diachronicity of the small baby trend from the Anglo-Saxon to later medieval periods may point to a population-specific genetic trait. It could also be argued that the small St. Peter's babies may suggest an unusually high prevalence of premature birth, although the use of multiple age indicators in their recording makes this a less likely scenario (Waldron, 2007). An important caveat must be made here regarding the St. Peter's population percentages in the 0-1 year (1.6% average) and 1-5 (14.2% average) year old categories. Waldron (2007:37) suggests that the unusually low number of 0-1 year olds may have been "an artefact, and some of those placed in the succeeding age group probably belong to this earlier stage". An unspecified number of 0-1 year olds mistakenly placed in the 1-5 year old category would skew analyses of femoral lengths and increase the impression of a cohort of smaller one year olds.

Mays (1999) suggests that the average age for cessation of breastfeeding at Wharram Percy was approximately 18 months, based on measurements of nitrogen isotopes. Although no such study has been performed on the Barton population, a possible adaptive breastfeeding strategy (mentioned above) may have been in place here; if babies at St. Peter's were born small and sickly, it may have influenced the practice of delaying full weaning in order to extend the immunological and nutritional benefits of breastfeeding. At Wharram Percy, the cessation of breastfeeding at an average of 18 months was correlated with a slowing of growth (Mays, 2007) and potentially increased child mortality. Breastfeeding and weaning practices may help to explain why average growth in the St. Peter's and Wharram Percy children differed so dramatically at one year, but reached near parity by the age of five. Isotopic study of the Barton skeletons would be required to support such hypotheses. It should, of course, be remembered that the growth profiles at both sites

are based upon children who died, and are therefore not necessarily representative of the healthy children who survived to adulthood (Mays, 2007; Waldron, 2007).

7.1.2(ii): General trends and summary

The total mortality profile (Figure 6.26) reflects a pattern that might be expected for antiquity, with peaks in childhood (1-6 years) and young adulthood (25-34), and remaining consistently high into middle and old adulthood (35+). The lowest mortality for both Fen and non-Fen sites was seen in the adolescent (13-17) age group. The survivorship and probability of death profiles suggest that very young (18-24 years) adult females were more at risk of dying than their male counterparts, which, given the risks associated with pregnancy, is to be expected. Increased male survivorship is commonly observed in pre-modern populations (Berin et al., 1989). The pattern is apparent for all periods, other than in the medieval non-Fen sample (Figure 6.25).

The significant difference ($p < 0.01$) between total Fen/non-Fen populations shown in Figures 6.26 suggests that location is significant in influencing age-at-death, although it was surprising to find that many of the target categories expected to show higher Fen mortality (the <1, 1-6, and 18-24 categories, for example) actually displayed the opposite relationship, with higher percentages in the non-Fen cemeteries. These percentages are not necessarily representative of the overall death rates in these populations; Figures 6.28 and 6.30 suggest that the overall survivorship and probability of death for Fen and non-Fen populations are similar, despite the apparent significance of location in determining the age-at-death. When examining mortality and survivorship by period, however, the picture that emerges from this small study seems to be of one adaptation to the environment, particularly in the Roman and Anglo-Saxon Fens. In these periods Fen population demographics consistently suggest a lower probability of death and better survivorship than for their non-Fen counterparts. It is likely that the Fen environment was as unwholesome as was frequently reported from the later literature, yet the populations seemed to be 'coping' with this constant challenge to their health. The high prevalence of Anglo-Saxon *cribra orbitalia* in Fen-associated populations (Gowland and Western, 2012) may be an indicator of this adaptation to a high-stress environment. It is, of course, very possible that *vivax* malaria played a part in the health status of Fen existence. However, it has not been possible through demographic

analysis to isolate *vivax* malaria from the epidemiological milieu that surely existed in the Roman and Anglo-Saxon Fens.

Health status in the Fens seems to have declined somewhat in the medieval period compared to the non-Fen populations, possibly due to new external pressures that were undoubtedly introduced during this period. As discussed in section 2.3, the 13th and 14th centuries were a time of upheaval in terms of climatic decline and disease epidemics. Since these factors would affect Fen and non-Fen alike, why would the former populations seem to suffer worse in terms of mortality? Defoe's 18th century accounts of marshland locals being 'seasoned to the place' (see section 3.6.3) is suggestive of adaptation to an inherently unhealthy environment, a state that perhaps existed in the Anglo-Saxon period (as evidenced by high *cribra* prevalence). Something may have occurred during the medieval period which upset this balance. Could it possibly be that *vivax* malaria made its appearance in the Fens at this time, adding further immunological stress to an already burdened, but coping, population? Perhaps increased instability resulting from a combination of environmental, demographic, and epidemiological changes had a greater impact upon Fen populations. It may be useful for future research to analyse the prevalence of medieval Fen non-specific stress markers, such as *cribra orbitalia*, to compare to the Anglo-Saxon data of Gowland and Western (2012). This type of analysis may reveal changing patterns in any possible adaptations. An analysis of post-medieval Fen and non-Fen demographics may also help to support Defoe's observations by investigating any possible return to adaptation in the Fens following medieval upheaval.

What does this limited analysis of Fen and non-Fen cemetery sites suggest concerning the presence of *P. vivax* malaria from the Roman to medieval periods? The overall demography shows no obviously higher Fen mortality rates in the expected groups most liable to succumb to infection. Location and the immediate environment may have had a significant influence on mortality for past and modern populations, as demonstrated in this study and others (e.g., Bull and Morton, 1978; Hertz et al., 1994; Lewis and Gowland, 2007). However, diachronic mortality and its representation within an attritional cemetery population certainly cannot be attributed to one extrinsic factor. Mortality is affected by a myriad of factors, geographical location being but one.

This is probably reflected by the fact that while cemetery location is a significant factor in mortality, there remains no significant difference between Fen and non-Fen in the distribution of age-at-death categories. *Plasmodium vivax* malaria would have to significantly influence mortality in select groups over an extended period for it to be evident in the mortality rates and profiles of an attritional Fen cemetery. The threat to life posed by comorbid *P. vivax* infection is well established, particularly in younger patients (Baird, 2007; Caulfield et al., 2004). Comorbidity almost certainly helps to explain the elevated post-medieval burial rates recorded in the marshland and Fenland parishes of eastern and south-eastern Britain (West, 1974; Wrigley and Schofield, 1981; Dobson, 1997), but unfortunately, such patterns may not be so readily observable in an attritional archaeological cemetery.

Although potentially interesting patterns in Fen and non-Fen mortality and survivorship have been revealed, the limited palaeodemographic analysis performed in this study cannot directly support the presence of *vivax* malaria in the Fens. The 'small' infant phenomenon encountered at St. Peter's Church may suggest an increased occurrence of IUGR, which is consistent with, but not evidence for, the long-term presence of *P. vivax*. Unfortunately, no other Fen site has yet yielded sufficient quantities of infant skeletons for any possible association between location and reduced infant size to be determined. While is highly likely that *vivax* malaria played an important part in the health status of past Fen inhabitants, it has not been possible through the analyses performed here to demonstrate the presence of the disease.

7.1.2(iii): Limitations

Appendix 2 Tables A2.1 and A2.2 show that 320 Fen and 325 non-Fen < 1 year old individuals were excavated from the selected sites, representing 9.8% and 11% of total cemetery populations, respectively. These figures may seem somewhat low, given the high infant mortality rates uniformly assumed for past societies. Here they probably, at least in part, represent the poor survival of inherently fragile bone, while also reflecting the large number of Anglo-Saxon cemeteries included in the study (a sampling bias first addressed in section 5.2), since it is unusual for Anglo-Saxon cemeteries to yield an expected number of foetal, neonate, and infant burials (Crawford, 1993). The under-representation of neonates

and infants in the archaeological record is a well-documented phenomenon, and forms an important source of sample biasing in palaeodemography (Hoppa and Vaupel, 2002; Chamberlain, 2006). While the problem is particularly acute for the Anglo-Saxon period (e.g., Figures 6.10, 6.11, 6.13, and 6.15), the Great Chesterford and St. Peter's (Barton-upon-Humber) cemeteries may represent more normalised Anglo-Saxon infant burial patterns (Inskip, 2008), as may the recent and ongoing (full demographic details yet to be published) excavations at Oakington, Cambridgeshire (Sayer et al., 2011).

This bias in the archaeological record is certainly reflected in many of the sites selected for this study (e.g., Figures 6.4 and 6.10; Tables 6.3 and 6.4), and may reflect poor bone preservation, or differential burial practice (Inskip, 2008). The former should, theoretically, be applicable to many lowland Fen-associated sites, where repeated exposure to constantly fluctuating groundwater levels may accelerate bone diagenesis (Jans et al., 2002). It has been argued that the acidity of peat soils may have a protective effect on buried bone in terms of reducing microbial attack (Manifold, 2012). Yet, there remains a demonstrated correlation between decreasing soil pH and bone deterioration (Gordon and Buikstra, 1981). Acidic soils may, at the very least, have a demineralising effect upon bone, thereby increasing the vulnerability to non-microbial diagenetic factors. In the most extreme cases, soil acidity can completely dissolve the mineral content of bone (Forbes, 2008). It would, of course, be foolish to assume that all Fen-associated sites have the same geology and would, therefore, display the same preservation patterns. Fen-edge sites, such as The Hoplands and Edix Hill, are likely to have different soil types to the Fen islands of Littleport and Ely, which would likely differ to the estuarine soils of Barton-upon-Humber. The same can also be said of the non-Fen sites, which represent a variety of different locations and, consequently, different geologies/soil types spread throughout Britain. As addressed in section 4.3.1, bone diagenesis and deterioration is influenced by a multitude of extrinsic and intrinsic factors which can result in differential intra-site and even intra-grave skeletal preservation.

A higher quantity of infants from Fen cemeteries may be expected due to increased mortality associated with living in unsanitary conditions (i.e., associated with the 'insalubrious' Fens). Post-medieval parish registers, for instance, show that 25% of infants born in Ely died in their first year, with only half surviving to 15 years of age (Dobson,

1997:167). Such high infant mortality patterns are observed in modern developing countries, where infant mortality rates can exceed that for neonates (Saunders and Barrans, 1999; Hill and Amouzou, 2006). This pattern, however, is not shown in this study. The reduced quantity of foetal/neonate bones in all sites from all periods are likely suggestive of a combination of many factors, including preservation bias and period-specific burial customs, such as those mentioned earlier for the Roman period.

An important limitation which affects most, if not all, palaeodemographic research based on cemetery populations, is the fact that the excavated skeletal series are rarely representative of the actual burial record (Wood et al., 1992; Waldron, 1994), for numerous reasons. These include, for example, differential burial practices and taphonomy, such as may have adversely affected the number of infant skeletons in the Fen populations analysed here. A further example would be an attritional cemetery (particularly if associated with a church), which might expect to receive many hundreds, if not thousands of burials over its use-life. This would result inevitably in the disturbance of earlier graves by later burials and a subsequent abundance of disarticulated remains upon excavation. It is also rare for modern commercial archaeological projects to be excavate entire cemeteries, since this would be extremely time consuming and expensive for the developer. A cemetery excavation would usually yield inhumed individuals only from areas under threat of destruction, thus leading to a biased sample. Even for sites that were not necessarily under threat of immediate development, such as St Peter's Church and Wharram Percy, the actual quantity of skeletons excavated would represent a fraction of the total number of burials in these locations. Bell and Beresford (1987), for instance, suggest that there may have been up to 10,000 burials in the Wharram Percy churchyard, yet excavations yielded less than a tenth of this figure (Mays, 2007). Reported mortality figures for this study are, therefore, actually prevalence rates of individuals from the excavated burial sample, rather than true mortality figures for the entire population. Any interpretations concerning cemetery populations must acknowledge these sample biases. Waldron (1991:24) neatly summarised the problem faced by palaeodemographers:

> "The underlying assumption that is inherent in any attempt to use a death assemblage to predict something about the living is that the dead population is representative – or at least typical – of the live population. Given all the non-random events that surround death and burial, not to mention preservation and recovery, this is at best an

> approximation, and at worst the two (the live and the dead) bear no epidemiological relation to each other whatsoever…"

The reliability of palaeodemographic reconstruction and interpretation is based upon accurate assessments of biological data (e.g., individual age and sex) in skeletal populations (White and Folkens, 2005). The accuracy of methods used to age adult skeletons has long been debated. Many of the commonly used ageing techniques in palaeopathology are based upon skeletal individuals of known age (Roberts and Manchester, 2005). The assumption is, therefore, that growth and deterioration, and the development of skeletal age 'markers', are universal processes. The application of ageing criteria derived from (often modern) skeletal individuals of known age to archaeological populations, therefore, fails to account for the complex variation of factors that influence ageing (Schmitt et al., 2002). In a reflection of the numerous methods available and the difficulties inherent in ageing adult skeletons, they are often attributed an age range based upon a variety of morphological changes (e.g., degeneration of joint surfaces in the pelvis, or dental attrition) (Roberts and Manchester, 2005). Given the difficulties of accurately ageing adult skeletons, it is vital that palaeopathologists detail their choice of analytical methods. However, as previously mentioned (Section 5.2.1), the range of lack of standards used for ageing immature and adult skeletons and the poor reporting of methods employed can impact upon the reliability of demographic analyses (Ubelaker, 2008). This study relied upon extant skeletal reports and was therefore dependent upon analyses performed by numerous people over an extended time period; some of these were compiled before the need for standardised recording techniques became apparent. Although some reports (e.g., at St. Peter's Church) made clear their use of multiple age indicators in an attempt to improve accuracy, many of the earlier reports (e.g., Anderson, 1996) in particular failed to specify exact methods used for age estimation. An ideal scenario would have been to reassess each skeleton in each report using standardised ageing criteria. As this was not possible for this small study, individuals were assigned an age category, where possible. The use of relatively broad age categories should have mitigated some of the issues associated with non-standardised ageing methodologies. The large overall sample sizes used in this study would have helped reduce some of the issues associated with poor ageing standardisation between reports.

The male and female adults who could not be placed into an age category must be considered when interpreting the adult percentages. Over 19% and 13% of Fen and non-Fen males and females, respectively, were recorded only as 'adult' (i.e., over 18 years of age). This could have significant implications for interpretations of the burial percentages, since these individuals could potentially fit into any of the adult age categories. These adults were not included in the mortality profiles, but the fact that up to a fifth of the excavated adult population is missing may lead to erroneous interpretations and conclusions based on the remaining aged individuals, especially if the unaged adults were biased towards one particular age group.

The sex of adolescent skeletons can be difficult to estimate due to a lack of sexually dimorphic characteristics (Roberts and Manchester, 2005). This means that both males and females are represented in the 12-17 year age category for this study. Although one of the smaller categories in terms of percentage of burial population (4.9% Fen, 5.4% non-Fen), the lack of sex differentiation has implications for comparing adolescent female mortality rates by location. This represents the age range in which adolescents reach sexual maturity, and there may, therefore, be an expected increase in pregnancy-related mortality in this group.

Although the Barton-upon-Humber site may offer the first evidence for intrauterine growth restriction in an archaeological population, IUGR as a result of *P. vivax* infection would be very difficult to identify, since the target skeletal demographic (i.e., neonates and infants) are groups with notoriously high mortality rates in all periods of antiquity. In order to potentially support the presence of *P. vivax*, observation of a phenomenon of comparatively 'small' neonates and infants in other Fen cemeteries would be required. A physical reappraisal (in the form of femoral measurements) of other cemetery populations with sufficient numbers of burials is required to support a possible link between Fen environment and small infants. Even then, the prevalence may not necessarily be attributable to *P. vivax*, since the unsanitary conditions associated with Fen environments may influence the development of IUGR. Early childhood catch-up growth in survivors of IUGR may also confound attempts at identification past the infant stage.

It should be remembered that since the main focus of this research project was the identification of biomolecular malaria markers, time permitted only a relatively small and

basic palaeodemographic study, rather than an exhaustive investigation. The resulting evidence deserves a thorough analysis over and above the search for indirect indicators of malaria. Such analysis was, unfortunately, beyond the scope for this study. Nevertheless, it has provided a useful starting point for further interpretation and possible demographic analyses of archaeological populations from Fen environs. Further cemeteries, such as Oakington in Cambridgeshire (Sayer et al., 2011) and Cuxon in Kent (Powers and Langthorne, 2006), have since been identified that could be included in any future comparisons of Fen and non-Fen demographics.

7.2: Histological preservation and implications for protein extraction

This section discusses the observed relationship between microscopic preservation and macroscopic preservation, before exploring the implications of histological preservation for extracted protein yield and preservation. Differential protein yields from element types are also discussed here.

7.2.1: Histological and macroscopic preservation

It is well established that bone diagenesis often occurs independently on the macroscopic and microscopic levels, and that external preservation state may provide no indication of histological preservation (Jans et al., 2002; Mays, 2010). The samples selected for this study support this, with numerous examples of externally well preserved samples displaying very poor histological preservation. This was particularly noticeable with the Highfield Farm, Littleport samples (Table 6.19), for which, as expected, poor macroscopic preservation often correlated with lower Histological Index (HI) scores (71% scored 0 or 1). However, 53% of samples displaying *good* macroscopic preservation also scored 0 or 1 on the HI, whereas only 12% scored 4 or 5. This complements earlier research by Hanson and Buikstra (1987), which demonstrated that the preservation of microscopic bone structure was often independent of the gross, cortical preservation.

As mentioned above (section 7.1.2(iii)), low-lying Fen-associated cemeteries may be more frequently exposed to fluctuating ground-water levels than non-Fen upland sites. Hydrological action is an important factor in bone deterioration, and has even been suggested to be the most significant extrinsic agent of diagenesis (Cattaneo et al., 1995). The

Littleport site provides a good example of the discrepancy between gross and microscopic preservation, and is a site where cyclical exposure to fluctuating ground-water was cited as being an important diagenetic agent, alongside generally shallow burial and root action (Western, 2007). At this site, macroscopic skeletal preservation was judged to be overall fair to good (Figure 7.1). Histological analysis, however, demonstrated that the sampled Littleport elements were generally very poorly preserved, with 82% of all samples being scored as 0-1 on the HI (Table 7.1).

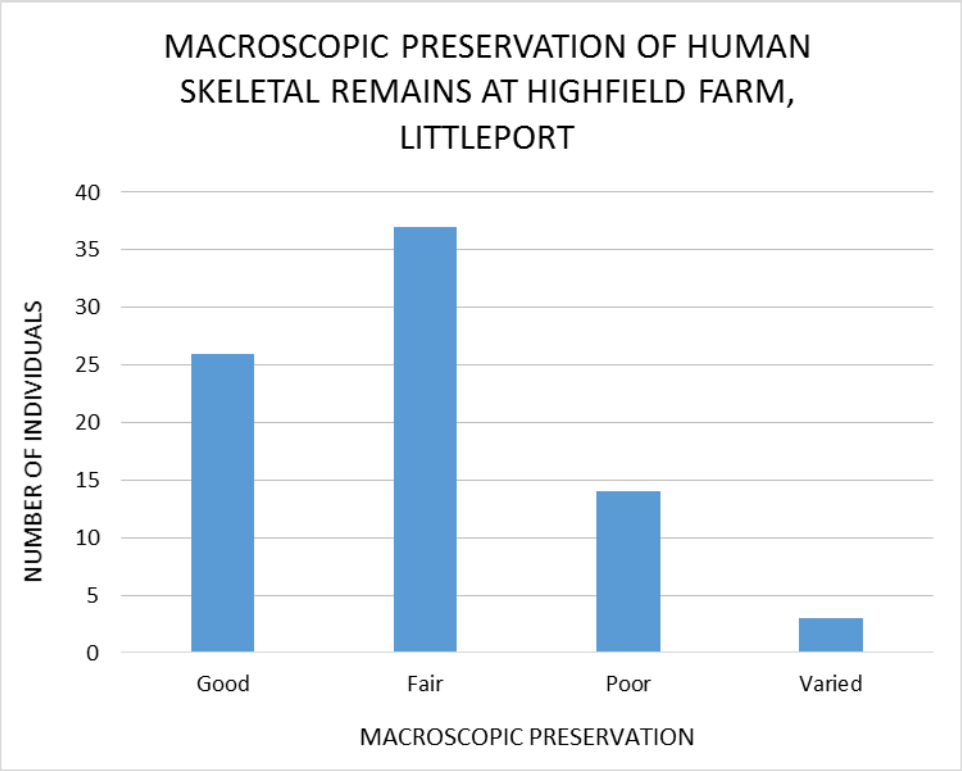

Figure 7.1: Observed macroscopic preservation of skeletal remains from Highfield Farm, Littleport (data reproduced with permission of G. Western).

OHI	Rib	Cranium	Hand Phalanx	Foot Phalanx
0	30	25	4	2
1	8	9	2	2
2	3	3	2	6
3	0	0	2	1
4	0	0	0	0
5	0	0	0	0

Table 7.1: Percentages of Littleport bon elements in each HI (histological preservation) category.

This study suggests that the underlying geology may be an important factor in histological bone preservation. The Edix Hill cemetery, for example, is located on a chalk knoll surrounded by clay lowlands (Malim and Hines, 1998). Overall, the histological bone preservation at this site was the highest of any selected for this research. Histological and proteomic analysis of sample EH156.3, for instance, demonstrated good preservation of both collagen and microscopic bone structure, which suggests optimal local conditions. Conversely, as discussed above, microscopic bone preservation at the Littleport site, which is situated on a clay and silt island (Holt, 2008), was exceptionally poor. This pattern of poor preservation is mirrored at Westfield Farm, which is situated on the south western extremity of Ely Island, on a geology of sand, silt, and clay (Newman, 2007).

One variable that may prove useful in the prediction of microscopic sample preservation is element type. Table 6.23 and Figure 6.33 suggest that, in this study, hand phalanges had the best chance of being well preserved (28% scored 4-5 on the OHI), whereas ribs and cranial samples displayed overall much poorer preservation (10% scored 4-5 on the OHI). This pattern is likely due to three main factors, the first being cortical thickness, the second being element size, and the third being anatomical position and location within the grave. Cortical thickness of each element type became apparent during sample cutting, when the cortex was exposed in cross section. Hand and foot phalanges displayed the thickest cortices, followed by the cranium (vault), and ribs. This is broadly reflected in the overall histological preservation of each element type (Table 6.23; Figure 6.33). Anatomical position and location within the grave may serve to protect hand phalanges (which in the author's experience tend to survive preferentially over foot phalanges), since they are often less prominent and therefore less prone to post-mortem

damage than the ribs or skull. Their small size may also help to protect them from mechanical damage.

These factors do not mean that all the phalanges were well preserved; 51% and 53% of hand and foot phalanges scored 0-1 on the OHI, respectively. They merely suggest that these elements seem to have a better chance of resisting diagenesis when compared to the other element types investigated in this study. Although these elements tend to be less frequently retrieved from the burial context, their increased histological preservation somewhat contradicts previous experimental research, which has demonstrated that small bones are less resistant to diagenesis (e.g., Von Endt and Ortner, 1984; Waldron, 1987c). It should be stated, however, that 'preservation' and rates of diagenesis can be measured in more ways than through macroscopic and microscopic analysis. Von Endt and Ortner (1984), for instance, measured artificially induced diagenesis rates through analysis of extant nitrogen in bone. In addition, the size of bones may be less influential than the age of the individual represented; small elements (e.g., phalanges) from sub-adults could be more prone to diagenesis than their adult counterparts, for instance. Purely in terms of macroscopic preservation, Bello and Andrews (2006) found that hand and foot phalanges were often well preserved, likely on account of their cortical thickness and reduced medullary cavity size – a suggestion supported by this research. Although always small in size, the hand and foot phalanges chosen in this study were often whole, closed systems, whereas cranial and rib samples were always fragmented. This increases exposure of the internal structures to diagenetic factors, so elements less prone to fragmentation should suffer less degradation.

As discussed in section 4.3.1, diagenesis and bone degradation are multifactorial processes and it cannot be assumed that macroscopic preservation or element type will correlate with microscopic preservation. While cortical thickness is an important factor in diagenetic resistance, the nature of the burial and the position of elements within the grave may also influence their microscopic degradation. The shallowness of burials at many sites, including Cleatham, Westfield Farm, and Littleport, increased the possibility of post-mortem damage, thus lowering the resistance of elements to extrinsic diagenetic influences. It is perhaps unsurprising that the rib and cranial samples displayed the poorest preservation of all elements, given their usual prominent positions and often fragmented state in the grave.

It might be expected that older samples would display poorer histological preservation, since they were exposed to taphonomic processes for longer. A comparison of percentages of elements in each OHI category from the Roman and Anglo-Saxon sites, however, shows the inverse is true for this set of samples. Figure 6.34 demonstrates that the Roman samples display a significantly (χ^2 <0.01) better overall level of preservation than the Anglo-Saxon samples. This suggests that microscopic preservation is independent of time since burial, at least for these samples. This conclusion is in line with other studies which have shown little correlation between preservation and burial period (e.g., Hedges et al., 1995). Histological preservation is also shown to be statistically independent (χ^2 p = 0.19) of the age category of the individual from which the elements were sampled (Figure 6.35), a conclusion which supports Manifold's (2013) research on sub-adult bone preservation.

These results may be used to support an argument that the immediate burial environment and its associated taphonomic processes have more fundamental effects on bone diagenesis than either the historical age of the bone, or the age-at-death of the individual from which the sample is taken. Pathological processes (e.g., conditions that may weaken the bone structure) may well contribute to the degradation of bone, but this was controlled for here through the selection of non-pathological bone samples. This could not, of course, guarantee a lack of pathology – it could only control for macroscopically observable pathological conditions.

7.2.2: Histological preservation and implications for protein extraction, yield, and preservation

The use of thiophilic adsorption chromatography (TAC) in this study has permitted the accurate recording of total and eluted protein yields from each P2, P3, and P4 extraction stage through spectrophotometry (yields read at 280nm). It has, therefore, been possible to compare protein yields from samples of differing histological preservation following a variety of extraction techniques, and to investigate correlations between histological preservation and protein yield. Protein yield and archaeological bone preservation have been previously investigated in association with collagen extraction for isotopic study or radiocarbon dating (e.g., Schoeninger et al., 1989; Pfeiffer and Varney, 2000), where the yield is measured by the percentage of extracted collagen, by the surviving carbon to

nitrogen ratios, or by the amino acid profile of the extracted proteins. This study represents the first to assess protein yields following immunoaffinity chromatography. Spectrophotometry cannot, of course, characterise the type or integrity of the extracted proteins; this would require analysis through mass spectrometry. It can, however, demonstrate which protocols and associated extraction stages are more efficient at releasing proteins from samples of differing histological preservation.

An immediate limitation to this approach is that not all of the HI categories are equally represented in the samples subjected to TAC (Table 7.2). For example, only one sample with an HI score of 1 was tested (sample HDJS4 in P2.3), while no samples with an HI score of 3 were investigated. Samples were often chosen to maximise the chance of investigating the hypothesis that non-collagenous proteins are preferentially retained over collagen in poorly preserved bone (after Masters, 1987). In order to efficiently evaluate this hypothesis, it was considered prudent to select the majority of samples from polar ends of the HI scale. Unfortunately, the very low throughput and extended time demands of subjecting three-stage extraction protocols to TAC meant that only a very limited number of total samples could be investigated.

HI	TAC samples
0	5
1	1
2	2
3	0
4	3
5	11

Table 7.2: Number of samples in each HI (histological preservation) category subjected to TAC.

When examining the graphs presented in Section 6.5, it should be recalled that yields from TAC elutions (measured in µg/ml) are those which result from the elution stage of the TAC protocol (e.g., Figures 6.68 and 6.71), which is the stage intended to elute bound IgGs from the columns. This should not be confused with total or average protein yields (e.g., Figures 6.66, 6.69, and 6.72), which include the yields from wash steps and elutions (measured in mg/ml).

Figures 6.66, 6.67, 6.69, 6.70, 6.72, 6.73, and Table 6.52 demonstrate a general pattern of P2 and P4 decreasing total and average protein yields as histological preservation increases. The only exception to this is the sample HDJS4 (HI: 1) extracted in P2.3, which produced unexpectedly low total and elution protein yields in comparison to other samples of relatively low HI. Although this sample seems to be an outlier in terms of fitting the overall observed yield pattern, the quantity of eluted protein is comparable to other samples of low preservation (Figures 6.67 and 6.68). The overall pattern observed in the P2 and P4 extractions supports Pfeiffer and Varney's (2000) finding that poorly preserved bone produces the highest protein yields. The P3 extractions do not entirely support this finding (Tables 6.54 and 6.56), since the best preserved samples released more protein in the second and third extraction stages. This may be due to the fact that only four samples were tested here, and all were well preserved (HI 4 or 5). The increased P3 second stage yields compare to the P2 and P4 third stage, and can be explained by the use of guanidine-HCl and EDTA at these stages. An elevated P3 third stage protein yield for sample CD120.2 (HI: 5) may indicate a correlation between increased protein retention and histological preservation. Further testing of a greater range of samples using the P3 protocol is necessary to clarify the reasons for this somewhat unexpected yield pattern.

The general correlation of increasing protein yield with decreasing histological preservation suggests that less chemical intervention is required to disassociate extant collagen fibrils from poorly preserved bioapatite matrix. Further to this, the integrity of the collagen itself is likely to be diminished in these cases of poor preservation, resulting in increasing protein yield early in the extraction process. These suppositions are supported by Tables 6.44 and 6.45, which suggest a slight decrease in collagen integrity from all P4 extraction stages in poorly preserved bone. This was assessed by comparing peptide numbers and sequence coverage through nLC-MS/MS analysis (section 6.4.5(i)). The investigation of correlations between histological preservation and protein yield suggests that screening of sample preservation should form an important precursor in sample selection if well preserved endogenous protein is a requirement for analysis. This is evidenced in Tables 6.39 (sample CD84.2, HI: 0) and 6.42 (sample EH156.3, HI: 5), which suggest a correlation between histological preservation and both NCP quantity and integrity.

7.2.3: Protein yield and quality by element type

Wiechmann et al. (1999) observed no significant difference in protein quality or quantity between extracts from the femur, rib, and phalanges, although no attempt was made to characterise the preservation status of their samples prior to extraction. It is difficult to make direct comparisons of protein yield and quality between this and Wiechmann et al.'s (1999) research, since the extraction methodologies differ quite significantly. The latter used a one-stage extraction (guanidine-HCl) followed by dialysis and, in some instances, ultracentrifugation. These final steps are intended to desalt, concentrate and remove small molecular components (e.g., heavily degraded collagen). This study used at least two extraction stages (depending upon the protocol followed) on bone of known microscopic preservation status and measured bulk protein yield from TAC purification. Thus, all non-resin-bound protein is being recorded in the yield, including the proteins which would be removed if following the methodology of Wiechmann et al. (1999).

It is interesting to note the differences in collagen and NCP quantity and integrity from P3 sample CD120.2 (hand phalanx, HI: 5) and P4 sample EH156.3 (cranium, HI: 5), as characterised by nLC-MS/MS analysis of TAC resin-bound proteins. Tables 6.45 and 7.4 suggest that well preserved cranial samples subjected to the P4 extraction protocol released collagen and NCPs in higher quantities and of higher quality, as shown by the increased diversity of resin-bound proteins, higher sequence coverages, and greater numbers of uniquely identified peptides. Tables 6.43 and 7.4 suggest that the same element type yields far less protein following the P3 protocol. When the results from all protocols and samples are averaged and histological preservation is disregarded (Table 6.55), the difference in total protein yield by element is small, a finding which may support the conclusion of Wiechmann et al. (1999).

Variation in the average elution percentage of total protein yields by element is interesting, in that ribs seem to display an inverse relationship between the two (Tables 6.53 and 6.54): they produced low protein yields, yet the highest resin-bound yields, particularly when following the P3 extraction protocol (Tables 6.49 to 6.52). It may be the case that well-preserved ribs represent the best target element for IgG extraction due their life-long haematopoietic function and the increased binding of extracted rib proteins to the TAC resins. However, given the observed propensity for non-specific collagen and NCP binding,

proteomic analysis of pre- and post-TAC rib samples would be required to confirm this hypothesis.

Hand phalanx samples CD112.3 (HI: 2) and LP4585.3 (HI: 2) demonstrate the influence of variables beyond element type and histological preservation, when assessing total protein yield (Table 6.52). Despite the similarity in microscopic preservation level, the Littleport sample (LP4585.3) yielded less than half the protein of its Castledyke contemporary (CD112.3). It is possible, then, that site location and local diagenetic factors may explain this disparity. Many Littleport samples, for instance, exhibited infiltration of an unidentified yellowish substance, which either destroyed or obscured much of the extant bone matrix. While Tables 6.52 and 6.59 suggest that protein yield is dependent upon both histological preservation and the choice of extraction protocol, the above example of CD112.3 and LP4585.3 shows that histological preservation may be useful in predicting yields and quality of extracting proteins, but should not be completely relied upon. Proteomic analysis of pre-TAC samples and their post-TAC elutions from all selected element types representing all histological preservation levels would potentially demonstrate which element represents the best target for NCP extraction.

7.2.4: Protein preservation

The importance of characterising the preservation of bone samples by histological analysis prior to protein extraction has been well established (e.g., Schoeninger et al., 1989; Schmidt-Schultz and Schultz, 2004). Histological preservation may be an important precursor in screening out contamination, but does it necessarily indicate which samples are more likely to provide the purest, most well-preserved protein yield? Although it may logically follow that histological preservation would provide a useful indication of the level of protein integrity, previous research on archaeological bone has demonstrated a distinct lack of correlation between histological integrity and the preservation of collagen, in particular (Pfeiffer and Varney, 2000). Nanospray LC-MS/MS analysis of a small number of excised P3 SDS-PAGE gel bands (Figures 6.61 and 6.62; Table 6.34) and P3 and P4 post-TAC samples (section 6.4.5) has provided interesting evidence that may be used to compare between studies and extraction techniques.

Three useful proteomic indicators of ancient protein survival and preservation are observed peptide number, confidence intervals, and peptide sequence coverage (De Hoffman and Stroobant, 2007). The first and second are, respectively, measures of how many peptides unique to individual proteins have been detected, and the level of confidence (e.g., 95%) that these peptides have been accurately identified. The sequence coverage (i.e., the percentage of peptide sequences that match a known protein sequence) resulting from proteomic analysis of samples with known histological preservation can provide a general indication of protein integrity. Accomplishing a 70% peptide sequence coverage is considered to represent a successful analysis using modern samples (De Hoffman and Stroobant, 2007), but there are a number of reasons why achieving a high peptide sequence coverage presents a significant challenge. Poor detection resulting in low coverage may be due to limited (e.g., <5000Da) or excessive size of peptides, which may cause them to either be lost from the LC column, or lost through adsorption or ineffective elution to and from gels or tubes (Wu et al., 2005). These problems would be exacerbated by peptide fragmentation, which may itself render the peptides 'unreadable' by the mass spectrometer (De Hoffman and Stroobant, 2007). Proteins of extremely low abundance in the sample may also be 'masked' by those of greater abundance. While samples can be analysed against exclusion lists in order to characterise proteins present in smaller quantities, this can be an extremely time consuming process (Adrian Brown, personal communication, March 2014), and was only attempted on two P3 post-TAC samples (see section 6.4.5).

It may be expected that confidently-identified peptides recovered from archaeological bone would be extremely small in number, with sequence coverage falling well below 70% due to protein diagenesis and fragmentation over an extended burial period. However, advances in proteomic technologies and extraction methodologies have provided extensive peptide sequences for ancient mammalian collagen (e.g., Buckley et al., 2010; 2011), the most abundant protein in bone, and a variety of non-collagenous proteins (e.g., Solazzo et al., 2008; Cappellini et al., 2012). NLC-MS/MS analysis of selected excised gel bands and post-TAC elutions (section 6.4.3(ii) and 6.4.5) in this study revealed collagen, keratin (the latter the result of extrinsic contamination), and a small quantity of non-collagenous (not including IgG, unfortunately) and non-human proteins. Proteomic analysis

may offer useful clues to the type and extent of degradation in samples of differential histological preservation.

7.2.5: Collagen preservation in SDS-PAGE band samples

Proteomic analysis suggests that collagen from gel band sample CD120.2 (HI 5) suffered less degradation than in EH198.1 (HI 4), providing at their highest a 72.8% and 34.5% sequence coverage, respectively (Table 6.34). The spectra from one CD120.2 gel band (Figure 7.2) with a relatively high coverage of 62.2% shows limited evidence of degradation/modification through deamidation, a spontaneous chemical reaction which removes an amide group, hence modifying protein functionality (Robinson, 2002). Here, deamidation has affected the amino-acid asparagine (N), which represents an extremely common non-enzymatic protein modification (Yang and Zubarev, 2010). Full modifications for all analysed samples can be seen in Appendix 3.

MFSFVDLRLLLLLAATALLTHGQEEGQVEGQDEDIPPITCVQNGLRYHDRDVWKPEPCRICV
CDNGKVLCDDVICDETKNCPGAEVPEGECCPVCPDGSESPTDQETTGVEGPKGDTGPRGPRG
PAGPPGRDGIPGQPGLPGPPGPPGPPGPPGLGGNFAPQLSYGYDEKSTGGISVPGPMGPSGPRG
LPGPPGAPGPQGFQGPPGEPGEPGASGPMGPRGPPGPPGKNGDDGEAGKPGRPGERGPPGPQ
GARGLPGTAGLPGMKGHRGFSGLDGAKGDAGPAGPKGEPGSPGENGAPGQMGPRGLPGER
GRPGAPGPAGAR**GNDGATGAAGPPGPTGPAGPPGFPGAVGAK**GEAGPQGPRGSEGPQGV
R**GEPGPPGPAGAAGPAGNPGADGQPGAKGANGAPGIAGAPGFPGARGPSGPQGPGGPP
GPK**GNSGEPGAPGSKGDTGAK**GEPGPVGVQGPPGPAGEEGKR**GARGEPGPTGLPGPPGE
R**GGPGSR**GFPGADGVAGPK**GPAGERGSPGPAGPKGSPGEAGRPGEAGLPGAKGLTGSPGSP
GPDGK*TGPPGPAGQDGRPGPPGPPGAR***GQAGVMGFPGPK**GAAGEPGKAGER*GVPGPPGAV
GPAGK***DGEAGAQGPPGPAGPAGER**GEQGPAGSPGFQGLPGPAGPPGEAGKPGEQGVPGDL
GAPGPSGARGERGFPGER**GVQGPPGPAGPR**GANGAPGNDGAKGDAGAPGAPGSQGAPGLQ
GMPGERGAAGLPGPKGDRGDAGPKGADGSPGKDGVR**GLTGPIGPPGPAGAPGDKGESGP
SGPAGPTGAR**GAPGDRGEPGPPGPAGFAGPPGADGQPGAKGEPGDAGAK**GDAGPPGPAGP
AGPPGPIGNVGAPGAK**GAR**GSAGPPGATGFPGAAGRVGPPGPSGNAGPPGPPGPAGK**E
GGKGPR**GETGPAGRPGEVGPPGPPGPAGEKGSPGADGPAGAPGTPGPQGIAGQR***GVVGL
PGQR*GER**GFPGLPGPSGEPGK**QGPSGASGER**GPPGPMGPPGLAGPPGESGR**EGAPGAEGS
PGRDGSPGAK**GDRGETGPAGPPGAPGAPGAPGPVGPAGK**SGDRGETGPAGPTGPVGPVG
ARGPAGPQGPR**GDKGETGEQGDRGIKGHR**GFSGLQGPPGPPGSPGEQGPSGASGPAGPRG
PPGSAGAPGKDGLNGLPGPIGPPGPR**GRTGDAGPVGPPGPPGPPGPPGPPSAGFDFSFLPQP
PQEKAHDGGRYYRADDANVVRDRDLEVDTTLKSLSQQIENIRSPEGSRKNPARTCRDLKMC
HSDWKSGEYWIDPNQGCNLDAIKVFCNMETGETCVYPTQPSVAQKNWYISKNPKDKRHVW
FGESMTDGFQFEYGGQGSDPADVAIQLTFLRLMSTEASQNITYHCKNSVAYMDQQTGNLKK
ALLLQGSNEIEIRAEGNSRFTYSVTVDGCTSHTGAWGKTVIEYKTTKTSRLPIIDVAPLDVGAP
DQEFGFDVGPVCFL

Figure 7.2: Complete sequence of human collagen alpha-1 chain with peptides identified by nLC-MS/MS analysis of P3 sample CD120.2 lane 4 upper gel band (62.2% coverage). **Bold** text - high-confidence matched peptides; *italic* text - medium confidence; underlined text – low-confidence; **N** – asparagine deamidation.

The six gel bands analysed from P1.2 (Figures 6.41 and 6.42; Table 6.24) show an average collagen peptide sequence coverage of only 35.8%, despite the samples HDAR3 and HDAR4 displaying HI scores of 4 and 5, respectively. The disparity in coverage between P1.2 and P3 may be the result of slightly different precipitation and sample preparation methods prior to SDS-PAGE, or it may be indicative of poor correlation between histological preservation and collagen integrity. Correspondingly low collagen peptide sequence coverage in the P3 post-TAC sample (Table 6.47) may support the former suggestion, since it represents the same sample (CD120.2) as shown above in Figure 7.2. Low sequence coverage from gel bands may also be the result of selecting bands representing proteins of specific molecular weights. Bands that possibly correspond to IgG fragments, for example, would also include any other proteins of that weight, including collagen fragments in various states of degradation.

7.2.6: Collagen and histological preservation in the P3 and P4 post-TAC samples

When assessing the impact of histological preservation on protein survival in this study, it should be recalled that P3 and P4 samples analysed by nLC-MS/MS represent post-TAC elutions. The protein yields from these elutions represent a fraction of the total pre-TAC yields, since the resins are designed to extract IgGs. Consequently, any non-IgG proteins present in the elutions have been bound non-selectively. It is uncertain exactly how these non-selectively bound proteins relate to the non-bound fractions, and why they were preferentially bound over others. It may be the result of protein degradation or unspecified interactions of buffer reagents which may increase the binding on non-IgG proteins (see sections 7.3.3 for further discussion of this).

Tables 6.47–6.49 (section 6.4.5(i)) display the characteristics of the resin-bound collagen from post-TAC P3 and P4 samples. Elutions from the P3 second stage were analysed due to the demonstrated elevated protein yield observed in extraction stages incorporating guanidine-HCl solublisation (see Table 6.50). Summary Table 7.3, and Tables 6.47-6.49 show that the P3 sample yielded a less diverse number of resin-bound collagen isoforms than P4 sample EH156.3, despite their comparative high level of histological preservation. Indeed, more diversity was seen in CD84.2 (HI: 0) than in CD120.2 (HI: 5). This may suggest that P4 is the more effective protocol for collagen extraction, perhaps in

part due to the absence of the HCl demineralisation stage in the P3 protocol. As discussed in section 7.7.5, HCl demineralisation increases the solubility of collagen (Cleland et al., 2012) and may induce a greater collagen yield in later extraction stages. The number of peptides and their sequence coverages are also much lower for the P3 sample. It is difficult to ascertain the reasons behind the relative lack of extracted collagen and its apparently poorer quality in the P3 sample. The two main variables here are bone element type and extraction protocol. It may be the case that the thick cortex of the CD120.2 (hand phalanx) required more intense chemical treatment (due to less efficient homogenisation) than provided by the P3 protocol in order to release well-preserved collagen, although it may be expected that sonication would be more than sufficient to release retained collagen. Conversely, the difference in collagen preservation between P3 and P4 may be an artifact of the non-specific TAC binding, perhaps related to differential impact of extraction techniques and reagents upon both the thiophilic resin and surviving collagen molecules.

In general, increased histological preservation seems to correlate with the retention of a greater diversity of collagen isoforms, although their successful acquisition may be just as dependent upon the inclusion of a demineralisation extraction stage. Full proteome analysis of pre-TAC samples would allow for a deeper investigation into the effects of histological preservation on the retention of collagen in bone.

Sample	CD120.2	EH156.3	CD84.2
Histological Index	5	5	0
Period	Anglo-Saxon	Anglo-Saxon	Anglo-Saxon
Location	Castledyke	Edix Hill	Castledyke
Element	Hand phalanx	Cranium	Cranium
Age	Very young adult	Young adult	Very young adult
Protocol	P3	P4	P4
Collagen isoforms	2	4	3
Average SC/P	30.1/16	75.2/77	74/82
Identified NCPs	2	12	6

Table 7.3: Comparison of variables and extracted peptide characteristics for nLC-MS/MS analysed post-TAC P3 and P4 samples. SC/P – sequence coverage (%)/peptides (95% confidence). NCPs represent only endogenous human proteins.

7.2.7: Non-collagenous protein (NCP) preservation

Table 7.4 demonstrates differences in the quantity of resin-bound NCPs between extraction protocols and histological preservation. NLC-MS/MS analyses suggests that bone displaying higher preservation (i.e., EH156.3, HI: 5) yields NCPs in greater quantity (Tables 7.7 and 7.8) and of better integrity (Table 7.4). The apparent lack of resin-bound collagen and NCPs from P3 sample CD120.2 (HI: 5) may suggest that this relationship is partially dependent upon the choice of extraction protocol. Possible reasons for differential collagen and NCP binding between protocols are explored in section 7.3.3. The observed correlation between protein quantity/quality suggests that well-preserved bone represents the best target for future NCP extractions, despite the fact that the NCPs characterised here (Table 7.4) represent TAC resin-bound proteins, rather than the full range of pre-TAC proteins. Firm conclusions based on these observations are difficult, however, due to the small sample numbers subjected to proteomic analysis.

	CD120.2 (HI: 5)		CD84.2 (HI: 0)		EH156.3 (HI:5)	
	P3		P4		P4	
Protein	**Sequence Coverage (%)**	**Peptides (95%)**	**Sequence Coverage (%)**	**Peptides (95%)**	**Sequence Coverage (%)**	**Peptides (95%)**
Terminal uridylyltransferase 4	6.5	1	-	-	-	-
Ankyrin repeat and SOCS box protein 18	9.2	1	-	-	-	-
Vitronectin	-	-	12.1	1	21.5	3
Chondroadherin	-	-	14.8	2	28.7	2
Pigment epithelium-derived factor	-	-	12.2	1	21.5	3
Biglycan	-	-	4.6	1	6.1	3
Prothrombin	-	-	12.9	1	17.8	2
Cystatin-A	-	-	38.8	1	-	-
Protein argonaute-4	-	-	-	-	7.9	1
Protein AHNAK2	-	-	-	-	11.1	1
Alpha-2-HS-glycoprotein	-	-	-	-	14.4	1
Osteomodulin	-	-	-	-	4.3	1
Lumican	-	-	-	-	5.6	1
Matrix Gla protein	-	-	-	-	36.9	4
Dermatopontin	-	-	-	-	9.5	1

Table 7.4: Comparison of extracted endogenous human NCP sequence coverage and peptide numbers for P3 and P4 post-TAC samples.

Further proteomic analysis of bones exhibiting a full range of histological preservations is required here to investigate a possible correlation between histological preservation and protein degradation. The extremely limited evidence presented may tentatively suggest that bone displaying better histological preservation may yield protein of higher quantity and integrity. This would be a logical conclusion if increased peptide sequence coverage is indicative of protein integrity, yet would contradict previous research (e.g., Pfeiffer and Varney, 2000), which failed to find a correlation between histological and protein preservation. It must, however, be stressed that the two studies used different methods of extracting protein and assessing preservation. An aim of Pfeiffer and Varney's (2000) research was to investigate whether histological preservation might be used to select bone samples suitable for palaeodietary isotopic analysis. They therefore used a technique designed for maximum retrieval of collagen and assessed protein integrity through examination of remaining carbon and nitrogen elemental values and amino-acid profiles (the

latter characterised by High Performance Liquid Chromatography). This study used extraction techniques designed to either maximise the extraction of non-collagenous protein (e.g., P1 and P3), or to extract as much protein as possible (e.g., P2 and P4). Integrity here was assessed though nLC-MS/MS analysis and identification of surviving protein peptide sequences. Direct comparison of conclusions between studies is, therefore, difficult.

7.3: Protein and immunoglobulin extraction and characterisation protocols

This section considers the different protein and immunoglobulin extraction protocols (P1-P5) and the subsequent protein characterisation methods employed in this study in terms of their effectiveness in extracting and characterising archaeological IgGs.

7.3.1: P1: adapted Schmidt-Schultz and Schultz (2004) extraction protocol

The first method employed to extract IgGs from archaeological bone (section 5.7.1) was based closely on a protocol developed by Schmidt-Schultz and Schultz (2004). This method was aimed specifically at disrupting the tight interaction between the bone mineral scaffold and non-collagenous proteins (NCP) through a process of demineralisation/chelation and denaturation designed to loosen the mineral scaffold and remove proteins not bound to the bioapatite. The final bone pellet solublisation stage destroys the scaffold, thus releasing remaining mineral-bound NCPs (Schmidt-Schultz and Schultz, 2004). The three-stage protocol discards supernatants following the first two stages and retains the final supernatant following bone pellet solublisation. This approach does, of course, rely upon the assumption that NCPs of interest are retained in the bone pellet until the final extraction stage. This research offered a highly promising method of extracting well-preserved IgGs from archaeological human bone. If successfully reproducible, the protocol could have been used to extract IgGs from target samples. These immunoglobulins could then have then been tested for reactivity and then against malaria antigens in order to detect the presence of the disease in past populations.

As mentioned in section 5.7.1, the method almost immediately required changing in terms of the specific protease inhibitors employed. Not only was the reported quantity of aprotinin clearly disproportionate, the authors also suspended PMSF in the aqueous

extraction buffers. PMSF is mostly insoluble in an aqueous solution, so its inhibitory properties would likely be severely negated without first dissolving it in a suitable medium (e.g., isopropanol). Perhaps more importantly, there was the distinct lack of strategy in the published protocol to account for the collagen invariably released during the solublisation. The presence of degraded collagen was confirmed through proteomic analysis and Western blots for the P1.2 and P1.3 samples, respectively (see below), even though the authors state that highly defined laboratory conditions are required to "produce collagen fragments larger than 10 kDa" (Schmidt-Schultz and Schultz, 2007:96). The copious quantities of degraded collagen resulting from all extractions in this study served to mask proteins of lower concentration and to cross-react with antibodies used in both the P1 Western Blot and IgG ELISA analyses. Cross-reactivity with collagen would almost certainly have occurred in the Western blots performed by Schmidt-Schultz and Schultz (2004; 2007), yet none was reported. It is, therefore, impossible to fully evaluate the efficacy of the Schmidt-Schultz and Schultz protocol without more in-depth reporting of their strategies for avoiding the extensive collagen cross-reactivity encountered when attempting to replicate their research. No method attempted here was able to reproduce the results of Schmidt-Schultz and Schultz (2004).

The SDS-PAGE gels analysing the P1.1 and P1.2 samples were affected quite strongly by smearing (Figures 6.39 and 6.40). This was particularly pronounced for the P1.2 gels, where only faint bands were visible through the smearing. Smearing is a relatively common problem in SDS-PAGE analysis, sometimes resulting from errors in gel preparation or over-loading of wells. It can also be caused through interference by residual chemicals used in sample preparation, such as EDTA (Cleland et al., 2012). EDTA is likely to be partly responsible for the smearing evident on the P1.1 and P1.2 gels, since it was required in the final solubilisation buffer. There may also have been residual EDTA in the lyophilised bone pellets prior to solubilisation, since removal of the chemical can require more than 15 washes (Cleland et al., 2012); the Schmidt-Schultz and Schultz (2004) protocol used only three washes. EDTA would also probably persist through SDS-PAGE sample preparation, since it is likely to precipitate alongside the proteins in TCA precipitation. Omission of EDTA from the final solublisation buffer and perhaps switching to HCl demineralisation may reduce any EDTA-associated smearing in future. However, as

discussed later, HCl demineralisation may not be suitable to extraction targeting archaeological IgGs.

Schmidt-Schultz and Schultz (2007) suggest that SDS-PAGE smearing from archaeological samples is the result of poorly-solubilised proteins failing to produce sharp bands. This may not, however, be completely accurate, since their solublisation protocol was exactly followed for the P1.2 samples, yet still resulted in significant smearing. Fragmented collagen has also been shown to increase smearing on SDS-PAGE gels of clinical samples (e.g., Acil et al., 2007; 2013), and research on collagen diagenesis by Dobberstein et al. (2009) encountered smearing in the oldest of their tested bone samples. The authors suggest that the smearing may be associated with the specific cleavage method employed, or partial hydrolysis of the collagen. It is possible that the mechanical grinding used in sample preparation throughout this study and by Schmidt-Schultz and Schultz (2004) had a significant degradation effect on the extant collagen, perhaps resulting in the increased smearing seen in the SDS-PAGE gels. This intense smearing was the reason for the use of collagenase in the subsequent P1.3 samples.

Bacterial collagenase was used in the P1.3 samples to remove the excess, interfering collagen that was extracted and characterised in P1.1 and P.1.2, and to reduce its potential masking effect on any low abundance NCPs (section 5.7.2). Research by Tuross and Stathoplos (1992) and Ostrom et al. (2001:1046) demonstrated significant reductions in gel lane smearing through the removal of "weakly staining collagenous degradation products". Figures 6.43 and 6.44 suggest that collagen digestion was rather less successful for the P1.3 samples. This failure to completely remove smearing was perhaps due to an insufficient collagenase concentration. The concentration used here was based on digestion of modern collagen (Hummelshoj et al., 2008), since figures were not explicitly stated by either Ostrom et al. (2001), or Tuross and Stathoplos (1992).

Collagenase digestion seems to have been differentially successful, depending upon the sample (Figures 6.43 and 6.44). The enzyme was particularly effective for samples AR6 and AN4, for example, but far less so for AP5 and AN2. It was initially thought that this may be an artifact of the preservation of collagen, in that the collagenase may be preferentially removing the poorly preserved protein, thus resulting in the reduction of smearing. However, if better microscopic bone preservation correlates with less degraded

collagen, the histological preservation of these four samples does not support this initial theory (AR6 = HI: 5; AN4 = HI:3; AP5 = HI:5; AN2 = HI:5). The reasons behind the protein banding and smearing for AP5 and AN2 following collagenase treatment are unclear, since the banding pattern certainly seems to match other samples which responded well to collagenase digestion. Residual EDTA may have reduced the effectiveness of the collagenase, since it acts as a collagenase inhibitor (Worthington, 1993).

It is interesting that in Western blot testing, non-collagenase treated AP5 and AN2 cross-reacted strongly with the secondary antibody, while their collagenase digested counterparts did not (Figures 6.45–6.51). Unfortunately, these gels were not silver stained. This process would have provided further band resolution, particularly in lanes where much of the smearing had been removed by collagenase. It may also have allowed for visualisation of the putative IgG positive discussed in section 7.3.1(ii).

Examination of the collagenase and non-collagenase Western blot membranes (Figures 6.45-6.51) shows that cross-reactions were ubiquitous with the latter samples, whereas very little evidence of cross-reactivity was seen with the former samples. This implies that, although the smearing often appeared slightly reduced on the SDS-PAGE gels, the main agents of cross-reactivity were actually removed by the collagenase. This subsequently suggests a correlation in reactivity between the secondary polyclonal antibody and highly degraded collagen, the latter of which was removed by collagenase treatment.

7.3.1(i): P1.2 MALDI-TOF and nLC-MS/MS analyses

Sections 5.7.3(iv) and 5.7.3(v) outline the methodologies used in the proteomic analysis of selected P1.2 gels bands (gels and bands shown in sections 6.4.1(ii) and 6.4.1(iii)). Peptide 'fingerprinting' by MALDI-TOF was the first analytical method attempted for these gel band samples, followed by nLC-MS/MS. Unfortunately, neither method successfully detected IgGs (section 6.4.1(iii)) in any of the six excised gel bands, The MALDI failed to detect any proteins (for possible reasons, see section 7.4.4, below), while the nLC-MS/MS analysis revealed collagen as the most prevalent protein in the bands (Table 6.24). The sequence coverage for these samples was somewhat low (the highest being 47.8%), thus demonstrating the degraded nature of the collagen fragments inadvertently excised in the gel bands.

In hindsight, the selected bands were probably not representative of the expected molecular weights of potentially degraded IgG heavy and light chains. The choice of bands for proteomic analyses was based on Schmidt-Schultz and Schultz's (2004; 2007) apparently successful Western blot analyses (Figure 6.37), which detected ancient IgG heavy chains with a molecular weight akin to modern, fresh samples (around 60kDa). It was assumed that following their published extraction protocol and using of well-preserved samples would produce gel bands of a similar weight. Western blot analysis (section 7.3.1(ii)) for later P1.3 samples supplied a possible positive for heavy chain IgG at around 40kDa (the disparity between this and the published analyses in terms of molecular weights is addressed in section 7.3.1(ii)). It was perhaps naïve to expect to be able to select bands analogous to the IgG chains apparently detected by Schmidt-Schultz and Schultz (2004), given the poor band definition and obvious smearing encountered in each gel lane. A more targeted approach to selecting gel bands possibly representing IgGs was required for proteomic analysis. This led to the inclusion of positive IgG controls in later SDS-PAGE analyses and excision of possible matching bands.

7.3.1(ii): P1.3 Western blot

The analysis of collagenase treated and non-collagenase treated samples allowed for a direct comparison by elucidating the effects of degraded collagen on potential masking of target proteins and cross-reactivity with the primary and secondary antibodies employed in the Western blot. Nitrocellulose membranes were exposed for periods of 10, 30, 60 seconds, and eight minutes, with x-ray records taken at each exposure stage. The results of the eight minute exposure of the non-collagenase treated samples were discounted due to over-exposure and subsequent poor band resolution.

The analysis yielded one possible positive for collagenase treated sample (HD)AP3 (Figures 6.48-6.51), which had an appearance very similar to the heavy chain IgG bands (Figure 6.37) identified by Schmidt-Schultz and Schultz (2004). The difference here, though, is that the band is suggestive of a protein fragment with a weight of approximately 40kDa, rather than the 55-60kDa detected by Schmidt-Schultz and Schultz (2004). This reduced molecular weight may be due to protein degradation in a sample of sub-optimal preservation: AP3 displayed a histological preservation score of 4, while the samples tested

by Schmidt-Schultz and Schultz (2004) were all very well preserved. It is unlikely that collagenase could have degraded the protein, since it seems to be present at the same molecular weight in the non-treated samples (Figures 6.46 and 6.47). Harper (1980) suggests that non-collagenous proteins should not be affected by collagenase, although Ostrom et al. (2001) experienced degradation of their target NCP, osteocalcin, and Wadsworth and Buckley (2014) lost NCPs during collagenase treatment. Clearly, any enzymatic effect on potentially degraded archaeological IgGs is unpredictable and cannot be completely ruled out. It is, of course, possible that the protein detected does not represent an IgG Fc chain at all, but rather a non-specific primary antibody/antigen binding. An example of this is the appearance of a band on the molecular weight marker chain at 29kDa (Figures 6.49 and 6.50). A commonly-used marker protein at this weight is bovine carbonic anhydrase, which has here likely cross-reacted with the primary antibody. However, the appearance of a clear band at a credible molecular weight (around 40 kDa) is suggestive of the presence of human IgG, and represents the most likely 'positive' from all of the P1.3 samples. Its appearance in both collagenase and non-collagenase treated samples over all exposure times suggests that the 'positive' was not the product of non-specific antibody binding to collagen. Very faint bands at approximately 25kDa on the collagenase 60 second exposure membrane for samples AR6, AR3, and AP5 (Figure 6.50) may tentatively suggest the presence of IgG light chains. Their appearance, however, would be the result of cross-reactivity, given the heavy-chain specificity of the primary antibody and anti-mouse IgG specificity of the secondary antibody.

A potential positive for sample HDAP3 prompts the question of why no further 'positives' from the other samples were encountered, particularly for those of similar histological preservation. It is possible that the Western blot methodology used can account for this dearth. Milk powder, rather than bovine serum albumin (BSA), may have been too strong of a blocking agent. If this was the case, it would have inhibited many primary antibody interactions with target IgGs (as well as potentially introducing bovine IgGs into the experiment). The alternative blocker, BSA, was, however, likely to cross-react with the primary antibody. Although BSA contributes a small percentage of protein in milk powder, the high concentration in analytical grade BSA would greatly increase the chances of cross-reactivity (Dr. Stephen Chivasa, personal communication, April 2014). It is for this latter

reason that milk powder was again chosen for the Western blots on the selected P2, P3, and P4 samples (section 5.7.10). Also, since the type of blocking buffer used by Schmidt-Schultz and Schultz (2004) was not specified, the use of milk powder followed clinical examples of human IgG heavy chain detection (e.g., Shiguekawa et al., 2000).

A further answer for the lack of positives may lie in the choice of a monoclonal primary antibody, which has specificity for a single target epitope. It may be the case that the 'positive' encountered in sample HDAP3 represents the only survival of the target epitope in all tested samples. The use of a polyclonal primary antibody with specificity to multiple IgG epitopes would increase the chances of detection, but would also correspondingly increase the likelihood of the type of nonspecific cross-reactions. However, the detection here of a potential IgG heavy chain using a monoclonal antibody increases the possibility that this is indeed a true positive. This may be in part why Schmidt-Schultz and Schultz (2004; 2007) detected IgG in all of their ancient samples; although not explicitly stated, it is likely that they employed a polyclonal primary antibody to increase the changes of positive interactions. Unfortunately, they do not explain how they interpreted any cross-reactions, although they are likely to have encountered them. They also only present cropped images of the 'positives' resulting from their Western blot analyses, rather than the entire membranes. A final reason for the lack of positives in the current study may be simply that IgGs were not present in the samples, and the HDAP3 'positive' was merely a cross-reaction with an unidentified protein.

The IgG ELISA (section 6.4.1(vi), Table 6.25) suggests an IgG concentration of approximately 1.66ng/ml for sample HDAP3. HDAR3 measured over 4ng/ml, yet the latter exhibited no apparent IgG in Western blot analysis. The reason for this disparity is unclear, but may be indicative of differential or incomplete protein transfer from gel to nitrocellulose membrane, or a possible false positive in the ELISA. It may also suggest that the HDAP3 'positive' on the Western Blot is an aberration. If these 'positives' are to be believed, it may be suggestive of differential levels of IgG in different bone elements (HDAP3 being an adult phalanx, HDAR3 being adult rib), although this simplistic conclusion would neglect a variety of other variables affecting IgG survival in bone.

The HDAP3 'positive' may be supported by silver staining of the P1.1 gel (Figure 6.39), which displays a possible band at approximately 40kDa. HDAP3 was also analysed

by SDS-PAGE in protocols P1.2 (Figure 6.40) and P2.2 (Figure 6.53). The former gel (Figure 6.40) does not display the same band around 40kDa, although the high quantity of smearing collagen may have obscured any IgG. The silver stained version (Figure 6.26), however, shows a clearer band at this weight. The P2.2 gel shows a very faint band around 40kDa (Figure 6.37), but this may match a band at the same weight in the collagen control samples on the same gel. Proteomic analysis was attempted on a band excised from the P1.2 gel (section 6.4.1(iii)), but not on the band shown in the P1.3 Western blot, which was performed subsequent to P1.2.

The analysis of collagenase and non-collagenase treated samples has allowed for loose characterisation through cross-reactivity of the collagen yield from the P1.3 extraction. The non-collagenase treated samples demonstrate beyond doubt that the vast majority of cross-reactivity is caused by the interaction of antibody with degraded collagen, although there are a number of other possible reasons for the increased number of bands detected for the non-collagenase treated samples. These include insufficient protein blocking, contamination during equipment preparation, or over-exposure of the film (as encountered here with exposure over 60 seconds). The most likely reasons here, though, are non-specific binding of the secondary antibody (a HRP-conjugated polyclonal anti-mouse IgG antibody) with degraded collagen, or too high of a secondary antibody concentration. The latter problem would be easiest to address by further dilution of the secondary antibody from 1:20000 used here, to 1:40000 suggested by Schmidt-Schultz and Schultz (2004). 1:20000 was used in this analysis to ensure sufficient conjugation with the primary antibody, but the concentration may have increased the prevalence of non-specific cross-reaction, particularly for the non-collagenase treated samples. This could be countered, although probably not completely ameliorated, by the use of a monoclonal secondary conjugated antibody at lower concentration. The monoclonal nature of the primary antibody should reduce the chances of cross-reactions with non-specific proteins at this stage, but at the risk of failing to detect IgGs with specific epitopes degraded beyond a detectable limit. Even with a monoclonal primary antibody, the possibility of such nonspecific interactions should not be discounted. The analysis of samples that have been subjected to antibody purification (e.g., thiophilic adsorption chromatography) should also improve detection by removing the majority of interfering collagen.

7.3.1(iii): P1 human IgG ELISA

Results for the P1 IgG ELISA (section 6.4.1(vi)) suggest that IgGs were detected at extremely low concentrations, with the highest concentration in sample HDAR3 (4.79ng/ml). This is, as expected, far below the level of normal human serum IgG, which ranges from 5-12mg/ml (Stoop et al., 1969). Unfortunately, these results were almost certainly affected by cross-reactivity. The polyclonal antibodies used to detect the numerous subclasses of human IgG may have resulted in the positives observed in the bovine collagen type III (3.53ng/ml) and animal bone sample AN7 (Table 6.25). The degraded nature of the samples may also have caused non-specific reactions with the ELISA antibodies. The fact that the blank third stage P1 buffer solution tested positive for human IgG (2.8ng/ml) is suggestive of exogenous contamination during sample preparation or testing (potentially from the standards, which were in close proximity to the blank buffer on the multi-well plate). Given the strong likelihood of non-specific cross reactions and exogenous contamination, positive results for any of the human samples should be considered with extreme caution. IgGs may well have been detected, but their positive detection cannot be separated from potential false positives resulting from contamination or cross-reactivity.

7.3.2: P2 and P4: adapted Jiang et al. (2007) protocols and thiophilic adsorption chromatography (TAC)

The chosen protocol for the P2 samples was based on Jiang et al.'s (2007) multi-stage protein extraction from fresh bone. It was felt that the exhaustive nature of these extractions would increase the chances of retrieving IgGs from archaeological bone samples if combined with an additional immunoglobulin purification stage. As described in section 5.7.4, the published methodology required minor alteration due to the disparity in sample type and downstream analytical applications. The fresh bone samples analysed by Jiang et al. (2007) required, for example, the initial removal of soft tissue and two strong acid (1.2M and 6M HCl) demineralisation steps. Their intention was to characterise proteins extracted from each stage by mass spectrometry, and they were therefore unconcerned if their extracted proteins were reduced to the peptide level, since this would be suitable for MS analysis. For this study, a more gentle demineralisation was required (in line with other archaeological protocols) in order to avoid hydrolysis of target IgGs and allow for their

purification using TAC. As explained in 5.7.4, the methodology changed slightly with each subsequent P2 extraction to mitigate for issues arising from the application of a protocol designed for fresh bone samples to ancient bone.

A strength of the Jiang et al. (2007) protocol over the Schmidt-Schultz and Schultz (2004) protocol is in the analysis of supernatants following each extraction stage.
It was hoped that the characterisation of proteins from each stage, rather than from a final pellet solublisation alone, would increase the chances of detecting target IgGs that may be released at any point during the extraction. Although Schmidt-Schultz and Schultz (2004; 2007) supposedly detected IgG following solublisation, they report no analysis of supernatants from their first two extraction stages. This is somewhat of a surprise, considering the research of Termine et al. (1981), which demonstrated that two thirds of mineral-bound NCP are released by extraction and solublisation with guanidine-HCl (the second stage employed by Schmidt-Schultz and Schultz). Given the uncertainty over at exactly which stage degraded IgGs might be extracted, it was felt prudent to analyse supernatants from each P2 and P4 extraction stage.

The Jiang et al. (2007) extraction methodology includes a precursory step designed to induce the solubilisation of mineral-bound proteins and start the release of proteins. HCl is a commonly used demineralising agent that increases bone matrix porosity, thus exposing and releasing mineral-bound protein (Cleland et al., 2012). Schmidt-Schultz and Schultz (2004) instead employed EDTA as the prime demineralizing agent. Both EDTA and HCl are effective demineralisers, each offering positives and negatives in terms of protein extraction and conservation. HCl demineralisation is much faster, negates the need for toxic protease inhibitors, and offers a 'cleaner' extraction. It is, therefore, ideal for downstream analysis such as mass spectrometry. The acidic conditions, however, increase the chances of target protein degradation or hydrolysis (Jiang et al., 2007; Cleland et al., 2012). Using EDTA takes far longer, but is a much gentler method of demineralisation (Cho et al., 2010). EDTA is also less compatible with many downstream applications and samples usually require extensive washing to remove it prior to analysis. The potential smearing effects of residual EDTA on SDS-PAGE gels can be seen in the P1 extractions (Figures 6.38-6.42), suggesting that the three washes advised by Schmidt-Schultz and Schultz (2004) were likely insufficient for complete EDTA removal, at least for these samples. The use of thiophilic

resins for the P2 and P4 protocols negated the requirement for EDTA removal from the second and third extraction stages, since the chemical should not be retained in the resins.

As can be seen in Figures 6.66, 6.69, and 6.72, total and average P2 and P4 protein yield increased with each subsequent extraction, which reflects the cumulative effects of chemical weakening of the bioapatite and release of mineral-associated proteins by HCl/EDTA demineralisation and chelation, and by guanidine-HCl denaturing and solubilisation. This pattern is demonstrated regardless of histological preservation, although poorer preservation generally correlated with higher protein yield for all extraction stages. The pattern is to be expected, as it should require less chemical intervention to release proteins (particularly degraded collagen) from samples displaying poorly preserved bone microstructure.

Figures 6.52-6.60 display the most 'successful' P2 post-TAC SDS-PAGE gels in terms of the visualisation of bands potentially corresponding to ancient IgG heavy and light chain fragments. Bands felt most likely to represent IgG fragments were excised and subjected to proteomic analysis, a choice facilitated by the introduction of a modern IgG control in P2.3. Unfortunately, no IgGs were detected in any of the excised bands, other than the control IgG bands excised from the P2.4 gel (see Figure 6.57, and Tables 6.29, and 6.30). Possible reasons for this failure are numerous, and are discussed in sections 7.3.3, 7.4.2, and 7.4.3.

7.3.3: TAC and non-specific protein binding

Thiophilic adsorption chromatography should, theoretically, be an optimal method for purifying IgGs. TAC offers a highly selective environment for retrieval of target immunoglobulins, yet avoids the harsh elution (and hence potentially degrading) conditions associated with, for example, traditional protein A columns. This study represents the first to attempt to apply TAC immunoaffinity to ancient samples. As with all modern analytical techniques adapted for archaeological samples, this does introduces a degree of uncertainty as to the effectiveness of the method. It was crucial, therefore, to analyse the elutions in order to both identify any eluted IgGs and to evaluate the method in terms of non-selective protein binding.

Protocol	Total TAC Protein Yield (mg/ml)	TAC Elution Protein %
P2	64.653	1.6
P3	18.764	4.1
P4	44.876	1.4

Table 7.5: Comparing total TAC protein yield and TAC elution protein percentage between protocols.

Spectrophotometric measurements of eluted protein concentrations were initially very promising. The very low percentage of eluted proteins (Table 7.5) suggested that the resins were binding very small amounts of protein, perhaps in quantities to be expected for archaeological IgGs. However, as described in sections 6.4.1(iii), 6.4.2(vi), and 6.4.3(ii), proteomic analysis of SDS-PAGE gel bands from eluted samples proved fruitless in terms of IgGs, revealing only collagen and exogenous keratin. This confirmed that the TAC resins were retaining other proteins, which was initially suggested by the appearance of gel bands for pure collagen samples (e.g., P2.2, Figure 6.53) and bands of higher molecular weight than those of the modern IgG positive controls (e.g., P2.3 and P2.4, Figures 6.54 and 6.56). Upon consideration of the likely degraded nature of any IgG and the subsequent difficulties in isolating appropriate gel bands for further analysis, it was decided to subject a selection of eluted samples to proteomic analysis. This should have allowed for characterisation of all proteins within each given elution and bypass any issues of potential IgG loss during SDS-PAGE analysis. nLC-MS/MS analysis of P3 and P4 post-TAC eluted samples demonstrates that the TAC resins did indeed retain a quantity of proteins other than the intended IgG target, while confirming that no IgG (at a detectable level) was successfully extracted or purified from these samples.

. This non-specific adsorption is perhaps unsurprising, given the degraded nature of the proteins in question and the use of an application that is unintended for archaeological samples. It is unlikely, for instance, that TAC resins have been tested against the range of degraded proteins or extraction reagents used in this study. An example of the latter may be seen in the reaction of 1st stage extracted (in 0.6M HCl) samples and the subsequent difficulty of sample introduction into the resins. Successful TAC requires samples to be above pH 8 to help prevent binding of proteins other than IgG. When these demineralised samples were brought from their acidic condition to pH 8, they became very viscous and proved difficult to introduce into the resins. This problem is unlikely to be encountered

when using modern, clinical samples. Further to this, the use of the denaturant guanidine-HCl may adversely affect the binding of protein during TAC (a potential problem discussed in section 7.7.6). The extent to which these unconventional conditions affected binding is uncertain, but it highlights the potential difficulties in using non-standard samples and reagents in clinical applications. Since whole proteomes were not studied here, comparisons of protein content with other archaeological proteome studies (e.g., Cappellini et al., 2012; Wadsworth and Buckley 2014) are difficult.

7.3.3(i): TAC-retained collagen

It is immediately clear from the Tables 6.47, 6.48, and 6.49 that the largest variety of collagen isoforms was detected in sample EH156.3. Their presence likely corresponds to the preservation level of this sample (HI: 5), since only three isoforms were identified in sample CD84.2 (HI: 0). Despite this difference, sequence coverage for both samples is surprisingly high, regardless of histological preservation. This may suggest that certain collagen isoforms resist diagenesis and when they do persist, it is in a relatively well-preserved state, even in poorly preserved bone, as previously suggested by Dobberstein et al (2009).

It is uncertain why the apparent variety and quality of collagen extracted from the P3 sample (CD120.2, HI: 5) is considerably lower than for the P4 samples. For unspecified reasons, higher quality collagen (and NCPs, see 7.3.3(ii) below) of more varied type was retained from the P4 extractions. It is possible that the higher quality collagen was extracted in the first P3 stage (not subjected to proteomic analysis) and is therefore not represented here. It is also possible that the P3 extraction conditions resulted in increased degradation of collagen; the third stage sonication, for instance, may be partly responsible for the low sequence coverage and peptide identifications seen in Table 7.3. The apparently poorer P3 collagen quality and lack of isoform variety may, of course, be an artifact of non-specific binding to the thiophilic resin, but exactly why poorly preserved collagen is retained here, rather than for the P4 samples, remains unknown.

7.3.3(ii): TAC-retained non-collagenous proteins (NCPs)

Despite the lack of IgGs purified in the TAC columns, nLC-MS/MS analysis of the P3 and P4 elutions offers glimpses of the types of extant NCPs extracted from each stage. It

also facilitates characterisation of NCPs released from the bones that display differing histological preservation. Summary Tables 7.6-7.8 show the range of NCPs (not including exogenous keratin) detected at the second and third extraction stages of P3 for sample CD120.2 (HI: 5), and all P4 extraction stage for samples CD84.2 (HI: 0) and EH156.3 (HI:5). Information concerning the location and function of each protein was taken from the National Center for Biotechnology Information database (NCBI, 2014). Full results of these analyses are shown in Appendix 3. It should once again be recalled that these represent NCPs that have bound non-specifically to the TAC resin, rather than an entire bone proteome. Any differences in extracted protein types between CD84.2 and EH156.3 cannot be attributed to methodology or element type, since both were young adult cranial vault fragments which were prepared, extracted and analysed using exactly the same techniques. The only variables here are histological preservation and diagenetic factors specific to the sites. Many of the NCPs in this study were identified based upon one peptide (Tables 6.41-6.64). Jiang et al. (2007) only considered identifications based on more than two peptides to represent confident protein matches, since this would reduce the chances of false positives.

Name	Location	Function	Stage
Dermcidin	Epidermis	Immune	2nd pre
Dermcidin	Epidermis	Immune	2nd post
Terminal uridylyltransferase 4	Cellular	Gene silencing	2nd post
Dermcidin	Epidermis	Immune	3rd pre
Terminal uridylyltransferase 4	Cellular	Gene silencing	3rd post
Dermcidin	Epidermis	Immune	3rd post
Ankyrin repeat and SOCS box protein 18	Cellular	Protein modification	3rd post

Table 7.6: Type and function of non-collagenous human proteins extracted from P3 sample CD120.2 (HI: 5), 2nd and 3rd extraction stages, pre-and post-exclusion. Bold entries are likely exogenous contaminants.

Name	Location	Function	Stage
Dermcidin	Epidermis	Immune	1st
MUC19 variant 12	ECM	Mucosal	1st
Vitronectin	Plasma	Multifunctional	2nd
Cystatin-A	Intra/extra cellular	Protease inhibition	2nd
Protein S100-A7	Cellular/epidermis	Regulation/immune	2nd
Chondroadherin	Cartilage	Mediation	2nd
Pigment epithelium-derived factor	ECM	Multifunctional	2nd
Dermcidin	Epidermis	Immune	3rd
Vitronectin	Plasma	Multifunctional	3rd
Chondroadherin	Cartilage	Mediation	3rd
Pigment epithelium-derived factor	ECM	Multifunctional	3rd
Hornerin	Epidermis	Immune	3rd
Biglycan	ECM	Bone mineralisation	3rd
Prothrombin	Plasma	Coagulation	3rd
Biglycan preproprotein variant (Fragment)	ECM	Bone mineralisation	3rd

Table 7.7: Non-collagenous human proteins extracted from sample CD84.2 (HI: 0), their types and functions, and extraction stage. Bold entries are likely exogenous contaminants. ECM – extra cellular matrix.

Name	Location	Function	Stage
Protein argonaute-4	Cell cytoplasm	Gene silencing	1st
Protein AHNAK2	Cell nucleus	Interacts with DYSF, a skeletal muscle protein	2nd
cDNA, FLJ94754, highly similar to Homo sapiens potassium inwardly-rectifying channel, subfamily J, member 2 (KCNJ2), mRNA	Cell membrane	Channel	2nd
Pigment epithelium-derived factor	ECM	Multifunctional	3rd
Biglycan	ECM	Bone mineralisation	3rd
Vitronectin	Plasma/ECM	Multifunctional	3rd
Chondroadherin	Cartilage	Mediation	3rd
Alpha-2-HS-glycoprotein	Plasma	Multifunctional	3rd
Osteomodulin	ECM	Biomineralisation	3rd
Lumican	ECM	Multifunctional	3rd
Biglycan preproprotein variant (Fragment)	ECM	Bone mineralisation	3rd
Matrix Gla protein	ECM	Calcification inhibition	3rd
Prothrombin	Plasma	Coagulation	3rd
cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein	Plasma	Multifunctional	3rd
Dermatopontin	ECM	ECM interactions	3rd
cDNA, FLJ93532, highly similar to Homo sapiens osteomodulin, mRNA	ECM	Biomineralisation	3rd

Table 7.8: Endogenous non-collagenous human proteins extracted from sample EH156.3 (HI: 5), their types and functions, and extraction stage. ECM – extra cellular matrix.

Before considering any implications of the extraction of these NCPs, possible reasons why these particular proteins bound to the TAC resins should be explored. Protein extraction and proteomic analyses of ancient mammalian bones (e.g., Buckley et al., 2011; Cappellini et al., 2012; Wadsworth and Buckley, 2014) have indicated the survival of a vast range of NCPs in samples from differing periods and from multiple environments. The range of NCPs extracted and characterised in this study must represent a small fraction of the whole extracted protein content, a product of non-specific binding in the search for IgG. It is likely that some of these NCPs were retained due to their close association with collagen. Biglycan, vitronectin, chondroadherin, and lumican, for instance, all bind to, interact with, or form complexes with collagen (Schvartz et al., 1999; Mansson et al., 2001; Wiberg et al., 2002; Nitokovic et al., 2008). Their presence could be explained by cross-linkage or complexing with resin-bound collagen, and by their release during trypsin digestion prior to mass spectrometry. The complexing of collagen with other organic molecules seems to be a common diagenetic phenomenon that can mask lower-abundance proteins from detection (Brandt et al., 2002). Their extremely small size (often only one peptide) and any potential masking effects of cross-linked collagen may help to explain the difficulties experienced in NCP detection by SDS-PAGE, Western blot, or ELISA.

Hardouin et al. (2007) reported non-specific TAC binding of alpha-2-HS-glycoprotein (A2HSG) in modern serum samples, so it is perhaps unsurprising to find it here. Interestingly, they also found significant TAC binding of serum albumin. Its absence here is, therefore, somewhat unexpected, given its relatively high abundance in serum, affinity for hydroxyapatite, apparent affinity for thiophilic resin, and common appearance in other proteomic analyses (Xu et al., 2009). Albumin was the second most prevalent NCP detected by Jiang et al. (2007), and was extracted and characterised in ancient samples using the same extraction method (Wadsworth and Buckley, 2014). Finally, the proteins dermcidin, hornerin, MUC19 variant 12, and S100-A7 are potentially exogenously derived, likely introduced during sample preparation. Although their degraded state and level of modification (see Appendix 3) may support endogenicity, their association with epidermal or mucosal cells means that external contamination cannot be ruled out. Indeed, their degradation in itself may provide a reason for their binding to the TAC resins.

The small range of NCPs characterised here is similar to the much larger proteome extracted by Wadsworth and Buckley (2014) following the Jiang et al. (2007) protocol, in that the majority of identified peptides represent extracellular matrix (ECM) proteins. The two P4 samples yielded peptides of 13 distinct NCPs, six of which are found in the ECM, and three found in blood serum. No bone-specific NCPs were retained on the TAC resins, although several that are functionally associated with bone (e.g., biglycan, osteomodulin, vitronectin, and A2HSG) were detected. Wadsworth and Buckley (2014) suggest that archaeological serum and ECM proteins may be easier to extract and detect than bone-specific proteins due to their higher abundance, their affinity to hydroxyapatite, and possible post-mortem adsorption to bone surfaces. Future proteome analysis of pre-TAC samples CD120.2, CD84.2 and EH156.3 should provide a much more extensive picture of the range of NCPs extant in these samples. These analyses exceeded the timeframe for this project, but are planned for future investigation.

The relative lack of collagen in the P3 sample may offer an explanation for a corresponding dearth of NCPs, if the latter are being indeed preferentially retained on the resins due to cross-linking or complexing with collagen. Alternatively, the extracted P3 NCPs may have been less degraded than their P4 counterparts, and thus resisted cross-linkage with collagen and non-specific resin binding. A majority of NCPs in the P3 sample may also have been released in the first extraction stage (not subjected to nLC-MS/MS analysis), which, in terms of reagents used, corresponds most closely to P2 and P4 third stage.

The research has been unable to support Masters' (1987) hypothesis concerning the preferential survival of NCPs due to their affinity with hydroxyapatite (section 1.2, research question 2) for two main reasons. Firstly, the choice analysis of TAC purified samples precluded an assessment of full bone sample proteomes. Secondly, and perhaps most interestingly, is the complete absence of osteocalcin from any samples. Osteocalcin represents the most abundant non-collagenous protein in human bone, and has a very high affinity for hydroxyapatite (Smith et al., 2005); if any NCP should be expected to preferentially survive diagenesis, it is osteocalcin. Although the protein data here represents non-specific TAC binding, it should perhaps be expected that osteocalcin should be present.

Its absence may call into question Masters' (1987) original supposition (Matthew Collins, personal communication, October 2014).

7.3.3(iii): TAC-retained non-human proteins

A small quantity of non-human peptides were detected through nLC-MS/MS analysis of post-TAC P4 samples and the matching of resulting peptide sequences to the Swiss-Prot database. This approach was useful for the identification of exogenous proteins, such as those from soil bacteria, that may have infiltrated the bone samples during diagenesis, or even proteins associated with exogenous pathogens. A problem with this approach is that reliable protein identification relies upon peptide matching to species with known genomes. When extracted peptide sequences are searched against a genomic database, a 'best fit' result may be given (Lubec and Afjehi-Sadat, 2007). Identification can, therefore, prove somewhat ambiguous, particularly when peptide sequences are extremely limited in number, or highly modified. This explains the detection of organismal proteins of unlikely presence in archaeological human bone. An example of this is a homolog protein matched to *Methanocaldococcus jannaschii* (Table 6.42), a eukaryotic organism only found in extreme environments, such as ocean floor hypothermal vents (Bult et al., 1996). Another peptide was matched to *Trichodesmium erythraeum* (Table 6.45), a tropical ocean-dwelling species of cyanobacteria, while *Halorubrum lacusprofundi* (Table 6.45) can only be found in one Antarctic lake (Ng et al., 2000). These results clearly represent 'best fit' matches for the peptides on the Swiss-Prot database, since they are highly unlikely to be associated with archaeological human remains.

Proteins from organisms whose association with inhumed bone is more credible include species from localised environmental sources which have infiltrated the samples through taphonomic and diagenetic action. Table 7.9 displays these organisms and their likely sources. All are from P4 samples, and all were detected in the first extraction (HCl demineralisation) stage elutions. Their release at this early stage suggests that they were not mineral bound, potentially supporting an exogenous source. Two species, *Streptomyces griseus*, and *Caenorhabditis elegans*, are known as a soil-dwelling microorganisms (Wood, 1998; Ohnishi et al., 2008). *Synechocystis sp.* cyanobacteria was likely introduced into the bone by groundwater infiltration, while the *Lactobacillus casei*, *Methanosarcina barkeri*,

and *Equine herpesvirus* are associated with livestock (Kaneko and Tabata, 1997; Cai et al., 2009; Telford et al., 1992; Grigoriev et al., 2012). The presence of all these can be credibly explained by extrinsic environmental contamination.

Cronobacter sakazakii is a pathogenic bacterium that can cause wound and urinary tract infections in human adults, and can be particularly dangerous to neonates, the elderly, and the immunocompromised. These bacteria are regarded as ubiquitous, very hardy, opportunistic pathogens, but their exact source in the environment remains unknown (Healy et al., 2010). Although its presence in the soil cannot be ruled out, *Cronobacter sakazakii* may tentatively represent the only detected non-human protein that was present in the ante-mortem bone, if individual CD120 was infected at the time of death.

When interpreting the presence of these TAC-retained non-human proteins, it should be acknowledged that they all are represented by a single peptide, many of which exhibit very low sequence coverages (e.g., only 3.6% for the *Cronobacter*). As Baldwin (2004:2) states, "the greater the number of peptides being matched to any one protein and the greater the sequence coverage, the greater the probability of a correct identification", although it is possible that archaeological proteins and their species can be correctly identified based on single peptides (e.g., Buckley et al., 2010). Although only peptides with a 95% confidence match to known genomic sequences are considered here, previous studies (e.g., Jiang et al., 2007; Cappellini et al., 2012) have discounted protein identifications based upon single peptides. If the species identified here *are* accurate, then their retention was likely due to non-selective binding, or cross-linkage to other proteins. They may be representative of a fraction of the non-human proteins from the pre-TAC samples. Proteomic analysis of the pre-TAC samples may have yielded evidence supporting positive species identifications in the form of multiple proteins from single organisms.

Species	Type	Source	SC/Pep	Sample	Protocol/Stage
Streptomyces griseus	Bacterium	Soil	13.2/1	CD84.2	P4/1st
Lactobacillus casei	Bacterium	Livestock?	10.6/1	CD84.2	P4/1st
Caenorhabditis elegans	Non-parasitic roundworm	Soil	3.3/1	CD84.2	P4/3rd
Synechocystis sp.	Cyanobacteria	Freshwater	6.3/1	EH156.3	P4/1st
Equine herpesvirus 1	Viral pathogen	Livestock	6.1/1	EH156.3	P4/1st
Cronobacter sakazakii	Bacterial pathogen	?	3.6/1	EH156.3	P4/1st
Methanosarcina barkeri	Archaea	Livestock	8.5/1	EH156.3	P4/1st

Table 7.9: Credible non-human microorganisms detected by nLC-MS/MS in post-TAC P4 samples CD84.2 and EH156.3. SC/Pep – Sequence coverage (%)/Peptide number (95% confidence).

7.3.4: P3: adapted Schmidt-Schultz and Schultz protocol with TAC purification

The P3 methodology followed the same extraction protocol as P1 with the addition of TAC intended to purify target IgGs and reduce potential masking by more abundant proteins (e.g., degraded collagen). As mentioned in section 5.7.6, multiple SDS-PAGE trials failed to produce sufficiently visible, resolved colloidal Coomassie-stained bands in post-TAC P3 samples. At this stage, the aim was to produce such bands, since previous proteomic analysis of P2 silver stained bands had invariably proven unsuccessful due to insufficient protein concentration. Smearing in gels (pre-TAC P3 samples) suggests the extraction of a quantity of degraded collagen in the 3rd P3 stage, a supposition supported by post-TAC proteomic analysis of sample CD120.2 (Table 6.36-6.40). As previously mentioned, smearing due to degraded collagen is commonly experienced in SDS-PAGE analyses of ancient samples (Wadsworth and Buckley, 2014). However, if poor band resolution and smearing is also due to insufficiently separated and solubilised proteins, as suggested by Schmidt-Schultz and Schultz (2004), it suggests possible issues with the extraction and detection methodology. Keratin also proved to be a significant contaminant for P3 bands and samples subjected to proteomic analysis (Table 6.34), far more so than for the P2 and P4 samples. This is surprising, considering that the same measures were taken to minimise external contamination for all samples in all protocols (e.g., the use nitrile gloves, fume cupboard, fresh reagents). The reasons for this elevated quantity of keratin remain unclear.

7.3.5: P2, P3, and P4 Western blot

A Western blot test was run on selected P2, P3, and P4 samples following TAC, precipitation, and SDS-PAGE. The protocol differed slightly from the Western blot described, reported and discussed in sections 5.7.3(ii), 6.4.1(v), and 7.3.1(ii), respectively. This later Western blot test (section 5.7.9) employed a longer primary antibody incubation time and initial exposure time due to the lower concentration of protein in these post-TAC samples. Unfortunately, the test failed to produce any results other than background staining/contamination after a five minute exposure. That the positive IgG control failed to be detected may suggest a problem with protein transfer to the nitrocellulose membrane. It is possible that the small gel pore size in the 15% gel slowed the transfer of proteins and they were therefore not given sufficient time to completely transfer to the membrane. The very low concentration of protein in these post-TAC samples is also a likely reason behind the negative result. In hindsight, the use of a positive control in lieu of a molecular weight ladder in this test was a mistake, since the markers would have been of sufficient concentration to demonstrate the effectiveness of transfer from a 15% gel; it is unlikely that the samples and the IgG positive control were at high enough concentrations (only 10µl per sample well) to survive the unavoidable loss of proteins during transfer. The milk powder blocking may also have been overwhelming for the inevitably low signal anticipated for these low-concentration samples (Dr. Stephen Chivasa, personal communication, April 2014).

Finally, the type of membrane used in the test may have had an effect upon the result. Nitrocellulose membranes represent the cheapest and most commonly employed type. Alternatively, polyvinylidene difluoride (PVDF) membranes can be used. These offer a higher binding capacity and sensitivity, but at a higher cost. Nitrocellulose membranes usually have a pore size of 0.45µm pore size, which is makes them less suitable for transferring proteins smaller than around 20kDa. It is possible that highly degraded archaeological IgGs might ‘escape’ through these larger pores during transfer, although this would not explain the non-detection of the positive control in this test.

Future Western blotting of post-TAC samples would benefit from attempting to increase protein concentration within the samples. This should be followed by comparing blots from 10%, 12%, and 15% gels, all with a molecular weight ladder and a positive

control in place. A comparison of results using different membrane types (nitrocellulose and PVDF) with differing pore sizes would also be informative. It is evident from both Western blot tests attempted in the study that the characterisation of degraded ancient proteins in this, as in all other attempted methods, presents a considerable challenge which requires systematic trial-and-error testing.

7.3.6: P5: adapted Cappellini et al. (2012) extraction protocol

The final extraction method attempted in this study was an adaptation of the groundbreaking protocol of Cappellini et al. (2012). This research successfully extracted non-collagenous proteins (NCPs), including IgG-related peptides from Pleistocene mammoth bones from permafrost and temperate environments. These proteins were identified through highly sensitive nanospray ESI-LC-MS/MS analysis following a simple extraction protocol, and protein concentration through the use of custom-made C-18 stage tips. This latter step was important in its novelty: the stage tips provided a rapid and simple method of concentrating peptides, while also removing potentially interfering salts from the final extraction buffer. Crucially, the mass spectrometric analysis demonstrated the presence of three peptides related to immunoglobulin G, as shown in the constructed human STRING network of orthologous mammoth proteins (Cappellini et al., 2012). That the Wadsworth and Buckley (2014) and Jiang et al. (2007) extractions and analyses failed to detect immunoglobulin-related peptides suggests that the Cappellini et al. (2012) extraction protocol may be better suited to their specific extraction.

A potential difficulty in replicating this research using the human bone in this study is that Cappellini et al.'s (2012) most successful extractions, in terms of recovering peptides, were made on Siberian samples. These permafrost-preserved samples yielded up to three times the protein of bones from temperate settings, thus confirming the importance of temperature in protein diagenesis and loss. Their research indicates that IgG may not preserve particularly well in mammalian remains from temperate regions, although it should be remembered that their samples were considerably older than the samples selected for this study.

A further complication in applying the Cappellini et al. (2012) protocol was that this study aimed to extract immunoreactive IgGs from archaeological bone in order to test them

against malaria antigens. As mentioned in section 5.7.11, the published protocol was not suitable for the gentle extraction of IgG, since the heating stage would completely denature the target protein, thus rendering the samples incompatible with TAC purification. Adaptation of the Cappellini et al. (2012) protocol in order to maximise the recovery of IgGs may require a lower second stage incubation temperature, possibly for a longer duration, to avoid heat-induced denaturing. Wiechmann et al. (1999) advocate the heating of samples at 55°C for 48 hours in order to facilitate the separation of ancient proteins, although this is during sample preparation for SDS-PAGE, rather than during extraction. However, since Vermeer and Norde (2000) demonstrated that whole IgGs quickly denature above 60°C, Wiechmann et al.'s (2000) 55°C incubation seems close to the limit of IgG stability, especially since the thermal stability of degraded, archaeological IgGs is unknown. Analysis of extractions following incubation at different temperatures may help to establish an optimal temperature for releasing IgGs into solution.

The high concentration of degraded collagen resulting from this extraction was, as expected, evident in the P5 SDS-PAGE gels (Figures 6.63-6.65). These display significant smearing for all extraction stages, which serves to obscure many bands of potential interest. Cappellini et al. (2012) encountered a masking of lower abundance proteins by collagen smearing in their 1D SDS-PAGE gels. They suggest that their results may be improved by the removal of collagen prior to analysis. Since collagenase has been demonstrated to deplete the NCP content of archaeological samples (Wadsworth and Buckley, 2014), the removal of excess collagen though immunoaffinity chromatography may remain the best solution for future analyses.

7.4: Observations on archaeological IgG purification and characterisation methods

As expected from examination of published attempts to extract and characterise non-collagenous biomolecules from ancient human remains (e.g., Grupe and Turban-Just, 1996; Wiechmann et al., 1999), all clinically-derived techniques require adaptation to account for the inevitably degraded nature of archaeological proteins. This is reflected in the adjustments made throughout the extraction protocols in this study. Although IgG was not conclusively identified in any samples in this study, results from attempts to purify, detect, and characterise them offer insights into the reasons behind this failure.

7.4.1: Thiophilic adsorption chromatography (TAC)

The failure of this study to successfully purify archaeological IgGs may call into question the efficacy of TAC for this purpose, despite its high affinity for the protein and gentle elution conditions. There a number of reasons why TAC may have been unsuccessful. Firstly, TAC is untested for use with archaeological samples. Despite its high affinity for fresh IgG, their ancient counterparts may be too degraded or altered during extraction to bind to the thiophilic support. The non-specific binding of collagen and certain NCPs is unsurprising, considering that Hardouin et al. (2007) experienced similar interactions in fresh biological samples. Non-specific binding may have been increased by the degraded nature of the extracted proteins, with the presence of single-peptide NCPs perhaps indicating their complexing/cross-linkage with resin-bound collagen. It may be encouraging that the eluted yields of resin-bound non-specific proteins were always extremely small, since a low protein concentration may reduce potential masking of target IgGs.

Secondly, the extraction buffers and their reagents may not be fully compatible with the resins. An example of this is guanidine-HCl (GuHCl), a chaotropic salt which may adversely affect the binding of IgG to the thiophilic support. The strong denaturing effect of GuHCl may also have caused extracted IgGs to aggregate with other proteins, thus negating their affinity to the resin. Once again, TAC manufacturers are unlikely to have tested the efficacy of thiophilic resins in the presence of the numerous chemical reagents used in this study. Future investigations could test the effect of these reagent used here on thiophilic resin IgG retention by testing solutions containing known IgG concentrations.

Thirdly, and perhaps most tellingly, is the complete lack of IgG-related peptides in any post-TAC sample analysed by nLC-MS/MS. This may suggest either an insufficient protein concentration for detection on the MS equipment (discussed in section 7.4.4), or that the chosen extraction protocols failed to release extant IgGs, resulting in no purification via TAC. The presence of IgG-related peptides in pre-TAC samples could, in future experiments, be assessed by mass spectrometry in order to screen out negatives prior to TAC purification.

7.4.2: SDS-PAGE

SDS-PAGE is a standard analytical tool employed in many fields, including biochemistry, genetics, and molecular biology. The early SDS-PAGE gels in this study (for P1, P2.1, and P2.2) were run by laboratory personnel in the Biological Sciences Department at Durham University following standard procedures used for modern samples (see sections 5.7.3(i) and 5.7.6). As can be seen in sections 6.4.1 and 6.4.2, resulting gels all display a significant level of smearing. This is particularly evident on the P1 silver stained gels. Such smearing serves to obscure bands that may potentially represent low abundance NCPs, such as IgGs, and is likely indicative of the high concentration of degraded collagen in these samples. It is possible that the highly acidic nature of the 20% trichloroacetic acid (TCA) precipitation exacerbated this smearing by further degrading large collagen molecules into smaller fragments. The low precipitation temperature (-20°C) may reduce the hydrolysis of collagen in fresh samples, but the effect on ancient degraded collagen is uncertain here. These early gels also display strongly concentrated bands at the interface between stacking and resolving phases. There are a number of reasons why this apparent failure of proteins to enter the resolving gel may occur. These include:

1. Single proteins or aggregated protein complexes are too large to enter the gel. This can be solved by decreasing the concentration of polyacrylamide to create gels with large pores, thus allowing entry of larger molecules. However, this increases the risk of losing proteins of smaller molecular weight from the gel.
2. Membrane-associated proteins, which may include certain collagen isoforms (e.g., Hagg et al., 1998), stack together with other proteins during electrophoresis, yet fail to enter the gel or fail to resolve into distinct bands (Hames, 1998).
3. Human error in hand-casting of gels. For instance, incorrect buffer pH can prevent many proteins from entering the resolving gel. This use of precast gels would provide a solution to this, although they are an expensive option.
4. The obstruction of proteins from entering the gel or efficiently migrating by "large and cross-linked organic substances in the extract, such as degraded collagen, mucopolysaccharides, and humic substances" (Wiechmann et al.,

1999:391). The presence of these substances is difficult to mitigate, although the extended heating of ancient samples in buffer, as advocated by Wiechmann et al. (1999), may reduce this problem.

Following the P1, P2.1, and P2.2 gels, it was decided to run further SDS-PAGE analyses in-house. This allowed for greater flexibility in terms of trialing different sample preparation techniques and buffer/gel recipes in order to optimise the protocol for characterising ancient IgGs. The introduction of a modern positive control was crucial at this stage, since it allowed for evaluation of the effects of different preparatory techniques on reduced IgGs. As mentioned in Section 4.4.4, SDS-PAGE has quite frequently been employed in attempts to separate and characterise ancient proteins, and therefore offers a level of standardisation unusual for most clinical protocols that have been adapted for archaeological studies. The choice of appropriate preparation and gel production methodologies can depend upon the target protein of interest. No published archaeological studies using SDS-PAGE have *specifically* attempted to identify only IgG, although Schmidt-Schultz and Schultz's (2004) protocol was apparently successful in detecting the protein (Figure 6.36).

The first stage of SDS-PAGE protein characterisation is sample preparation prior to introduction into the gel, which usually consists of precipitation and subsequent denaturation of proteins in appropriate reagents and buffers. Commonly used reagents that precipitate a wide variety of proteins include trichloroacetic acid (TCA), acetone, or high-salt reagents (e.g., ammonium sulphate). Although the latter is commonly employed in the clinical precipitation of IgG, often resulting in high yields (Page and Thorpe, 2002), it was not attempted in this study due to the comparative technical difficulty of the methodology, the potential interference of residual salt with downstream analysis, and the success of Schmidt-Schultz and Schultz (2004) in identifying IgG through SDS-PAGE (following TCA precipitation) and Western blot. As the use of a modern positive control in this study demonstrated that both acetone and TCA precipitation resulted in detectable IgG, it was assumed, perhaps naively, that either method would also work for ancient IgGs. It would, however, be useful to attempt ammonium sulphate precipitation in future attempts to characterise ancient IgGs through SDS-PAGE.

Protein precipitation is a complex phenomenon which relies on the aggregation of proteins by hydrophobic interaction with the chosen solution. Formation of a protein pellet through aggregation, which can be subsequently solubilised into SDS sample buffer, requires a sufficiently high sample protein concentration (Hames, 1982). Samples of low protein concentration (e.g., the post-TAC samples in this study) may not efficiently form pellets, resulting in loss of protein. Chemicals within the sample may also aggregate along with any protein, such as was encountered with acetone precipitation of post-TAC samples. This inevitably resulted in large phosphate pellets, which were very difficult to solubilise in SDS sample buffer, thus resulting in a decreased quantity of pelletised proteins. Precipitation, therefore, inevitably leads to a certain degree of protein loss. The process has been described as 'inefficient' for degraded archaeological proteins by Cappellini et al. (2012), who avoided it in favour of stage tip purification. Precipitation may not, therefore, represent the most efficient method of concentrating low-abundance archaeological proteins (e.g., NCPs), since loss of even a small portion of these would detrimentally affect efforts to characterise them. This inefficiency may provide a reason for the lack of IgGs detected through SDS-PAGE or Western blot in this study.

The second stage for SDS-PAGE is preparation of an appropriate sample buffer into which the precipitated protein pellet can be solubilised. Most published archaeological SDS-PAGE protocols follow a recipe based on Laemmli (1970), which includes a reducing agent (usually DTT or mercaptoethanol), glycerol, tris, and SDS (e.g., Freundorfer et al., 1995; Grupe and Turban-Just, 1996; Wiechmann et al., 1999; Brandt et al., 2000; Ostrom et al., 2000). Many employ a higher concentration of SDS in order to increase denaturation of potentially cross-linked proteins. For this study, it was decided to use and adapt a sample buffer recipe constructed by Page and Thorpe (2002), which is optimised for SDS-PAGE characterisation of fresh IgGs, and adapt it by increasing the concentration of SDS in line with archaeological protocols. This adapted buffer used bicine and sucrose, rather than glycerol, since it reportedly results in clearer resolution of IgG light chains (Page and Thorpe, 2002). A number of archaeological protocols use iodoacetamide in their sample buffers. This powerful cysteine inhibitor helps to maintain the reduced state of proteins following boiling of samples in SDS buffer. Although not used in this study due its absence in the Page and Thorpe (2002) recipe, further trials with archaeological samples could

include iodoacetamide to assess its efficacy and allow for comparisons between methodologies.

Wiechmann et al. (1999) advocate the extended heating of samples (at 55°C for 48 hours) in SDS buffer in order to destroy products of decomposition, such as protein cross-links, and to free proteins that might be encapsulated by humic substances. They suggest that the traditional method of boiling samples for a short period fails to result in adequate separation of ancient proteins. A comparison of the extended heating and traditional short boil approaches was attempted with three P2.3 post-TAC samples (section 5.7.6(ii)). Although the gel required silver staining for visualisation (section 6.4.2(iii), Figure 6.54), it clearly shows that the quick-boiled sample produced stronger bands. This suggests an increase in protein loss for the samples exposed to longer heating, possibly due to heat-associated degradation or associated increase in protease activity. Since proteomic analysis failed to identify any peptides in the excised bands (Figure 6.55), it is difficult to assess exactly how Wiechmann et al.'s (1999) extended heating might affect archaeological NCPs. The apparent loss of proteins through extended heating and the success of Schmidt-Schultz and Schulz (2004) in characterising IgGs after traditional sample boiling influenced the decision to continue with the traditional method.

After multiple trials utilizing slightly different precipitation techniques and buffer/gel recipes, it was found that acetone precipitation followed by introduction into a 5% stacking/15% resolving gel resulted in the clearest bands for IgG (positive control) heavy and light chains. However, as the acetone precipitation proved incompatible with post-TAC eluted samples (due to high salt content), 8% TCA precipitation was instead adopted (after Schmidt-Schultz and Schultz, 2004). The majority of post-P2.2 gels presented in chapter 6 used 8% TCA precipitation and a 15% resolving gel. This configuration generally resulted in the clearest bands of both the positive control and resolved ancient proteins.

The main problem encountered using SDS-PAGE in this study was the difficulty in producing a high enough protein yield for visualisation using Coomassie staining. Attempts at proteomic analysis (e.g., section 6.4.2(iv)) of silver stained bands often proved fruitless due to the extremely low concentration of proteins in post-Coomassie silver stained bands. It is likely that the low protein concentration in the eluted TAC samples is mostly responsible for this problem, although the inevitable loss of protein associated with inefficient

precipitation and loss and degradation during gel electrophoresis surely contributed. The latter may be suggested by nLC-MS/MS results (Table 6.31) showing relatively low peptide sequence coverage of the excised P2.4 IgG positive control bands; one might expect these fresh antibodies to show less degradation and greater sequence coverage, although the IgG fractions did remain at the expected molecular weights (approximately 50 and 25 kDa). The fact that modern IgG subjected to SDS-PAGE showed this much peptide fragmentation may indicate that the method (at least as attempted here) is somewhat unsuitable for visualizing already-degraded archaeological IgG.

The problem of matching degraded IgG fractions to their modern IgG counterparts was shown in a lack of successful detection using proteomic analysis of selected gel bands. Following Schmidt-Schultz and Schultz's (2004) supposed detection of ancient IgGs at a molecular weight approximating 55-60kDa, it was assumed that proteomic analysis of excised bands at or around this weight range would produce positive results. It is, however, more likely that any extracted IgGs would survive in a degraded state of uncertain molecular weight. This would confound attempts to identify them in excised bands matching a positive control, as can be seen in this study by the detection of keratin or collagen at weights matching the IgG positive control. If ancient IgG was present in these bands, then it was probably either masked by more abundant proteins, or at too low a concentration for proteomic detection.

The experience gained in attempting to tailor a methodology for successful SDS-PAGE characterisation of ancient IgGs has led to the conclusion that the technique is probably not entirely suitable for detecting these extremely low-abundance proteins. This is mainly due to the difficulties in concentrating the target proteins to a suitable level for visualisation and downstream analysis, combined with the inevitable protein loss during sample preparation and electrophoresis, and uncertainties concerning the molecular weight of degraded target proteins. Masking by more abundant proteins, such as collagen, is also a potential issue. A possible way forward for SDS-PAGE of archaeological IgGs would be in combination with Western blotting and subsequent proteomic analysis of any positive acquired, if an initially sufficient protein concentration could be achieved.

7.4.3: Western blot

Western blotting, like SDS-PAGE, is a commonplace analytical technique in many modern biological fields, and although employed less frequently for archaeological samples, it can potentially detect very low abundance proteins not necessarily visible by post-SDS-PAGE gel staining. The use of conjugated antibodies to interact with target proteins increases detection sensitivity far above that of traditional gel staining. The process is also reasonably straightforward, with few changes to established techniques required to adapt standardised Western blotting protocols to archaeological samples. The most important of these adaptations is in the choice of primary and secondary antibodies, which should be dictated by the nature of the target protein, taking into account the likely presence of potential sources of cross-reactivity in the samples.

On the surface, Western blotting should provide the ideal technique for detection of ancient IgGs, since it should allow for detection of degraded fractions of unknown molecular weight, thus eliminating a major problem with SDS-PAGE characterisation, discussed above. This assumes, of course, that IgGs have been successfully extracted, precipitated, electrophoresed, transferred during blotting, and detected by the primary antibody. Unfortunately, these numerous stages present a myriad of opportunities for protein loss and degradation beyond detectability, and it is perhaps not surprising that only one potential positive was detected in this study (section 6.4.1(v)). As discussed above (section 7.3.1(ii)), this single detection represents the most likely true positive from any other presented here. The gel was not retained by the Biological Sciences department for further analysis, which is unfortunate since this early sample (from P1.3) may have been of sufficiently high concentration for proteomic confirmation. Later Western blotting of P2, P3, and P4 post-TAC samples failed to yield any positives (section 6.4.6), possibly due to inefficient transfer of proteins from a high percentage gel, or protein degradation beyond the point of antibody interaction.

7.4.4: Proteomics

Recent proteomic technological advances and methodological refinements have produced new insights into the vast range of NCPs extant in archaeological mammalian bone (e.g., Cappellini et al., 2012; Wadsworth and Buckley, 2014). However, attempts at

characterising ancient bone proteomes have had varied success in terms of detecting IgG-related peptides. As previously mentioned, the detection of specific NCPs is heavily dependent upon the choice of extraction and purification methodologies. The technology utilised in their accurate detection and characterisation is of no less importance. These factors can both be seen in Table 7.12 (section 7.7) which summarises protocols which have reportedly extracted and detected archaeological IgG. Wadsworth and Buckley (2014) analysed archaeological bone proteomes using equipment of similar specification to that used in this study (see section 5.7.3(v)), following the Jiang et al. (2007) extraction and sample preparation protocols. The failure of the three studies to detect IgGs suggests that the extraction or purification methods used were unsuited to the task and/or that the MS equipment used was insufficiently sensitive to detect the target peptides. Cappellini et al. (2012) identified IgG-related peptides using MS equipment of higher resolution and sensitivity, following a relatively simple extraction protocol and purification by customised C-18 tips. Despite the apparent identification of NCPs based on single peptides in this study, the MS equipment employed may not have been appropriately sensitive to detect fragmented IgG peptides in post-TAC samples (Adrian Brown, personal communication, April 2014). As can be seen in Appendix 3, most single-peptide protein identifications required a sequence of at least eight amino acids. It is possible that IgGs were fragmented into smaller sequences and were not identified by the MS equipment.

This study has shown that MALDI-TOF may not be an ideal method for detecting low-abundance proteins in ancient samples, particularly when excised SDS-PAGE bands are analysed. As can be seen in Table 6.30, which shows the results of MALDI analysis of selected colloidal Coomassie-stained P2.4 gel bands, the analysis failed to detect any peptides in two of the six tests. This is likely due to the low concentration of proteins in the bands, other than the IgG positive controls. MALDI analysis also failed to detect any proteins in excised silver stained bands from post-TAC samples. Since MALDI uses extremely small samples sizes and usually requires at least 10 peptides for identification, many excised gel bands evidently contained insufficient proteins for detection (particularly of NCPs, many of which were identified by nLC-MS/MS based on far fewer peptides than required for MALDI). This likely explains the failure of MALDI to identify proteins in the excised bands from the P1.2 (section 6.4.1(iii)) and P2.3 (Section 6.4.2(iv)) gels. SDS-

PAGE gel band analysis may not, therefore, represent the most ideal format for detecting ancient proteins.

As mentioned in section 7.2.4, it is possible to create exclusion lists following nLC-MS/MS. These are lists of peptides resulting from sample analysis which are ignored by the MS equipment during subsequent analyses of the same samples, thus increasing the chance of detecting low-abundance proteins. The creation of such lists is extremely time-consuming, especially for samples which contain many different protein isoforms (slightly different forms of the same protein), each of which requires manual entry onto the list. This was attempted for P3 post-TAC sample CD120.2 (Tables 6.36-6.40), and proved relatively effective in 'screening out' much of the collagen (Table 6.47) and improving the detection of NCPs (Table 7.6). Considering the failure of the Jiang et al. (2007) protocol to detect IgG-related peptides from fresh bone, and the time constraints of the project, it was decided not to create exclusion lists and reanalyse the six P4 samples.

High resolution proteomic analysis may provide the first proof that IgGs survive within a chosen sample. With a known positive, extraction methodology could be refined in an attempt to isolate IgGs without reducing them to the peptide level. This is the goal for studies such as this, which require the extraction of immunoreactive IgGs to test against pathogenic antigens. Prior proteomic confirmation of the presence of IgG would also aid in the verification of any positive immunological results.

7.5: Malaria ELISA

The second ELISA to be run in this study was the Malaria Total Antibody EIA (Lab 21), which is designed to detect IgM, IgA, and IgG antibodies to all human malaria species. Selected samples from the P3, P4, and P5 protocols were tested, alongside controls in the form of protocol buffers, ultrapure water, and animal bone samples (Table 6.60). The P3 and P4 samples were TAC elutions with known protein concentrations. Samples were run in duplicate with average absorbance above 0.382 indicating a positive reaction. Before assessing the results, it should be stated that that the manufacturer guidelines suggest that negative readings should be less than 0.080 (with a maximum cut-off value of 0.180) for the test be considered valid. Table 6.60 shows that in this case, both negative control readings were significantly higher than recommended, giving a cut-off of 0.382. As none of the

buffer controls and a vast majority of the samples registered lower than the recommended maximum cut-off value (0.180), it is possible that the established cut-off value remains valid for this test. However, in light of the potentially high cut-off value, caution should be taken when interpreting the results.

Table 7.10 shows that a small number of positives were recorded for this ELISA. While it is potentially encouraging that the vast majority of positives are from human samples, the occurrence of a positive for an animal control (HPAN5) suggests the presence of an interfering contaminant that has cross-reacted with the anti-malaria antibodies. Placental mammalian antibody structure shows little variation across the clade, so it may be the case that extracted antibodies in sample HPAN5 have degraded or been modified into a form that cross-reacted with the test antigens. The human samples most likely to have produced true positives are CD165.1 and EH133.1 (Table 7.10), since the duplicates are relatively close in value. The other human positives demonstrate a large difference between duplicates (e.g., CD112.2), suggesting either contamination or cross-reactivity. This demonstrates the importance of running ancient samples at least in duplicate to help rule out false positives. Since both EH133.1 and CD165.1 present values close to the cut-off, however, the 'true' positives should be treated with caution.

Sample	Type	HI	Protocol	Stage	Protein conc. (mg/ml)	First Read	Second Read	Average Absorbance
Positive	Control	-	-	-	-	**2.639**	**2.53**	**2.585**
CD165.1	Human	5	3	3	0.112	0.358	**0.57**	**0.464**
CD112.3	Human	2	4	3	0.059	0.202	**2.08**	**1.141**
EH156.3	Human	5	4	3	0.044	**0.839**	0.169	**0.504**
EH133.1	Human	1	5	1	-	**0.396**	0.319	0.358
OL1104.2	Human	5	5	2	-	0.252	**1.067**	**0.66**
HPAN5	Animal	5	5	2	-	**0.696**	**0.521**	**0.609**

Table 7.10: Positive results from malaria ELISA. HI - histological preservation. Stage is extraction stage. Cut off value is 0.382. Positive samples when read at A_{450} in bold.

The type of ELISA employed here *may* be more suitable for use with ancient samples than the IgG ELISA discussed above. The use of a secondary polyclonal antibody in the earlier IgG test (Figure 7.3) increases the IgG binding capacity, as the antibody is capable of recognizing many epitopes on the bound sample IgGs. Yet this would also increase the prevalence of reactions with non-specific molecules in the degraded sample

(Brandt et al., 2002). The malaria sandwich ELISA does not rely on a secondary polyclonal antibody (Figure 7.4) to detect the bound sample IgGs, thus theoretically reducing the chances of cross-reactivity. The well plates are coated with antigens, which should specifically bind to antibodies in the samples, which in turn should also bind specifically to the conjugated secondary malaria antigens. However, the potential for nonspecific binding of the antigens to other proteins present in the samples should not be discounted. Degraded collagen, for instance, is capable of forming complexes with other proteins, or humic substances. These complexes can either confound antibody/antigen reactions by blocking epitope/paratope interactions, or can cause nonspecific cross-reactions (Brandt et al., 2002). Either possibility cannot be ruled out in this instance. The human 'positives' encountered here for CD165.1 and EH133.1 could be supported by future independent ELISA and analysis of different skeletal elements.

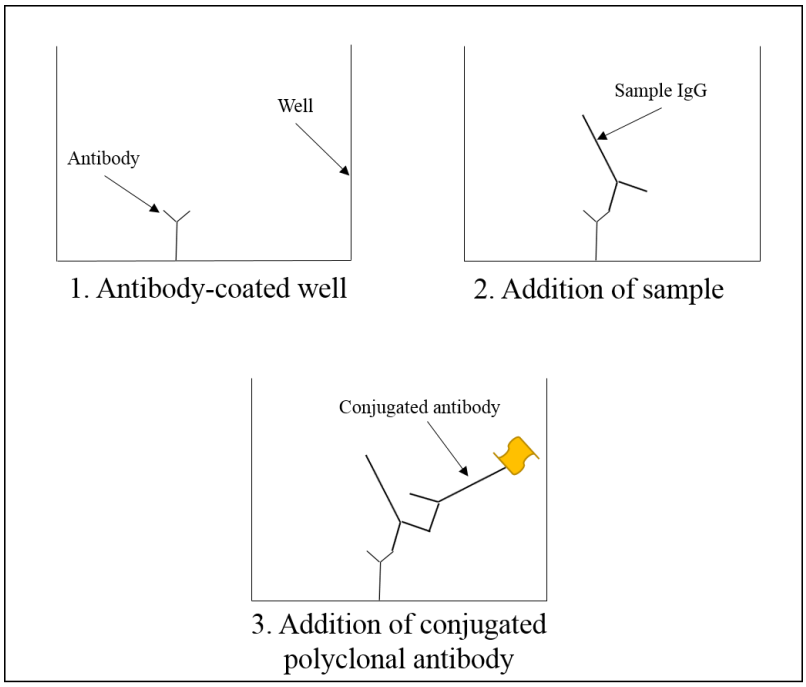

Figure 7.3: IgG ELISA. Unreacted material is removed by washing between stages.

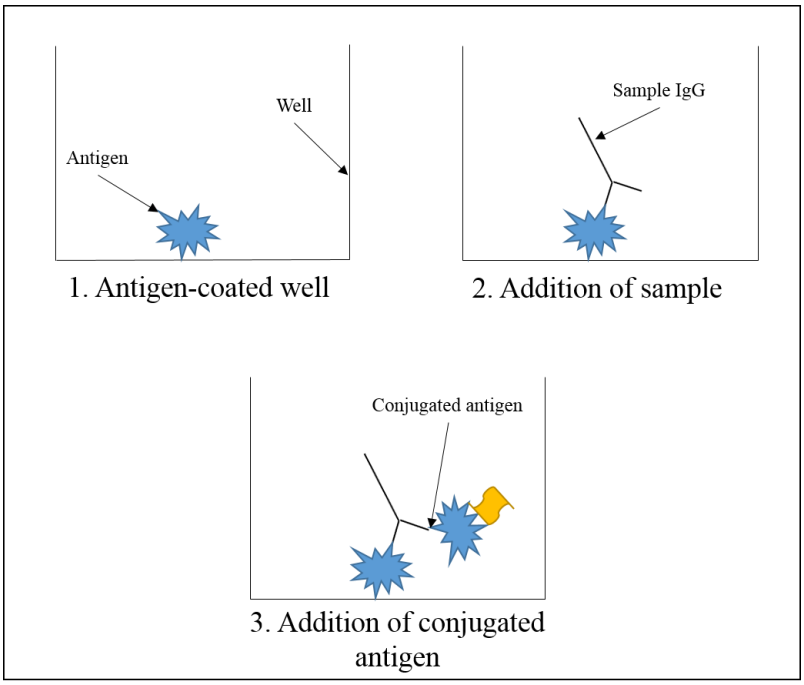

Figure 7.4: Malaria sandwich ELISA. Unreacted material is removed by washing between stages.

7.6: Malaria rapid testing

The lack of any positive rapid test results from the Fornaciari et al. (2010) protocol and P5 samples most likely suggests that the malaria antigen was not present in any of these samples, and that reagents used in these protocols do not induce cross reactions with this particular RDT. However, if the antigen was present, its concentration may have been too low to register a positive. Since RDTs are designed to be used on modern, fresh serum samples, they only require relatively low sensitivity levels. Surviving antigens in archaeological bone may not be of a high enough concentration to be detected by rapid testing.

The positive P2.5 results (see Table 6.61) may tentatively suggest evidence of malaria infection in the selected individuals. These results must, however, be considered with extreme caution. Cross contamination resulting in false positives must first be ruled out. These may arise from a number of sources which interact unexpectedly with the RDT antibodies. These sources may include the chemical reagents used in the preparation of samples, degraded endogenous and exogenous biomolecules, or extrinsic soil-based components that have infiltrated the bone (Brown and Brown, 2011). As with all clinical

immunological tests, RDTs are not designed to be used to detect ancient, degraded proteins. Neither are they evaluated for cross reactivity against biomolecules found in association with ancient samples, or against the range of chemical reagents used in archaeological protein extraction (Brandt et al., 2002). The polyclonal nature of the RDT antibodies used to detect multiple species of malaria may only serve to increase the likelihood of cross-reaction with non-specific targets. It should also be stated that none of the 'positives' encountered here developed within the manufacturer-specified 20 minutes following testing, with many of them taking up to 24 hours to register positive. By manufacturer standards, none of the results should be considered true positives. The extended development time of the positives may, of course, be an artifact of low antigenic (be they specific or non-specific reactions) concentration.

	Fornaciari et al. (2010)	**P2.5**	**P5**
Stage 1	Phosphate buffered saline	0.6M HCL	0.5M EDTA
Stage 2	-	100mM Tris; 6M guanidine-HCl; 5mM benzamidine; 10mM aminocaproic acid	50mM ammonium bicarbonate
Stage 3	-	100mM Tris; 6M guanidine-HCl; 0.5M EDTA; 5mM benzamidine; 10mM aminocaproic acid	0.6M HCl

Table 7.11: Comparison of reagents used in the three RDT protocols. Stage – extraction stage.

The first potential source of cross-reactivity to be ruled out is chemical. Table 7.11 summarises the reagents used in each protocol. As Table 6.61 and Appendix 2 Figures A2.7 and A2.8 demonstrate, P2.5 positives only resulted in samples from the first and second extraction stages. The first stage utilised only 0.6M HCl to demineralise the samples. It is interesting that a number of the samples from this stage produced positives, while none from the P5 third stage (also 0.6M HCl) samples reacted positively (Figure A2.9). This suggests that HCl is not a source of reactivity. The fact that reactivity is sporadic between samples from the first and second extractions suggests that that none of the reagents caused cross-reactivity, as does the lack of positives recorded for the third extraction stage and for the post-TAC P2.5 samples (Figure A2.10). This latter group were very unlikely to register as

positive, since any extant antigen should not bind to the thiophilic resin, and would therefore not be eluted from the columns.

Could these intermittent reactions recorded in the first two P2.5 extraction stages be suggestive of true malaria positives, despite their delay in development far beyond the recommended 20 minutes? LP3819D, a sample of powdered dentine, is interesting. Dentine is slightly more highly mineralised than bone (Goldberg et al., 2011) and *may* potentially afford increased protection to any surviving malaria antigens over bone samples. Unfortunately, histological preservation was not assessed for this sample. The nature of the positives for the Littleport samples and the collagen control sample may, however, be suggestive of cross-reactivity. None of the Littleport samples produced positive results outside of the P2.5 protocol. Given the lack of corresponding positives, it is more likely that an unidentified contaminant present in the bone was released during first two P2.5 extraction stages that was perhaps not released in the other two protocols. This may be the very same substance as mentioned in section 7.2.3. A similar contamination scenario could explain why sample HP154.1 failed to test positive in the Fornaciari et al. (2010) protocol tests, yet registered positive following the P2.5 protocol.

Positive reactions showing mixed malaria infections for the LP3760.1 and LP4585.3 samples (Table 7.11) also point to cross-reactivity, since it is rather implausible that these individuals would have died while infected with both tropical *P. falciparum* and temperate *vivax* malaria. As the collagen control tested using the Fornaciari et al. (2010) protocol failed to elicit a false positive, it seems unlikely that the positives encountered in the P2.5 tests can be attributed to cross-reactivity with intact collagen. It is somewhat surprising, then, that the collagen control sample (Figure A2.11) from the second P2.5 stage tested positive. It is possible that the test reacted specifically to well-preserved bovine collagen, rather than the degraded human collagen. Beyond this possibility, it remains unclear why this cross reaction occurred.

A positive rapid test result here would require the survival of circulating antigenic biomolecules in the bone samples. In order for the tests to detect *Plasmodium vivax* infection, survival of the *vivax*-specific lactate dehydrogenase enzyme (LDH) in the bone samples would be necessary. No published research has investigated the extent to which this enzyme may persist in archaeological bone, if at all. Unlike anti-malaria antibodies, LDH

does not persist in the body following the clearance of parasites (Iqbal et al., 2004). The patient would, therefore, have to have perished during, or within approximately three weeks (Kakkilaya, 2003) of, infection for the antigens to remain in the circulatory system upon burial. While it is possible that at least some of the positives encountered in the P2.5 rapid tests represent true malaria infections (LP3819D being the most likely), the chances of sampling an individual with surviving LDH are remote. Clinical immunological tests that depend upon antigen/antibody interactions should be supported by secondary, confirmatory testing in order to verify claims of positive reactions (Child and Pollard, 1992; Brandt et al., 2002). Proteomic testing of the positive samples encountered here are planned for the near future, since it was beyond the timeframe for this project.

7.7: Archaeological IgG as a target biomolecule: combining past and present for a brighter future

Do the results of this study and the history of research aimed at extracting archaeological IgGs support Cattaneo et al.'s (1992) conclusion that ancient IgG represents a poor choice for biomolecular analysis? Their early research is one of the few published attempts at specifically extracting immunoreactive immunoglobulins from archaeological bone, and their conclusion may have led to a dearth of similar research until Kolman et al.'s (1999) and Schmidt-Schultz and Schultz's (2004) apparently successful extraction and detection of IgGs. A comparison of the Cattaneo et al. (1992) methodology with more recent attempts at extracting IgGs highlights the evolution of the technique and offers potential lessons for such future extractions (Table 7.12). It also provides insights into the likely reasons why Cattaneo et al. (1992) struggled to isolate IgG from their samples. Their cellulose-based filtration stage, for instance, probably bound and removed many extracted IgGs before detection (see Walsh and Coles, 1980). Even prior to this, mechanical sample grinding and extraction without protease inhibitors may have severely degraded extant IgGs. Their methodology also seems to assume that diagenesis would affect their target molecules (albumin and IgG) equally, and that their chosen extraction methodology was suitable for both types. Subsequent research into diagenesis, protein survival and extraction techniques has led to a growing appreciation of the importance of adapting methodologies depending

upon the choice of specific protein target (Cleland et al., 2012). Table 7.12 compares four techniques that have reportedly extracted and detected IgGs or IgG-related peptides and a proposed new methodology based upon the published research, and this current study (discussed below, section 7.8).

	Cattaneo et al., 1992	**Kolman et al., 1999**	**Schmidt-Schultz and Schultz, 2004**	**Cappellini et al., 2012**	**2014**
Elements tested	Vertebral body	Femur	Long bones/cranium	Femur	Various
Histology	No	No	Yes	No	Yes
Sample size	10g	15g	1g	75mg	100mg
Grinding	Mechanical	Mechanical	Mechanical under nitrogen	Hand powdered	Hand powdered under nitrogen
Low temperature	Partly	Yes	Yes	Partly	Partly
Demineralisation	EDTA	EDTA	EDTA	EDTA	EDTA
Solublisation	No	No	Guanidine; sonication	Ammonium bicarbonate	Ammonium bicarbonate; sonication
Protease inhibition	No	No	Yes	No	Yes
Dialysis	Yes	Yes	No	No	No
Purification	Cellulose filter	Filtration; HPLC; protein A	No	In house C-18 stage tips	Stage tips; thiophilic adsorption
Characterisation	ELISA	ELISA	Immunological	nLC-MS/MS	Immunological; proteomic

Table 7.12: Comparison of techniques reported to have extracted and detected archaeological IgGs. 2014 – this thesis: detailed in section 7.8.

7.7.1: Element selection

Table 7.12 shows that studies attempting to extract archaeological IgGs have tested a variety of bone elements. Many studies favoured long bones, such as the femur, due to their inherently thicker cortices and inferred resistance to diagenesis. Others, such as Cattaneo et al. (1992) tested blood-rich haematopoietic vertebral bodies in the anticipation that these would contain higher concentrations of IgG. These elements, however, often display thinner cortices, rendering them potentially more vulnerable to diagenetic degradation. The TAC purifications performed in this study may support the research of Wiechmann et al. (1999:384), who found there to be no significant differences [between elements]…in terms of protein yield and quality", since there is little observable difference in gross protein yields

between element types. Based on TAC elutions and nLC-MS/MS analysis of samples in this study, cranial bone may at first appear to present a good target for IgG extraction, yet both this and the Jiang et al. (2007) research failed to detect IgG-related peptides from cranial samples. However, since Schmidt-Schultz and Schultz (2004) apparently detected ancient IgGs in cranial bone, the failure may be methodologically-based, perhaps influenced by HCl hydrolysis, inefficient purification, or insufficiently sensitive detection equipment.

Upon consideration of the different elements tested in reported IgG extractions, and non-standardisation of extraction and detection methods used, it would seem prudent that future IgG extractions test a range of bone elements. Crucially, the microscopic preservation of the elements should first be assessed: this study has supported Schmidt-Schultz and Schultz's (2004) research by indicating that that increased quantities of higher quality NCPs can be obtained from well-preserved bone. It is likely, therefore, that microscopic preservation may be a more reliable predictor of NCP survival (including IgG) than bone element type alone. Unfortunately, beyond histological analysis, little can be done to predict the preservation state of archaeological IgGs, if, indeed, they survive at all.

7.7.2: Histological preservation

While not a necessary precondition for the successful extraction of archaeological IgGs, histological analysis provides important information concerning the state of sample preservation prior to analysis. It helps to control for the selection of well-preserved samples that are less likely to be damaged by microbial attack. Characterisation of microscopic preservation would, for instance, have further supported Kolman et al.'s (1999) positive antibody/antigen reactions by ruling out extensive microbial damage and reducing the chances of cross-contamination with diagenetically introduced contaminants. It may also have allowed them to target samples displaying better preservation, potentially allowing them to destroy far less than their 15g of bone per sample. Histological analysis also allows for comparison of proteomes and protein yields extracted from different bone elements displaying different levels of preservation. It could, therefore, provide an important base from which to select samples.

The observed inverse relationship between protein yield and histological preservation in this study suggests that the chosen protein extraction methodology should

reflect sample preservation. It is likely that samples displaying the best preservation may require more intensive extractions to disrupt the bone matrix and release proteins of interest. However, if the end goal is to identify and characterise target protein(s) by methods that require well preserved molecules (e.g., immunological testing), then all care should be taken not to further degrade proteins during the extraction process. The extraction protocol could potentially be modified by increasing the duration of exposure to chemical reagents, rather than the relative strengths of the reagents. It seems prudent that further research should first identify the target proteins in well-preserved bone before applying the successful extraction method to more poorly preserved samples. This study made the mistake of attempting to extract and characterise IgGs from samples displaying a range of preservations before positively identifying them in any samples; it was naïvely assumed that following of published extraction protocols (P1, in particular) would result in successful IgG extraction.

7.7.3: Sample preparation and size

The choice of initial sample preparatory steps and methods of preparing samples for extraction are important precursors in the subsequent extraction, detection, and characterisation of NCPs. Many of the earlier studies homogenised samples to increase the surface area exposed to reagents during the extraction process. While grinding certainly increases protein yield, it is known to significantly degrade collagen (Collins and Galley, 1998). Crucially, Jiang et al. (2007) found that bone homogenisation releases large quantities of collagen, which may obscure lower-abundance proteins. Although IgG was detected in ground bone samples by Cattaneo et al. (1992) and Kolman et al. (1999), the effects of grinding on NCP quantity and quality are unknown. The rise in temperatures particularly associated with mechanical homogenisation may be adversely detrimental to IgG survival. Hand grinding (such as performed by Cappellini et al. 2012) may prove less damaging, especially if performed following immersion in liquid nitrogen, which is shown to promote the release of proteins while decreasing the rate of degradation (Wu et al., 2009).

Table 7.12 suggests a general trend over time towards testing smaller samples. Whereas earlier research used up to 15g of powdered bone (Kolman et al., 1999), the latest proteomic studies (e.g., Cappellini et al., 2012; Wadsworth and Buckley, 2014) require less than 100mg of sample. This change reflects both the early choice of extraction and

characterisation methods, and the recent technological advancements which require only tiny samples for protein identification. The early attempts at IgG detection understandably used larger quantities of sample, since their target protein was likely to be present in extremely low abundance, and detection methods were less sensitive than in their more modern analytical counterparts. Cattaneo et al.'s (1992) ELISA, for instance, had a detection limit of 10ng. If the IgG ELISA results from this study were accurate, the highest detected IgG yield was less than 5ng. Thus, if IgGs are normally this low in abundance in archaeological samples, it may indicate why Cattaneo et al.'s results were limited by their choice of analytical test. Subsequent improvements in extraction techniques which saw increases in protein yield and purity (e.g., Brand et al., 2002; Schmidt-Schultz and Schultz, 2004), permitted the use of smaller samples, thus easing pressure on a finite skeletal resource.

Considering the difficulties encountered in this study in extracting IgGs from archaeological material, it is difficult to propose exactly what sample size might be appropriate for their successful extraction. It may ultimately depend upon the choice of characterisation method. Proteomic identification, for instance, has been shown to require much less initial sample than immunological detection (Table 7.12). It seems advisable to test a range of sample sizes, depending upon the availability of laboratory equipment suitable for extraction. For example, larger samples usually require larger receptacles, which subsequently require equipment capable of receiving them (e.g., refrigerated centrifugation of 15ml, rather than 1.5ml test tubes). This equipment availability was a major factor in dictating sample sizes in this study. The future proposed extraction protocol (see section 7.8) suggests the distribution of numerous 120mg sample fractions between tubes, depending upon availability of processing equipment. The use of numerous fractions from the sample should increase the overall yield of extracted proteins and allow sufficient material for numerous downstream analyses (Cleland et al., 2012), thus maximizing the chances of ancient IgG detection.

7.7.4: Extraction temperatures

The temperature at which protein extraction take places is a further variable to take into consideration when proposing a method for archaeological IgG extraction. All of the

published studies in Table 7.12 perform the demineralisation stage at 4°C in order to slow protein degradation. However, analysis of the research suggests a transition to low temperature sample processing throughout the extraction and most of the characterisation stages. This change may be related to an increasing effort to follow clinical protein extraction practices and a growing appreciation of the potential range of proteolytic biomolecules (both extrinsic and intrinsic) that may survive in bone alongside target NCPs. Given the relationship between elevated temperature and protein degradation, it seems wise for any future IgG extractions to keep samples at a low temperature throughout processing and testing to prevent further deterioration of already degraded archaeological IgGs.

7.7.5: Demineralisation

The most commonly employed agents of bone demineralisation in biomolecular archaeological studies are EDTA and HCl. Both have benefits and drawbacks. In comparison to HCl demineralisation, EDTA provides a more gentle method. However, it takes much longer, requiring the presence of protease inhibitors and extensive desalting of samples. Despite HCl demineralisation leading to the 'purest' and cleanest archaeological protein extractions (Cleland et al., 2012), no protocol employing this method has resulted in detectable IgGs. HCl demineralisation is optimised for the extraction of acid-soluble collagen and collagen-associated proteins through the dissolution of the inorganic bone content and induction of "'swelling' of the collagen matrix [which] increases the ability of both collagen I and collagen-associated proteins to go into solution" (Cleland et al., 2012:4). Although 0.6M HCl is proposed to reduce protein hydrolysis and degradation during demineralisation, the low pH (approximately 0.2) may make the method unsuitable for extractions specifically aimed at IgGs. Vermeer and Norde (2000) demonstrated that IgG denaturation is proportional to decreasing pH, with significant denaturation occurring at pH 3.5 at 20°C. Smejkal et al. (2007) experienced significant protein loss in the 55-60kDa range (approximating IgG heavy chain molecular mass) following HCl demineralisation of fresh ostrich cortical bone. While most HCl demineralisations of archaeological bone take place at 4°C in order to reduce protein degradation, the harsh acidic conditions may help to explain why no IgGs have been detected following this demineralisation technique.

EDTA demineralisation is more time consuming than HCl, but the reaction is not dependent upon pH: the former reagent removes calcium from the bioapatite by chelation, rather than dissolution, meaning that the pH of the extraction solution can be regulated. This is potentially crucial for IgG extraction. The length of time required for EDTA demineralisation and the gentle conditions require extraction at low temperature in the presence of protease inhibitors. Increased temperature has been demonstrated to significantly accelerate demineralisation rate (e.g., Cho et al., 2010), but this may not be advisable for extracting already-degraded IgGs. It is difficult to determine the effect of HCl demineralisation on the quantity and quality of NCPs, since studies employing the method have tended to assess the yield and purity of extracted collagen. It may be significant that no research (either on modern or ancient bone) that has used HCl demineralisation has subsequently reported the presence of extracted IgGs or IgG-related peptides: all of the published protocols that reported the presence of extracted IgGs in Table 7.12 used EDTA, as did Delmas et al. (1984) in their extraction of the protein from modern bovine bones. While HCl and EDTA have been shown to selectively extract certain NCPs from modern bone, "EDTA solutions at neutral pH were as efficient as the strongest acid in dissociating the mineral phase" (Gerstenfeld et al., 1994:231). It seems prudent, therefore, for future NCP extractions to employ EDTA demineralisation, given the gentle nature of the process in comparison to HCl, and the apparent success of the reagent in extraction IgGs from both modern and archaeological bone.

7.7.6: Solubilisation

The solubilisation of proteins is commonly performed with archaeological samples, often following demineralisation. The decision as to exactly which agent of solubilisation to employ may have important consequences on the outcome of extractions targeting archaeological IgG. The most commonly employed reagents are guanidine-HCl (GuHCl) or ammonium bicarbonate, both of which effectively solubilise proteins following the removal of bone mineral (Cleland et al., 2012). The former is an extremely strong denaturant, which solubilises protein through denaturation. This usually does not represent a problem, since many proteins can refold back into a functional unit following solubilisation. However, the oligomeric nature of IgGs means that they often fail to refold, which can result in

irreversible aggregation with other proteins and loss of functionality (Maede et al., 1996). Although IgG has been detected following extractions which included GuHCl denaturing (e.g., Schmidt-Schultz and Schultz, 2004), the omission of a denaturant may result in an increased IgG yield, particularly following purification. Purification following GuHCl denaturation may not be effective, since the inherent affinity of the IgG for the chosen ligand may not survive in a denatured state. This may help to explain why Kolman et al. (1999) managed to extract immunoreactive antibodies, although their choice of protein A columns required a low pH (3.0-3.5) elution, which may have reduced their IgG yield through acid-induced degradation or denaturation.

The choice of downstream purification or analytical techniques should influence the choice of solublisation agent. Thiophilic adsorption, the purification technique advocated here, relies on the presence of non-chaotropic salts (e.g., potassium sulphate) to increase efficient immunoglobulin binding to the thiophilic support (Thermo Scientific, 2011). GuHCl is a strong chaotropic salt, and its presence in solutions may adversely affect the binding efficiency of the thiophilic resins, depending upon the interaction of GuHCl with water molecules. GuHCl also reduces the efficiency of tryptic digestion in sample preparation for mass spectrometry analysis (Proc et al., 2010), and samples therefore require an additional GuHCl-removal step if this type of analytical approach is chosen. It is for these reasons that ammonium bicarbonate has been proposed as the solublisation agent of choice when attempting to extract archaeological IgG from bone (section 7.8). However, it may prove useful to be run a tandem extraction using GuHCl, in order to assess both resulting extractions for the presence of IgG.

Schmidt-Schultz and Schultz (2004) advocate the use of sample sonication as an additional solubilisation step, claiming that it results in a more efficient release of strongly mineral-bound NCPs. nLC-MS/MS analysis of P3 sample CD120.2 (post-sonication) does not necessarily support this step, since it produced only two cellular NCPs (Table 6.40). Its effectiveness cannot be fully evaluated, however, because of the post-TAC nature of the sample tested; many non-resin-bound NCPs would have been lost during TAC purification. Sonication has been included in the proposed protocol (section 7.8) in order to more accurately assess its efficacy.

7.7.7: Dialysis

Dialysis during (e.g., Kolman et al., 1999), or following (e.g., Cattaneo et al., 1992) extractions is an extended process undertaken to remove contaminants, such as excess salts or residual EDTA from samples, in preparation for downstream analyses. Dialysis is particularly effective for samples containing high salt concentrations and for removing interfering contaminants that might precipitate along with proteins during sample preparation (Cleland et al., 2012). The process is, however, time consuming and introduces an extra preparatory stage, which may increase the loss of target proteins, particularly if they have a tendency to adsorb to any of the dialysis equipment or filters. Schmidt-Schultz and Schultz (2004) advise against dialysis due to this latter concern. Contaminant removal through dialysis or washing was not required for samples subjected to thiophilic adsorption, since contaminants should wash away during the TAC process. The resulting elutions in sodium phosphate buffer, however, may require desalting, depending upon chosen downstream analytical protocol.

7.7.8: Purification

In a process similar to dialysis, protein purification techniques following extraction are often designed to both remove contaminants and high abundance proteins from samples, thereby purifying the target protein(s). This step is particularly important when target proteins, such as immunoglobulins, are naturally of low abundance. All of the published extraction protocols in Table 7.12 use some form of protein purification, other than Schmidt-Schultz and Schultz (2004), who state that the use of purification or concentration techniques results in loss of NCPs. While this is likely true to an extent, the authors offer no solution for eliminating the ubiquitous NCP-masking collagen inevitably extracted alongside any NCPs. Kolman et al. (1999) demonstrate the importance of IgG purification by comparing extracted IgG reactivity in purified and unpurified samples. They found that the latter failed to produce immunological confirmation of IgG, whereas the purified samples reacted positively. This suggests that IgG is susceptible to masking by more abundant proteins, such as collagen or albumin.

TAC purification in this study resulted in the non-specific binding of a small quantity of collagen and possibly cross-linked non-collagenous proteins (not including IgG). This is

likely due to the failure of the preceding stages (P2 and P4 in particular) for extracting IgG from the bone samples, rather than a failure of the TAC to purify the target protein. TAC represents a gentle, highly specific method of purifying immunoglobulins and further extractions are required from archaeological bone to truly test the efficacy of thiophilic binding of degraded target IgGs. It would be useful to analyse pre-TAC samples using high-resolution nLC-MS/MS to identify samples containing IgG-related peptides. Positive samples could then be subjected to TAC to assess the suitability of the purification method. Alternatively, the HPLC/protein A purification method employed by Kolman et al. (1999) could be attempted, with the results compared to matching samples that have been TAC purified. If proteome analysis (perhaps prior to TAC) is to be performed, it may be advisable to follow the C18 stage tip purification and concentration method of Cappellini et al. (2012). Bona et al., (2014) also used stage tips to concentrate proteins in their apparent detection of protein biomarkers specific to osteogenic sarcoma in archaeological bone. That they also detected heavily degraded IgG heavy chains (around 12kDa) may support the potential of using stage tips in future analyses.

7.7.9: Detection and characterisation

The choice of final IgG detection technique is crucial in developing a suitable extraction methodology. A purely proteomic approach, for instance, does not require the extraction of functional, immunoreactive IgGs for identification. It also benefits from the use of compatible extraction reagents, since additional sample clean-up steps may result in the loss of target IgGs. Extraction for proteomic analysis also requires only a very small initial bone sample quantity.

When considering Cappellini et al.'s (2012) success in detecting ancient IgGs using high-resolution nLC/MS-MS, it should be remembered that they analysed Pleistocene mammoth bone from both polar and temperate regions. Unsurprisingly, the samples preserved in permafrost produced more detectable IgG-related peptides of better preservation compared to the temperate samples (Table 7.13). However, their innovative purification and concentration technique and use of high-resolution proteomics offers hope for future analysis of archaeological samples, since it demonstrates that the proteins can survive extended periods in temperate conditions. Their success also shows that newly

developed, highly sensitive technology can characterise ancient proteins of extremely low concentration – proteins that may previously have been present, but undetectable by less sensitive techniques (a problem discussed by Tran et al., 2011).

Burial environment	Protein	Sequence coverage (%)	Unique peptides
Permafrost	PREDICTED: IgG heavy chain	8.8	3
Permafrost	PREDICTED: IgG heavy chain	10.4	3
Temperate	PREDICTED: IgG heavy chain	5.6	2

Table 7.13: Mammoth IgG-related peptides from permafrost and temperate samples (Cappellini et al., 2012).

Theoretically, more recent bone should yield greater quantities of IgG than Cappellini et al.'s (2012) mammoth samples. However, their research suggests that, should IgGs be present, they will likely be at extremely low abundance and of potentially poor quality. It is, therefore, crucial to first identify extracted IgGs by the most sensitive, accurate, and reliable methods available. At the current time, high-resolution proteomics seems the best method to achieve this. Once IgG-containing samples are identified, testing using other characterisation methods (e.g., ELISA or Western blot) can be considered.

7.8: A proposed protocol for future ancient IgG extractions

Bearing in mind the problems encountered in this study, the experience gained from attempting to extract archaeological IgGs, and a reanalysis of published protocols that have reportedly extracted them, a new protocol (summarised in Table 7.12) has been devised for future IgG extractions. It represents a combination of extraction stages from Schmidt-Schultz and Schultz (2004) and Cappellini et al. (2012), both of which reported successful extraction of IgGs or IgG-related peptides. This protocol is adapted specifically for the extraction of any extant IgGs from well-preserved samples, with an emphasis on reducing possible further damage to already degraded target proteins. The extraction will result in three separate supernatant fractions which could potentially be subjected to numerous analyses to investigate IgG presence. This is one reason for preparing multiple samples from one bone of known histological preservation. Fractions could potentially be purified using

stage tips and characterised by nLC-MS/MS in order to identify IgG-containing samples. Corresponding fractions could then be subjected to TAC to evaluate the effectiveness of this purification method on samples with known IgG content.

1. Select bone of known good histological preservation.

2. Clean bone surfaces, as in section 5.6.1. Immerse bone in liquid nitrogen.

3. Wrap bone sample in clean aluminium foil and powder with a hammer. This should offer more control over any excessive heating associated with mechanical grinding, and possibly reduce NCP degradation. It should also result in lower quantities of extracted collagen.

4. Take multiple 100mg bone sample into microtubes and suspend in 1.5ml of 0.5M EDTA (pH 8) in the presence of protease inhibitors for 48 hours at 4°C, with gentle agitation.

5. Centrifuge for 15 minutes in a microcentrifuge at maximum speed (e.g., 17,000g) at below 8°C. Retain supernatant (Fraction A) at -20°C.

6. Wash bone pellet twice in 0.75ml of analytical grade water, then suspend in 1.2ml of 50mM ammonium bicarbonate, in the presence of protease inhibitors, pH 7.4.

7. Incubate sample for 48 hours at 45°C with gentle agitation.

8. Centrifuge sample for 15 minutes in a microcentrifuge at maximum speed (e.g., 17,000g) below 8°C. Retain supernatant (Fraction B) at -20°C.

9. Wash pellet twice in 0.75ml of analytical grade water then lyophilise.

10. Take 20mg of lyophilised bone pellet and suspend in 1.5ml solubilisation buffer (20mM NaH_2PO_4, 30mM Na_2HPO_4, and protease inhibitors 1mM benzamidine, 10mM aminocaproic acid, 10mM EDTA, pH 7.4).

11. Sonicate sample in twice over ice for 7 seconds. Centrifuge for 15 minutes in a bench top microcentrifuge at maximum speed (e.g., 17,000g) below 8°C. Retain supernatant (Fraction C) and bone pellets at -20°C.

Protein purification:

1. As mentioned above, C-18 stage tip purification following the Cappellini et al. (2012) protocol could be employed at this point to prepare sample fractions for nLC-MS/MS analysis.

IgG purification by TAC:

1. Add 87mg reagent grade crystalline potassium sulphate per ml of sample fractions, before gentle mixing.

2. Centrifuge sample at 10,000g for 20 minutes below 8°C. Carefully remove supernatant through 0.45µm low-protein binding filters (using 5ml syringe, filter, and 20 gauge needle). Bring aspirated supernatant to pH8.

3. Equilibrate thiophilic columns and buffers to room temperature. Uncap columns and allow to drain. Equilibrate with 12ml of binding buffer, discarding the flow-through fractions.

4. Apply sample supernatants to the columns and allow to completely enter the resin.

5. Wash columns with consecutive 3ml volumes of binding buffer. Collect flow-through fractions after each wash and monitor absorbance of each at 280nm against pure binding buffer using a spectrophotometer. This step will determine when all non-bound material is removed from the column.

6. Elute bound proteins from each column using as many elution buffer washes as is necessary, until no further protein is measurable at 280nm against pure elution buffer. Retain eluted fractions containing protein at -20°C. Discard fractions containing no measurable protein.

7. Lyophilise retained protein-containing fractions (expect the tubes to contain a high quantity is dried phosphate). The remaining protein pellet can then be resuspended in a buffer appropriate for chosen downstream analytical technique. Alternatively, the samples could be desalted prior to lyophilisation.

If this protocol proves successful in extracting IgGs from archaeological bone, the integrity of the proteins will be investigated through high resolution mass spectrometry. This would provide some idea of the potential functionality of the extracted IgGs. In the case of poorly preserved proteins, the extraction technique could be altered to see if preservation is being negatively affected during the

extraction process. Should preservation be adequate, the next stage would be to assess the functionality (or immunoreactivity) of the isolated IgGs. Immunoreactivity could potentially be assessed by employing a human IgG ELISA or Western blot. If immunoreactive IgGs are detected, then they will be tested against *P. vivax* antigens to assess the original thesis hypothesis concerning the presence of malaria in the past English Fens.

CHAPTER 8: CONCLUSIONS

8.1: Summary of research findings

The aim of this study was to detect the presence of temperate *Plasmodium vivax* malaria in the past English Fens (from the Roman to late medieval periods) through a variety of indirect and direct methods. *P. vivax* is known to have affected post-medieval marshland populations in south east England, but its presence has yet to be confirmed prior to this. This is despite the existence of favourable environmental and demographic conditions for many preceding centuries. These conditions were particularly suitable for endemic *vivax* malaria in the Fens of Lincolnshire and Cambridgeshire, yet very little palaeoepidemiological research has concentrated in these areas. The reservations of some researchers concerning the presence of malaria in England prior to the post-medieval period are likely based on a lack of documentary sources pertaining to the disease, beyond vague symptomatic descriptions. There are, however, few justifiable reasons why *P. vivax* malaria should *not* have been present before the 16th century. The results of this study in relation to the research questions posed in Section 1.2 can be summarised thus:

1. This research was unable to extract or detect extracted antibodies from archaeological human bone. Consequently, it cannot assess the proposal that ancient *P. vivax* malaria can be detected through testing of extracted anti-malarial antibodies.
2. The failure to extract IgGs suggests that the methods of extraction and detection were insufficient, or that IgG is not preferentially retained in bone, or both. However, the study has confirmed that bone of higher histological preservation yields a more diverse range of NCPs. The lack of osteocalcin may indicate that NCPs do not preferentially survive in archaeological bone due to a high affinity to hydroxyapatite.
3. Analysis of published skeletal reported revealed no evidence of genetic anaemia in the form of β thalassaemia in the small Fen-associated sample (through analysis of published reports). This suggests that *P. vivax*, if present, did not exert sufficient selective pressure to drive the development of thalassaemia, or that the nature of the disease and poor skeletal preservation precluded its manifestation in the archaeological record.

4. Palaeodemographic comparison of Fen/non-Fen populations through analysis of published skeletal reports could not support the presence of *P. vivax*. However, analysis of mortality patterns did suggest evidence of 'healthy adaptation' to the Fen environment, which supports earlier research (Gowland and Western, 2012). One Fen population also displayed possible evidence for intra-uterine growth restriction, a condition strongly linked with endemic *P. vivax*.

The first indirect method of tracing *P. vivax* malaria entailed the analysis of osteoarchaeological reports in order to identify putative sequelae of the haemoglobinopathy β thalassaemia in populations from potentially malarious areas (third research question). This analysis followed recent refining of diagnostic criteria for identifying the condition in skeletal remains (Lewis, 2010). Identification of this disease in past Fen populations would suggest that malaria supplied a significant enough selective pressure to drive the selection of genetic resistance. One individual from the Littleport site displayed two possible changes associated with thalassaemia (porotic hyperostosis and 'hair-on-end' diplöic arrangement), but further macroscopic and radiographic analysis yielded no further suggestive pathologies (see sections 6.1.1 and 6.1.4). Unfortunately, an overall lack of reported sequelae cannot currently support the presence of thalassaemia in the past in the English Fens. Nor can it support, by proxy, the hypothesised malarial selective pressure. It may be more likely that genetic resistance in the form of G6PD deficiency would have developed in response to long-term *P. vivax* exposure, such as has been recently observed in Southeast Asia (Louicharoen et al., 2009). While macroscopic identification of this condition in archaeological bone remains impossible, recent developments in aDNA technology may prove useful in identifying preserved genomic markers of G6PD deficiency.

There can be little doubt that the past Fens and marshlands of England presented significant epidemiological and environmental challenges to their inhabitants. Post-medieval parish registers from eastern and south eastern England, for instance, clearly demonstrate the significant health burden placed upon marshland-associated populations, which suffered considerably higher mortality rates than their upland counterparts (Dobson, 1997). The year-round resources offered by these locations were, however, worth the risk associated with their continual utilisation. The seasonal pattern to this increased mortality is highly

suggestive of endemic *P. vivax* malaria, likely comorbid with other diseases ubiquitous to what were inherently unhealthy environments. Although until recently considered a 'benign' infection in comparison to its tropical cousin *P. falciparum*, *vivax* malaria in modern settings is well known to increase morbidity and mortality in the most vulnerable groups, namely infants and mothers. In pre-medicalised antiquity, *P. vivax* infection would have been chronic and debilitating, and would have added to the plethora of diseases and conditions that served to increase infant and maternal mortality. It was the intention of this study to investigate whether *P. vivax* presence could be inferred from comparing the mortality patterns of Fen and non-Fen cemetery populations, with particular emphasis on these most vulnerable groups (fourth research question).

Despite the acknowledged limitations of cemetery population-based palaeodemographic analyses, some interesting, and somewhat unexpected patterns emerged concerning Fen/non-Fen survivorship and probability of death. Statistical analysis showed that location was a significant factor influencing age-at-death and it was expected that Fen-associated populations would show lower survivorship and increased force of mortality compared to their non-Fen counterparts. The opposite relationship was, however, observed in most of the 'vulnerable' groups for all but the medieval period, with Roman and Anglo-Saxon Fen populations seemingly coping better with their environments. Although unexpected, this pattern may suggest that these relatively isolated populations had adapted to the constant epidemiological pressures of their unwholesome locations. The observed increased prevalence of *cribra orbitalia* in Anglo-Saxon marshland-associated populations (Gowland and Western, 2012) may support such a 'healthy adaptation' hypothesis. It is possible that an acquired, rather than genetic immunity to malaria was present here. It is unknown why this pattern reverses in the later medieval period. The economic boom and favourable climatic conditions of the 12th and 13th centuries certainly saw an exponential increase in Fen population and subsequent exploitation. It could, theoretically, be the case that this expansion (including migrants unadapted to the Fens) increased contact with either local parasite vectors or new, externally introduced pathogens. The latter could, of course, include malaria.

Perhaps tellingly in terms of identifying *vivax* malaria through increased mortality rates, demographic analysis showed no significant difference in Fen/non-Fen age-at-death

category distribution, where it might be expected that *vivax* malaria would increase Fen mortality in certain vulnerable groups. It remains uncertain whether *P. vivax*, if present at all, provided a force of mortality significant enough to be inferable through analysis of cemetery demography. One potential indirect indicator, however, was observed in the St. Peter's, Barton-upon-Humber population, which showed an unusually high prevalence of small infants (as recorded though femoral diaphyseal measurement). This may be a potential indicator of intrauterine growth restriction (IUGR), a condition strongly linked to endemic *P. vivax* malaria in modern populations. IUGR does have alternative aetiologies that cannot be discounted here. However, its putative presence has not before been documented in archaeological populations, and its strong association with *vivax* malaria presents an intriguing possible link to the disease affecting an otherwise apparently 'healthy' population. The further research required to substantiate this would, unfortunately, be complicated by an overall dearth of infant burials from Fen contexts. Although the palaeodemographic analysis attempted in this study has been unable to confidently identify the presence of *P. vivax* malaria as a driver of mortality, it has offered a hitherto unforeseen glimpse of the health status of past Fenlanders, a group potentially well adapted to the epidemiological pressures associated with life in 'demon-haunted' (Colgrave, 1956:78, translating Felix), miasmic wilderness of the pre-modern Fens.

Direct evidence of *P. vivax* malaria was sought though the biomolecular analysis of human skeletal remains from potentially malarious areas of the Lincolnshire and Cambridgeshire Fens (first research question). Since published attempts at detecting pathogenic *P. vivax*-related molecules had failed, it was hypothesised that the extraction and detection of biomolecules associated with the human immune response to *P. vivax* infection would prove more successful. The biomolecule of choice was immunoglobulin G (IgG), the most abundant serum antibody, which has been clinically shown to circulate long after infection (and associated pathogenic molecules) has been cleared from the body (Wipasa et al., 2010). It was hypothesized that the extraction of functional anti-malaria antibodies from archaeological bone would offer a direct method of detecting the presence of the disease in past populations.

The inherent stability of IgGs and their naturally negative charge should influence strong adsorption to bioapatite following death, thereby providing increased protection

against diagenetic factors. Theoretically then, IgG should persist in well-preserved archaeological bone. Each collected bone sample was initially subjected to histological analysis in order to characterise the level of preservation. This provided an important baseline for selecting samples for protein extraction, and provided useful information regarding the relationships between histological preservation and the quantity and integrity of proteins yielded.

Numerous protein extraction and characterisation techniques were attempted in this research, each based on published protein extraction techniques using either archaeological (e.g., Schmidt-Schultz and Schultz, 2004) or modern (e.g., Jiang et al., 2007) bone. This study was also the first to attempt to purify archaeological IgGs using thiophilic adsorption chromatography (TAC). This technique was introduced to eliminate collagen, which was responsible for masking of lower abundance proteins, a phenomenon confirmed by proteomic analysis. Although no IgG was identified in the TAC-purified samples, nLC-MS/MS analysis of post-TAC elutions demonstrated non-specific binding of collagen and a small quantity of endogenous and exogenous NCPs, along with one possibly from a pathogenic organism (*Cronobacter sakazakii*). Since many of these protein identifications were based upon single peptides, further testing of pre-TAC samples is required for more secure identifications. The study also found that Schmidt-Schultz and Schultz's (2004) 'successful' extraction and detection of archaeological IgG could be not reproduced, which may call into question the authenticity of this published research; future work based on this research should regard the reported findings with extreme caution.

The research was unable to fully support Masters' (1987) theory that non-collagenous proteins (NCPs) are preferentially retained in archaeological bone due to a high affinity with hydroxyapatite (second research question), since the nature of the proteomic analyses (nLC-MS/MS of post-thiophilic resin samples) did not allow for a full evaluation of entire sample proteomes. Many of the identified NCPs may have been cross-linked to collagen, rather than associated with bone mineral. Also, the lack of expected osteocalcin in any analysed sample calls into question the original hypothesis concerning preferential NCP survival. The study did, however, support earlier research (Schmidt-Schultz and Schultz, 2004) by indicating that increased quantities of higher quality NCPs can be obtained from well-preserved bone, thus demonstrating the importance of histological screening of samples

prior to analysis. The observed inverse relationship between protein yield and histological preservation also suggests that the chosen protein extraction methodology should reflect sample preservation. It is likely, for instance, that samples displaying the best preservation may require more intensive extractions to disrupt the bone matrix and release proteins of interest. This research joins other studies (e.g., Cappellini et al., 2012) in supporting Cleland et al.'s (2012) suggestion that extraction and purification methods should be tailored to the specific target protein. Consequently, the experience gained during this research has led to the proposal of a protocol specifically aimed at specific extraction of archaeological IgGs. This protocol will be implemented in the near future, alongside investigations into the diagenesis and retention of IgGs in modern mammalian bone.

8.2: Summary of limitations

The main limitations of this research can be summarised thus:

1. Assessment of the first research question was limited by the inability to extract IgG from archaeological bone following published protocols.
2. Although a range of NCPs were characterised and the relationship between high histological preservation and NCP yield was confirmed, the nature of the analyses (nLC-MS/MS on eluted TAC samples) meant that full proteomes were not revealed. High resolution mass spectrometry of pre-elution samples would be required for this.
3. Evidence for genetic anaemia in associated with *P. vivax* was limited by the reliance on sufficiently detailed reporting of suggestive sequelae in skeletal reports, and the small sample size in terms of cemetery sites. Limitations also included the likely poor representation of thalassaemia in the archaeological record, and current difficulties in detecting G6PD deficiency (a condition more likely to be associated with *P. vivax* than β thalassaemia) in skeletal samples.
4. Palaeodemographic analysis of mortality and survivorship patterns is inherently limited by the lack of correlation between living and cemetery populations in terms of demographic structure. Furthermore, many recent palaeodemographic studies have reassessed skeletal assemblages using statistically-based methods in an attempt to both standardise and increase the accuracy of ageing adult skeletons. The large sample sizes analysed in this study and time-constraints precluded the reassessment

of reported skeletal populations, each of which would have originally been recorded using different criteria.

8.3: Future research directions

Despite the limitations inherent in such analyses, the paleodemographic element of this study revealed interesting and unexpected patterns in Fen mortality, as well as possible evidence for IUGR at the St. Peter's, Barton-upon-Humber site. Since this research relied completely on skeletal reports produced by many palaeopathologists over a number of decades, the lack of standardisation in recording limits the usefulness of interpretations based upon this data. The potential insights into past Fen health and adaptation suggested here warrants deeper investigation, perhaps incorporating reassessment of skeletal populations using a standardised recording criteria. This would be particularly useful for the St. Peter's Barton-upon-Humber population, since there remains uncertainty concerning the possible under-ageing of infants. This presents a possible confounding factor for the presence of IUGR that only a reassessment of these individuals could address.

This study has confirmed the lack of a standardised methodology for extracting IgGs from archaeological bone. It is, therefore, recommended that future attempts should first extract IgGs from modern mammalian bone, perhaps through trialing a number of different techniques (including the methods proposed in Section 7.8) and monitoring the presence of IgGs in each extract. Fresh bone samples could also be buried and tested sequentially over time to assess the degradation of IgGs. The successful extraction of immunoreactive IgGs from archaeological bone offers enormous potential in the detection and characterisation of past disease, either through the confirmation of a suspected diagnosis, or the identification of latent conditions. The challenge, as highlighted by this study, remains the extraction, detection, and the ultimate utilisation of an incredibly elusive biomolecule that offers such high potential for palaeopathology. Although this study was unsuccessful in extracting archaeological IgGs, it has explored and advanced new methodologies in their extraction, purification, and characterisation. The resulting newly-proposed technique for retrieving IgG from archaeological bone can now be implemented. It is hoped that this new methodology, in combination with high-resolution proteomic analysis, will ultimately lead to a successful, replicable technique of ancient, reactive IgG isolation.

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Appendix 1: Thiophilic adsorption chromotography (TAC) results

Appendix 1 presents in full results of the thiophilic adsorption chromotography (TAC) tests performed on P2, P3, and P4 samples. All rinse and elution figures are mg/ml. HI is histological preservation.

P2.1

Sample	HI	Wash	1st stage rinse	1st stage elution	2nd stage rinse	2nd stage elution	3rd stage rinse	3rd stage elution	4th stage rinse	4th stage elution
HDAP 5	5	1	1.16	0.01	1.22	0	1.27	0.01	0.23	0
		2	0.13	0.01	0.08	0	0.67	0.004	0.24	0
		3	0	0.01	0.04	0	0.03	0.01	0	0
		4	0	0.008	0.03	-	0	0.001	0	-
		5	0	0	0	-	0	0	0	-
		6	-	0	0	-	0	0	-	-
		7	-	0	0	-	-	0	-	-
AN1	5	1	0.04	0.007	2.45	0	0	0	0	0
		2	0	0.002	0.45	0.006	0.007	0	0	0
		3	0	0.004	0	0	0	0	0	0
		4	0	0	0	0	0	-	-	-
		5	-	0	0	0	0	-	-	-
		6	-	0	-	-	-	-	-	-

Table A1.1: P2 TAC results.

P2.2

Sample	HI	Wash	1st stage rinse	1st stage elution	2nd stage rinse	2nd stage elution	3rd stage rinse	3rd stage elution
HDAR9	5	1	0.188	0	0.625	0.01	1.424	0.01
		2	0	0	0.071	0.015	0.474	0.013
		3	0	0	0.015	0.024	0.018	0.004
		4	0	-	0	0.035	0.007	0
		5	-	-	0	0.019	0.007	0
		6	-	-	0	0	0	0
		7	-	-	-	0	0	-
		8	-	-	-	0	0	-
HDAP3	5	1	0.006	0.137	0.207	0.01	0.171	0.004
		2	0.038	0	0.052	0.013	0.796	0.014
		3	0.006	0	0.02	0.032	0.011	0.004
		4	0	0	0.013	0.016	0.003	0
		5	0	-	0.002	0.014	0.003	0
		6	0	-	0	0	0	0
		7	-	-	0	0	0	-
		8	-	-	0	0	0	-
AN2	5	1	0.024	0.06	0.58	0.009	2.954	0
		2	0.045	0	0.434	0.021	0.202	0.009
		3	0	0	0.025	0.009	0.011	0
		4	0	0	0.003	0.007	0.003	0
		5	0	-	0.003	0.01	0.007	0
		6	-	-	0	0.016	0	-
		7	-	-	0	0	0	-
		8	-	-	0	0	0	-
		9	-	-		0	-	-
COLLAGEN	-	1	0	0.003	0.078	0.016	0.097	0
		2	0	0	0.047	0.017	0.248	0
		3	0	0	0.001	0.015	0.003	0
		4	-	0	0	0.014	0	-
		5	-	-	0	0	0	-
		6	-	-	0	0	0	-
		7	-	-	-	0	-	-

Table A1.2: P2 TAC results

P2.3

Sample	HI	Wash	1st stage rinse	1st stage elution	2nd stage rinse	2nd stage elution	3rd stage rinse	3rd stage elution
HDAP5	5	1	0.45	0.08	0.344	0	0.798	0.01
		2	0.876	0.02	0.07	0	0.303	0.02
		3	0.768	0	0.037	0	0.011	0.01
		4	0.084	0	0.003	-	0.005	0.001
		5	0.035	0	0	-	0	0
		6	0	-	0	-	0	0
		7	0	-	0	-	0	0
		8	0	-	-	-	-	-
HDAR2	5	1	0.37	0	0.513	0	0.257	0.023
		2	0.405	0	0.668	0.005	0.062	0
		3	0.027	0	0.05	0	0	0.003
		4	0.007	0.001	0.011	0	0.017	0
		5	0.003	0	0.002	0	0.004	0
		6	0	0	0	-	0	0
		7	0	0	0	-	0	-
		8	0	-	0	-	0	-
HDAR3	4	1	0.209	0	0.438	0.004	0.937	0.027
		2	0.375	0.004	0.272	0.01	0.373	0.007
		3	0.051	0	0.017	0.004	0	0.006
		4	0.01	0	0.007	0	0.008	0.004
		5	0.004	0	0.003	0	0.005	0.011
		6	0	-	0	0	0	0.01
		7	0	-	0	-	0	0.007
		8	0	-	0	-	0	0
		9	-	-	-	-	-	0
		10	-	-	-	-	-	0
HDJS3	1	1	0.211	0.022	0.641	0.003	1.274	0.024
		2	0.167	0.014	0.111	0	0.122	0.018
		3	0.084	0.004	0.009	0	0.003	0.007
		4	0.024	0	0.003	0	0	0.006
		5	0.015	0	0.001	-	0	0
		6	0	0	0	-	0	0
		7	0	-	0	-	-	0
		8	0	-	0	-	-	-

Table A1.3: P3 TAC results.

P2.4

Sample	HI	Wash	1st stage rinse	1st stage elution	2nd stage rinse	2nd stage elution	3rd stage rinse	3rd stage elution
WM2316.1	5	1	0.065	0.01	0.184	0	0.624	0.002
		2	0.033	0.021	0.066	0	0.119	0.016
		3	0.008	0.01	0	0	0	0.004
		4	0.007	0.009	0	-	0	0
		5	0.01	0.01	0	-	0	0
		6	0.008	0		-	-	0
		7	0.005	0	-	-	-	-
		8	0	0	-	-	-	-
		9	0	-	-	-	-	-
		10	0	-	-	-	-	-
CD127.2	5	1	0.029	0.011	0.2	0	1.16	0
		2	0.064	0.012	0.076	0	1.5	0.009
		3	0.009	0.014	0	0	0.056	0
		4	0	0.006	0	-	0.027	0.001
		5	0	0.003	0	-	0.018	0
		6	0	0	-	-	0.014	0
		7	-	0	-	-	0	0
		8	-	0	-	-	0	-
		9	-	-	-	-	0	-
LP3845.1	0	1	0.017	0.012	2.469	0	3	0.006
		2	0.223	0.011	0.346	0	2.8	0.012
		3	0.013	0.019	0.825	0	0.077	0.009
		4	0.009	0.013	0.038	-	0.019	0.037
		5	0.006	0.018	0	-	0.011	0.024
		6	0.004	0	0	-	0	0
		7	0	0	0	-	0	0
		8	0	0	-	-	0	0
		9	0	-	-	-	-	-
AN5	0	1	0.043	0.013	0.202	0	2.73	0.039
		2	0.254	0.013	0.42	0	2.76	0.021
		3	0.026	0.006	0	0	0.012	0.018
		4	0.004	0.007	0	-	0	0.005
		5	0	0.006	0	-	0	0.001
		6	0	0.009	-	-	0	0
		7	0	0	-	-	-	0
		8	-	0	-	-	-	0
		9	-	0	-	-	-	-

Table A1.4: P2.4 TAC results.

P2.5

Sample	HI	Wash	1st stage rinse	1st stage elution	2nd stage rinse	2nd stage elution	3rd stage rinse	3rd stage elution
HP154.1	5	1	0.071	0.023	0.336	0	2.325	0.005
		2	0.023	0.018	0.075	0.005	0.053	0.013
		3	0.002	0	0	0	0	0.001
		4	0.008	0	0	0.001	0	0
		5	0	0	0	0.025	0	0
		6	0	-	-	0.011	-	0
		7	0	-	-	0.011	-	-
		8	-	-	-	0	-	-
		9	-	-	-	0	-	-
		10	-	-	-	0	-	-
LP3760.1	0	1	1.596	0.007	1.083	0.01	3	0.016
		2	0.842	0	0.451	0	0.664	0
		3	0.22	0	0.086	0	0.108	0
		4	0.075	0	0.04	0	0	0
		5	0.041	-	0.026	-	0	-
		6	0.006	-	0	-	0	-
		7	0	-	0	-	-	-
		8	0	-	0	-	-	-
		9	0	-	-	-	-	-
LP4585.3	2	1	0.285	0	1.46	0	2.98	0.005
		2	0.213	0	0.267	0	0.374	0.01
		3	0.032	0	0.004	0	0	0
		4	0.006	-	0	-	0	0
		5	0.011	-	0	-	0	0
		6	0.004	-	0	-	-	-
		7	0	-	-	-	-	-
		8	0	-	-	-	-	-
		9	0	-	-	-	-	-
OL1104.2	5	1	0	0.002	0.148	0	1.388	0.007
		2	0	0	0.115	0	0.03	0.008
		3	0	0	0	0.004	0	0
		4	0	0	0	0.005	0	0
		5	0	-	0	0.004	0	0
		6	-	-	-	0	-	-
		7	-	-	-	0	-	-
		8	-	-	-	0	-	-

Table A1.5: P2.5 TAC results.

P3

Sample	HI	Wash	1st stage rinse	1st stage elution	2nd stage rinse	2nd stage elution	3rd stage rinse	3rd stage elution
EH198.1	4	1	1.172	0.038	1.414	0.038	0.869	0.011
		2	0.424	0.039	0.204	0.034	0.053	0.016
		3	0	0.016	0	0.026	0.02	0.013
		4	0	0.024	0	0.007	0.017	0.005
		5	0	0	0	0	0	0.01
		6	-	0	-	0	0	0
		7	-	0	-	0	0	0
		8	-	-	-	-	-	0
CD120.2	5	1	1.243	0.009	2.526	0.015	1.33	0.009
		2	0.741	0.043	0.173	0.025	0.369	0.022
		3	0.011	0.003	0.051	0.014	0.037	0.014
		4	0.003	0	0.03	0	0	0.004
		5	0	0	0	0	0	0.007
		6	0	0	0	0	0	0.02
		7	0	-	0	-	-	0.008
		8	-	-	-	-	-	0
		9	-	-	-	-	-	0
		10	-	-	-	-	-	0
CD165.1	5	1	1.188	0.008	1.802	0.048	1.432	0.018
		2	0.123	0.007	0.132	0.038	0.196	0.039
		3	0.002	0.006	0.027	0.017	0.004	0.022
		4	0.012	0	0.019	0	0.001	0.016
		5	0	0	0	0	0	0.017
		6	0	0	0	0	0	0
		7	0	-	0	-	0	0
		8	-	-	-	-	-	0
OL1104.2	5	1	0.302	0.008	0.837	0.009	0.749	0.005
		2	0.171	0.009	0.094	0.008	0	0.007
		3	0.004	0.007	0.073	0.007	0	0.002
		4	0	0	0.039	0	0	0
		5	0	0	0	0	-	0.007
		6	0	0	0	0	-	0
		7	-	0	0	-	-	0
		8	-	-	-	-	-	0

Table A1.6: P3 TAC results.

P4

Sample	HI	Wash	1st stage rinse	1st stage elution	2nd stage rinse	2nd stage elution	3rd stage rinse	3rd stage elution
CD84.2	0	1	1.175	0.012	2.942	0.031	3.129	0.028
		2	1.831	0.006	0.972	0.024	2.267	0.023
		3	0.463	0.007	0	0.017	0.092	0.01
		4	0.126	0	0	0.008	0.055	0.003
		5	0.077	0	0	0	0.028	0
		6	0.038	0	-	0	0.027	0
		7	0.033	-	-	0	0.012	0
		8	0	-	-	-	0	-
		9	0	-	-	-	0	-
		10	0	-	-	-	0	-
CD112.3	2	1	0.981	0.021	2.948	0.027	3.17	0.015
		2	1.64	0.013	1.359	0.025	2.785	0.028
		3	0.278	0	0.054	0.015	0.093	0.012
		4	0.045	0	0.003	-	0.042	0.004
		5	0.015	0	0	-	0.009	0
		6	0	-	0	-	0	0
		7	0	-	0	-	0	0
		8	0	-		-	0	-
EHA4	4	1	0.926	0.006	2.194	0.014	3.032	0.013
		2	0.522	0.011	0.59	0.01	1.323	0.02
		3	0.073	0.008	0	0.008	0.086	0.007
		4	0.004	0	0	0.005	0.023	0
		5	0	0	0	0	0.014	0
		6	0	0	-	0	0	0
		7	0	-	-	0	0	-
		8	-	-	-	-	0	-
EH156.3	5	1	1.494	0.012	2.003	0.025	2.742	0.004
		2	0.707	0.017	0.833	0.033	0.743	0.025
		3	0.161	0.021	0	0.021	0.034	0.01
		4	0.038	0	0	0.014	0.007	0.005
		5	0.015	0	0	0	0	0
		6	0.005	0	-	0	0	0
		7	0	-	-	0	0	0
		8	0	-	-	-	-	-
		9	0	-	-	-	-	-

Table A1.7: P4 TAC results.

Appendix 2: Palaeodemography, histology, and rapid test data

Appendix 2 presents complete tables showing the Fen (Table A2.1) and non-Fen (Table A2.2) sites selected for palaeodemographic analysis. These tables include the numbers of individuals in each age category and the reference for the osteological report associated with each site. This is followed by full results of histological analysis of the individuals selected for biomolecular analysis (Tables A2.3-2.16); individual sample numbers, element type, and associated histological preservation (or HI) is shown for each site. Finally, Figures A2.1-2.14 display the photographed results of malaria rapid testing (photographs by author).

Site	Period	< 1 month	0-12 months	1-6 years	7-12 years	13-17 years	Male 18-24 years	Female 18-24 years	Male 25-34 years	Female 25-34 years	Male 35-49 years	Female 35-49 years	Male 50+	Female 50+	Male adult 18+	Female adult 18+	Reference
Durobrivae, Cambs	Rom	0	0	2	3	1	0	0	0	1	2	3	1	0	2	1	Duhig 1999
Hoplands, Sleaford, Lincs	Rom	2	0	5	2	3	0	1	4	5	7	7	3	5	0	0	Western 2011
Prickwillow Road, Ely	Rom	0	0	2	0	0	0	1	1	2	3	2	4	0	0	1	Anderson 2003
The Parks, Godmanchester	Rom	0	0	3	9	5	2	3	8	4	0	0	8	7	2	1	Brickley 2003
Watersmeet, Huntingdon	Rom	2	3	3	4	0	0	0	4	5	6	5	2	6	1	4	Phillips 2006
Barton Bendish, Norfolk	A-S	0	3	14	6	0	4	2	10	4	9	2	5	1	3	5	Stroud 1984
Baston, Lincs	A-S	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	Manchester 1976
Burgh Castle, Norfolk	A-S	0	1	9	9	11	8	7	9	13	29	17	24	13	7	13	Anderson and Birkett 1989
Caistor-on-Sea, Norfolk	A-S	0	7	6	12	7	0	9	5	5	6	6	15	17	4	1	Anderson 1991
Castle Mall, Norwich	A-S	3	4	9	6	3	3	10	2	5	10	17	3	2	3	2	Anderson 1996
Castledyke South, Lincs	A-S	0	1	15	13	13	5	14	11	13	8	18	13	17	12	6	Drinkall and Foreman 1998
Cleatham, Lincs	A-S	0	1	4	2	0	2	4	12	8	3	4	4	0	3	2	Leahy 2007

Edix Hill, Cambs	A-S	1	2	13	8	14	12	7	7	18	7	6	11	5	8	2	Duhig 1998
Haddenham, Cambs	A-S	0	0	0	3	0	2	0	0	0	0	0	0	0	1	3	Robinson and Duhig 1992
Highfield Farm, Littleport	A-S	3	1	3	7	6	2	4	5	4	6	6	0	5	0	0	Western 2007
Market Deeping Bypass, Cambs	A-S	0	1	1	1	0	1	1	3	3	1	0	2	0	0	2	Gowland 2000
Monkton, Kent	A-S	0	0	1	0	0	1	1	2	1	2	1	0	0	7	2	Richardson 2005
Quarrington, Lincs	A-S	0	0	2	1	2	1	3	3	0	1	0	1	0	1	0	Dickinson 2004
Ramsgate, Kent	A-S	0	6	8	6	3	4	7	12	7	10	6	1	1	8	7	Richardson 2005
Rivenhall, Suffolk	A-S	0	2	4	4	3	2	1	6	3	2	1	2	2	20	12	O'Connor 1993
Snodland, Kent	A-S	0	0	2	0	2	1	2	9	6	5	2	0	1	1	0	Richardson 2005
St Peter's, Barton-upon-Humber, Lincs	A-S	9	92	80	45	14	18	26	65	65	16	4	91	50	83	70	Waldron 2007
Staunch Meadow, Suffolk	A-S	6	10	9	12	9	11	13	13	12	15	13	9	8	8	8	Anderson 1990
Thetford, Norfolk	A-S	0	7	20	13	5	2	2	3	5	9	6	4	5	2	3	Stroud 1993
Ulwell, Dorset	A-S	0	0	1	9	0	4	3	2	6	3	3	4	4	1	3	Waldron 1987a
Westfield Farm, Ely	A-S	0	0	1	1	2	1	0	3	1	2	2	0	0	0	1	Hills and Dodwell 2007
Orchard Lane, Huntingdon	Med	0	1	10	5	3	4	3		2	2	0	3	2	5	4	Duhig 1996
Rivenhall, Suffolk	Med	0	2	14	16	4	3	6	3	3	3	3	14	6	32	31	O'Connor 1993
St Peter's, Barton-upon-Humber, Lincs	Med	8	116	88	92	56	16	27	61	51	27	5	59	32	76	135	Waldron 2007
Stonar, Kent	Med	17	9	24	13	0	5	2	16	6	11	8	8	4	7	7	Bayley and Eley 1975

Table A2.1: Numbers of individuals from Fen sites. Rom - Roman; A-S – Anglo-Saxon; Med – Medieval.

Site	Period	< 1 month	1-12 months	1-6 years	7-12 years	13-17 years	Male 18-24 years	Female 18-24 years	Male 25-34 years	Female 25-34 years	Male 35-49 years	Female 35-49 years	Male 50+	Female 50+	Male adult 18+	Female adult 18+	Reference
Ancaster, Lincs	Rom	34	6	19	22	11	8	11	42	16	33	19	13	14	33	23	Cox 1989
Babraham Institute, Cambs	Rom	0	2	5	2	1	0	0	1	2	15	9	0	0	2	3	Armour et al. 2007
Bainesse Farm, Catterick	Rom	0	0	1	5	1	2	0	1	1	3	2	3	2	0	2	Waldron 1985
Baldock, Herts	Rom	25	9	1	0	0	3	0	1	0	0	1	0	2	2	1	Henderson 1982
Mangiovinium, Bucks	Rom	1	7	1	0	0	0	1	1	2	1	3	0	2	4	3	Henderson 1981
Newarke Street, Leicester	Rom	1	1	1	5	0	0	4	2	4	1	1	5	1	1	1	Cooper 1996
Queenford Farm, Oxon	Rom	11	3	33	15	3	6	7	9	23	17	14	32	15	3	8	Chambers et al. 1987
Rudston Villa, Yorks	Rom	10	3	6	1	0	0	2	2	0	1	0	0	0	0	0	Bayley 1980a
St. Albans, Herts	Rom	7	1	0	0	1	1	2	0	0	0	0	0	0	0	0	Keepax 1973
Barnstaple Castle	A-S	0	5	9	4	1	5	10	12	4	2	6	0	0	5	4	Bayley 1976
Buckland, Kent	A-S	1	1	19	21	20	10	22	33	41	23	25	31	13	19	13	Richardson 2005
Castle Green, Hereford	A-S	5	10	8	0	0	6	4	6	4	12	0	3	0	5	9	Bayley 1980b
Charlton Plantation, Wilts	A-S	0	0	3	3	3	2	2	4	2	2	4	3	1	0	1	Henderson 1983a
Coddenham, Suffolk	A-S	0	0	9	2	2	3	2	3	0	2	1	2	1	3	4	Anderson 2011
Darenth Park, Kent	A-S	0	0	2	0	1	1	0	0	2	1	2	0	0	0	0	Richardson 2005
Dunton Green, kent	A-S	0	0	18	10	9	7	12	16	4	4	2	12	4	4	2	Richardson 2005
Empingham II, Rutland	A-S	0	2	20	17	15	18	16	11	10	6	6	1	2	6	3	Mays 1990
Filton, Bristol	A-S	2	0	2	5	3	3	4	1	2	0	5	3	1	4	2	Caffell and Holst 2005
George Street, Aylesbury	A-S	0	0	0	1	1	1	1	1	2	0	0	2	0	6	6	Henderson 1983b
Great Chesterford, Cambs	A-S	17	38	11	10	2	7	9	7	10	8	17	8	6	5	1	Inskip 2008
Henley Wood, Berks	A-S	0	4	2	4	3	8	6	9	5	4	3	2	0	1	2	Bayley 1973
Mill Hill, Kent	A-S	0	0	7	6	11	5	6	2	0	5	4	6	7	2	2	Richardson 2005
Norton, Cleveland	A-S	0	2	11	10	14	16	14	12	10	4	3	2	2	0	0	Marlow 1992

Orpington, Kent	A-S	0	0	3	1	1	1	5	5	5	5	4	2	0	1	5	Richardson 2005
School Street, Ipswich	A-S	0	2	4	8	4	5	6	8	5	3	8	11	1	8	8	Mays 1989
Sewerby, Yorks	A-S	0	0	1	3	2	1	1	3	5	4	1	1	1	3	3	Bayley 1974
West Heslerton, Yorks	A-S	1	2	11	6	8	2	2	5	9	4	1	0	3	4	8	Cox 1990
Blackfriars Street, Carlisle	Med	0	1	9	3	7	15	7	14	7	5	10	11	5	45	22	Henderson 1984
Brighton Hill South, Hamps	Med	3	6	16	2	0	0	0	1	1	6	3	4	1	2	2	Waldron 1987b
Corbridge, Northumberland	Med	0	1	1	0	0	1	0	0	0	1	1	1	0	0	0	Holst 2006
Wharram Percy, Yorks	Med	48	53	131	66	35	28	23	26	19	24	19	75	44	39	15	Mays 2007

Table A2.2: Numbers of individuals from non-Fen sites. Rom - Roman; A-S – Anglo-Saxon; Med – Medieval.

Sample	**Type**	**HI**	**Sample**	**Type**	**HI**
HDAR1	Adult rib	4	HDAP3	Adult phalanx	5
HDAR2	Adult rib	5	HDAP4	Adult phalanx	5
HDAR3	Adult rib	4	HDAP5	Adult phalanx	5
HDAR4	Adult rib	5	HDJR1	Sub-adult rib	1
HDAR5	Adult rib	4	HDJR2	Sub-adult rib	2
HDAR6	Adult rib	5	HDJR3	Sub-adult rib	2
HDAR7	Adult rib	2	HDJS1	Sub-adult cranium	2
HDAR8	Adult rib	1	HDJS2	Sub-adult cranium	1
HDAR9	Adult rib	5	HDJS3	Sub-adult cranium	1
HDAR10	Adult rib	3	AN1	Animal rib	5
HDAS1	Adult cranium	3	AN2	Animal rib	5
HDAS2	Adult cranium	0	AN3	Animal rib	4
HDAS3	Adult cranium	2	AN4	Animal rib	3
HDAS4	Adult cranium	1	AN5	Animal rib	0
HDAS5	Adult cranium	0	AN6	Animal rib	4
HDAP1	Adult phalanx	2	AN7	Animal rib	5
HDAP2	Adult phalanx	3	AN8	Animal rib	5

Table A2.3: Control sample (Hanging Ditch site) histological index (HI).

Sample	Age	Sex	Element	Macroscopic Condition	HI
DU2.1	Adolescent	?	Rib	Good	2
DU2.2	Adolescent	?	Cranium	Good	0
DU4.1	Child	?	Rib	Fair	2
DU4.2	Child	?	Rib	Fair	2
DU536.1	Middle adult	Female	Rib	Fair	3
DU536.2	Middle adult	Female	Cranium	Fair	2
DU601	Very young adult	?	Rib	Good	3
DU661	Very young adult	?	Rib	Fair	4
DUA2	Animal	N/A	Rib	Fair	0
DUA3	Animal	N/A	Rib	Fair	2

Table A2.4: Histological index (HI) and observed macroscopic condition of bone samples from the *Durobrivae* Roman site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
GM12.1	Juvenile	?	Rib	Fair	1
GM12.2	Juvenile	?	Rib	Fair	0
GM18.1	Very young adult	Female	Rib	Fair	0
GM18.2	Very young adult	Female	Hand phalanx	Fair	0
GM18.3	Very young adult	Female	Cranium	Fair	0
GM20.1	Juvenile	?	Rib	Fair	0
GM20.2	Juvenile	?	Hand phalanx	Fair	0
GM23.1	Child	?	Rib	Fair	1
GM23.2	Child	?	Cranium	Fair	0
GM25.1	Very young adult	Female	Rib	Fair	0
GM25.2	Very young adult	Female	Foot phalanx	Fair	0
GM25.3	Very young adult	Female	Cranium	Good	0
GM29.1	Child	?	Rib	Fair	0
GM29.2	Child	?	Cranium	Good	0
GM32.1	Very young adult	Female	Rib	Fair	3
GM32.2	Very young adult	Female	Hand phalanx	Good	5
GM32.3	Very young adult	Female	Cranium	Good	1
GM39.1	Juvenile	?	Rib	Good	3
GM39.2	Juvenile	?	Rib	Good	2
GM4.1	Juvenile	?	Rib	Fair	2
GM4.2	Juvenile	?	Hand phalanx	Fair	1
GM4.3	Juvenile	?	Cranium	Good	2
GM42.1	Juvenile	?	Rib	Good	0
GM42.2	Juvenile	?	Hand phalanx	Good	0
GM49.1	Juvenile	?	Rib	Fair	2
GM49.2	Juvenile	?	Hand phalanx	Fair	0
GM51.1	Juvenile	?	Rib	Fair	3
GM51.2	Juvenile	?	Rib	Fair	2
GM58	Adolescent	Female?	Cranium	Good	4
GM59.1	Very young adult	?	Rib	Good	0
GM59.2	Very young adult	?	Foot phalanx	Good	1
GM59.3	Very young adult	?	Cranium	Good	0
GMA1	Animal	N/A	Rib	Fair	0
GMA2	Animal	N/A	Rib	Fair	2
GMA4	Animal	N/A	Rib	Fair	1
GMA5	Animal	N/A	Rib	Good	0

Table A2.5: Histological index (HI) and observed macroscopic condition of bone samples from The Parks, Godmanchester Roman site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
HP23.2	Young adult	Female?	Foot phalanx	Good	0
HP23.2	Young adult	Female?	Foot phalanx	Good	0
HP104.1	Child	?	Rib	Fair	4
HP104.2	Child	?	Rib	Fair	3
HP113.1	Adolescent	?	Rib	Good	1
HP113.2	Adolescent	?	Hand phalanx	Good	1
HP117.1	Child	?	Rib	Good	0
HP136.1	Adolescent	?	Rib	Good	2
HP136.2	Adolescent	?	Hand phalanx	Good	1
HP136.3	Adolescent	?	Hand phalanx	Good	1
HP154.1	Young adult	Female	Rib	Good	5
HP154.2	Young adult	Female	Hand phalanx	Good	5
HP154.3	Young adult	Female	Hand phalanx	Good	5
HP157.1	Young adult	Female?	Rib	Good	0
HP157.2	Young adult	Female?	Rib	Fair	2
HP179.1	Adolescent	?	Rib	Good	0
HP179.2	Adolescent	?	Hand phalanx	Good	4
HP179.3	Adolescent	?	Foot phalanx	Good	4
HP214.1	Very young adult	Female	Rib	Good	0
HP214.2	Very young adult	Female	Hand phalanx	Good	1
HP214.3	Very young adult	Female	Foot phalanx	Good	1
HP217.1	Young adult	Female	Rib	Good	0
HP222.1	Young adult	Female	Rib	Good	0
HP222.2	Young adult	Female	Foot phalanx	Good	5
HP227.1	Child	?	Rib	Good	0
HP227.2	Child	?	Rib	Good	2
HPA1	Animal	N/A	Rib	Good	3
HPA2	Animal	N/A	Rib	Good	1
HPA3	Animal	N/A	Rib	Good	5
HPA4	Animal	N/A	Rib	Good	0
HPA5	Animal	N/A	Rib	Good	1

Table A2.6: Histological index (HI) and observed macroscopic condition of bone samples from the Hoplands, Sleaford Roman site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
PW13.1	Young adult	Female	Rib	Good	3
PW13.2	Young adult	Female	Rib	Good	4
PW13.3	Young adult	Female	Hand phalanx	Good	5
PWA1	N/A	Animal	Rib	Good	1
PWA2	N/A	Animal	Rib	Fair	1

Table A2.7: Histological index (HI) and observed macroscopic condition of bone samples from the Prickwillow Road, Ely Roman site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
WM2104.1	Young adult	Female	Rib	Fair	3
WM2104.2	Young adult	Female	Hand phalanx	Fair	0
WM2125.1	Child	?	Rib	Fair	1
WM2125.2	Child	?	Rib	Fair	1
WM2221.1	Young adult	Female?	Rib	Fair	3
WM2221.2	Young adult	Female?	Hand phalanx	Fair	5
WM2244.1	Young adult	Female	Rib	Fair	0
WM2244.2	Young adult	Female	Hand phalanx	Fair	5
WM2283.1	Young adult	Female	Rib	Good	0
WM2283.2	Young adult	Female	Cranium	Good	0
WM2291.1	Juvenile	?	Rib	Good	0
WM2291.2	Juvenile	?	Foot phalanx	Good	0
WM2291.3	Juvenile	?	Cranium	Good	2
WM2293.1	Juvenile	?	Rib	Fair	3
WM2293.2	Juvenile	?	Cranium	Fair	0
WM2297.1	Child	?	Rib	Fair	0
WM2297.2	Child	?	Rib	Fair	0
WM2316.1	Adolescent	?	Rib	Fair	2
WM2316.2	Adolescent/	?	Foot phalanx	Fair	5
WMA1	Animal	N/A	Rib	Good	2
WMA3	Animal	N/A	Rib	Fair	0
WMA4	Animal	N/A	Rib	Fair	0

Table A2.8: Histological index (HI) and observed macroscopic condition of bone samples from the Watersmeet, Huntingdon Roman site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
BS54.1	Very young adult	Female	Rib	Fair	1
BS54.2	Very young adult	Female	Hand phalanx	Fair	2
BSA1	Animal	N/A	Rib	Fair	0
BSA2	Animal	N/A	Rib	Good	1
BSA3	Animal	N/A	Rib	Good	1

Table A2.9: Histological index (HI) and observed macroscopic condition of bone samples from the Baston Anglo-Saxon site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
CD14.1	Juvenile	?	Rib	Poor	1
CD14.2	Juvenile	?	Cranium	Fair	0
CD16.1	Child	?	Rib	Poor	3
CD16.2	Child	?	Cranium	Fair	0
CD20	Juvenile	?	Cranium	Poor	1
CD28.1	Juvenile	?	Rib	Fair	4
CD32.1	Juvenile	?	Rib	Good	1
CD32.2	Juvenile	?	Cranium	Fair	5
CD34.1	Juvenile	?	Rib	Good	3
CD34.2	Juvenile	?	Cranium	Good	0
CD34.3	Juvenile	?	Foot phalanx	Fair	2
CD37.1	Juvenile	?	Cranium	Poor	3
CD61.1	Child	?	Rib	Poor	0
CD61.2	Child	?	Cranium	Fair	0
CD68.1	Child	?	Rib	Fair	0
CD68.2	Child	?	Cranium	Fair	3
CD76.1	Juvenile	?	Rib	Good	0
CD76.2	Juvenile	?	Cranium	Good	1
CD77.1	Adolescent	?	Rib	Poor	3
CD77.2	Adolescent	?	Cranium	Poor	3
CD84.1	Very young adult	Female	Rib	Fair	1
CD84.2	Very young adult	Female	Cranium	Good	0
CD84.3	Very young adult	Female	Foot phalanx	Good	1
CD88.1	Very young adult	Female	Rib	Good	1
CD96.1	Very young adult	Female	Rib	Fair	0
CD96.2	Very young adult	Female	Rib	Fair	1
CD107.1	Very young adult	Female	Rib	Poor	4
CD107.2	Very young adult	Female	Cranium	Poor	3
CD112.1	Very young adult	Female	Rib	Good	1
CD112.2	Very young adult	Female	Cranium	Good	1

CD112.3	Very young adult	Female	Hand phalanx	Good	2
CD120.1	Very young adult	Female	Rib	Fair	0
CD120.2	Very young adult	Female	Hand phalanx	Good	5
CD127.1	Very young adult	Female	Rib	Fair	4
CD127.2	Very young adult	Female	Cranium	Poor	5
CD134.1	Very young adult	Female	Rib	Fair	2
CD134.2	Very young adult	Female	Foot phalanx	Fair	0
CD155.1	Very young adult	Female	Rib	Good	2
CD155.2	Very young adult	Female	Cranium	Fair	1
CD156.1	Adolescent	?	Rib	Fair	2
CD156.2	Adolescent	?	Cranium	Poor	4
CD156.3	Adolescent	?	Hand phalanx	Fair	5
CD164.1	Very young adult	Female?	Rib	Fair	1
CD164.2	Very young adult	Female?	Cranium	Poor	4
CD165.1	Child	?	Rib	Good	5
CD165.2	Child	?	Cranium	Fair	4
CD172.1	Child	?	Rib	Good	2
CD172.2	Child	?	Cranium	Good	1
CD183.1	Very young adult	Female	Rib	Fair	2
CD183.2	Very young adult	Female	Cranium	Good	0
CD183.3	Very young adult	Female	Foot phalanx	Good	0
CDA1	Animal	N/A	Rib	Good	0
CDA2	Animal	N/A	Rib	Good	0
CDA3	Animal	N/A	Rib	Good	0
CDA4	Animal	N/A	Rib	Good	2
CDA5	Animal	N/A	Rib	Good	0

Table A2.10: Histological index (HI) and observed macroscopic condition of bone samples from the Castledyke South Anglo-Saxon site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
CL11.1	Young adult	Female	Rib	Fair	4
CL11.2	Young adult	Female	Cranium	Poor	0
CL13.1	Adolescent	Female	Rib	Poor	0
CL13.2	Adolescent	Female	Cranium	Poor	0
CL13.3	Adolescent	Female	Hand phalanx	Poor	0
CL26.1	Young adult	Female	Rib	Poor	0
CL26.2	Young adult	Female	Cranium	Fair	0
CL27.1	Adolescent	Male	Rib	Fair	0
CL27.2	Adolescent	Male	Cranium	Fair	1
CL27.3	Adolescent	Male	Hand phalanx	Good	1
CL28.1	Adolescent	Male	Rib	Poor	0
CL28.2	Adolescent	Male	Cranium	Poor	1
CL28.3	Adolescent	Male	Hand phalanx	Fair	0
CL35.1	Child	?	Cranium	Poor	3
CL50.1	Young adult	Female	Rib	Fair	1
CL50.2	Young adult	Female	Cranium	Poor	1
CL54.1	Young adult	Female	Rib	Poor	0
CL54.2	Young adult	Female	Cranium	Fair	0
CL58	Juvenile	?	Cranium	Poor	0
CL61.1	Adolescent	Female	Rib	Fair	1
CL61.2	Adolescent	Female	Cranium	Poor	1

Table A2.11: Histological index (HI) and observed macroscopic condition of bone samples from the Cleatham Anglo-Saxon site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
EH19.1	Very young adult	Male	Rib	Fair	0
EH19.2	Very young adult	Male	Foot phalanx	Good	4
EH45.1	Very young adult	Female	Cranium	Good	3
EH45.2	Very young adult	Female	Hand phalanx	Good	4
EH119.1	Young adult	Female	Rib	Fair	0
EH119.2	Young adult	Female	Hand phalanx	Fair	2
EH128.1	Adolescent	Female	Cranium	Fair	2
EH128.2	Adolescent	Female	Hand phalanx	Fair	3
EH133.1	Child	?	Rib	Good	1
EH133.2	Child	?	Cranium	Good	2
EH156.1	Young adult	Female	Rib	Poor	3
EH156.2	Young adult	Female	Foot phalanx	Fair	0
EH156.3	Young adult	Female	Cranium	Fair	5
EH172.1	Juvenile	?	Rib	Fair	3
EH172.2	Juvenile	?	Cranium	Good	0
EH198.1	Very young adult	Male	Rib	Fair	4
EH198.2	Very young adult	Male	Foot phalanx	Good	1
EH436.1	Very young adult	Female	Rib	Good	0
EH436.2	Very young adult	Female	Foot phalanx	Good	1
EH436.3	Very young adult	Female	Cranium	Good	2
EH584.1	Child	?	Rib	Good	0
EH584.2	Child	?	Cranium	Good	0
EH586.1	Juvenile	?	Rib	Fair	3
EH586.2	Juvenile	?	Hand phalanx	Fair	1
EH586.3	Juvenile	?	Cranium	Fair	4
EHA1	Animal	N/A	Rib	Fair	0
EHA2	Animal	N/A	Rib	Fair	2

Table A2.12: Histological index (HI) and observed macroscopic condition of bone samples from the Edix Hill Anglo-Saxon site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
HA5.1	?	?	Rib	Fair	4
HA5.2	?	?	Cranium	Fair	0

Table A2.13: Histological index (HI) and observed macroscopic condition of bone samples from the Haddenham Anglo-Saxon site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
LP3311.1	Juvenile	?	Rib	Good	0
LP3311.2	Juvenile	?	Cranium	Good	0
LP3687.1	Young adult	Female	Rib	Fair	1
LP3687.2	Young adult	Female	Foot phalanx	Fair	2
LP3690.1	Young adult	?	Rib	Good	0
LP3690.2	Young adult	?	Cranium	Good	1
LP3690.3	Young adult	?	Hand phalanx	Good	0
LP3745.1	Young adult	Female	Rib	Good	1
LP3745.2	Young adult	Female	Cranium	Good	0
LP3745.3	Young adult	Female	Foot phalanx	Fair	2
LP3760.1	Juvenile	?	Rib	Good	0
LP3760.2	Juvenile	?	Cranium	Good	0
LP3770.1	Child	?	Rib	Fair	0
LP3770.2	Child	?	Cranium	Fair	0
LP3819.1	Adult	Female?	Rib	Fair	0
LP3819.2	Adult	Female?	Cranium	Fair	2
LP3819.3	Adult	Female?	Hand phalanx	Fair	0
LP3845.1	Adolescent	?	Rib	Fair	0
LP3845.2	Adolescent	?	Cranium	Fair	0
LP4037.1	Child	?	Cranium	Fair	2
LP4037.2	Child	?	Cranium	Fair	1
LP4046.1	Adolescent	?	Rib	Good	1
LP4046.2	Adolescent	?	Cranium	Fair	2
LP4046.3	Adolescent	?	Foot phalanx	Good	0
LP4067.1	Middle adult	Female	Rib	Fair	2
LP4067.2	Middle adult	Female	Cranium	Fair	1
LP4067.3	Middle adult	Female	Foot phalanx	Fair	3
LP4085.1	Young adult	Female?	Rib	Good	0
LP4085.2	Young adult	Female?	Foot phalanx	Fair	2
LP4092.1	Young adult	Male?	Rib	Good	1
LP4092.2	Young adult	Male?	Cranium	Fair	1
LP4092.3	Young adult	Male?	Hand phalanx	Fair	3
LP4112.1	Adolescent	?	Rib	Fair	2
LP4112.2	Adolescent	?	Cranium	Good	0
LP4116.1	Juvenile	?	Rib	Good	0
LP4116.2	Juvenile	?	Cranium	Good	0
LP4116.3	Juvenile	?	Foot phalanx	Fair	1
LP4144.1	Juvenile	?	Rib	Fair	0
LP4144.2	Juvenile	?	Hand phalanx	Fair	1
LP4155.1	Juvenile	?	Cranium	Fair	0

LP4156.1	Adolescent	?	Rib	Fair	0
LP4156.2	Adolescent	?	Cranium	Good	0
LP4167.1	Juvenile	?	Rib	Fair	0
LP4167.2	Juvenile	?	Rib	Fair	1
LP4174.1	Adolescent	?	Rib	Fair	0
LP4174.2	Adolescent	?	Cranium	Good	1
LP4174.3	Adolescent	?	Foot phalanx	Fair	2
LP4371.1	Adolescent	?	Rib	Poor	0
LP4371.2	Adolescent	?	Cranium	Fair	0
LP4373.1	Young adult	Female?	Rib	Fair	0
LP4373.2	Young adult	Female?	Cranium	Good	1
LP4373.3	Young adult	Female?	Foot phalanx	Fair	0
LP4395.1	Middle adult	Female	Rib	Good	0
LP4395.2	Middle adult	Female	Cranium	Good	0
LP4395.3	Middle adult	Female	Hand phalanx	Good	0
LP4494.1	Juvenile	?	Cranium	Fair	0
LP4585.1	Middle adult	Female?	Rib	Good	1
LP4585.2	Middle adult	Female?	Cranium	Good	0
LP4585.3	Middle adult	Female?	Hand phalanx	Good	2
LP4751.1	Adolescent	?	Rib	Fair	0
LP4751.2	Adolescent	?	Cranium	Fair	1
LP4751.3	Adolescent	?	Foot phalanx	Fair	2
LP4848.1	Child	?	Rib	Good	0
LP4848.2	Child	?	Cranium	Good	0
LP5252.1	Child	?	Rib	Fair	0
LP5252.2	Child	?	Cranium	Fair	0
LPA1	Animal	N/A	Rib	Fair	3
LPA3	Animal	N/A	Rib	Fair	3

Table A2.14: Histological index (HI) and observed macroscopic condition of bone samples from the Highfield Farm, Littleport Anglo-Saxon site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
WF25.2	Young adult	Female	Hand phalanx	Poor	1
WF28.1	Adolescent	?	Rib	Poor	1
WF45.2	Adolescent	Female?	Foot phalanx	Poor	2
WF51.1	Juvenile	?	Rib	Poor	0
WF57.2	Child	?	Rib	Poor	0
WF57.3	Child	?	Rib	Poor	0
WF25.1	Young adult	Female?	Rib	Poor	2
WF28.2	Adolescent	?	Foot phalanx	Poor	2
WF45.1	Adolescent	Female?	Rib	Poor	2
WF51.2	Juvenile	?	Rib	Poor	1
WF57.1	Child	?	Rib	Poor	1

Table A2.15: Histological index (HI) and observed macroscopic condition of bone samples from the Westfield Farm, Ely Anglo-Saxon site.

Sample	Age	Sex	Element	Macroscopic Condition	HI
OL1071.1	Adolescent	Female?	Rib	Fair	0
OL1071.2	Adolescent	Female?	Hand phalanx	Good	4
OL1072.1	Very young adult	Female	Rib	Good	0
OL1072.2	Very young adult	Female	Hand phalanx	Good	1
OL1072.3	Very young adult	Female	Cranium	Good	0
OL1094.1	Juvenile	?	Rib	Fair	4
OL1094.2	Juvenile	?	Hand phalanx	Fair	1
OL1094.3	Juvenile	?	Cranium	Fair	1
OL1100.1	Child	?	Rib	Good	0
OL1100.2	Child	?	Cranium	Good	1
OL1104.1	Young adult	Female	Rib	Fair	2
OL1104.2	Young adult	Female	Hand phalanx	Fair	5
OL1104.3	Young adult	Female	Cranium	Fair	1
OL1123.2	Juvenile	?	Hand phalanx	Fair	5
OL1125.1	Child	?	Rib	Good	1
OL1125.2	Child	?	Cranium	Good	1
OL1130.1	Very young adult	Female	Rib	Fair	0
OL1130.2	Very young adult	Female	Cranium	Fair	0
OLA1	Animal	N/A	Rib	Good	2
OLA2	Animal	N/A	Rib	Fair	2

Table A2.16: Histological index (HI) and observed macroscopic condition of bone samples from the Orchard Lane, Huntingdon medieval site.

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Fornaciari et al. (2010) protocol:

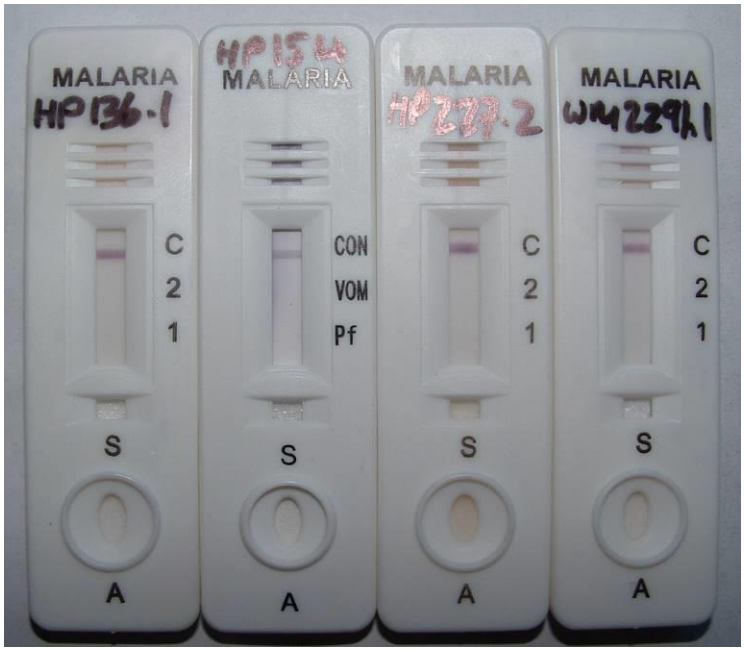

Figure A2.1: Malaria rapid test results following Fornaciari et al. (2010) protocol. CON – control line; VOM or 2 – *P. vivax, P. malariae,* or *P. ovale*; Pf or 1 – *P. falciparum*.

Figure A2.2: Malaria rapid test results following Fornaciari et al. (2010) protocol. CON – control line; 2 – *P. vivax, P. malariae,* or *P. ovale*; 1 – *P. falciparum*.

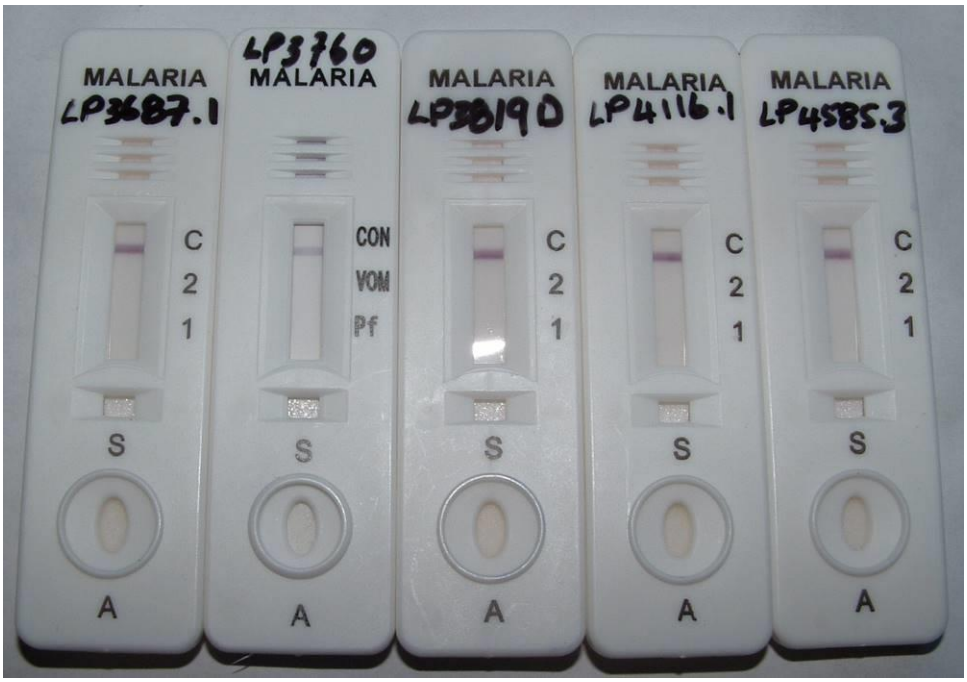

Figure A2.3: Malaria rapid test results following Fornaciari et al. (2010) protocol. CON – control line; VOM or 2 – *P. vivax, P. malariae,* or *P. ovale*; Pf or 1 – *P. falciparum*.

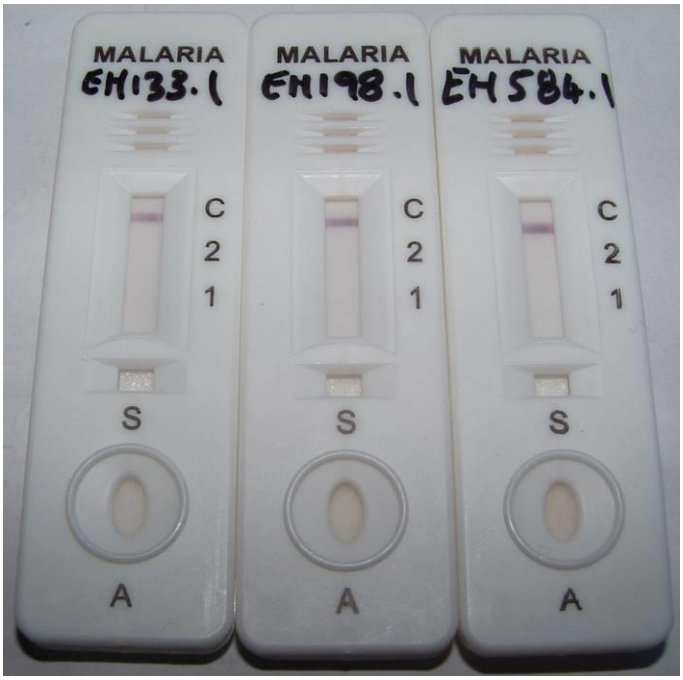

Figure A2.4: Malaria rapid test results following Fornaciari et al. (2010) protocol. CON – control line; 2 – *P. vivax, P. malariae,* or *P. ovale*; 1 – *P. falciparum*.

Figure A2.5: Malaria rapid test results following Fornaciari et al. (2010) protocol. CON – control line; 2 – *P. vivax, P. malariae,* or *P. ovale*; 1 – *P. falciparum.*

Figure A2.6: Malaria rapid test results following Fornaciari et al. (2010) protocol. Collagen control. CON – control line; 2 – *P. vivax, P. malariae,* or *P. ovale*; 1 – *P. falciparum*.

P2.5 samples

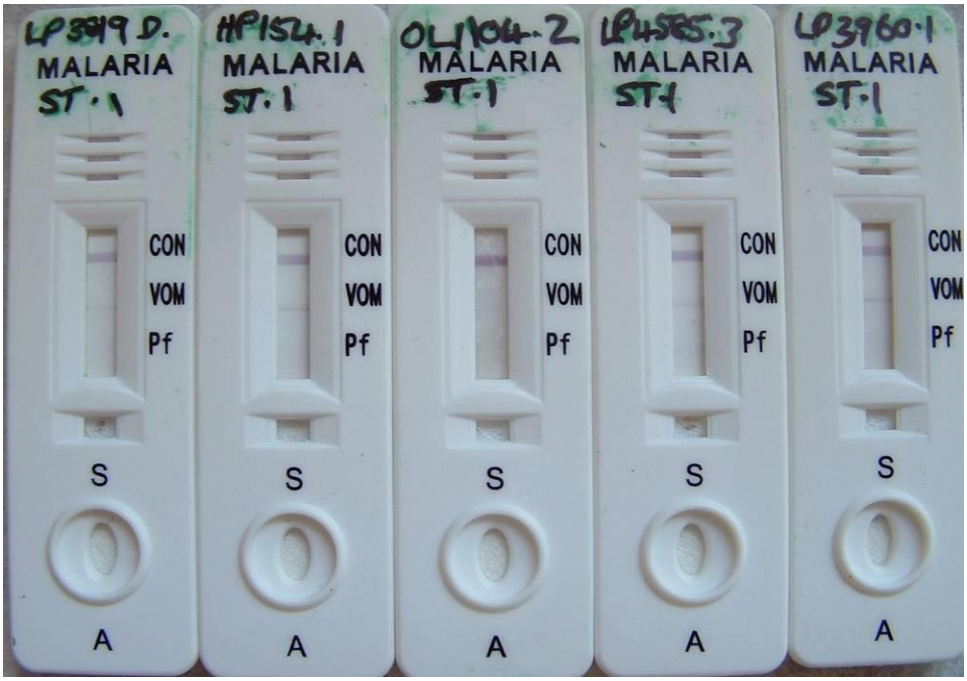

Figure A2.7: Malaria rapid test results from P2.5 samples, 1st extraction stage. CON – control line; VOM – *P. vivax, P. malariae,* or *P. ovale*; Pf – *P. falciparum.*

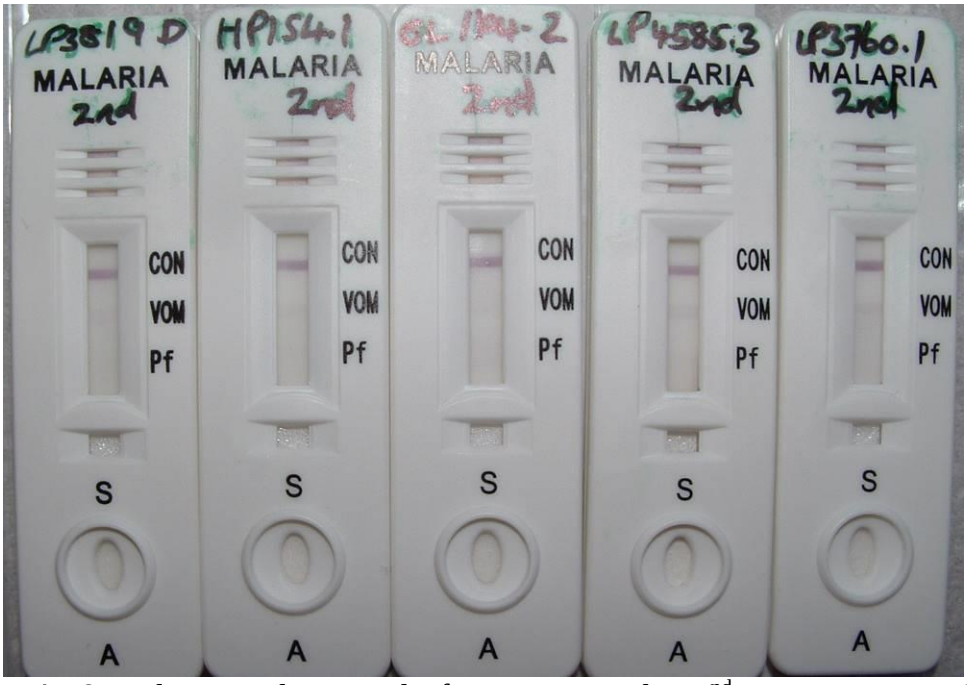

Figure A2.8: Malaria rapid test results from P2.5 samples, 2nd extraction stage. CON – control line; VOM – *P. vivax, P. malariae,* or *P. ovale*; Pf – *P. falciparum.*

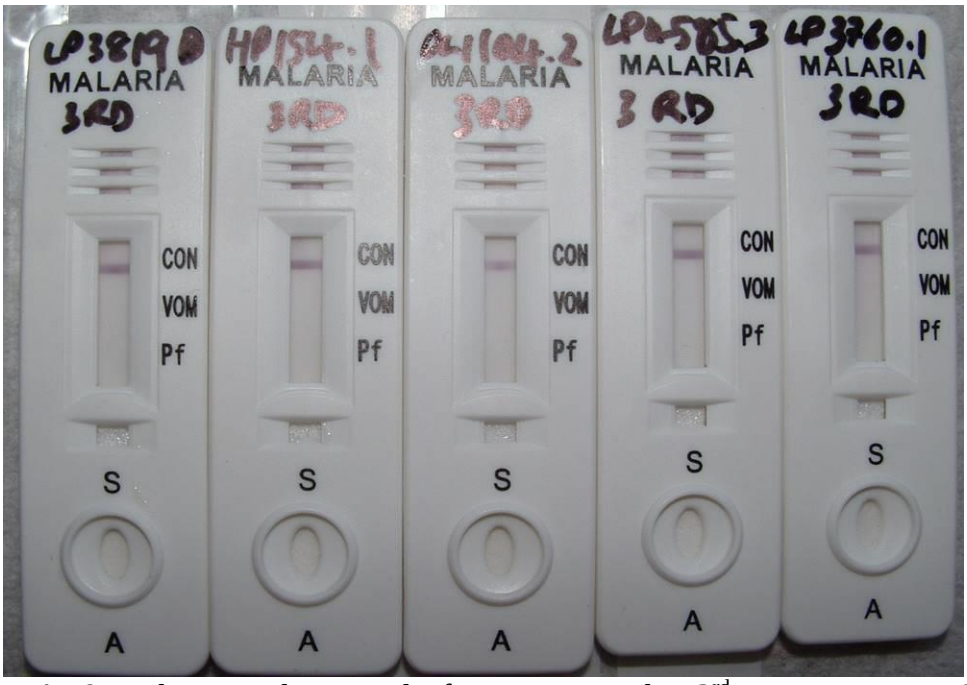

Figure A2.9: Malaria rapid test results from P2.5 samples, 3rd extraction stage. CON – control line; VOM – *P. vivax, P. malariae,* or *P. ovale*; Pf – *P. falciparum*.

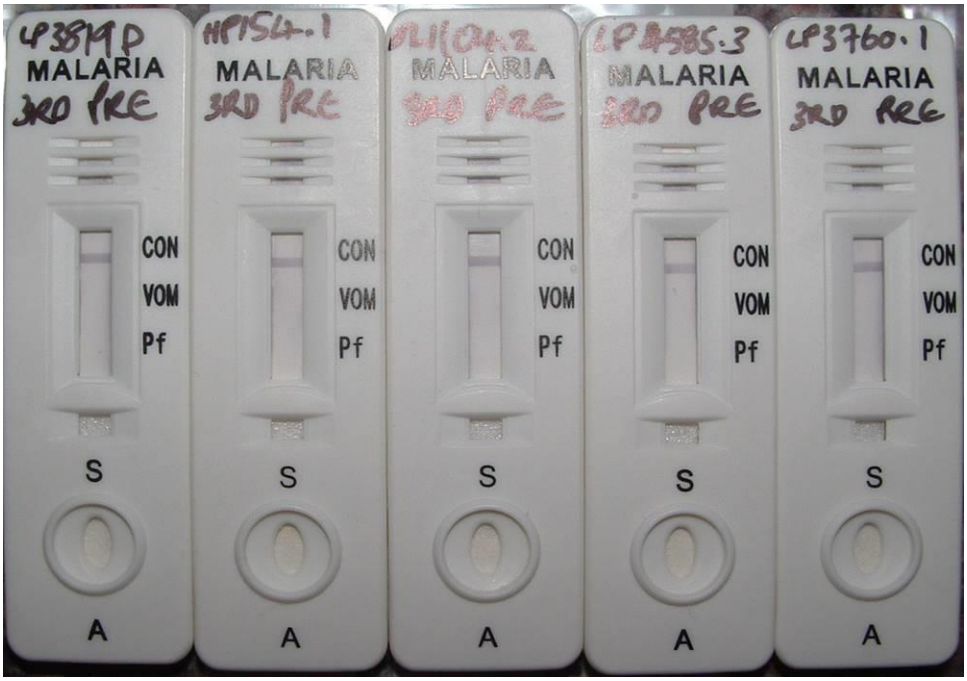

Figure A2.10: Malaria rapid test results from P2.5 samples following thiophilic chromatography adsorption. CON – control line; VOM – *P. vivax, P. malariae,* or *P. ovale*; Pf – *P. falciparum*.

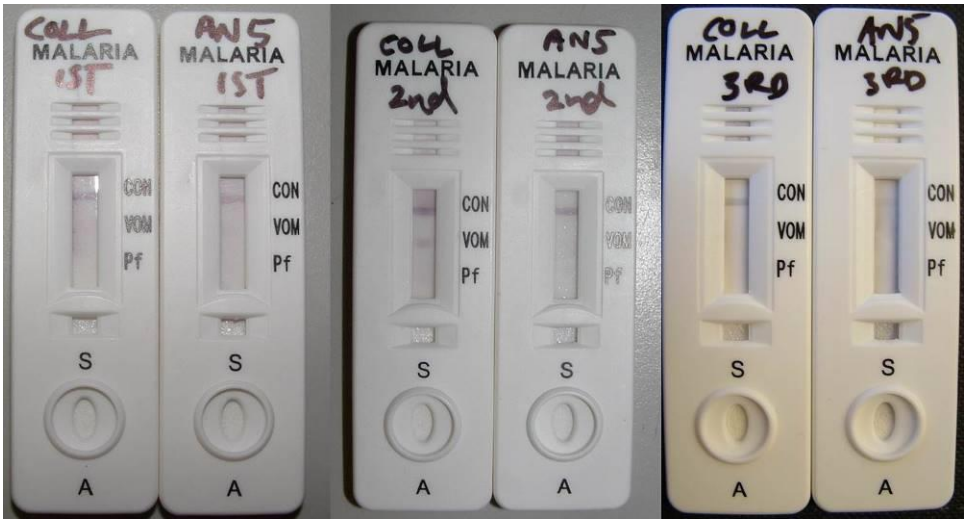

Figure A2.11: Malaria rapid test results for adapted Jiang et al. protocol using collagen (COLL) and animal bone (AN5) controls. CON – control line; VOM – *P. vivax, P. malariae,* or *P. ovale*; Pf – *P. falciparum*.

P5 samples

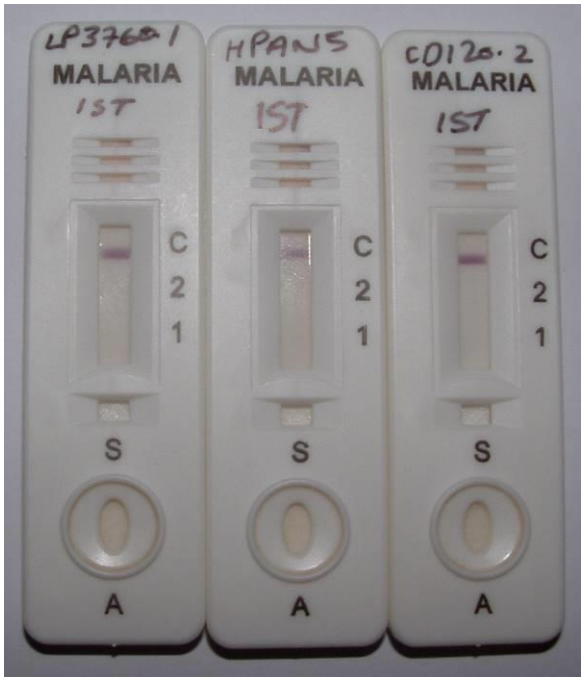

Figure A2.12: Malaria rapid test results for adapted P5 Cappellini et al. protocol, 1st extraction. HPAN5 – control animal sample. CON – control line; 2 – *P. vivax, P. malariae,* or *P. ovale*; 1 – *P. falciparum*.

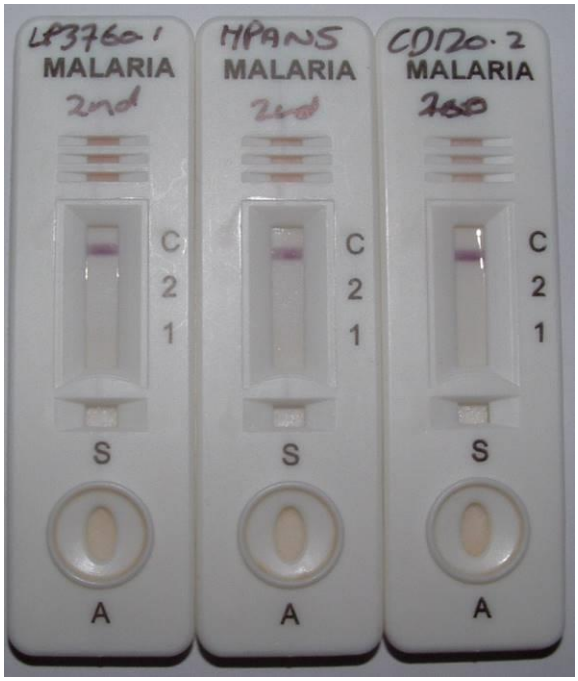

Figure A2.13: Malaria rapid test results for adapted P5 Cappellini et al. protocol, 2nd extraction. HPAN5 – control animal sample. CON – control line; 2 – *P. vivax, P. malariae,* or *P. ovale*; 1 – *P. falciparum*.

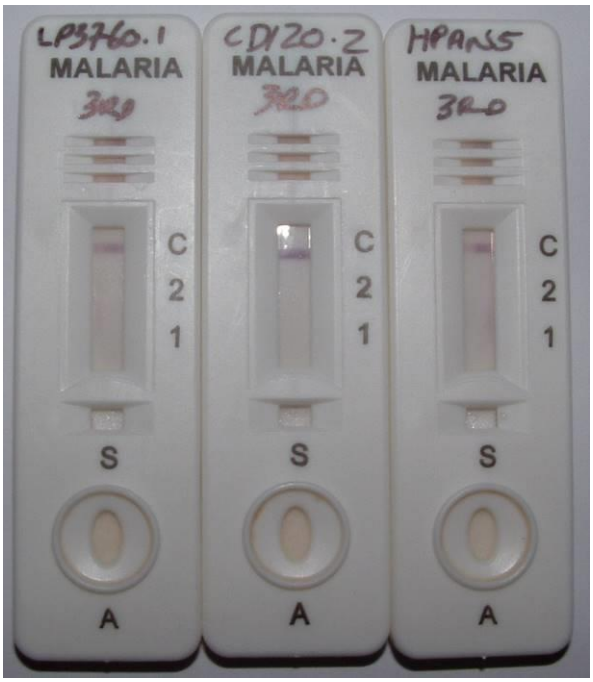

Figure A2.14: Malaria rapid test results for adapted P5 Cappellini et al. protocol, 3rd extraction. HPAN5 – control animal sample. CON – control line; 2 – *P. vivax, P. malariae,* or *P. ovale*; 1 – *P. falciparum*.

Appendix 3: nLCMS/MS proteomic results

Appendix 3 presents in-depth information on the results of nLC-MS/MS analysis of post-TAC elutions from the P3 and P4 protocols (section 6.45). Groups identified with 95% confidence and above (for human non-contaminating proteins, and non-human proteins of interest) are shown, with modifications and cleavages, where present. Trypsin is a remnant from pre-analysis digestion.

Table column title key:

N – Protein group
SC – Sequence coverage (%)
Prec MW – Precursor molecular weight

CD120.2, P3 2nd extraction, pre-exclusion, TrEMBL database

N	SC	Name	Peptides (95%)
1	58.9	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	24
2	51.3	Collagen, type I, alpha 1, isoform CRA_a OS=Homo sapiens GN=COL1A1 PE=4 SV=1	19
3	26.6	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	6
4	25	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	5
5	32	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	3
6	20.5	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	2
7	29.1	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2	2

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GETGPSGPVGPAGAVGPR			1561.8
GIPGPVGAAGATGAR	Oxidation(P)@5		1266.6
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.7
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@8		1253.6

GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Oxidation(P)@14; Oxidation(P)@21		2114.1
GPNGEAGSAGPPGPPGLR	Deamidated(N)@3; Oxidation(P)@12; Oxidation(P)@15		1619.7
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@11; Oxidation(P)@21; Oxidation(P)@24		2958.4
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@3; Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1751.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1844.9
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@11		1200.6
GENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Deamidated(N)@21; Oxidation(P)@24		2567.2

Group 2: Collagen, type I, alpha 1, isoform CRA

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@8; Oxidation(P)@15; Oxidation(P)@24		2280.1
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.1
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15	missed K-G@18	2868.4
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@5; Oxidation(M)@6; Oxidation(P)@15		1831.9
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9

CD120.2, P3 2nd extraction, post-exclusion, TrEMBL database

N	SC	Name	Peptides (95%)
1	25.9	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	3
2	24.4	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	2
3	25.5	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2	1
4	8.7	Terminal uridylyltransferase 4 OS=Homo sapiens GN=ZCCHC11 PE=1 SV=3	1

Group 4: Terminal uridylyltransferase 4

Sequence	Modifications	Cleavages	Prec MW
MDDFQLKGIVEEKFVK	Oxidation(M)@1; Lys->Allysine(K)@7	missed K-G@7; missed K-F@13	1939.9

CD120.2, P3 3nd extraction, pre-exclusion, TrEMBL database

N	SC	Name	Peptides (95%)
1	44.6	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	15
2	35.9	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	12
3	30.3	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	10
4	40.9	Collagen, type I, alpha 1, isoform CRA_a OS=Homo sapiens GN=COL1A1 PE=4 SV=1	7
5	27.1	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	8
6	25	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	6
7	22.4	Keratin, type I cytoskeletal 15 OS=Homo sapiens GN=KRT15 PE=1 SV=3	2
8	32.8	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	12
9	10	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GETGPSGPVGPAGAVGPR			1561.8
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Oxidation(P)@14; Oxidation(P)@21		2114.1
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@11; Oxidation(P)@21; Oxidation(P)@24		2958.4
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@3; Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1751.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.7
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7

Group 4: Collagen, type I, alpha 1, isoform CRA

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
GETGPAGPAGPVGPVGAR			1545.8
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.7

CD120.2, P3 3nd extraction, post-exclusion, TrEMBL database

N	SC	Name	Peptides (95%)
1	31.5	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	7
2	21.3	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	3
3	15.4	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	2
4	28.5	Collagen, type I, alpha 1, isoform CRA_a OS=Homo sapiens GN=COL1A1 PE=4 SV=1	2
5	22.4	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	1
6	25.2	cDNA FLJ16494 fis, clone CTONG3004576, highly similar to Keratin, type I cytoskeletal 15 OS=Homo sapiens PE=2 SV=1	1
7	6.5	Terminal uridylyltransferase 4 OS=Homo sapiens GN=ZCCHC11 PE=1 SV=3	1
8	10	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2	1
9	6.1	Protein LOC100652824 (Fragment) OS=Homo sapiens GN=LOC100652824 PE=2 SV=1	1
10	9.2	Ankyrin repeat and SOCS box protein 18 OS=Homo sapiens GN=ASB18 PE=2 SV=2	1

Group 4: Collagen, type I, alpha 1, isoform CRA

Sequence	Modifications	Cleavages	Prec MW
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0

Group 7: Terminal uridylyltransferase 4

Sequence	Modifications	Cleavages	Prec MW
MDDFQLKGIVEEKFVK	Dioxidation(M)@1; Dehydrated(D)@2; Deamidated(Q)@5	missed K-G@7; missed K-F@13	1939.9

Group 9: Protein LOC100652824 (Fragment)

Sequence	Modifications	Cleavages	Prec MW
EGKVYGQAEAAIGK		missed K-V@3	1419.7

Group 10: Ankyrin repeat and SOCS box protein 18

Sequence	Modifications	Cleavages	Prec MW
GAHVDARNGRGETALSAACGAAR		missed R-N@7; missed R-G@10	2210.1

CD84.2, P4 1st extraction, Swiss-Prot database

N	SC	Name	Peptides (95%)
1	70.3	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	62
2	66.3	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	89
3	31.1	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	11
4	25.6	Keratin, type II cytoskeletal 1 OS=Pan troglodytes GN=KRT1 PE=1 SV=1	10
5	28.6	Trypsin OS=Sus scrofa PE=1 SV=1	14
6	23.5	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	5
7	67.2	Collagen alpha-1(I) chain OS=Rattus norvegicus GN=Col1a1 PE=1 SV=5	52
8	8.1	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	2
10	66.1	Collagen alpha-1(I) chain OS=Bos taurus GN=COL1A1 PE=1 SV=3	67
11	62.2	Collagen alpha-2(I) chain OS=Canis familiaris GN=COL1A2 PE=2 SV=2	26
12	69.7	Collagen alpha-2(I) chain OS=Mammut americanum PE=1 SV=3	23
13	13.2	L-glutamine:scyllo-inososе aminotransferase OS=Streptomyces griseus GN=stsC PE=1 SV=1	1
14	58.8	Collagen alpha-2(I) chain OS=Mus musculus GN=Col1a2 PE=2 SV=2	20
15	59.5	Collagen alpha-1(II) chain OS=Rattus norvegicus GN=Col2a1 PE=1 SV=2	4
16	10.6	Probable tRNA sulfurtransferase OS=Lactobacillus casei (strain ATCC 334) GN=thiI PE=3 SV=1	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEAGAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.4
GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@3; Deamidated(N)@9; Deamidated(Q)@12; Oxidation(P)@15		2147
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Deamidated(N)@3; Deamidated(N)@21; Oxidation(P)@24		2568.2
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.7
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@9		1200.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GESGNKGEPGSAGPQGPPGPSGEEGK	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2395.1

GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21		2052
GHNGLDGLK	Deamidated(N)@3		910.5
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(H)@15; Oxidation(P)@21		2800.3
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.7
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6		1252.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@21		2114.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(M)@15		1782.8
GPNGEAGSAGPPGPPGLR	Deamidated(N)@3; Oxidation(P)@12; Oxidation(P)@15		1619.8
GPPGAAGAPGPQGF	Oxidation(P)@3; Oxidation(P)@9	cleaved F-Q@C-term	1211.6
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.4
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@3; Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1751.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
GPSGPPGPDGNKGEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@33	missed K-G@12	3212.5
GPSGPQGIR			867.5
GSDGSVGPVGPAGPIGSAGPPGFPGAPGPK	Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@27		2588.2
HGNRGETGPSGPVGPAGAVGPR		missed R-G@4	2025.9
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.6
PGPIGPAGAR	Oxidation(P)@1	cleaved R-P@N-term	907.5
PGPVGAAGATGAR	Oxidation(P)@1	cleaved I-P@N-term	1096.6
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
QGPAGEPGEPGQTGPAGAR	Oxidation(P)@7; Oxidation(P)@10	cleaved F-Q@N-term	1764.8
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1844.9

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGER			1689.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@5; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@21		2149.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.1
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.1
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12		2688.3
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.9
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@11		1192.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8

GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9

Group 13: *Streptomyces griseus*

Sequence	Modifications	Cleavages	Prec MW
LSELGMTAQATAPGTTARAYYRYLVR	Deamidated(Q)@9	missed R-A@18; missed R-Y@22	2860.3

Group 16: *Lactobacillus casei*

Sequence	Modifications	Cleavages	Prec MW
LNGNVTRALHEFPNLTIRPK	Deamidated(N)@4	missed R-A@7	2290.2

CD84.2, P4 1st extraction, TrEMBL database

N	SC	Name	Peptides 95%
1	77.1	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	63
2	72.4	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	105
3	29.5	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	10
4	30.4	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	10
5	34.2	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	5
6	14.6	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	2
7	69.9	Collagen, type I, alpha 1, isoform CRA_a OS=Homo sapiens GN=COL1A1 PE=4 SV=1	71
8	27.3	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2	1
9	2.6	MUC19 variant 12 OS=Homo sapiens GN=MUC19 PE=2 SV=1	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEAGAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3
GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@3; Deamidated(N)@9; Deamidated(Q)@12; Oxidation(P)@15		2147.0
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Deamidated(N)@3; Deamidated(N)@21; Oxidation(P)@24		2568.2
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.7
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@9		1200.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GESGNKGEPGSAGPQGPPGPSGEEGK	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2395.1
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21		2051.9
GEVGPAGPNGFAGPAGAAGQPGAKGER	Deamidated(N)@9; Oxidation(P)@21; Oxidation(K)@24	missed K-G@24	2409.2
GHNGLDGLK	Deamidated(N)@3		910.5
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(H)@15; Oxidation(P)@21		2800.3
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.7
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6		1252.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Cation:Na(E)@11; Oxidation(P)@14; Oxidation(P)@21		2136.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(M)@15		1766.8
GPNGEAGSAGPPGPPGLR	Deamidated(N)@3; Oxidation(P)@12; Oxidation(P)@15		1619.8
GPPGAAGAPGPQGF	Oxidation(P)@3; Oxidation(P)@9	cleaved F-Q@C-term	1211.6
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.4
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@3; Oxidation(P)@9;		1751.8

	Deamidated(N)@12; Oxidation(P)@15		
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
GPSGPPGPDGNKGEPGVVGAVG TAGPSGPSGLPGER	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@33	missed K-G@12	3212.5
GPSGPQGIR			867.5
GSDGSVGPVGPAGPIGSAGPPGF PGAPGPK	Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@27		2588.2
HGNRGETGPSGPVGPAGAVGPR		missed R-G@4	2025.9
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.7
PGPIGPAGAR	Oxidation(P)@1	cleaved R-P@N-term	907.5
PGPVGAAGATGAR	Oxidation(P)@1	cleaved I-P@N-term	1096.6
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
QGPAGEPGEPGQTGPAGAR	Oxidation(P)@7; Oxidation(P)@10	cleaved F-Q@N-term	1764.8
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1828.9
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9		1221.6

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GDAGAPGAPGSQGAPGLQ GMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2214.0
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAPG APGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GEPGPPGPAGAAGPAGNPG ADGQPGAK	Oxidation(P)@5; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.0
GEPGPPGPAGFAGPPGADG QPGAK	Oxidation(P)@5; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEG K	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEG KR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0

GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.1
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12		2688.3
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@9		1257.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.9
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(R)@27		2454.2
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(P)@9		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@5		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6		897.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Deamidated(Q)@9; Oxidation(P)@13; Dioxidation(P)@19		2057.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9

CD84.2, P4 2nd extraction, Swiss-Prot database

N	SC	Name	Peptides (95%)
1	74.2	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	86
2	61.7	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	87
3	37.2	Trypsin OS=Sus scrofa PE=1 SV=1	12
4	59.5	Collagen alpha-2(I) chain OS=Mus musculus GN=Col1a2 PE=2 SV=2	28
5	55.3	Collagen alpha-1(I) chain OS=Mus musculus GN=Col1a1 PE=1 SV=4	48
6	67	Collagen alpha-2(I) chain OS=Bos taurus GN=COL1A2 PE=1 SV=2	32
7	30.7	Protein S100-A7 OS=Homo sapiens GN=S100A7 PE=1 SV=4	2
8	5.9	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	2
9	64.7	Collagen alpha-2(I) chain OS=Rattus norvegicus GN=Col1a2 PE=1 SV=3	25
10	54.6	Collagen alpha-1(I) chain OS=Cynops pyrrhogaster GN=COL1A1 PE=2 SV=1	3
11	59.4	Collagen alpha-1(II) chain OS=Mus musculus GN=Col2a1 PE=1 SV=2	4
12	15	Chondroadherin OS=Bos taurus GN=CHAD PE=1 SV=1	1
13	82.5	Collagen alpha-1(I) chain OS=Mammut americanum GN=COL1A1 PE=1 SV=4	51
14	62.4	Collagen alpha-2(I) chain OS=Canis familiaris GN=COL1A2 PE=2 SV=2	30
15	5.8	Pigment epithelium-derived factor OS=Bos taurus GN=SERPINF1 PE=1 SV=1	1
16	4.2	Vitronectin OS=Homo sapiens GN=VTN PE=1 SV=1	1
17	28.6	Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1	1
18	13.5	Keratin, type I cytoskeletal 10 OS=Mus musculus GN=Krt10 PE=1 SV=3	1
19	27.4	Collagen alpha-1(X) chain OS=Bos taurus GN=COL10A1 PE=2 SV=1	1
20	9.2	Protein pelota homolog OS=Methanocaldococcus jannaschii (strain ATCC 43067 / DSM 2661 / JAL-1 / JCM 10045 / NBRC 100440) GN=pelA PE=3 SV=1	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
EGPVGLPGIDGRPGPIGPAGAR	Oxidation(P)@7; Oxidation(P)@13		2071.1
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@17		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGE AGAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3
GAPGPDGNNGAQGPPGPQGVQ GGK	Oxidation(P)@5; Deamidated(N)@9; Oxidation(P)@15		2146.0
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR			1546.8
GENGVVGPTGPVGAAGPAGPN GPPGPAGSR	Deamidated(N)@3; Deamidated(N)@21; Oxidation(P)@24		2568.2

GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.7
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@9		1200.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@3; Oxidation(P)@21		2136.0
GEQGPPGPPGFQGLPGPSGPAGEVGKPGER	Formyl@N-term; Oxidation(P)@17; Oxidation(P)@27		2869.3
GESGNKGEPGSAGPQGPPGPSGEEGK	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2395.0
GESGNKGEPGSAGPQGPPGPSGEEGKR	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6; missed K-R@26	2551.1
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Oxidation(P)@21		2051.0
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9		1221.6
GHNGLDGLK	Deamidated(N)@3		910.4
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@21		2800.4
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.7
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6		1252.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Oxidation(P)@14; Oxidation(P)@21		2114.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@17		1766.8
GPNGEAGSAGPPGPPGLR	Oxidation(P)@12; Oxidation(P)@15		1618.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.3
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1750.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.7
GPSGPPGPDGNKGEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@8; Oxidation(P)@15; Oxidation(P)@33	missed K-G@12	3212.5
GPSGPQGIR			867.5
GSDGSVGPVGPAGPIGSAGPPGFPGAPGPK	Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@27		2588.2
GYPGNIGPVGAAGAPGPHGPVGPAGK	Oxidation(P)@3; Oxidation(H)@18		2283.1
HGNRGETGPSGPVGPAGAVGPR	Deamidated(N)@3	missed R-G@4	2027.0
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.7
PGPIGPAGAR		cleaved R-P@N-term	891.5

PGPVGAAGATGAR	Oxidation(P)@1	cleaved I-P@N-term	1096.6
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
QGPAGEPGEPGQTGPAGAR	Oxidation(P)@7; Oxidation(P)@10	cleaved F-Q@N-term	1764.8
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1844.9
GHAGLAGAR			808.4
GPSGEAGTAGPPGTPGPQGLLGAPGILGLPGSR	Oxidation(P)@15; Oxidation(P)@17; Oxidation(P)@24; Oxidation(P)@30		2956.4

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.7
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2213.9
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2496.2
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFSGLDGAK	Dehydrated(S)@3		832.4

GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGTAGLPGMK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(M)@11		1145.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.8
GPSGPQGPGGPPGPK	Oxidation(P)@2; Oxidation(P)@12		1317.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2056
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
GVVGLPGQR	Oxidation(P)@6		897.5
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@9		1257.6

Group 16: Vitronectin

Sequence	Modifications	Cleavages	Prec MW
FEDGVLDPDYPR			1421.6

Group 17: Cystatin

Sequence	Modifications	Cleavages	Prec MW
SLPGQNEDLVLTGYQVDK			1975

CD84.2, P4 2nd extraction, TrEMBL database

N	SC	Name	Peptides (95%)
1	79.8	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	95
2	75.9	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	93
3	74	Collagen, type I, alpha 1, isoform CRA_a OS=Homo sapiens GN=COL1A1 PE=4 SV=1	67
4	30.7	Protein S100-A7 OS=Homo sapiens GN=S100A7 PE=1 SV=4	2
5	22	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	2
6	20.3	Chondroadherin OS=Homo sapiens GN=CHAD PE=2 SV=2	1
7	7.9	Vitronectin OS=Homo sapiens GN=VTN PE=2 SV=1	1
8	9.8	Pigment epithelium-derived factor OS=Homo sapiens GN=SERPINF1 PE=1 SV=4	1
9	38.8	CSTA protein OS=Homo sapiens GN=CSTA PE=2 SV=1	1
10	14.2	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
EGPVGLPGIDGRPGPIGPAGAR	Oxidation(P)@7; Oxidation(P)@13		2071.1
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@17		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEAGAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3
GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@5; Deamidated(N)@9; Oxidation(P)@15		2146.0
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Deamidated(N)@3; Deamidated(N)@21; Oxidation(P)@24		2568.2
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.7
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@9		1200.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@3; Oxidation(P)@21		2136.0
GEQGPPGPPGFQGLPGPSGPAGEVGKPGER	Formyl@N-term; Oxidation(P)@17; Oxidation(P)@27		2869.3
GESGNKGEPGSAGPQGPPGPSGEEGK	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2395.1
GESGNKGEPGSAGPQGPPGPS	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6;	2551.1

GEEGKR		missed K-R@26	
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Cation:Na(E)@2; Oxidation(P)@8; Deamidated(N)@9; Oxidation(P)@21		2088.9
GEVGPAGPNGFAGPAGAAGQPGAKGER	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21; Oxidation(K)@24	missed K-G@24	2410.1
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9		1221.6
GHNGLDGLK	Deamidated(N)@3		910.5
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@21		2800.4
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.7
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@8		1253.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Cation:Na(E)@11; Oxidation(P)@12; Oxidation(P)@21		2136.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@17		1766.8
GPNGEAGSAGPPGPPGLR	Deamidated(N)@3; Oxidation(P)@12; Oxidation(P)@15		1619.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.3
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1735.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
GPSGPPGPDGNKGEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@33	missed K-G@12	3212.5
GPSGPQGIR			867.5
GSDGSVGPVGPAGPIGSAGPPGFPGAPGPK	Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@27		2588.3
GYPGNIGPVGAAGAPGPHGPVGPAGK	Oxidation(P)@3; Oxidation(H)@18		2283.2
HGNRGETGPSGPVGPAGAVGPR	Deamidated(N)@3	missed R-G@4	2027.0
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.7
PGPIGPAGAR		cleaved R-P@N-term	891.5
PGPVGAAGATGAR	Oxidation(P)@1	cleaved I-P@N-term	1096.6
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
QGPAGEPGEPGQTGPAGAR	Oxidation(P)@7; Oxidation(P)@10	cleaved F-Q@N-term	1764.8
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1844.9

GHAGLAGAR			808.4
GPSGEAGTAGPPGTPGPQGLL GAPGILGLPGSR	Oxidation(P)@15; Oxidation(P)@17; Oxidation(P)@24; Oxidation(P)@30		2956.4

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GDAGAPGAPGSQGAPGLQ GMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2214.0
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAPG APGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GEPGPPGPAGAAGPAGNPG ADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGFAGPPGADG QPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEG K	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEG KR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.0
GETGPAGRPGEVGPPGPPG PAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGP SGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15		2704.3
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGTAGLPGMK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(M)@11		1145.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2

GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@20; Dioxidation(P)@23		2454.2
GPSGPQGPGGPPGPK	Oxidation(P)@2; Oxidation(P)@12		1317.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6		897.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9

Group 3: Collagen, type I, alpha 1, isoform CRA

Sequence	Modifications	Cleavages	Prec MW
GETGPAGPAGPVGPVGAR			1545.8
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
DGEAGAQGPPGPAGPAGER			1689.8
DGEAGAQGPPGPAGPAGER	Cation:K(E)@3; Oxidation(P)@10		1743.7
DGEAGAQGPPGPAGPAGER	Deamidated(Q)@7; Oxidation(P)@10		1706.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@15; Cation:K@C-term		1583.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8

DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8

GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2214.0
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:K(D)@2; Oxidation(P)@8; Oxidation(P)@17; Oxidation(P)@24		2318.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@11; Oxidation(P)@21	missed R-G@3	2480.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2512.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2496.2
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@21		2149.0
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1546.8
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.0
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.0

GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@5; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@20		2231.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Cation:K(E)@11; Oxidation(P)@15; Oxidation(P)@20		2253.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12		2688.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15		2704.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15; Cation:K(E)@17		2742.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@12; Oxidation(P)@15		2688.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12		2688.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9	missed K-G@18	2852.4
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4

GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6		1815.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@15		1815.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.8
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@2; Lys->Allysine(K)@11; Oxidation(P)@18; Dioxidation(P)@23		2452.3
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@20; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Dioxidation(P)@9; Deamidated(N)@15; Dioxidation(P)@23		2470.2
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(D)@6; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@23		2121.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Cation:Na(D)@6; Oxidation(P)@12		2126.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12;		2105.0

	Deamidated(Q)@23		
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15		2120.0
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6		897.5
GVVGLPGQR	Oxidation(P)@6		897.5
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10; Oxidation(K)@16	cleaved R-P@N-term	1505.7
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9;		1742.7

	Oxidation(P)@12		
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFSGLDGAK	Dehydrated(S)@3		832.4
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGTAGLPGMK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(M)@11		1145.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPSGPQGPGGPPGPK	Oxidation(P)@2; Oxidation(P)@12		1317.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2056.0
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.7
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Pro->pyro-Glu(P)@6; Oxidation(P)@12; Oxidation(P)@15		1583.8
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@5;		2315.0

	Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Pro->pyro-Glu(P)@3; Oxidation(P)@12		1844.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12; Oxidation(P)@14		1318.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.7
GQAGVMGFPGPK	Deamidated(Q)@2; Oxidation(M)@6; Oxidation(P)@9		1177.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Deamidated(Q)@9; Dioxidation(P)@16; Oxidation(P)@19		2057.0
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2056.0

Group 6: Chondroadherin

Sequence	Modifications	Cleavages	Prec MW
FSDGAFLGVTTLK			1354.7

Group 7: Vitronectin

Sequence	Modifications	Cleavages	Prec MW
FEDGVLDPDYPR			1421.4

Group 8: Pigment epithelium-derived factor

Sequence	Modifications	Cleavages	Prec MW
DTDTGALLFIGK			1249.6
DTDTGALLFIGK			1249.7

Group 9: CSTA protein

Sequence	Modifications	Cleavages	Prec MW
SLPGQNEDLVLTGYQVDK			1975

CD84.2 P4 3rd extraction, Swiss-prot database

N	SC	Name	Peptides (95%)
1	72.5	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	72
2	66	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	93
3	35.7	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	23
4	34.3	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	13
5	28.3	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	13
6	35.5	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	12
7	44.6	Trypsin OS=Sus scrofa PE=1 SV=1	14
8	60	Collagen alpha-2(I) chain OS=Bos taurus GN=COL1A2 PE=1 SV=2	30
9	64.3	Collagen alpha-1(I) chain OS=Bos taurus GN=COL1A1 PE=1 SV=3	75
10	64.9	Collagen alpha-1(I) chain OS=Rattus norvegicus GN=Col1a1 PE=1 SV=5	56
11	6.9	Chondroadherin OS=Bos taurus GN=CHAD PE=1 SV=1	2
12	54.9	Collagen alpha-1(II) chain OS=Bos taurus GN=COL2A1 PE=1 SV=4	5
13	20.9	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5	6
14	18.4	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4	2
15	52.3	Collagen alpha-2(I) chain OS=Mus musculus GN=Col1a2 PE=2 SV=2	23
16	8.2	Vitronectin OS=Homo sapiens GN=VTN PE=1 SV=1	1
17	49.6	Collagen alpha-2(I) chain OS=Rattus norvegicus GN=Col1a2 PE=1 SV=3	22
18	19.9	Keratin, type II cytoskeletal 2 epidermal OS=Canis familiaris GN=KRT2 PE=2 SV=1	6
19	66.3	Collagen alpha-2(I) chain OS=Canis familiaris GN=COL1A2 PE=2 SV=2	24

20	6.7	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2	1
21	10.3	Pigment epithelium-derived factor OS=Homo sapiens GN=SERPINF1 PE=1 SV=4	1
22	10	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2	1
23	4.6	Biglycan OS=Homo sapiens GN=BGN PE=1 SV=2	1
24	12.9	Prothrombin OS=Homo sapiens GN=F2 PE=1 SV=2	1
25	3.3	Suppressor of presenilin protein 4 OS=Caenorhabditis elegans GN=spr-4 PE=4 SV=2	1
26	54.5	Collagen alpha-1(I) chain OS=Gallus gallus GN=COL1A1 PE=1 SV=3	37

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEAGAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3
GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@3; Deamidated(N)@8; Deamidated(N)@9; Oxidation(P)@15		2147.0
GAPGPHGPVGPAGK	Oxidation(P)@3	cleaved A-G@N-term	1213.6
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1532.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Deamidated(N)@3; Deamidated(N)@21; Oxidation(P)@24		2568.2
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.7
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@9		1200.6
GEPGPLGIAGPPGAR	Oxidation(P)@3; Oxidation(P)@12	cleaved V-G@N-term	1376.7
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GEQGPPGPPGFQGLPGPSGPAGEVGKPGER	Formyl@N-term; Deamidated(Q)@12; Oxidation(P)@15; Oxidation(P)@27		2870.3
GESGNKGEPGSAGPQGPPGPSGEEGK	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2395.0
GESGNKGEPGSAGPQGPPGPSGEEGKR	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6; missed K-R@26	2552.1
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21		2052.0
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9		1221.6
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@21		2800.4

GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.8
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6		1252.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Cation:Na(E)@11; Oxidation(P)@14; Oxidation(P)@21		2136.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.5
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@17		1782.8
GPNGEAGSAGPPGPPGLR	Deamidated(N)@3; Oxidation(P)@12; Oxidation(P)@15		1619.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.4
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1735.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
GPSGPPGPDGNKGEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@33	missed K-G@12	3212.5
GSDGSVGPVGPAGPIGSAGPPGFPGAPGPK	Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@27		2588.2
GYPGNIGPVGAAGAPGPHGPVGPAGK	Pro->pyro-Glu(P)@3; Deamidated(N)@5; Oxidation(P)@17		2282.1
HGNRGETGPSGPVGPAGAVGPR	Deamidated(N)@3	missed R-G@4	2027.0
PGPHGPVGPAGK	Oxidation(P)@1	cleaved A-P@N-term	1085.6
PGPIGPAGAR		cleaved R-P@N-term	891.5
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1844.9
GPSGPQGIR			867.4
PGAPGPK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	654.3
EGPVGLPGIDGRPGPIGPAGAR	Oxidation(P)@7; Oxidation(P)@18		2071.1

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.2
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAP	Oxidation(P)@12; Oxidation(P)@15;	missed R-G@3	2512.2

GPVGPAGK	Oxidation(P)@18; Oxidation(P)@21		
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2003.9
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.9
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15		2120.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0

VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
GVVGLPGQR	Oxidation(P)@6		897.5

Group 16: Vitronectin

Sequence	Modifications	Cleavages	Prec MW
FEDGVLDPDYPR			1421.6

Group 21: Pigment epithelium-derived factor

Sequence	Modifications	Cleavages	Prec MW
DTDTGALLFIGK			1249.7

Group 23: Biglycan

Sequence	Modifications	Cleavages	Prec MW
VPSGLPDLK			924.5

Group 24: Prothrombin

Sequence	Modifications	Cleavages	Prec MW
ELLESYIDGR			1193.6

Group 25: *Caenorhabditis elegans*

Sequence	Modifications	Cleavages	Prec MW
KGDSIPPGVK		missed K-G@1	996.5

CD84.2, P4 3rd extraction, TrEMBL database

N	SC	Name	Peptides (95%)
1	76.2	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	73
2	70.4	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	97
3	39.3	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	23
4	34.8	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	14
5	35.8	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	13
6	37.2	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	12
7	18.6	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4	3
8	67.6	Collagen, type I, alpha 1, isoform CRA_a OS=Homo sapiens GN=COL1A1 PE=4 SV=1	65
9	14.8	Chondroadherin OS=Homo sapiens GN=CHAD PE=2 SV=2	2
10	30.5	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5	5

11	12.3	Vitronectin OS=Homo sapiens GN=VTN PE=2 SV=1	1
12	9.8	Biglycan preproprotein variant (Fragment) OS=Homo sapiens PE=2 SV=1	1
13	22.2	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3	3
14	12.2	Hornerin OS=Homo sapiens GN=HRNR PE=2 SV=1	1
15	12.2	Pigment epithelium-derived factor OS=Homo sapiens GN=SERPINF1 PE=1 SV=4	1
16	17.3	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEAGAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3
GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@3; Deamidated(N)@9; Oxidation(P)@15		2146.0
GAPGPHGPVGPAGK	Oxidation(P)@5	cleaved A-G@N-term	1213.6
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Deamidated(N)@3; Deamidated(N)@21; Oxidation(P)@24		2568.2
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.7
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@9		1200.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GESGNKGEPGSAGPQGPPGPSGEEGK	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2396.0
GESGNKGEPGSAGPQGPPGPSGEEGKR	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6; missed K-R@26	2552.1
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Oxidation(P)@21		2051.0
GEVGPAGPNGFAGPAGAAGQPGAKGER	Deamidated(N)@9; Oxidation(P)@21; Oxidation(K)@24	missed K-G@24	2409.1
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9		1221.6
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@21		2800.4
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.8

GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6		1252.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Oxidation(P)@14; Oxidation(P)@21		2114.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.5
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(M)@15		1782.8
GPNGEAGSAGPPGPPGLR	Deamidated(N)@3; Oxidation(P)@12; Oxidation(P)@15		1619.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.3
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@3; Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1751.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
GPSGPPGPDGNKGEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@33	missed K-G@12	3212.5
GPSGPQGIR			867.5
GSDGSVGPVGPAGPIGSAGPPGFPGAPGPK	Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@27		2588.2
GYPGNIGPVGAAGAPGPHGPVGPAGK	Deamidated(N)@5; Methyl(I)@6; Oxidation(H)@18		2282.1
HGNRGETGPSGPVGPAGAVGPR	Deamidated(N)@3	missed R-G@4	2027.0
PGPHGPVGPAGK	Oxidation(P)@1	cleaved A-P@N-term	1085.6
PGPIGPAGAR		cleaved R-P@N-term	891.5
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1828.9

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
AGRPGEAGLPGAK	Oxidation(P)@4; Oxidation(P)@10	cleaved E-A@N-term	1211.6
DGEAGAQGPPGPAGPAGER			1689.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.7
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2214.0
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7

GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.8
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2003.9
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2168.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15		2688.3
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9	missed K-G@18	2852.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2454.2
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6		897.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
TGPPGPAGQDGRPGPPGPPGA	Oxidation(P)@4; Oxidation(P)@13;		2056.0

R	Oxidation(P)@16; Oxidation(P)@19		
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
GAPGDRGEPGPPGPAGFAGPP GADGQPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.2

Group 8: Collagen, type I, alpha 1, isoform CRA

Sequence	Modifications	Cleavages	Prec MW
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@9; Oxidation(P)@15		1545.8
GAPGDRGEPGPPGPAGFAGPP GADGQPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.2
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12		1847.8
DGEAGAQGPPGPAGPAGER	Cation:K(E)@3; Oxidation(P)@10		1743.7
GVVGLPGQR	Oxidation(P)@6		897.5
GFSGLDGAK			850.4
GNDGATGAAGPPGPTGPAGPP GFPGAVGAK	Deamidated(N)@2; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2548.2
TGPPGPAGQDGRPGPPGPPGA R	Oxidation(P)@4; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@19		2056.0
GETGPAGPAGPVGPVGAR			1545.8
SGDRGETGPAGPAGPVGPVGA R		missed R-G@4	1961.0
AGRPGEAGLPGAK	Oxidation(P)@4; Oxidation(P)@10	cleaved E-A@N-term	1211.6
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.7
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNPGAD GQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.8
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2003.9
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFSGLDGAK	Dehydrated(S)@3		832.4
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPP GFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9;		1654.8

	Oxidation(P)@15		
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
DGEAGAQGPPGPAGPAGER			1689.8
DGEAGAQGPPGPAGPAGER	Deamidated(Q)@7; Oxidation(P)@10		1706.7
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Dioxidation(P)@15; Cation:K@C-term		1583.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@13		1561.8
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2214.0
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:K(D)@2; Oxidation(P)@11; Oxidation(P)@15; Oxidation(P)@24		2318.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:K(D)@2; Oxidation(P)@15; Oxidation(P)@24		2302.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2512.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0

GEPGPPGPAGFAGPPGADGQP GAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPPGPAGFAGPPGADGQP GAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@21		2149.0
GESGPSGPAGPTGAR			1296.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPAGPVGPVGAR	Pro->pyro-Glu(P)@11		1559.8
GETGPAGPPGAPGAPGAPGPV GPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2168.0
GETGPAGPPGAPGAPGAPGPV GPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.0
GETGPAGRPGEVGPPGPPGPA GEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GETGPAGRPGEVGPPGPPGPA GEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@8		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFSGLQGPPGPPGSPGEQGPSG ASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15		2704.2
GFSGLQGPPGPPGSPGEQGPSG ASGPAGPR	Oxidation(P)@9; Cation:K(E)@17		2710.2
GFSGLQGPPGPPGSPGEQGPSG ASGPAGPR	Oxidation(P)@9; Oxidation(P)@12		2688.3
GFSGLQGPPGPPGSPGEQGPSG ASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GFSGLQGPPGPPGSPGEQGPSG ASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@18		2705.2
GFSGLQGPPGPPGSPGEQGPSG ASGPAGPR	Oxidation(P)@9; Oxidation(P)@15		2688.3
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGES GPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15; Cation:K(D)@17	missed K-G@18	2906.3
GLTGPIGPPGPAGAPGDKGES GPSGPAGPTGAR	Oxidation(P)@9	missed K-G@18	2852.4
GLTGPIGPPGPAGAPGDKGES GPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6		1815.8
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@15		1815.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9;		1831.8

	Oxidation(P)@15		
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2454.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2454.2
GSAGPPGATGFPGAAGR	Methyl(S)@2; Oxidation(P)@6; Oxidation(P)@12		1472.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15		2120.0
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6		897.5
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GEPGPPGPAGAAGPAGNPGADGQPGAK	Dioxidation(P)@6; Oxidation(P)@18; Deamidated(Q)@23; Oxidation(P)@24		2316.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@9		1257.6
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6

GPSGPQGPGGPPGPK	Methyl(Q)@6; Oxidation(P)@8; Oxidation(P)@12		1331.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12; Oxidation(P)@14		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@5; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@5; Oxidation(P)@14		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@14		1317.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Deamidated(Q)@9; Oxidation(P)@15; Dioxidation(P)@19		2057.0

Group 9: Chondroadherin

Sequence	Modifications	Cleavages	Prec MW
FSDGAFLGVTTLK			1354.7
SIPDNAFQSFGR	Deamidated(N)@5		1338.6

Group 11: Vitronectin

Sequence	Modifications	Cleavages	Prec MW
FEDGVLDPDYPR			1421.6

Group 12: Biglycan preproprotein variant (fragment)

Sequence	Modifications	Cleavages	Prec MW
VPSGLPDLK			924.5

Group 15: Pigment epithelium-derived factor

Sequence	Modifications	Cleavages	Prec MW
DTDTGALLFIGK			1249.7

EH156.3, P4 1st extraction, Swiss-Prot database

N	SC	Name	Peptides (95%)
1	77.8	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	97
2	73.8	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	129
3	33.3	Trypsin OS=Sus scrofa PE=1 SV=1	6
4	70.5	Collagen alpha-1(I) chain OS=Mus musculus GN=Col1a1 PE=1 SV=4	65
5	67.7	Collagen alpha-1(II) chain OS=Bos taurus GN=COL2A1 PE=1 SV=4	8
6	75.2	Collagen alpha-2(I) chain OS=Bos taurus GN=COL1A2 PE=1 SV=2	35
7	69.3	Collagen alpha-2(I) chain OS=Mus musculus GN=Col1a2 PE=2 SV=2	26
8	72.7	Collagen alpha-2(I) chain OS=Canis familiaris GN=COL1A2 PE=2 SV=2	38
9	71.1	Collagen alpha-1(I) chain OS=Gallus gallus GN=COL1A1 PE=1 SV=3	43
10	5.7	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	1
11	88.8	Collagen alpha-2(I) chain OS=Mammut americanum PE=1 SV=3	35
12	66.3	Collagen alpha-1(II) chain OS=Mus musculus GN=Col2a1 PE=1 SV=2	8
13	74.1	Collagen alpha-2(I) chain OS=Rattus norvegicus GN=Col1a2 PE=1 SV=3	25
14	34	Collagen alpha-1(XXVIII) chain OS=Homo sapiens GN=COL28A1 PE=2 SV=2	1
15	52	Collagen alpha-1(XIX) chain OS=Homo sapiens GN=COL19A1 PE=1 SV=3	0
16	3.6	HTH-type transcriptional regulator MalT OS=Cronobacter sakazakii (strain ATCC BAA-894) GN=malT PE=3 SV=1	1
17	8.5	Dihydroxy-acid dehydratase OS=Methanosarcina barkeri (strain Fusaro / DSM 804) GN=ilvD PE=3 SV=1	1
18	6.3	Diflavin flavoprotein A 1 OS=Synechocystis sp. (strain PCC 6803 / Kazusa) GN=dfa1 PE=1 SV=1	1
19	6.1	DNA polymerase catalytic subunit OS=Equine herpesvirus 1 (strain V592) GN=30 PE=3 SV=1	1
20	58.5	Collagen alpha-1(X) chain OS=Bos taurus GN=COL10A1 PE=2 SV=1	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
EGPVGLPGIDGRPGPIGPAGAR	Oxidation(P)@7		2055.1
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@17		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEAGAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.4
GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@3; Deamidated(N)@9; Oxidation(P)@15		2146.0
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPN	Deamidated(N)@3;		2568.2

GPPGPAGSR	Deamidated(N)@21; Oxidation(P)@24		
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.8
GEPGNIGFPGPK	Oxidation(P)@3; Deamidated(N)@5; Oxidation(P)@9		1201.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@3; Oxidation(P)@21		2136.1
GESGNKGEPGSAGPQGPPGPSGEEGK	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2395.1
GESGNKGEPGSAGPQGPPGPSGEEGKR	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6; missed K-R@26	2551.2
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.3
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21		2052.0
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9		1221.6
GHAGLAGAR			808.4
GHNGLDGLK	Deamidated(N)@3		910.5
GHNGLDGLKGQPGAPGVK	Deamidated(N)@3; Deamidated(Q)@11; Oxidation(P)@12; Oxidation(P)@15	missed K-G@9	1734.9
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@21		2800.4
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.8
GLPGLKGHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Oxidation(P)@3; Oxidation(H)@8; Oxidation(P)@15; Oxidation(P)@27	missed K-G@6	3397.8
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6		1252.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Cation:K(E)@11; Oxidation(P)@12; Oxidation(P)@21		2152.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@12		1766.8
GPNGEAGSAGPPGPPGLR	Deamidated(N)@3; Oxidation(P)@12; Oxidation(P)@15		1619.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Cation:K(E)@23; Oxidation(P)@24		2980.4
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@3; Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1751.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFY	Oxidation(P)@14; Oxidation(P)@17; Oxidation(P)@20; Oxidation(P)@21	cleaved Y-R@C-term	3572.6
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFYR	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21		3728.7
GPSGEAGTAGPPGTPGPQGLLGAPGILGLPGSR	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@24; Oxidation(P)@30		2956.5
GPSGPPGPDGNKGEPGVVGAV	Oxidation(P)@6; Oxidation(P)@15;	missed K-G@12	3212.6

GTAGPSGPSGLPGER	Oxidation(P)@33		
GPSGPQGIRGDKGEPGEK	Oxidation(P)@15	missed R-G@9; missed K-G@12	1780.9
GPTGDPGKNGDKGHAGLAGAR	Oxidation(P)@6; Deamidated(N)@9	missed K-N@8; missed K-G@12	1949.0
GSDGSVGPVGPAGPIGSAGPPGFPGAPGPK	Oxidation(P)@21; Oxidation(P)@27		2572.3
GVGLGPGPMGLMGPR	Oxidation(M)@9		1410.7
GYPGNIGPVGAAGAPGPHGPVGPAGK	Oxidation(P)@3; Oxidation(P)@15		2283.2
HGNRGETGPSGPVGPAGAVGPR	Deamidated(N)@3	missed R-G@4	2027.0
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.7
PGNIGFPGPK	Oxidation(P)@1; Oxidation(P)@7	cleaved E-P@N-term	1014.5
PGNIGPVGAAGAPGPHGPVGPAGK	Oxidation(P)@1; Oxidation(H)@16	cleaved Y-P@N-term	2063.1
PGPIGPAGAR		cleaved R-P@N-term	891.5
PGPVGAAGATGAR	Oxidation(P)@1	cleaved I-P@N-term	1096.6
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
QGPAGEPGEPGQTGPAGAR	Oxidation(P)@7; Oxidation(P)@10	cleaved F-Q@N-term	1764.8
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1828.9
PGTPGLPGFK	Oxidation(P)@1; Oxidation(P)@4; Oxidation(P)@7	cleaved F-P@N-term	1017.5
GVVGPQGAR			839.5
PGAPGPKGEIGAVGNAGPAGPAGPR		cleaved F-P@N-term; missed K-G@7	2148.1

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.3
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2198.0
GDAGPAGPKGEPGSPGENGAPGQMGPR	Oxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2509.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:Na(D)@2; Oxidation(P)@8; Oxidation(P)@15; Oxidation(P)@24		2302.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2

GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGDAGAKGDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@3; Oxidation(P)@15; Oxidation(P)@24; Oxidation(P)@33	missed K-G@9	3078.5
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@21		2149.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.1
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGKQGPSGASGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(Q)@15	missed K-Q@14	2255.0
GFSGLDGAK			850.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GNDGATGAAGPPGPTGPAGPPGFPGAVGAKGEAGPQGPR	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@35	missed K-G@30	3412.7
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6		1815.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Oxidation(P)@15		2104.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6

PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGLPGPSGEPGK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	1123.6
PGPMGPSGPR	Oxidation(M)@4	cleaved V-P@N-term	967.5
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@1; Oxidation(P)@4; Deamidated(N)@7; Oxidation(P)@19	cleaved P-P@N-term; missed K-N@6	2193.1
PGPTGLPGPPGER	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved E-P@N-term	1278.6
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
PPGPAGQDGRPGPPGPPGAR	Oxidation(P)@2; Oxidation(P)@11; Oxidation(P)@14; Oxidation(P)@17	cleaved G-P@N-term	1897.9
SGDRGETGPAGPTGPVGPVGAR	Delta:H(2)C(2)@N-term; Dehydrated(T)@13	missed R-G@4	1998.9
SGPQGPGGPPGPK	Oxidation(P)@10	cleaved P-S@N-term	1147.6
STGGISVPGPMGPSGPR	Oxidation(P)@8		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@15; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1827.9
GVVGLPGQR	Oxidation(P)@6; Deamidated(Q)@8		898.5
AGRPGEAGLPGAK	Oxidation(P)@4; Oxidation(P)@10	cleaved E-A@N-term	1211.6
GAAGLPGPKGDRGDAGPK	Oxidation(P)@6; Oxidation(P)@17	missed K-G@9; missed R-G@12	1651.9
GAAGLPGPK	Oxidation(P)@6		782.4

Group 14: Collagen alpha-1(XXVIII) chain

Sequence	Modifications	Cleavages	Prec MW
GEPGPPGPYGSPGAPGIGQQGIK	Oxidation(P)@3; Oxidation(P)@6; Deamidated(Q)@20		2145

Group 16: *Cronobacter sakazakii*

Sequence	Modifications	Cleavages	Prec MW
EQLLEIGSQQLAFTHQEAR	Deamidated(Q)@10; Deamidated(Q)@16		2199

Group 17: *Methanosarcina barkeri*

Sequence	Modifications	Cleavages	Prec MW
APNRSLLKATGVTDSEMR	Oxidation(M)@17	missed R-S@4; missed K-A@8	1961

Group 18: *Synechocystis sp.*

Sequence	Modifications	Cleavages	Prec MW
VAKAEGLTAVHHR		missed K-A@3	1387.8

Group 19: Equine herpesvirus 1

Sequence	Modifications	Cleavages	Prec MW
EAGVVNSMRGTAQNPVVTKT ARPQPK	Glu->pyro-Glu@N-term; Deamidated(N)@14	missed R-G@9; missed K-T@19	2718.3

EH156.3, P4 1st extraction, TrEMBL database

N	SC	Name	Peptides (95%)
1	83.4	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	117
2	77.7	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	148
3	76.9	Collagen, type I, alpha 1, isoform CRA_a OS=Homo sapiens GN=COL1A1 PE=4 SV=1	93
4	65.8	Collagen alpha-1(XIX) chain OS=Homo sapiens GN=COL19A1 PE=1 SV=3	0
5	79.1	Collagen alpha-1(II) chain OS=Homo sapiens GN=COL2A1 PE=1 SV=3	9
6	79.2	Collagen alpha-6(IV) chain OS=Homo sapiens GN=COL4A6 PE=2 SV=3	1
7	56	Collagen, type XI, alpha 1, isoform CRA_c OS=Homo sapiens GN=COL11A1 PE=4 SV=1	1
8	17.2	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	1
9	94.1	cDNA FLJ56576, highly similar to Collagen alpha-2(I) chain OS=Homo sapiens PE=2 SV=1	14
10	7.9	Protein argonaute-4 OS=Homo sapiens GN=AGO4 PE=1 SV=2	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
EGPVGLPGIDGRPGPIGPAGAR	Oxidation(P)@7		2055.1
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEAGA AGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3

GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@3; Deamidated(N)@9; Oxidation(P)@15		2146.0
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Deamidated(N)@3; Deamidated(N)@21; Oxidation(P)@24		2568.2
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.8
GEPGNIGFPGPK	Oxidation(P)@3; Deamidated(N)@5; Oxidation(P)@9		1201.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@3; Oxidation(P)@21		2136.0
GESGNKGEPGSAGPQGPPGPSGEEGK	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2396.1
GESGNKGEPGSAGPQGPPGPSGEEGKR	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6; missed K-R@26	2552.2
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21		2052.0
GEVGPAGPNGFAGPAGAAGQPGAKGER	Deamidated(N)@9; Oxidation(P)@21; Oxidation(K)@24	missed K-G@24	2409.1
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9		1221.6
GFPGTPGLPGFKGIR	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9; Carbamyl(K)@12	missed K-G@12	1590.8
GHAGLAGAR			808.5
GHNGLDGLK	Deamidated(N)@3		910.5
GHNGLDGLKGQPGAPGVK	Deamidated(N)@3; Deamidated(Q)@11; Oxidation(P)@12; Oxidation(P)@15	missed K-G@9	1734.9
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@21		2800.4
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.8
GLPGLKGHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Oxidation(P)@3; Oxidation(H)@8; Oxidation(P)@15; Oxidation(P)@27	missed K-G@6	3397.7
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@8		1253.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Oxidation(P)@14; Oxidation(P)@21		2114.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9;		1766.8

	Oxidation(P)@12		
GPNGEAGSAGPPGPPGLR	Deamidated(N)@3; Oxidation(P)@12; Oxidation(P)@15		1619.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.3
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@3; Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1751.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFY	Deamidated(Q)@6; Dioxidation(P)@21; Cation:K(D)@29; Oxidation(F)@30	cleaved Y-R@C-term	3595.5
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFYR	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21		3728.6
GPSGEAGTAGPPGTPGPQGLLGAPGILGLPGSR	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@24; Oxidation(P)@30		2956.5
GPSGPPGPDGNKGEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@33	missed K-G@12	3212.5
GPSGPQGIR			867.5
GPSGPQGIRGDKGEPGEK	Oxidation(P)@15	missed R-G@9; missed K-G@12	1780.9
GPTGDPGKNGDKGHAGLAGAR	Oxidation(P)@6; Deamidated(N)@9	missed K-N@8; missed K-G@12	1949.0
GSDGSVGPVGPAGPIGSAGPPGFPGAPGPK	Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@27		2588.2
GSDGSVGPVGPAGPIGSAGPPGFPGAPGPKGEIGAVGNAGPAGPAGPR	Myristoyl(K)@30	missed K-G@30	4280.1
GVGLGPGPMGLMGPR			1391.7
GYPGNIGPVGAAGAPGPHGPVGPAGK	Oxidation(P)@3; Oxidation(P)@15		2283.2
HGNRGETGPSGPVGPAGAVGPR	Deamidated(N)@3	missed R-G@4	2027.0
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.7
PGNIGFPGPK	Oxidation(P)@1; Oxidation(P)@7	cleaved E-P@N-term	1014.5
PGNIGPVGAAGAPGPHGPVGPAGK	Oxidation(P)@1; Oxidation(H)@16	cleaved Y-P@N-term	2063.1
PGPIGPAGAR	Oxidation(P)@1	cleaved R-P@N-term	907.5
PGPVGAAGATGAR	Oxidation(P)@1	cleaved I-P@N-term	1096.6
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.6
PGTPGLPGFK	Oxidation(P)@1; Oxidation(P)@4; Oxidation(P)@7	cleaved F-P@N-term	1017.6
QGPAGEPGEPGQTGPAGAR	Oxidation(P)@7; Oxidation(P)@10	cleaved F-Q@N-term	1764.8
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.7
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1828.9
GVVGPQGAR			839.5
PGAPGPKGEIGAVGNAGPAGPAGPR		cleaved F-P@N-term; missed K-G@7	2148.0

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
AGRPGEAGLPGAK	Oxidation(P)@4; Oxidation(P)@10	cleaved E-A@N-term	1211.7
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
GAAGLPGPKGDRGDAGPK	Oxidation(P)@6; Oxidation(P)@17	missed K-G@9; missed R-G@12	1651.9
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.7
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Dioxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@27	missed R-G@6	2718.2
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2213.9
GDAGPAGPKGEPGSPGENGAPGQMGPR	Oxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2509.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGDAGAKGDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@3; Oxidation(P)@15; Oxidation(P)@24; Oxidation(P)@33	missed K-G@9	3078.4
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Cation:K(E)@17		1868.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGSPGENGAPGQMGPR	Dioxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12; Deamidated(Q)@14		1743.7
GEQGPAGSPGFQGLPGPAGPPGEAGKPGEQGVPGDLGAPGPSGAR	Oxidation(P)@9; Deamidated(Q)@30; Phospho(D)@35		4112.9
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.7
GFPGLPGPSGEPGKQGPSGASGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(Q)@15	missed K-Q@14	2255.0
GFSGLDGAK			850.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7

GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGPPGAPGPQGFQGPPGEPGEPGASGPMGPR	Dioxidation(P)@6; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		3083.4
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@11	missed K-G@18	2852.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GNDGATGAAGPPGPTGPAGPPGFPGAVGAKGEAGPQGPR	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24; Oxidation(K)@30	missed K-G@30	3412.6
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9		1831.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@21	missed K-N@8	2347.1
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GSPGPAGPKGSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(K)@9; Dioxidation(P)@18; Oxidation(P)@24	missed K-G@9	2435.2
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVVGLPGQR	Oxidation(P)@6		897.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGLPGPSGEPGK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	1123.6
PGPMGPSGPR	Oxidation(M)@4	cleaved V-P@N-term	967.5
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.6
PGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@1; Oxidation(P)@4; Deamidated(N)@7; Oxidation(P)@19	cleaved P-P@N-term; missed K-N@6	2193.1
PGPTGLPGPPGER	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved E-P@N-term	1278.6
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
SGDRGETGPAGPTGPVGPVGAR	Carboxy(D)@3; Dehydrated(T)@7; Dehydrated(T)@13	missed R-G@4	1999.0

SGPQGPGGPPGPK	Oxidation(P)@10	cleaved P-S@N-term	1147.6
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
GAAGLPGPK	Oxidation(P)@6		782.5

Group 3: Collagen, type I, alpha 1, isoform CRA

Sequence	Modifications	Cleavages	Prec MW
GAAGLPGPK	Oxidation(P)@6		782.5
GISVPGPMGPSGPR		cleaved G-G@N-term	1307.7
GNDGATGAAGPPGPTGPAGPPGFPGAVGAKGEAGPQGPR	Oxidation(P)@17; Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@38	missed K-G@30	3412.5
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Ubiquitination GG(K)@8; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2445.0
GETGPAGPAGPVGPVGAR			1545.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
GFPGLPGPSGEPGK	Oxidation(P)@12		1311.6
GETGPAGPAGPVGPVGAR			1545.8
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
AGRPGEAGLPGAK	Oxidation(P)@4; Oxidation(P)@10	cleaved E-A@N-term	1211.7
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.7
GDAGPAGPKGEPGSPGENGAPGQMGPR	Oxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2509.1
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Cation:K(E)@17		1868.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGSPGENGAPGQMGPR	Dioxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12; Deamidated(Q)@14		1743.7
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFSGLDGAK			850.4
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6;	cleaved F-Q@C-	1295.6

	Oxidation(P)@9	term	
GLPGPPGAPGPQGFQGPPGEPGEPGASGPMGPR	Dioxidation(P)@6; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		3083.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GNDGATGAAGPPGPTGPAGPPGFPGAVGAKGEAGPQGPR	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24; Oxidation(K)@30	missed K-G@30	3412.6
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@21	missed K-N@8	2347.1
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GSPGPAGPKGSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(K)@9; Dioxidation(P)@18; Oxidation(P)@24	missed K-G@9	2435.2
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGPMGPSGPR	Oxidation(M)@4	cleaved V-P@N-term	967.5
PGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@1; Oxidation(P)@4; Deamidated(N)@7; Oxidation(P)@19	cleaved P-P@N-term; missed K-N@6	2193.1
PGPTGLPGPPGER	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved E-P@N-term	1278.6
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
SGDRGETGPAGPTGPVGPVGAR	Carboxy(D)@3; Dehydrated(T)@7; Dehydrated(T)@13	missed R-G@4	1999.0
SGPQGPGGPPGPK	Oxidation(P)@10	cleaved P-S@N-term	1147.6
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2056.0
GAPGERGETGPPGPAGFAGPPGADGQPGAK	Methyl(E)@5; Oxidation(P)@12; Dioxidation(P)@21; Deamidated(Q)@26	missed R-G@6	2719.2
KGEPGPAGPQGAPGPAGEEGK	Oxidation(P)@4; Dehydrated(E)@18	cleaved P-K@N-term; missed K-G@1	1884.8
DGEAGAQGPPGPAGPAGER			1689.8
DGEAGAQGPPGPAGPAGER	Cation:K(E)@3; Oxidation(P)@10		1743.7
DGEAGAQGPPGPAGPAGER	Deamidated(Q)@7; Oxidation(P)@10		1706.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8

DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8
DGLNGLPGPIGPPGPR	Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1560.8
GAAGLPGPKGDRGDAGPK	Oxidation(P)@6; Oxidation(P)@17	missed K-G@9; missed R-G@12	1651.9
GAPGDRGEPGPPGPAGFAGPPG ADGQPGAK	Dioxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@27	missed R-G@6	2718.2
GAPGDRGEPGPPGPAGFAGPPG ADGQPGAK	Oxidation(D)@5; Dioxidation(P)@12; Oxidation(P)@21; Deamidated(Q)@26	missed R-G@6	2703.2
GAPGDRGEPGPPGPAGFAGPPG ADGQPGAK	Oxidation(D)@5; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.2
GDAGAPGAPGSQGAPGLQGMP GER	Delta:H(2)C(2)@N-term; Carboxy(D)@2; Oxidation(P)@15; Oxidation(P)@21		2236.0
GDAGAPGAPGSQGAPGLQGMP GER	Oxidation(P)@6; Oxidation(P)@9; Deamidated(Q)@18; Dioxidation(M)@20		2198.9
GDAGAPGAPGSQGAPGLQGMP GER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2213.9
GDAGAPGAPGSQGAPGLQGMP GER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2198.0
GDAGPPGPAGPAGPPGPIGNVG APGAK	Cation:K(D)@2; Oxidation(P)@11; Oxidation(P)@15; Oxidation(P)@24		2318.1
GDAGPPGPAGPAGPPGPIGNVG APGAK	Cation:Na(D)@2; Oxidation(P)@8; Oxidation(P)@15; Oxidation(P)@24		2302.1
GDAGPPGPAGPAGPPGPIGNVG APGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDAGPPGPAGPAGPPGPIGNVG APGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2512.2
GDRGETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2512.2

GDRGETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GDRGETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GEPGDAGAKGDAGPPGPAGPAG PPGPIGNVGAPGAK	Oxidation(P)@3; Oxidation(P)@15; Oxidation(P)@24; Oxidation(P)@33	missed K-G@9	3078.4
GEPGPPGPAGFAGPPGADGQPG AK	Dioxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20; Oxidation(P)@21		2149.9
GEPGPPGPAGFAGPPGADGQPG AK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPPGPAGFAGPPGADGQPG AK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPPGPAGFAGPPGADGQPG AK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@21		2149.0
GEQGPAGSPGFQGLPGPAGPPGE AGKPGEQGVPGDLGAPGPSGAR	Oxidation(P)@9; Deamidated(Q)@30; Phospho(D)@35		4112.9
GESGPSGPAGPTGAR			1296.6
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPPGAPGAPGAPGPVG PAGK	Oxidation(P)@9; Oxidation(P)@12		2152.0
GETGPAGPPGAPGAPGAPGPVG PAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.0
GETGPAGPPGAPGAPGAPGPVG PAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.0
GETGPAGPPGAPGAPGAPGPVG PAGK	Oxidation(P)@9; Oxidation(P)@15		2152.1
GETGPAGRPGEVGPPGPPGPAGE K	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.1
GETGPAGRPGEVGPPGPPGPAGE K	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GETGPAGRPGEVGPPGPPGPAGE K	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18; Oxidation(K)@24		2231.1
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@12		1327.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@12		1327.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@8		1343.6
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.7
GFPGLPGPSGEPGKQGPSGASGE R	Oxidation(P)@3; Oxidation(P)@6; Deamidated(Q)@15	missed K-Q@14	2255.0
GFSGLQGPPGPPGSPGEQGPSGA SGPAGPR	Deamidated(Q)@6; Oxidation(P)@9; Oxidation(P)@15		2689.3
GFSGLQGPPGPPGSPGEQGPSGA SGPAGPR	Oxidation(P)@11; Oxidation(P)@12		2688.3
GFSGLQGPPGPPGSPGEQGPSGA SGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Cation:Na(E)@17		2710.2
GFSGLQGPPGPPGSPGEQGPSGA SGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15; Cation:K(E)@17		2742.2

GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Cation:Na(E)@17		2710.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Cation:Na(E)@17		2710.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@18		2705.2
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@11	missed K-G@18	2852.4
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@11; Cation:K(D)@17	missed K-G@18	2890.4
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@15; Cation:K(D)@17; Oxidation(K)@18	missed K-G@18	2906.4
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6		1815.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9		1831.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@15		1815.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9		1815.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.8
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Dioxidation(K)@11; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2470.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Dioxidation(P)@9; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2470.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@2; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Deamidated(Q)@23		2089.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Oxidation(P)@15		2104.0

GSPGADGPAGAPGTPGPQGIAG QR	Oxidation(P)@3; Cation:Na(D)@6; Oxidation(P)@12		2126.0
GSPGADGPAGAPGTPGPQGIAG QR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGADGPAGAPGTPGPQGIAG QR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105.0
GSPGADGPAGAPGTPGPQGIAG QR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15		2120.0
GSPGADGPAGAPGTPGPQGIAG QR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@23		2121.0
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.7
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.5
GVVGLPGQR	Oxidation(P)@6		897.5
GVVGLPGQR	Oxidation(P)@6; Deamidated(Q)@8		898.5
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGLPGPSGEPGK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	1123.6
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.6
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.6
PGPPGAVGPAGK	Oxidation(P)@4	cleaved V-P@N-term	1019.6
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1827.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGERGETGPPGPAGFAGPPG ADGQPGAK	Methyl(E)@8; Oxidation(P)@12; Oxidation(P)@21; Deamidated(Q)@26	missed R-G@6	2703.2
GEPGPPGPAGAAGPAGNPGADG	Dioxidation(P)@8; Oxidation(P)@18;		2316.1

QPGAK	Deamidated(Q)@23; Oxidation(P)@24		
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12		1847.8
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.8
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GFPGADGVAGPK	Oxidation(P)@3		1087.6
GFPGADGVAGPK	Oxidation(P)@3		1087.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@9		1257.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Cation:K(D)@3; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@24		2585.2
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Cation:Na(D)@3; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2569.2
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Deamidated(N)@2; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2548.2
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Dioxidation(P)@21	missed K-N@8	2363.1
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.7
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.7
GPSGPQGPGGPPGPK	Oxidation(P)@2; Oxidation(P)@12		1317.7
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GQAGVMGFPGPK	Deamidated(Q)@2; Oxidation(M)@6		1161.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
STGGISVPGPMGPSGPR			1552.8
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
STGGISVPGPMGPSGPR	Oxidation(P)@8		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Deamidated(Q)@9; Dioxidation(P)@16; Oxidation(P)@19		2057.0
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@15;		2056.0

	Oxidation(P)@16; Oxidation(P)@19		

Group 5: Collagen alpha-1(II) chain

Sequence	Modifications	Cleavages	Prec MW
GAAGLPGPK	Oxidation(P)@6		782.5
GISVPGPMGPSGPR		cleaved G-G@N-term	1307.7
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK GEAGPQGPR	Oxidation(P)@17; Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@38	missed K-G@30	3412.5
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Ubiquitination GG(K)@8; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2445.0
GETGPAGPAGPVGPVGAR			1545.8
GFPGLPGPSGEPGK	Oxidation(P)@12		1311.6
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
GAPGERGETGPPGPAGFAGPPGADGQPGAK	Methyl(E)@5; Oxidation(P)@12; Dioxidation(P)@21; Deamidated(Q)@26	missed R-G@6	2719.2
KGEPGPAGPQGAPGPAGEEGK	Oxidation(P)@4; Dehydrated(E)@18	cleaved P-K@N-term; missed K-G@1	1884.8
GAPGERGETGPPGPAGFAGPPGADGQPGAK	Methyl(E)@8; Oxidation(P)@12; Oxidation(P)@21; Deamidated(Q)@26	missed R-G@6	2703.2
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@12		1327.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@12		1327.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@8		1343.6
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.7
PGLPGPSGEPGK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	1123.6
AGRPGEAGLPGAK	Oxidation(P)@4;	cleaved E-A@N-term	1211.7

	Oxidation(P)@10		
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
GAAGLPGPKGDRGDAGPK	Oxidation(P)@6; Oxidation(P)@17	missed K-G@9; missed R-G@12	1651.9
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.7
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Dioxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@27	missed R-G@6	2718.2
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2213.9
GDAGPAGPKGEPGSPGENGAPGQMGPR	Oxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2509.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGDAGAKGDAGPPGPAGPAGPPGPIGNV GAPGAK	Oxidation(P)@3; Oxidation(P)@15; Oxidation(P)@24; Oxidation(P)@33	missed K-G@9	3078.4
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Cation:K(E)@17		1868.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGSPGENGAPGQMGPR	Dioxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12;		1743.7

	Deamidated(Q)@14		
GEQGPAGSPGFQGLPGPAGPPGEAGKPGEQGVPGDLGAPGPSGAR	Oxidation(P)@9; Deamidated(Q)@30; Phospho(D)@35		4112.9
GESGPSGPAGPTGAR			1296.6
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGKQGPSGASGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(Q)@15	missed K-Q@14	2255.0
GFSGLDGAK			850.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGPPGAPGPQGFQGPPGEPGEPGASGPMGPR	Dioxidation(P)@6; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		3083.4
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@11	missed K-G@18	2852.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GNDGATGAAGPPGPTGPAGPPGFPGAVGAKGEAGPQGPR	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24; Oxidation(K)@30	missed K-G@30	3412.6
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9		1831.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@21	missed K-N@8	2347.1
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2

GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GSPGPAGPKGSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(K)@9; Dioxidation(P)@18; Oxidation(P)@24	missed K-G@9	2435.2
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVVGLPGQR	Oxidation(P)@6		897.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPMGPSGPR	Oxidation(M)@4	cleaved V-P@N-term	967.5
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.6
PGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@1; Oxidation(P)@4; Deamidated(N)@7; Oxidation(P)@19	cleaved P-P@N-term; missed K-N@6	2193.1
PGPTGLPGPPGER	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved E-P@N-term	1278.6
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
SGDRGETGPAGPTGPVGPVGAR	Carboxy(D)@3; Dehydrated(T)@7; Dehydrated(T)@13	missed R-G@4	1999.0
SGPQGPGGPPGPK	Oxidation(P)@10	cleaved P-S@N-term	1147.6
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9

DGEAGAQGPPGPAGPAGER			1689.8
DGEAGAQGPPGPAGPAGER	Cation:K(E)@3; Oxidation(P)@10		1743.7
DGEAGAQGPPGPAGPAGER	Deamidated(Q)@7; Oxidation(P)@10		1706.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8
DGLNGLPGPIGPPGPR	Oxidation(P)@7; Oxidation(P)@12;		1560.8

	Oxidation(P)@13		
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Oxidation(D)@5; Dioxidation(P)@12; Oxidation(P)@21; Deamidated(Q)@26	missed R-G@6	2703.2
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Oxidation(D)@5; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.2
GDAGAPGAPGSQGAPGLQGMPGER	Delta:H(2)C(2)@N-term; Carboxy(D)@2; Oxidation(P)@15; Oxidation(P)@21		2236.0
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Deamidated(Q)@18; Dioxidation(M)@20		2198.9
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2198.0
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:K(D)@2; Oxidation(P)@11; Oxidation(P)@15; Oxidation(P)@24		2318.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:Na(D)@2; Oxidation(P)@8; Oxidation(P)@15; Oxidation(P)@24		2302.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2512.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18;	missed R-G@3	2512.2

	Oxidation(P)@21		
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GEPGPPGPAGAAGPAGNPGADGQPGAK	Dioxidation(P)@8; Oxidation(P)@18; Deamidated(Q)@23; Oxidation(P)@24		2316.1
GEPGPPGPAGFAGPPGADGQPGAK	Dioxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20; Oxidation(P)@21		2149.9
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@21		2149.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12		1847.8
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.8
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12		2152.0
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.0
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@15		2152.1
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.1
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18; Oxidation(K)@24		2231.1

GFPGADGVAGPK	Oxidation(P)@3		1087.6
GFPGADGVAGPK	Oxidation(P)@3		1087.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Deamidated(Q)@6; Oxidation(P)@9; Oxidation(P)@15		2689.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12		2688.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Cation:Na(E)@17		2710.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15; Cation:K(E)@17		2742.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Cation:Na(E)@17		2710.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Cation:Na(E)@17		2710.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@18		2705.2
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@11; Cation:K(D)@17	missed K-G@18	2890.4
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@15; Cation:K(D)@17; Oxidation(K)@18	missed K-G@18	2906.4
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@9		1257.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Cation:K(D)@3; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@24		2585.2
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Cation:Na(D)@3; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2569.2
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Deamidated(N)@2; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2548.2

GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6		1815.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@15		1815.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9		1815.9
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.8
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Dioxidation(P)@21	missed K-N@8	2363.1
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Dioxidation(K)@11; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2470.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Dioxidation(P)@9; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2470.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@2; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.7
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.7
GPSGPQGPGGPPGPK	Oxidation(P)@2; Oxidation(P)@12		1317.7
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GQAGVMGFPGPK	Deamidated(Q)@2; Oxidation(M)@6		1161.6

GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Deamidated(Q)@23		2089.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Oxidation(P)@15		2104.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Cation:Na(D)@6; Oxidation(P)@12		2126.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15		2120.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@23		2121.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.7
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.5
GVVGLPGQR	Oxidation(P)@6; Deamidated(Q)@8		898.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.6
PGPPGAVGPAGK	Oxidation(P)@4	cleaved V-P@N-term	1019.6
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
STGGISVPGPMGPSGPR			1552.8
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
STGGISVPGPMGPSGPR	Oxidation(P)@8		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Deamidated(Q)@9; Dioxidation(P)@16; Oxidation(P)@19		2057.0
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@15; Oxidation(P)@16; Oxidation(P)@19		2056.0

VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1827.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9

Group 6: Collagen alpha-6(IV)

Sequence	Modifications	Cleavages	Prec MW
VGPPGDPGFPGMKGKAGPR	Oxidation(P)@4; Methyl(D)@6	missed K-G@13; missed K-A@15	1850.8

Group 7: Collagen, type XI, alpha 1, isoform CRA

Sequence	Modifications	Cleavages	Prec MW
EGRQGEKGAKGE AGAEGP	Deamidated(R)@3	cleaved A-E@N-term; cleaved P-P@C-term; missed R-Q@3; missed K-G@7; missed K-G@10	1727.7

Group 9: cDNA FLJ56576, highly similar to Collagen alpha-2(I)

Sequence	Modifications	Cleavages	Prec MW
TGIGAPRTRNT		cleaved T-S@C-term; missed R-T@7; missed R-N@9	1142.6

Group 10: Protein argonaute-4

Sequence	Modifications	Cleavages	Prec MW
RPGLGTVGKPIR	Deamidated(R)@1; Oxidation(P)@2		1266.6

EH156.3, P4 2nd extraction, Swiss-Prot database

N	SC	Name	Peptides (95%)
1	78.8	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	122
2	72.3	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	123
3	70.5	Collagen alpha-1(I) chain OS=Rattus norvegicus GN=Col1a1 PE=1 SV=5	68
4	8.7	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	4
5	19.9	Trypsin OS=Sus scrofa PE=1 SV=1	6
6	71.3	Collagen alpha-2(I) chain OS=Bos taurus GN=COL1A2 PE=1 SV=2	41
7	58.2	Collagen alpha-1(I) chain OS=Cynops pyrrhogaster GN=COL1A1 PE=2 SV=1	6
8	68.7	Collagen alpha-1(I) chain OS=Gallus gallus GN=COL1A1 PE=1 SV=3	42
9	71.2	Collagen alpha-2(I) chain OS=Mus musculus GN=Col1a2 PE=2 SV=2	35
10	46.2	Collagen alpha-2(IV) chain OS=Caenorhabditis elegans GN=let-2 PE=1 SV=2	1
11	71.7	Collagen alpha-2(I) chain OS=Canis familiaris GN=COL1A2 PE=2 SV=2	44
12	94.3	Collagen alpha-1(I) chain OS=Mammut americanum GN=COL1A1 PE=1 SV=4	69
13	80.9	Collagen alpha-2(I) chain OS=Mammut americanum PE=1 SV=3	40
14	65.4	Collagen alpha-1(II) chain OS=Homo sapiens GN=COL2A1 PE=1 SV=3	5
15	7.2	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	1
16	5.2	Imidazoleglycerol-phosphate dehydratase OS=Trichodesmium erythraeum (strain IMS101) GN=hisB PE=3 SV=1	1
17	7.9	30S ribosomal protein S3Ae OS=Halorubrum lacusprofundi (strain ATCC 49239 / DSM 5036 / JCM 8891 / ACAM 34) GN=rps3ae PE=3 SV=1	1
18	3.3	Inward rectifier potassium channel 2 OS=Canis familiaris GN=KCNJ2 PE=2 SV=1	1
19	6	Receptor tyrosine-protein kinase erbB-3 OS=Mus musculus GN=Erbb3 PE=1 SV=2	1
20	63.1	Collagen alpha-2(I) chain OS=Rattus norvegicus GN=Col1a2 PE=1 SV=3	32

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
EGPVGLPGIDGRPGPIGPAGAR	Oxidation(P)@7; Oxidation(P)@13		2071.1
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@17		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEA GAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3
GAPGPDGNNGAQGPPGPQGVQ GGK	Oxidation(P)@3; Cation:K(D)@6; Deamidated(N)@9; Oxidation(P)@15		2183.9
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPN	Deamidated(N)@3;		2568.2

GPPGPAGSR	Deamidated(N)@21; Oxidation(P)@24		
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.7
GEPGNIGFPGPK	Oxidation(P)@3; Deamidated(N)@5; Oxidation(P)@9		1201.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GEPGSAGPQGPPGPSGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	1978.9
GEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@3; Oxidation(P)@21		2136.0
GEQGPPGPPGFQGLPGPSGPAGEVGKPGER	Formyl@N-term; Oxidation(P)@17; Oxidation(P)@27		2869.3
GESGNKGEPGSAGPQGPPGPSGEEGK	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2396.1
GESGNKGEPGSAGPQGPPGPSGEEGKR	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6; missed K-R@26	2552.2
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21		2052.0
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@9		1205.6
GHAGLAGAR			808.4
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@21		2800.4
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.6
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.8
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@8		1253.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Cation:Na(E)@11; Oxidation(P)@14; Oxidation(P)@21		2136.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@12		1766.8
GPNGEAGSAGPPGPPGLR	Oxidation(P)@12; Oxidation(P)@15		1618.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.4
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1735.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.8
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFY	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@20; Oxidation(P)@21	cleaved Y-R@C-term	3572.6
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFYR	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@20; Oxidation(P)@21		3728.6
GPSGEAGTAGPPGTPGPQGLLGAPGILGLPGSR	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@24; Oxidation(P)@30		2956.5
GPSGPPGPDGNKGEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@33	missed K-G@12	3212.6

GPSGPQGIR			867.5
GPTGDPGKNGDKGHAGLAGAR	Oxidation(P)@6; Deamidated(N)@9	missed K-N@8; missed K-G@12	1948.9
GSDGSVGPVGPAGPIGSAGPPGFPGAPGPK	Oxidation(P)@20; Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@27		2604.3
GVGLGPGPMGLMGPR	Oxidation(M)@9		1410.7
GVVGPQGAR			839.5
GYPGNIGPVGAAGAPGPHGPVGPAGK	Oxidation(P)@3; Deamidated(N)@5; Oxidation(H)@18		2284.1
HGNRGETGPSGPVGPAGAVGPR	Deamidated(N)@3	missed R-G@4	2027.0
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.7
PGNIGFPGPK	Oxidation(P)@1; Oxidation(P)@7	cleaved E-P@N-term	1014.5
PGPIGPAGAR		cleaved R-P@N-term	891.5
PGPVGAAGATGAR	Oxidation(P)@1	cleaved I-P@N-term	1096.6
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
PGTPGLPGFK	Oxidation(P)@1; Oxidation(P)@4; Oxidation(P)@7	cleaved F-P@N-term	1017.5
PGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@1; Cation:K(E)@9; Oxidation(P)@19	cleaved L-P@N-term	1966.0
QGPAGEPGEPGQTGPAGAR	Oxidation(P)@7; Oxidation(P)@10	cleaved F-Q@N-term	1764.8
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.8
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1844.9
GAPGPDGNNGAQGP	Deamidated(N)@8; Deamidated(N)@9	cleaved P-P@C-term	1209.5
PGAPGPKGEIGAVGNAGPAGPAGPR		cleaved F-P@N-term; missed K-G@7	2147.6

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGER	Deamidated(Q)@7; Oxidation(P)@10		1706.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
GAAGLPGPK	Oxidation(P)@6		782.4
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.2
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2198.0
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2

GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.8
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1741.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.1
GETGPAGPTGPVGPVGAR	Formyl@N-term; Dehydrated(T)@3; Dehydrated(T)@9		1567.8
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.1
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9	missed K-G@18	2852.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Deamidated(N)@2; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2548.2
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(P)@9		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.5
GVVGLPGQR	Oxidation(P)@6		897.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4

PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGEQGPSGASGPAGPR	Oxidation(P)@1	cleaved S-P@N-term	1436.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGLPGPSGEPGK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	1123.6
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPTGLPGPPGER	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved E-P@N-term	1278.6
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
QGPPGEPGEPGASGPMGPR	Oxidation(P)@4; Oxidation(P)@7; Oxidation(P)@10; Oxidation(M)@16	cleaved F-Q@N-term	1837.8
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPP GAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPA GK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
SGDRGETGPAGPTGPVGPV GAR	Delta:H(2)C(2)@N-term; Dehydrated(T)@13	missed R-G@4	1998.9

Group 14: Collagen alpha-1(II) chain

Sequence	Modifications	Cleavages	Prec MW
TGPPGPAGQDGRPGPAGPP GAR	Oxidation(P)@4; Carbamyl(R)@12; Oxidation(P)@18; Oxidation(P)@19		2057.0
DGLNGLPGPIGPPGPR	Oxidation(P)@7; Oxidation(P)@15		1544.8
GPSGPQGPGGPPGPK	Oxidation(P)@12; Cation:K@C-term		1339.6
GFSGLDGAK			850.4
GFSGLDGAK			850.4
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15; Cation:K(E)@17		2742.2
SGDRGETGPAGPTGPVGPV GAR	Delta:H(2)C(2)@N-term; Dehydrated(T)@13	missed R-G@4	1998.9
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Cation:K(D)@3; Oxidation(P)@20; Oxidation(P)@21; Oxidation(P)@24		2585.2
GEAGPQGARGSEGPQGVR	Oxidation(P)@5; Deamidated(Q)@6; Oxidation(P)@14; Deamidated(Q)@15	missed R-G@9	1742.7
PGERGAAGLPGAK	Oxidation(P)@1; Oxidation(P)@10	cleaved M-P@N-term; missed R-G@4	1211.6
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GAPGERGETGPPGPAGFAG PPGADGQPGAK	Pro->pyro-Glu(P)@3; Oxidation(P)@12; Oxidation(P)@21; Deamidated(Q)@26	missed R-G@6	2703.2
GFPGLPGPSGEPGK	Oxidation(P)@3		1311.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.7

GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
PGLPGPSGEPGK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	1123.6
DGEAGAQGPPGPAGPAGER	Deamidated(Q)@7; Oxidation(P)@10		1706.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
GAAGLPGPK	Oxidation(P)@6		782.4
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.2
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2198.0
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPAGVQGPPGPAGEEGK	Carbamyl@N-term; Oxidation(P)@12		1845.9
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.8
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1741.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPAGPIGPAGAR	Pro->pyro-Glu(P)@14		1545.7
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.1
GETGPAGPTGPVGPVGAR	Formyl@N-term; Dehydrated(T)@3; Dehydrated(T)@9		1567.8
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.1
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9	missed K-G@18	2852.4

GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Deamidated(N)@2; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2548.2
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGSAGSPGKDGLNGLPGPIGPPGPR	Deamidated(N)@15; Oxidation(P)@18; Oxidation(P)@23; Oxidation(P)@24		2454.2
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(P)@9		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.5
GVVGLPGQR	Oxidation(P)@6		897.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGEQGPSGASGPAGPR	Oxidation(P)@1	cleaved S-P@N-term	1436.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPTGLPGPPGER	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved E-P@N-term	1278.6
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
QGPPGEPGEPGASGPMGPR	Oxidation(P)@4; Oxidation(P)@7; Oxidation(P)@10; Oxidation(M)@16	cleaved F-Q@N-term	1837.8
SGDRGETGPAGPAGPAGPAGVR	Pro->pyro-Glu(P)@15; Pro->pyro-Glu(P)@18	missed R-G@4	1961.0
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
DGEAGAQGPPGPAGPAGER			1689.8
DGEAGAQGPPGPAGPAGER	Cation:K(E)@3; Oxidation(P)@10		1743.7
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8

DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Cation:K(D)@2; Oxidation(P)@15; Oxidation(P)@24		2302.1
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAP GAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GEPGPPGPAGFAGPPGADG QPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPPGPAGFAGPPGADG QPGAK	Oxidation(P)@3; Oxidation(P)@8; Oxidation(P)@15; Cation:K(D)@18		2170.9
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEE GK	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12		1847.9
GEPGPVGVQGPPGPAGEE GK	Oxidation(P)@3; Oxidation(P)@12; Cation:Na(E)@17		1868.9
GEPGPVGVQGPPGPAGEE GKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0

GETGPAGPAGPIGPAGAR	Pro->pyro-Glu(P)@14		1545.8
GETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.1
GETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@9; Oxidation(P)@18		2152.1
GETGPAGRPGEVGPPGPPG PAGEK	Oxidation(P)@5; Oxidation(P)@15; Oxidation(P)@17		2215.0
GETGPAGRPGEVGPPGPPG PAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GETGPAGRPGEVGPPGPPG PAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@20		2231.1
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15		2704.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@12; Oxidation(P)@15; Cation:K(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@18		2705.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15		2688.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15; Cation:Na(E)@17		2710.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15; Deamidated(Q)@18		2689.3
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15; Cation:K(D)@17	missed K-G@18	2906.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15; Cation:Na(E)@20	missed K-G@18	2890.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15; Lys->Allysine(K)@18	missed K-G@18	2867.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15; Lys->Allysine(K)@18	missed K-G@18	2867.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@11; Oxidation(P)@15	missed K-G@18	2884.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@9		1257.6
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6		1815.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9		1831.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.9
GPPGPMGPPGLAGPPGESG	Oxidation(P)@3; Oxidation(P)@15		1815.9

R			
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9		1815.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@21	missed K-N@8	2347.1
GPPGSAGSPGKDGLNGLPGPIGPPGPR	Deamidated(N)@15; Oxidation(P)@18; Oxidation(P)@23; Oxidation(P)@24		2454.2
GPPGSAGSPGKDGLNGLPGPIGPPGPR	Deamidated(N)@15; Oxidation(P)@18; Oxidation(P)@23; Oxidation(P)@24		2454.2
GPSGPQGPGGPPGPK	Cation:K@C-term		1323.6
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@14		1317.6
GQAGVMGFPGPK	Deamidated(Q)@2; Oxidation(M)@6		1161.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Oxidation(P)@9		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Deamidated(Q)@23		2089.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Oxidation(P)@15		2104.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Cation:Na(D)@6; Oxidation(P)@12		2126.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@18; Deamidated(Q)@23		2106.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15		2120.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@23		2121.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6; Deamidated(Q)@8		898.5
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7;	cleaved R-P@N-term	1489.7

	Oxidation(P)@10		
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@12	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2055.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9

EH156.3, P4 2nd extraction, TrEMBL database

N	SC	Name	Peptides (95%)
1	81.7	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	146
2	77.3	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	143
3	20	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	4
4	76.5	Collagen, type I, alpha 1, isoform CRA_a OS=Homo sapiens GN=COL1A1 PE=4 SV=1	96
5	76.3	Collagen alpha-1(II) chain OS=Homo sapiens GN=COL2A1 PE=1 SV=3	6
6	11.1	Protein AHNAK2 OS=Homo sapiens GN=AHNAK2 PE=1 SV=2	1
7	14.1	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	1
8	11.2	cDNA, FLJ94754, highly similar to Homo sapiens potassium inwardly-rectifying channel, subfamily J, member 2 (KCNJ2), mRNA OS=Homo sapiens PE=2 SV=1	1
9	56.7	Collagen alpha-2(XI) chain OS=Homo sapiens GN=COL11A2 PE=2 SV=1	0
10	48.4	Collagen alpha-4(IV) chain OS=Homo sapiens GN=COL4A4 PE=1 SV=3	0

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
EGPVGLPGIDGRPGPIGPAGAR	Oxidation(P)@7; Oxidation(P)@13		2071.1
ENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Phospho(T)@8; Deamidated(N)@20; Oxidation(P)@25	cleaved G-E@N-term	2590.2
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@17		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEAGAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3
GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@3; Deamidated(N)@9; Oxidation(P)@14		2146.0
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPNGPPG	Deamidated(N)@3;		2568.2

PAGSR	Deamidated(N)@21; Oxidation(P)@24		
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.7
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@9		1200.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GEPGSAGPQGPPGPSGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	1978.9
GEPGVVGAVGTAGPSGPSGLPGER	Oxidation(P)@3; Oxidation(P)@21		2136.0
GEQGPPGPPGFQGLPGPSGPAGEVGKPGER	Formyl@N-term; Oxidation(P)@17; Oxidation(P)@27		2869.3
GESGNKGEPGSAGPQGPPGPSGEEGK	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2396.0
GESGNKGEPGSAGPQGPPGPSGEEGKR	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6; missed K-R@26	2552.2
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Dioxidation(P)@18; Deamidated(N)@21; Oxidation(K)@27		2434.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21		2051.9
GEVGPAGPNGFAGPAGAAGQPGAKGER	Deamidated(N)@9; Oxidation(P)@21; Oxidation(K)@24	missed K-G@24	2409.1
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@9		1205.6
GHAGLAGAR			808.4
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Deamidated(Q)@6; Oxidation(P)@9; Oxidation(P)@21		2801.4
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.7
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.8
GLPGLKGHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Dioxidation(P)@3; Deamidated(N)@9; Oxidation(H)@21; Oxidation(P)@27	missed K-G@6	3397.7
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6		1252.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Cation:Na(E)@11; Oxidation(P)@14; Oxidation(P)@21		2136.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Oxidation(P)@9; Oxidation(P)@12		1765.8
GPNGEAGSAGPPGPPGLR	Oxidation(P)@12; Oxidation(P)@15		1618.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.3
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1735.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.7
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFY	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@18; Cation:K(D)@29	cleaved Y-R@C-term	3594.5

GPQGHQGPAGPPGPPGPPGPPGVSG GGYDFGYDGDFYR	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@20; Oxidation(P)@21		3728.5
GPSGEAGTAGPPGTPGPQGLLGAPGI LGLPGSR	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@24; Oxidation(P)@30		2956.5
GPSGPPGPDGNKGEPGVVGAVGTAG PSGPSGLPGER	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@33	missed K-G@12	3212.5
GPSGPQGIR			867.5
GPTGDPGKNGDKGHAGLAGAR	Oxidation(P)@6; Deamidated(N)@9	missed K-N@8; missed K-G@12	1949.0
GSDGSVGPVGPAGPIGSAGPPGFPGA PGPK	Oxidation(P)@20; Oxidation(P)@21; Oxidation(P)@24; Oxidation(P)@27		2604.3
GSDGSVGPVGPAGPIGSAGPPGFPGA PGPKGEIGAVGNAGPAGPAGPR	Biotin(K)@30	missed K-G@30	4295.1
GVGLGPGPMGLMGPR	Oxidation(M)@9		1410.7
GVVGPQGAR			839.5
GYPGNIGPVGAAGAPGPHGPVGPAG K	Oxidation(P)@3; Oxidation(P)@15		2283.1
HGNRGETGPSGPVGPAGAVGPR	Deamidated(N)@3	missed R-G@4	2027.0
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.7
PGNIGFPGPK	Oxidation(P)@1; Deamidated(N)@3; Oxidation(P)@7	cleaved E-P@N-term	1015.5
PGPIGPAGAR	Oxidation(P)@1	cleaved R-P@N-term	907.5
PGPVGAAGATGAR	Oxidation(P)@1	cleaved I-P@N-term	1096.6
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
PGTPGLPGFK	Oxidation(P)@1; Oxidation(P)@4; Oxidation(P)@7	cleaved F-P@N-term	1017.5
PGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@1; Cation:K(E)@9; Oxidation(P)@19	cleaved L-P@N-term	1966.0
QGPAGEPGEPGQTGPAGAR	Oxidation(P)@7; Oxidation(P)@10	cleaved F-Q@N-term	1764.8
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.8
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1828.9
VGLPGIDGRPGPIGPAGAR	Cation:Cu[I](D)@7; Oxidation(P)@10	cleaved P-V@N-term	1833.9
GFPGTPGLPGFKGIR	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9; Carbamyl(K)@12	missed K-G@12	1590.8

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8

GAAGLPGPK	Oxidation(P)@6		782.4
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Dioxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@27	missed R-G@6	2718.2
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2213.9
GDAGPAGPKGEPGSPGENGAPGQMGPR	Dioxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2525.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.8
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1741.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(R)@8; Oxidation(P)@15; Oxidation(P)@17		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Cation:K(E)@17		2710.2
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGPPGAPGPQGFQGPPGEPGEPGASGPMGPR	Dioxidation(P)@5; Oxidation(P)@11; Oxidation(P)@21; Oxidation(P)@24		3083.4
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2

GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@15		1815.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.6
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@15; Oxidation(K)@18		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6		897.5
NGLPGPIGPPGPRGR	Deamidated(N)@1; Oxidation(P)@4; Oxidation(P)@10; Oxidation(P)@12	cleaved L-N@N-term; missed R-G@13	1489.7
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGEQGPSGASGPAGPR	Oxidation(P)@1	cleaved S-P@N-term	1436.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGLPGPSGEPGK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	1123.6
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@1; Oxidation(P)@4; Deamidated(N)@7; Oxidation(P)@16	cleaved P-P@N-term; missed K-N@6	2193.0
PGPTGLPGPPGER	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved E-P@N-term	1278.6
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
QGPPGEPGEPGASGPMGPR	Oxidation(P)@4; Oxidation(P)@7; Oxidation(P)@10; Oxidation(M)@16	cleaved F-Q@N-term	1837.8
QGPPGPAGPR	Oxidation(P)@4	cleaved V-Q@N-term	948.5
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
GFPGPKGAAGEPGK	Oxidation(P)@3; Oxidation(P)@5; Carbamyl(K)@14	cleaved M-G@N-term; missed K-G@6	1343.6
SGDRGETGPAGPTGPVGPVGAR	Delta:H(2)C(2)@N-term; Dehydrated(T)@13	missed R-G@4	1998.9

Group 4: Collagen, type I, alpha 1, isoform CRA

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGE R	Cation:K(E)@3; Oxidation(P)@10		1743.7
DGEAGAQGPPGPAGPAGE R			1689.8
DGEAGAQGPPGPAGPAGE R	Deamidated(Q)@7; Oxidation(P)@10		1706.7
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Dioxidation(P)@15; Deamidated(R)@16		1562.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Dioxidation(P)@15		1577.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7;		1561.8

	Oxidation(P)@12; Oxidation(P)@15		
GAAGLPGPK	Oxidation(P)@6		782.4
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.7
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Dioxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@27	missed R-G@6	2718.2
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Oxidation(D)@5; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.2
GAPGERGETGPPGPAGFAGPPGADGQPGAK	Methyl(E)@8; Oxidation(P)@12; Oxidation(P)@21; Deamidated(Q)@26	missed R-G@6	2703.2
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2213.9
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2197.9
GDAGPAGPKGEPGSPGENGAPGQMGPR	Dioxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2525.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:K(D)@2; Oxidation(P)@15; Oxidation(P)@24		2302.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:K(D)@2; Oxidation(P)@11; Oxidation(P)@15; Oxidation(P)@24		2318.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGAAGPAGNPGADGQPGAK	Dioxidation(P)@8; Oxidation(P)@18; Deamidated(Q)@23; Oxidation(P)@24		2316.0
GEPGPPGPAGFAGPPGADGQPGAK	Dioxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20; Oxidation(P)@21		2149.9
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@8; Oxidation(P)@15; Cation:K(D)@18		2170.9
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12		1847.8
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12; Cation:Na(E)@17		1868.9

GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.8
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1741.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPAGPVGPVGAR	Cation:Na(E)@2		1567.8
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1545.7
GETGPAGPAGPVGPVGAR			1545.7
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1544.8
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.0
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@18		2152.0
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.1
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18; Oxidation(K)@24		2231.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(R)@8; Oxidation(P)@15; Oxidation(P)@17		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3		1311.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@12; Oxidation(P)@15; Cation:K(E)@17		2726.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Cation:K(E)@17		2710.2
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15; Deamidated(Q)@18		2689.2

GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15		2704.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@18		2705.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@11; Dioxidation(P)@15; Cation:K(E)@17		2742.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15		2688.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGPPGAPGPQGFQGPPG EPGEPGASGPMGPR	Dioxidation(P)@5; Oxidation(P)@11; Oxidation(P)@21; Oxidation(P)@24		3083.4
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15; Cation:Na(E)@20	missed K-G@18	2890.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Carboxy(D)@17; Delta:H(2)C(2)(K)@18	missed K-G@18	2906.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@9	missed K-G@18	2852.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15; Oxidation(K)@18	missed K-G@18	2884.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@15; Lys->Hydroxyallysine(K)@18	missed K-G@18	2867.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@15; Lys->Hydroxyallysine(K)@18	missed K-G@18	2867.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24; Oxidation(K)@30		2563.2
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Deamidated(N)@2; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2548.2
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9		1831.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6		1815.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(P)@9		1815.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(P)@15		1815.9
GPPGPMGPPGLAGPPGESG	Oxidation(P)@3; Oxidation(M)@6;		1831.8

R	Oxidation(P)@15		
GPPGPPGKNGDDGEAGKP GRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGPPGKNGDDGEAGKP GRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Dioxidation(P)@21	missed K-N@8	2363.1
GPPGPPGKNGDDGEAGKP GRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@21	missed K-N@8	2347.1
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Dioxidation(K)@11; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2470.2
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2454.2
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@14		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Oxidation(P)@9		1160.6
GQAGVMGFPGPK	Deamidated(Q)@2; Oxidation(M)@6		1161.6
GQAGVMGFPGPK	Oxidation(P)@9		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.6
GSPGADGPAGAPGTPGPQ GIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105.0
GSPGADGPAGAPGTPGPQ GIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@18; Deamidated(Q)@23		2106.0
GSPGADGPAGAPGTPGPQ GIAGQR	Oxidation(P)@12; Deamidated(Q)@23		2089.0
GSPGADGPAGAPGTPGPQ GIAGQR	Oxidation(P)@12		2088.0
GSPGADGPAGAPGTPGPQ GIAGQR	Oxidation(P)@12; Oxidation(P)@15		2104.0
GSPGADGPAGAPGTPGPQ GIAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGADGPAGAPGTPGPQ GIAGQR	Oxidation(P)@3; Cation:Na(D)@6; Oxidation(P)@12		2126.0
GSPGADGPAGAPGTPGPQ GIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@23		2121.0
GSPGADGPAGAPGTPGPQ GIAGQR	Oxidation(D)@6; Oxidation(P)@12; Oxidation(P)@15		2120.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8

GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@15; Oxidation(K)@18		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6; Deamidated(Q)@8		898.5
GVVGLPGQR	Oxidation(P)@6		897.5
NGLPGPIGPPGPRGR	Deamidated(N)@1; Oxidation(P)@4; Oxidation(P)@10; Oxidation(P)@12	cleaved L-N@N-term; missed R-G@13	1489.7
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGEQGPSGASGPAGPR	Oxidation(P)@1	cleaved S-P@N-term	1436.7
PGERGAAGIAGPK	Oxidation(R)@4; Oxidation(P)@12	cleaved M-P@N-term; missed R-G@4	1211.6
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10; Oxidation(K)@16	cleaved R-P@N-term	1505.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@12; Oxidation(K)@16	cleaved R-P@N-term	1505.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@12	cleaved R-P@N-term	1489.7
PGLPGPSGEPGK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	1123.6
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@1; Oxidation(P)@4; Deamidated(N)@7; Oxidation(P)@16	cleaved P-P@N-term; missed K-N@6	2193.0
PGPTGLPGPPGER	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved E-P@N-term	1278.6
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
QGPPGEPGEPGASGPMGPR	Oxidation(P)@4; Oxidation(P)@7; Oxidation(P)@10; Oxidation(M)@16	cleaved F-Q@N-term	1837.8
QGPPGPAGPR	Oxidation(P)@4	cleaved V-Q@N-term	948.5
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Deamidated(Q)@9; Oxidation(P)@15; Dioxidation(P)@19		2056.9
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0

TGPPGPAGQDGRPGPPGPP GAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2055.9
VGPPGPSGNAGPPGPPGPA GK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
VGPPGPSGNAGPPGPPGPA GK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGPKGAAGEPGK	Oxidation(P)@3; Oxidation(P)@5; Carbamyl(K)@14	cleaved M-G@N-term; missed K-G@6	1343.6
GFSGLDGAK			850.4
SGDRGETGPAGPTGPVGPV GAR	Delta:H(2)C(2)@N-term; Dehydrated(T)@13	missed R-G@4	1998.9
DGLNGLPGPIGPPGPR	Oxidation(P)@7; Oxidation(P)@15		1544.8
GFSGLDGAK			850.4
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Cation:K(D)@3; Oxidation(P)@20; Oxidation(P)@21; Oxidation(P)@24		2585.1
GPSGPQGPGGPPGPK	Oxidation(P)@12; Cation:K@C-term		1339.6
GETGPAGPPGAPGAPGAPG PVGPAGK	Cation:Na(E)@2; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2190.0
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Cation:Na(D)@3; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2569.2

Group 5: Collagen alpha-1(II) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGE R	Cation:K(E)@3; Oxidation(P)@10		1743.7
DGEAGAQGPPGPAGPAGE R			1689.8
DGEAGAQGPPGPAGPAGE R	Deamidated(Q)@7; Oxidation(P)@10		1706.7
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Dioxidation(P)@15; Deamidated(R)@16		1562.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7;		1545.8

	Oxidation(P)@15		
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Dioxidation(P)@15		1577.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GAAGLPGPK	Oxidation(P)@6		782.4
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.7
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GAPGDRGEPGPPGPAGFA GPPGADGQPGAK	Dioxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@27	missed R-G@6	2718.2
GAPGDRGEPGPPGPAGFA GPPGADGQPGAK	Oxidation(D)@5; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.2
GAPGERGETGPPGPAGFAG PPGADGQPGAK	Methyl(E)@8; Oxidation(P)@12; Oxidation(P)@21; Deamidated(Q)@26	missed R-G@6	2703.2
GDAGAPGAPGSQGAPGLQ GMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2197.9
GDAGAPGAPGSQGAPGLQ GMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2213.9
GDAGPAGPKGEPGSPGEN GAPGQMGPR	Dioxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2525.1
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Cation:K(D)@2; Oxidation(P)@11; Oxidation(P)@15; Oxidation(P)@24		2318.1
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Cation:K(D)@2; Oxidation(P)@15; Oxidation(P)@24		2302.1
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1

GDRGETGPAGPPGAPGAP GAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GDRGETGPAGPPGAPGAP GAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNP GADGQPGAK	Dioxidation(P)@8; Oxidation(P)@18; Deamidated(Q)@23; Oxidation(P)@24		2316.0
GEPGPPGPAGAAGPAGNP GADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGFAGPPGADG QPGAK	Oxidation(P)@3; Oxidation(P)@8; Oxidation(P)@15; Cation:K(D)@18		2170.9
GEPGPPGPAGFAGPPGADG QPGAK	Dioxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20; Oxidation(P)@21		2149.9
GEPGPPGPAGFAGPPGADG QPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPPGPAGFAGPPGADG QPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEE GK	Oxidation(P)@3; Oxidation(P)@12; Cation:Na(E)@17		1868.9
GEPGPVGVQGPPGPAGEE GK	Oxidation(P)@3; Oxidation(P)@12		1846.8
GEPGPVGVQGPPGPAGEE GK	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12		1847.8
GEPGPVGVQGPPGPAGEE GKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGPVGVQGPPGPAGEE GKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1741.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPAGPVGPVGAR			1544.8
GETGPAGPAGPVGPVGAR	Cation:Na(E)@2		1567.8
GETGPAGPAGPVGPVGAR			1545.7
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1545.7
GETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.0
GETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@9; Oxidation(P)@18		2152.0
GETGPAGPPGAPGAPGAPG PVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.0
GETGPAGRPGEVGPPGPPG PAGEK	Oxidation(R)@8; Oxidation(P)@15; Oxidation(P)@17		2215.0
GETGPAGRPGEVGPPGPPG	Oxidation(P)@9; Oxidation(P)@15;		2215.1

PAGEK	Oxidation(P)@18		
GETGPAGRPGEVGPPGPPG PAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18; Oxidation(K)@24		2231.0
GETGPAGRPGEVGPPGPPG PAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3		1311.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@12; Oxidation(P)@15; Cation:K(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15; Deamidated(Q)@18		2689.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@11; Dioxidation(P)@15; Cation:K(E)@17		2742.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12; Oxidation(P)@15		2704.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@18		2705.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Cation:K(E)@17		2710.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15		2688.2
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGPPGAPGPQGFQGPPG EPGEPGASGPMGPR	Dioxidation(P)@5; Oxidation(P)@11; Oxidation(P)@21; Oxidation(P)@24		3083.4
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15	missed K-G@18	2868.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Carboxy(D)@17; Delta:H(2)C(2)(K)@18	missed K-G@18	2906.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@9; Oxidation(P)@15; Oxidation(K)@18	missed K-G@18	2884.4

GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@9	missed K-G@18	2852.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@15; Lys->Hydroxyallysine(K)@18	missed K-G@18	2867.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@15; Lys->Hydroxyallysine(K)@18	missed K-G@18	2867.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15; Cation:Na(E)@20	missed K-G@18	2890.4
GLTGSPGSPGPDGK	Oxidation(P)@6		1241.6
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Deamidated(N)@2; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2548.2
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24; Oxidation(K)@30		2563.2
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(P)@15		1815.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6		1815.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9		1831.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(P)@9		1815.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
GPPGPPGKNGDDGEAGKP GRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Dioxidation(P)@21	missed K-N@8	2363.1
GPPGPPGKNGDDGEAGKP GRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@21	missed K-N@8	2347.1
GPPGPPGKNGDDGEAGKP GRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Dioxidation(K)@11; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2470.2
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2454.2
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@14		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Oxidation(P)@9		1160.6

GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Deamidated(Q)@2; Oxidation(M)@6		1161.6
GQAGVMGFPGPK	Oxidation(P)@9		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Cation:Na(D)@6; Oxidation(P)@12		2126.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@23		2121.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(D)@6; Oxidation(P)@12; Oxidation(P)@15		2120.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Deamidated(Q)@23		2089.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@18; Deamidated(Q)@23		2106.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Oxidation(P)@15		2104.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@15; Oxidation(K)@18		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6; Deamidated(Q)@8		898.5
GVVGLPGQR	Oxidation(P)@6		897.5
NGLPGPIGPPGPRGR	Deamidated(N)@1; Oxidation(P)@4; Oxidation(P)@10; Oxidation(P)@12	cleaved L-N@N-term; missed R-G@13	1489.7
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.5
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.8
PGEQGPSGASGPAGPR	Oxidation(P)@1	cleaved S-P@N-term	1436.7
PGERGAAGIAGPK	Oxidation(R)@4; Oxidation(P)@12	cleaved M-P@N-term; missed R-G@4	1211.6

PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10; Oxidation(K)@16	cleaved R-P@N-term	1505.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@12	cleaved R-P@N-term	1489.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@12; Oxidation(K)@16	cleaved R-P@N-term	1505.7
PGLPGPSGEPGK	Oxidation(P)@1; Oxidation(P)@4	cleaved F-P@N-term	1123.6
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@1; Oxidation(P)@4; Deamidated(N)@7; Oxidation(P)@16	cleaved P-P@N-term; missed K-N@6	2193.0
PGPTGLPGPPGER	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved E-P@N-term	1278.6
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
QGPPGEPGEPGASGPMGPR	Oxidation(P)@4; Oxidation(P)@7; Oxidation(P)@10; Oxidation(M)@16	cleaved F-Q@N-term	1837.8
QGPPGPAGPR	Oxidation(P)@4	cleaved V-Q@N-term	948.5
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
STGGISVPGPMGPSGPR			1552.8
STGGISVPGPMGPSGPR	Oxidation(M)@11		1568.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@18; Oxidation(P)@19		2055.9
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Deamidated(Q)@9; Oxidation(P)@15; Dioxidation(P)@19		2056.9
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGPKGAAGEPGK	Oxidation(P)@3; Oxidation(P)@5; Carbamyl(K)@14	cleaved M-G@N-term; missed K-G@6	1343.6
GFSGLDGAK			850.4
SGDRGETGPAGPTGPVGPVGAR	Delta:H(2)C(2)@N-term; Dehydrated(T)@13	missed R-G@4	1998.9
DGLNGLPGPIGPPGPR	Oxidation(P)@7; Oxidation(P)@15		1544.8
GFSGLDGAK			850.4

GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Cation:K(D)@3; Oxidation(P)@20; Oxidation(P)@21; Oxidation(P)@24		2585.1
GPSGPQGPGGPPGPK	Oxidation(P)@12; Cation:K@C-term		1339.6
GETGPAGPPGAPGAPGAPG PVGPAGK	Cation:Na(E)@2; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2190.0
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Cation:Na(D)@3; Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2569.2

Group 6: Protein AHNAK2

Sequence	Modifications	Cleavages	Prec MW
GLQEDAPGRQGS AGR	Deamidated(Q)@10; Dehydrated(S)@12	cleaved F-G@N-term; missed R-Q@9	1480.7

Group 8: cDNA, FLJ94754, highly similar to Homo sapiens potassium inwardly-rectifying channel

Sequence	Modifications	Cleavages	Prec MW
LATMAVANGFGNGK			1349.6

EH156.3, P4 3rd extraction, Swiss-Prot database

N	SC	Name	Peptides (95%)
1	78.3	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	94
2	68.4	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	104
3	16.6	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	7
4	18.7	Pigment epithelium-derived factor OS=Homo sapiens GN=SERPINF1 PE=1 SV=4	6
5	37.2	Trypsin OS=Sus scrofa PE=1 SV=1	9
6	20.1	Biglycan OS=Homo sapiens GN=BGN PE=1 SV=2	3
7	12.6	Vitronectin OS=Homo sapiens GN=VTN PE=1 SV=1	3
8	68.2	Collagen alpha-2(I) chain OS=Canis familiaris GN=COL1A2 PE=2 SV=2	30
9	9.4	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	2
10	58.8	Collagen alpha-1(II) chain OS=Homo sapiens GN=COL2A1 PE=1 SV=3	5
11	24.3	Matrix Gla protein OS=Sus scrofa GN=MGP PE=2 SV=1	2
12	64.1	Collagen alpha-1(I) chain OS=Rattus norvegicus GN=Col1a1 PE=1 SV=5	58
13	19.8	Chondroadherin OS=Homo sapiens GN=CHAD PE=2 SV=2	4
14	12.5	Prothrombin OS=Pongo abelii GN=F2 PE=2 SV=1	2
15	81	Collagen alpha-1(I) chain OS=Mammut americanum GN=COL1A1 PE=1 SV=4	60
16	9.6	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	1
17	16.9	Dermatopontin OS=Mus musculus GN=Dpt PE=2 SV=1	1
18	64.6	Collagen alpha-2(I) chain OS=Mus musculus GN=Col1a2 PE=2 SV=2	28
19	14.4	Alpha-2-HS-glycoprotein OS=Homo sapiens GN=AHSG PE=1 SV=1	1
20	11.9	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	1
21	59	Collagen alpha-1(I) chain OS=Gallus gallus GN=COL1A1 PE=1 SV=3	39
22	4.3	Osteomodulin OS=Homo sapiens GN=OMD PE=1 SV=1	1
23	68.8	Collagen alpha-2(I) chain OS=Bos taurus GN=COL1A2 PE=1 SV=2	31
24	75.5	Collagen alpha-2(I) chain OS=Mammut americanum PE=1 SV=3	29
25	10.1	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5	3
26	10.9	Lumican OS=Homo sapiens GN=LUM PE=1 SV=2	1
27	13.8	Keratin, type I cytoskeletal 17 OS=Bos taurus GN=KRT17 PE=2 SV=1	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
EGPVGLPGIDGRPGPI	Oxidation(P)@7; Dehydrated(D)@10; Oxidation(P)@13	cleaved I-G@C-term	1543.8
EGPVGLPGIDGRPGPIGPAGAR	Oxidation(P)@7		2055.1
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@17		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGE	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3

AGAAGPAGPAGPR			
GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@3; Deamidated(N)@9; Oxidation(P)@15		2146.0
GAPGPHGPVGPAGK	Oxidation(P)@3	cleaved A-G@N-term	1213.6
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7
GEAGAAGPAGPAGPR			1234.5
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Deamidated(N)@3; Deamidated(N)@21; Oxidation(P)@24		2568.2
GEPGAPGENGTPGQTGAR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1700.7
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@9		1200.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GEQGPPGPPGFQGLPGPSGPAGEVGKPGER	Pro->pyro-Glu(P)@8; Pro->pyro-Glu(P)@9; Oxidation(P)@20; Oxidation(P)@27		2869.3
GESGNKGEPGSAGPQGPPGPSGEEGK	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2395.1
GESGNKGEPGSAGPQGPPGPSGEEGKR	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6; missed K-R@26	2551.2
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21		2052.0
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9		1221.6
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@21		2800.4
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.6
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.7
GLPGLKGHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Oxidation(P)@3; Oxidation(H)@8; Oxidation(P)@15; Oxidation(P)@27	missed K-G@6	3396.7
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6		1252.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Cation:Na(E)@11; Oxidation(P)@12; Oxidation(P)@21		2136.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@12		1766.8
GPNGEAGSAGPPGPPGLR	Oxidation(P)@12; Oxidation(P)@15		1618.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.4
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@3; Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1751.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.7
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFY	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@18; Oxidation(P)@20	cleaved Y-R@C-term	3572.5

GPQGHQGPAGPPGPPGPPGPP GVSGGGYDFGYDGDFYR	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@17; Oxidation(P)@21		3728.6
GPSGEAGTAGPPGTPGPQGLL GAPGILGLPGSR	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@24; Oxidation(P)@30		2956.5
GPSGPPGPDGNKGEPGVVGAV GTAGPSGPSGLPGER	Oxidation(P)@8; Oxidation(P)@15	missed K-G@12	3196.6
GPTGDPGKNGDKGHAGLAGA R	Deamidated(N)@9	missed K-N@8; missed K-G@12	1932.9
GSDGSVGPVGPAGPIGSAGPP GFPGAPGPK	Oxidation(P)@21; Oxidation(P)@27		2572.2
GVGLGPGPMGLMGPR	Oxidation(M)@9		1410.7
GYPGNIGPVGAAGAPGPHGPV GPAGK	Oxidation(P)@3; Oxidation(P)@15		2283.1
HGNRGETGPSGPVGPAGAVGP R	Deamidated(N)@3	missed R-G@4	2027.0
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.6
PGAPGPKGEIGAVGNAGPAGP AGPR		cleaved F-P@N-term; missed K-G@7	2148.1
PGNIGFPGPK	Oxidation(P)@1; Oxidation(P)@7	cleaved E-P@N-term	1014.5
PGPIGPAGAR		cleaved R-P@N-term	891.5
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
PGTPGLPGFK	Oxidation(P)@1; Oxidation(P)@4; Oxidation(P)@7	cleaved F-P@N-term	1017.5
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1844.9
GHNGLDGLK	Deamidated(N)@3		910.4

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGE R	Deamidated(Q)@7; Oxidation(P)@10		1706.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
GAAGLPGPK	Oxidation(P)@6		782.4
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAG PPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.3
GDAGAPGAPGSQGAPGLQ GMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2198.0
GDAGPAGPKGEPGSPGEN GAPGQMGPR	Oxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2509.1
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAPG	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2

APGPVGPAGK			
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@8; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.1
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.1
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@18		2705.3
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@11	missed K-G@18	2852.4
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@11		1257.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6		1815.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6		897.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4

PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9

Group 4: Pigment epithelium-derived factor

Sequence	Modifications	Cleavages	Prec MW
DTDTGALLFIGK			1249.6
LAAAVSNFGYDLYR	Deamidated(N)@7		1559.8
TSLEDFYLDEER			1515.7
TVQAVLTVPK			1054.6
DTDTGALLFIGK			1249.7
DTDTGALLFIGK	Cation:Na(D)@3		1271.6
LAAAVSNFGYDLYR			1560.8

Group 6: Biglycan

Sequence	Modifications	Cleavages	Prec MW
LGLGHNQIR	Deamidated(N)@6		1007.6
PVPYWEVQPATFR		cleaved N-P@N-term	1588.8
VPSGLPDLK			924.5
VPSGLPDLK			924.5
VPSGLPDLK			924.5

Group 7: Vitronectin

Conf	Sequence	Modifications	Cleavages	Prec MW
99	DVWGIEGPIDAAFTR			1645.8
99	FEDGVLDPDYPR			1421.6
99	RVDTVDPPYPR		missed R-V@1	1313.7
99	FEDGVLDPDYPR			1421.7
99	FEDGVLDPDYPR			1421.6

Group 10: Collagen alpha-1(II) chain

Sequence	Modifications	Cleavages	Prec MW
GAPGERGETGPPGPAGFAGPP GADGQPGAK	Pro->pyro-Glu(P)@3; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@27	missed R-G@6	2718.2

Group 13: Chondroadherin

Sequence	Modifications	Cleavages	Prec MW
FSDGAFLGVTTLK			1350.7
SIPDNAFQSFGR	Deamidated(N)@5		1338.6
FSDGAFLGVTTLK			1354.7
FSDGAFLGVTTLK			1354.7
FSDGAFLGVTTLK			1354.7
SIPDNAFQSFGR	Deamidated(N)@5; Deamidated(Q)@8		1339.6
SIPDNAFQSFGR	Deamidated(Q)@8		1338.6

Group 19: Alpha-2-HS-glycoprotein

Sequence	Modifications	Cleavages	Prec MW
HTLNQIDEVK	Deamidated(N)@4		1196.6
HTLNQIDEVK	Deamidated(N)@4		1196.6

Group 22: Osteomodulin

Sequence	Modifications	Cleavages	Prec MW
LLLGYNEISK	Deamidated(N)@6		1149.629
LLLGYNEISK	Deamidated(N)@6		1149.636

Group 26: Lumican

Sequence	Modifications	Cleavages	Prec MW
FNALQYLR	Deamidated(N)@2		1024.5

EH156.2, P4 3rd extraction, TrEMBL database

N	SC	Name	Peptides (95%)
1	79.5	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE=1 SV=7	100
2	76.4	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5	117
3	20.5	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1	7
4	24.6	Pigment epithelium-derived factor OS=Homo sapiens GN=SERPINF1 PE=1 SV=4	6
5	24.7	Biglycan preproprotein variant (Fragment) OS=Homo sapiens PE=2 SV=1	4
6	21.5	Vitronectin OS=Homo sapiens GN=VTN PE=2 SV=1	3
7	36.9	Matrix Gla protein OS=Homo sapiens GN=MGP PE=1 SV=2	4
8	12.2	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	2
9	73.1	Collagen, type I, alpha 1, isoform CRA_a OS=Homo sapiens GN=COL1A1 PE=4 SV=1	78
10	28.7	Chondroadherin OS=Homo sapiens GN=CHAD PE=2 SV=2	2
11	17.8	Prothrombin OS=Homo sapiens GN=F2 PE=1 SV=2	2
12	74.8	Collagen alpha-1(II) chain OS=Homo sapiens GN=COL2A1 PE=1 SV=3	6
13	18.5	cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein OS=Homo sapiens PE=2 SV=1	2
14	9.5	Dermatopontin OS=Homo sapiens GN=DPT PE=2 SV=2	1
15	20.7	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	1
16	8.8	cDNA, FLJ93532, highly similar to Homo sapiens osteomodulin (OMD), mRNA OS=Homo sapiens PE=2 SV=1	1
17	61.5	Collagen alpha-2(V) chain OS=Homo sapiens GN=COL5A2 PE=1 SV=3	1
18	18.5	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	1
19	5.6	Lumican variant (Fragment) OS=Homo sapiens PE=2 SV=1	1
20	21.3	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5	3
21	11.3	Collagen alpha-1(XII) chain OS=Homo sapiens GN=COL12A1 PE=2 SV=1	0
22	20.8	Keratin, type I cytoskeletal 17 OS=Homo sapiens GN=KRT17 PE=1 SV=2	1

Group 1: Collagen alpha-2(I) chain

Sequence	Modifications	Cleavages	Prec MW
EGPVGLPGIDGR	Oxidation(P)@7	cleaved R-P@C-term	1181.6
EGPVGLPGIDGRPGPIGPAGAR	Oxidation(P)@7; Oxidation(P)@15		2071.1
GAAGLPGVAGAPGLPGPR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@17		1561.8
GAPGAVGAPGPAGATGDR	Oxidation(P)@3; Oxidation(P)@9		1509.7
GAPGAVGAPGPAGATGDRGEAGAAGPAGPAGPR	Oxidation(P)@3; Oxidation(P)@9	missed R-G@18	2726.3
GAPGPDGNNGAQGPPGPQGVQGGK	Oxidation(P)@3; Deamidated(N)@9; Oxidation(P)@15		2146.0
GAPGPHGPVGPAGK	Oxidation(H)@6	cleaved A-G@N-term	1213.6
GDGGPPGMTGFPGAAGR	Oxidation(P)@6; Oxidation(M)@8; Oxidation(P)@12		1548.7

GEAGAAGPAGPAGPR			1234.6
GEIGAVGNAGPAGPAGPR	Deamidated(N)@8		1547.8
GENGVVGPTGPVGAAGPAGPNGPPGPAGSR	Deamidated(N)@3; Deamidated(N)@21; Oxidation(P)@24		2568.2
GEPGNIGFPGPK	Oxidation(P)@3; Oxidation(P)@9		1200.6
GEPGSAGPQGPPGPSGEEGK	Oxidation(P)@3; Oxidation(P)@12		1822.8
GEQGPPGPPGFQGLPGPSGPAGEVGKPGER	Pro->pyro-Glu(P)@8; Pro->pyro-Glu(P)@9; Oxidation(P)@20; Oxidation(P)@27		2869.3
GESGNKGEPGSAGPQGPPGPSGEEGK	Deamidated(N)@5; Oxidation(P)@9; Oxidation(P)@18	missed K-G@6	2396.0
GESGNKGEPGSAGPQGPPGPSGEEGKR	Oxidation(P)@9; Oxidation(P)@18	missed K-G@6; missed K-R@26	2551.2
GETGPSGPVGPAGAVGPR			1561.8
GEVGLPGLSGPVGPPGNPGANGLTGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@18; Deamidated(N)@21		2418.2
GEVGPAGPNGFAGPAGAAGQPGAK	Deamidated(N)@9; Deamidated(Q)@20; Oxidation(P)@21		2052.0
GEVGPAGPNGFAGPAGAAGQPGAKGER	Deamidated(N)@9; Oxidation(P)@21; Oxidation(K)@24	missed K-G@24	2409.1
GFPGTPGLPGFK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9		1221.6
GFPGTPGLPGFKGIR	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9; Carbamyl(K)@12	missed K-G@12	1590.8
GHNGLDGLK	Deamidated(N)@3		910.4
GHNGLQGLPGIAGHHGDQGAPGSVGPAGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@21		2800.3
GIPGPVGAAGATGAR	Oxidation(P)@3		1266.6
GLHGEFGLPGPAGPR	Oxidation(P)@9		1476.7
GLPGSPGNIGPAGK	Oxidation(P)@3; Oxidation(P)@6		1252.6
GLPGVAGAVGEPGPLGIAGPPGAR	Oxidation(P)@3; Cation:K(E)@11; Oxidation(P)@12; Oxidation(P)@21		2152.1
GLVGEPGPAGSK	Oxidation(P)@6		1083.6
GPAGPSGPAGKDGR	Carbamyl(K)@11		1265.6
GPNGDAGRPGEPGLMGPR	Deamidated(N)@3; Oxidation(P)@9; Oxidation(P)@12		1766.8
GPNGEAGSAGPPGPPGLR	Oxidation(P)@12; Oxidation(P)@15		1618.8
GPPGAAGAPGPQGFQGPAGEPGEPGQTGPAGAR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@21; Oxidation(P)@24		2958.4
GPPGAVGSPGVNGAPGEAGR	Oxidation(P)@9; Deamidated(N)@12; Oxidation(P)@15		1735.8
GPPGESGAAGPTGPIGSR	Oxidation(P)@3		1579.7
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFY	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@18; Oxidation(P)@20	cleaved Y-R@C-term	3572.5
GPQGHQGPAGPPGPPGPPGPPGVSGGGYDFGYDGDFYR	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@17; Oxidation(P)@21		3728.6

GPSGEAGTAGPPGTPGPQGLLGAPG ILGLPGSR	Oxidation(P)@12; Oxidation(P)@17; Oxidation(P)@24; Oxidation(P)@30		2956.5
GPSGPPGPDGNKGEPGVVGAVGTA GPSGPSGLPGER	Oxidation(P)@8; Oxidation(N)@11	missed K-G@12	3196.6
GPSGPQGIR			867.5
GPTGDPGKNGDKGHAGLAGAR	Deamidated(N)@9	missed K-N@8; missed K-G@12	1932.9
GSDGSVGPVGPAGPIGSAGPPGFPG APGPK	Oxidation(P)@21; Oxidation(P)@27		2572.2
GSDGSVGPVGPAGPIGSAGPPGFPG APGPKGEIGAVGNAGPAGPAGPR	Biotin(K)@30; Deamidated(N)@38	missed K-G@30	4296.1
GVGLGPGPMGLMGPR	Oxidation(M)@9		1410.7
GYPGNIGPVGAAGAPGPHGPVGPA GK	Oxidation(P)@3; Deamidated(N)@5; Oxidation(H)@18		2284.1
HGNRGETGPSGPVGPAGAVGPR	Deamidated(N)@3	missed R-G@4	2027.0
LGAPGILGLPGSR	Oxidation(P)@4; Oxidation(P)@10	cleaved L-L@N-term	1238.6
PGNIGFPGPK	Oxidation(P)@1; Oxidation(P)@7	cleaved E-P@N-term	1014.5
PGPIGPAGAR		cleaved R-P@N-term	891.5
PGSPGNIGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved L-P@N-term	1082.5
PGTPGLPGFK	Oxidation(P)@1; Oxidation(P)@4; Oxidation(P)@7	cleaved F-P@N-term	1017.5
RGPNGEAGSAGPPGPPGLR	Deamidated(N)@4; Oxidation(P)@13; Oxidation(P)@16	missed R-G@1	1775.9
TGEVGAVGPPGFAGEK	Oxidation(P)@10		1487.7
TGHPGTVGPAGIR	Oxidation(P)@4		1234.6
TGPPGPSGISGPPGPPGPAGK	Oxidation(P)@3; Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1844.9

Group 2: Collagen alpha-1(I) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
GAAGLPGPK	Oxidation(P)@6		782.4
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAG PPGADGQPGAK	Oxidation(D)@5; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.3
GDAGAPGAPGSQGAPGLQ GMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2198.0
GDAGPAGPKGEPGSPGENG APGQMGPR	Oxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2509.1
GDAGPPGPAGPAGPPGPIG NVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDRGETGPAGPPGAPGAPG APGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2512.2
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7

GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@8; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.1
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15		2688.3
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGPPGAPGPQGFQGPPGEPGEPGASGPMGPR	Dioxidation(P)@5; Oxidation(P)@9; Dioxidation(P)@18; Oxidation(P)@24		3099.4
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	Oxidation(P)@11	missed K-G@18	2852.4
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@11		1257.6
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVVGLPGQR	Oxidation(P)@6; Deamidated(Q)@8		898.5

PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
GLPGTAGLPGMKGHR	Oxidation(P)@9; Oxidation(M)@11; Formyl(K)@12; Deamidated(R)@15	missed K-G@12	1508.7
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7

Group 4: Pigment epithelium-derived factor

Sequence	Modifications	Cleavages	Prec MW
DTDTGALLFIGK			1249.6
LAAAVSNFGYDLYR	Deamidated(N)@7		1559.8
TSLEDFYLDEER			1515.7
TVQAVLTVPK			1054.6
DTDTGALLFIGK			1249.7
DTDTGALLFIGK	Cation:Na(D)@3		1271.6
LAAAVSNFGYDLYR			1560.8

Group 5: Biglycan preproprotein variant (fragment)

Sequence	Modifications	Cleavages	Prec MW
LGLGHNQIR	Deamidated(N)@6		1007.6
PLENSGFEPGAFDGLK	Deamidated(N)@4	cleaved N-P@N-term	1677.8
PVPYWEVQPATFR		cleaved N-P@N-term	1588.8
VPSGLPDLK			924.5
VPSGLPDLK			924.5
VPSGLPDLK			924.5

Group 6: Vitronectin

Sequence	Modifications	Cleavages	Prec MW
DVWGIEGPIDAAFTR			1645.8
FEDGVLDPDYPR			1421.6
RVDTVDPPYPR		missed R-V@1	1313.7
FEDGVLDPDYPR			1421.7
FEDGVLDPDYPR			1421.6

Group 7: Matrix Gla protein

Sequence	Modifications	Cleavages	Prec MW
RNANTFISPQQR	Deamidated(N)@2; Deamidated(N)@4	missed R-N@1	1432.7
YAMVYGYNAAYNR	Deamidated(N)@8; Deamidated(N)@12		1556.7
NANTFISPQQR	Deamidated(N)@1; Deamidated(N)@3		1276.6
YAMVYGYNAAYNR	Oxidation(M)@3; Deamidated(N)@8; Deamidated(N)@12		1572.7

Group 9: Collagen, type I, alpha 1, isoform CRA

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGE R	Deamidated(Q)@7; Oxidation(P)@10		1706.8
DGEAGAQGPPGPAGPAGE R			1689.8
DGEAGAQGPPGPAGPAGE R	Cation:K(E)@3; Oxidation(P)@10		1743.7
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGE R	Oxidation(P)@10		1705.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8

DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
GAAGLPGPK	Oxidation(P)@6		782.4
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Oxidation(D)@5; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.3
GAPGERGETGPPGPAGFAGPPGADGQPGAK	Pro->pyro-Glu(P)@3; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@27	missed R-G@6	2718.2
GDAGAPGAPGSQGAPGLQGMPGER	Dioxidation(P)@9; Deamidated(Q)@12; Oxidation(P)@15; Dioxidation(M)@20		2215.0
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2198.0
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2214.0
GDAGPAGPKGEPGSPGENGAPGQMGPR	Oxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2509.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:K(D)@2; Oxidation(P)@11; Oxidation(P)@15; Oxidation(P)@24		2318.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2512.2
GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@8; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGAAGPAGNPGADGQPGAK	Dioxidation(P)@6; Oxidation(P)@18; Deamidated(Q)@23; Oxidation(P)@24		2316.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0

GEPGPPGPAGFAGPPGADGQPGAK	Dioxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20; Oxidation(P)@21		2150.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Dioxidation(M)@15		1758.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.1
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@15		2152.0
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.1
GETGPAGPPGAPGAPGAPGPVGPAGK	Cation:Na(E)@2; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2190.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.1
GFPGADGVAGPK	Oxidation(P)@3; Cation:K(D)@6		1125.5
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFSGLDGAK			850.4
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@18		2705.3

GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Acetyl(S)@3; Carboxy(E)@17		2742.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12		2688.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@12; Oxidation(P)@15; Cation:K(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15		2688.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Cation:K(E)@17		2710.2
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGPPGAPGPQGFQGPPG EPGEPGASGPMGPR	Oxidation(P)@3; Dioxidation(P)@11; Oxidation(P)@21; Oxidation(P)@24		3083.4
GLPGPPGAPGPQGFQGPPG EPGEPGASGPMGPR	Dioxidation(P)@5; Dioxidation(P)@11; Oxidation(P)@21; Oxidation(P)@24		3099.4
GLPGPPGAPGPQGFQGPPG EPGEPGASGPMGPR	Dioxidation(P)@5; Oxidation(P)@9; Dioxidation(P)@18; Oxidation(P)@24		3099.4
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(D)@17; Oxidation(K)@18; Cation:Na(E)@20	missed K-G@18	2890.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@15; Lys->Hydroxyallysine(K)@18	missed K-G@18	2867.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Delta:H(2)C(2)@N-term; Carboxy(D)@17	missed K-G@18	2906.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11	missed K-G@18	2852.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15	missed K-G@18	2868.4
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@11		1257.6
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6		1815.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(P)@15		1815.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.9
GPPGPPGKNGDDGEAGKP GRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGPPGKNGDDGEAGKP GRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2

GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Dioxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2470.2
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLP GPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Dioxidation(P)@18; Dioxidation(P)@23		2470.2
GPSGPQGPGGPPGPK	Oxidation(P)@12; Oxidation(P)@14		1317.6
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@2; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@14		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GQAGVMGFPGPK	Deamidated(Q)@2; Oxidation(M)@6		1161.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQG IAGQR	Oxidation(P)@12; Deamidated(Q)@23		2089.0
GSPGADGPAGAPGTPGPQG IAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGADGPAGAPGTPGPQG IAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105.0
GSPGADGPAGAPGTPGPQG IAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@23		2121.0
GSPGADGPAGAPGTPGPQG IAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15		2120.0
GSPGADGPAGAPGTPGPQG IAGQR	Oxidation(P)@12		2088.0
GSPGADGPAGAPGTPGPQG IAGQR	Oxidation(P)@3; Cation:Na(D)@6; Oxidation(P)@12		2126.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6

GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6		897.5
GVVGLPGQR	Oxidation(P)@6		897.5
GVVGLPGQR	Oxidation(P)@6; Deamidated(Q)@8		898.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Deamidated(Q)@9; Oxidation(P)@15; Dioxidation(P)@19		2057.0
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GESGPSGPAGPTGAR			1296.6
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPSGLAGPKGANGDPGRPGEPGLPGAR	Oxidation(N)@12; Cation:Na(D)@14; Oxidation(P)@15	missed K-G@9	2492.2
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Dioxidation(P)@12		1561.8

GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFSGLDGAK			850.4
GLPGTAGLPGMKGHR	Oxidation(P)@9; Oxidation(M)@11; Formyl(K)@12; Deamidated(R)@15	missed K-G@12	1508.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@8		1327.6
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7

Group 10: Chondroadherin

Sequence	Modifications	Cleavages	Prec MW
FSDGAFLGVTTLK			1350.7
SIPDNAFQSFGR	Deamidated(N)@5		1338.6
FSDGAFLGVTTLK			1354.7
FSDGAFLGVTTLK			1354.7
FSDGAFLGVTTLK			1354.7
SIPDNAFQSFGR	Deamidated(N)@5		1338.6

Group 11: Prothrombin

Sequence	Modifications	Cleavages	Prec MW
ELLESYIDGR			1193.6
ETAASLLQAGYK			1250.6
ELLESYIDGR			1193.6
ELLESYIDGR			1193.6

Group 12: Collagen alpha-1 (II) chain

Sequence	Modifications	Cleavages	Prec MW
DGEAGAQGPPGPAGPAGER			1689.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGER	Oxidation(P)@10		1705.8
DGEAGAQGPPGPAGPAGER	Deamidated(Q)@7; Oxidation(P)@10		1706.8
DGEAGAQGPPGPAGPAGER	Cation:K(E)@3; Oxidation(P)@10		1743.7
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8

DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@15		1545.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@15		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.8
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@12; Oxidation(P)@13		1561.7
GAAGLPGPK	Oxidation(P)@6		782.4
GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GANGAPGIAGAPGFPGAR	Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1584.8
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	Oxidation(D)@5; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@21	missed R-G@6	2702.3
GAPGERGETGPPGPAGFAGPPGADGQPGAK	Pro->pyro-Glu(P)@3; Oxidation(P)@12; Oxidation(P)@21; Oxidation(P)@27	missed R-G@6	2718.2
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Dioxidation(M)@20		2214.0
GDAGAPGAPGSQGAPGLQGMPGER	Dioxidation(P)@9; Deamidated(Q)@12; Oxidation(P)@15; Dioxidation(M)@20		2215.0
GDAGAPGAPGSQGAPGLQGMPGER	Oxidation(P)@6; Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@21		2198.0
GDAGPAGPKGEPGSPGENGAPGQMGPR	Oxidation(P)@12; Oxidation(P)@15; Deamidated(N)@18; Dioxidation(M)@24	missed K-G@9	2509.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Cation:K(D)@2; Oxidation(P)@11; Oxidation(P)@15; Oxidation(P)@24		2318.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Oxidation(P)@24		2280.1
GDAGPPGPAGPAGPPGPIGNVGAPGAK	Oxidation(P)@6; Oxidation(P)@15; Deamidated(N)@20; Oxidation(P)@24		2281.1
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@18	missed R-G@3	2480.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18; Oxidation(P)@21	missed R-G@3	2512.2
GDRGETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@21	missed R-G@3	2496.2

GEAGRPGEAGLPGAK	Oxidation(P)@6; Oxidation(P)@12	cleaved P-G@N-term	1397.7
GEPGPPGPAGAAGPAGNPGADGQPGAK	Dioxidation(P)@6; Oxidation(P)@18; Deamidated(Q)@23; Oxidation(P)@24		2316.0
GEPGPPGPAGAAGPAGNPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@8; Oxidation(P)@18; Oxidation(P)@24		2315.1
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20		2134.0
GEPGPPGPAGFAGPPGADGQPGAK	Dioxidation(P)@6; Oxidation(P)@15; Deamidated(Q)@20; Oxidation(P)@21		2150.0
GEPGPPGPAGFAGPPGADGQPGAK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@15		2133.0
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPTGLPGPPGER	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@12		1464.7
GEPGPVGVQGPPGPAGEEGK	Oxidation(P)@3; Oxidation(P)@12		1846.9
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Deamidated(Q)@9; Oxidation(P)@12	missed K-R@20	2004.0
GEPGPVGVQGPPGPAGEEGKR	Oxidation(P)@3; Oxidation(P)@12	missed K-R@20	2003.0
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Dioxidation(M)@15		1758.7
GEPGSPGENGAPGQMGPR	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@12		1742.7
GEQGPSGASGPAGPR		cleaved P-G@N-term	1323.6
GESGPSGPAGPTGAR			1296.6
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPAGPVGPVGAR			1545.8
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2168.1
GETGPAGPPGAPGAPGAPGPVGPAGK	Cation:Na(E)@2; Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@18		2190.0
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Oxidation(P)@18		2184.1
GETGPAGPPGAPGAPGAPGPVGPAGK	Oxidation(P)@9; Oxidation(P)@15		2152.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.0
GETGPAGRPGEVGPPGPPGPAGEK	Oxidation(P)@9; Oxidation(P)@15; Oxidation(P)@18		2215.1
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGADGVAGPK	Oxidation(P)@3		1087.5
GFPGADGVAGPK	Oxidation(P)@3; Cation:K(D)@6		1125.5
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6		1327.6

GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFPGLPGPSGEPGK	Oxidation(P)@6		1311.6
GFSGLDGAK	Dehydrated(S)@3		832.4
GFSGLDGAK			850.4
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@11; Oxidation(P)@12		2688.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@18		2705.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@12; Oxidation(P)@15; Cation:K(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15; Cation:Na(E)@17		2726.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Acetyl(S)@3; Carboxy(E)@17		2742.2
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@15		2688.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Oxidation(P)@12; Oxidation(P)@15		2704.3
GFSGLQGPPGPPGSPGEQG PSGASGPAGPR	Oxidation(P)@9; Cation:K(E)@17		2710.2
GLPGPPGAPGPQGF	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@9	cleaved F-Q@C-term	1295.6
GLPGPPGAPGPQGFQGPPG EPGEPGASGPMGPR	Dioxidation(P)@5; Dioxidation(P)@11; Oxidation(P)@21; Oxidation(P)@24		3099.4
GLPGPPGAPGPQGFQGPPG EPGEPGASGPMGPR	Dioxidation(P)@5; Oxidation(P)@9; Dioxidation(P)@18; Oxidation(P)@24		3099.4
GLPGPPGAPGPQGFQGPPG EPGEPGASGPMGPR	Oxidation(P)@3; Dioxidation(P)@11; Oxidation(P)@21; Oxidation(P)@24		3083.4
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9; Oxidation(P)@15		1589.8
GLTGPIGPPGPAGAPGDK	Oxidation(P)@9		1573.8
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11; Oxidation(P)@15	missed K-G@18	2868.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(D)@17; Oxidation(K)@18; Cation:Na(E)@20	missed K-G@18	2890.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@11	missed K-G@18	2852.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Oxidation(P)@15; Lys->Hydroxyallysine(K)@18	missed K-G@18	2867.4
GLTGPIGPPGPAGAPGDKG ESGPSGPAGPTGAR	Delta:H(2)C(2)@N-term; Carboxy(D)@17	missed K-G@18	2906.4
GLTGSPGSPGPDGK	Oxidation(P)@6; Oxidation(P)@11		1257.6
GNDGATGAAGPPGPTGPA GPPGFPGAVGAK	Oxidation(P)@14; Oxidation(P)@21; Oxidation(P)@24		2547.2
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(P)@15		1815.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.9
GPPGPMGPPGLAGPPGESG R	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@15		1831.8
GPPGPMGPPGLAGPPGESG	Oxidation(P)@3; Oxidation(M)@6		1815.9

R			
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1831.9
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGPPGKNGDDGEAGKPGRPGER	Oxidation(P)@3; Oxidation(P)@6; Deamidated(N)@9; Oxidation(P)@18	missed K-N@8	2347.1
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Dioxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@24		2470.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Dioxidation(P)@18; Dioxidation(P)@23		2470.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPPGSAGAPGKDGLNGLPGPIGPPGPR	Oxidation(P)@3; Deamidated(N)@15; Oxidation(P)@18; Dioxidation(P)@23		2454.2
GPSGPQGPGGPPGPK	Deamidated(Q)@6; Oxidation(P)@12		1302.6
GPSGPQGPGGPPGPK	Oxidation(P)@12; Oxidation(P)@14		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@2; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@14		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@8; Oxidation(P)@12		1317.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GPSGPQGPGGPPGPK	Oxidation(P)@12		1301.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GQAGVMGFPGPK	Oxidation(M)@6; Oxidation(P)@9		1176.6
GQAGVMGFPGPK	Oxidation(P)@11		1160.6
GQAGVMGFPGPK	Deamidated(Q)@2; Oxidation(M)@6		1161.6
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSAGPPGATGFPGAAGR	Oxidation(P)@6; Oxidation(P)@12		1458.7
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12; Deamidated(Q)@23		2089.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Deamidated(Q)@23		2105.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@12		2088.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15		2120.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12		2104.0
GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Cation:Na(D)@6; Oxidation(P)@12		2126.0

GSPGADGPAGAPGTPGPQGIAGQR	Oxidation(P)@3; Oxidation(P)@12; Oxidation(P)@15; Deamidated(Q)@23		2121.0
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GSPGEAGRPGEAGLPGAK	Oxidation(P)@3; Oxidation(P)@9; Oxidation(P)@15		1654.8
GVPGPPGAVGPAGK	Oxidation(P)@3		1175.6
GVPGPPGAVGPAGK	Oxidation(P)@6		1175.6
GVPGPPGAVGPAGK	Oxidation(P)@3; Oxidation(P)@6		1191.6
GVQGPPGPAGPR	Deamidated(Q)@3; Oxidation(P)@6		1105.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVQGPPGPAGPR	Oxidation(P)@6		1104.6
GVVGLPGQR	Oxidation(P)@6		897.5
GVVGLPGQR	Oxidation(P)@6		897.5
GVVGLPGQR	Oxidation(P)@6; Deamidated(Q)@8		898.5
PGADGVAGPK	Oxidation(P)@1	cleaved F-P@N-term	883.4
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEAGRPGEAGLPGAK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@13	cleaved S-P@N-term	1510.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGEVGPPGPPGPAGEK	Oxidation(P)@1; Oxidation(P)@7; Oxidation(P)@10	cleaved R-P@N-term	1489.7
PGPPGAVGPAGK	Oxidation(P)@1; Oxidation(P)@4	cleaved V-P@N-term	1035.5
PGPVGVQGPPGPAGEEGKR	Oxidation(P)@1; Oxidation(P)@10	cleaved E-P@N-term; missed K-R@18	1816.9
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
SGDRGETGPAGPAGPVGPVGAR		missed R-G@4	1961.0
STGGISVPGPMGPSGPR			1552.8
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Deamidated(Q)@9; Oxidation(P)@15; Dioxidation(P)@19		2057.0
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
TGPPGPAGQDGRPGPPGPPGAR	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16; Oxidation(P)@19		2056.0
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Deamidated(N)@9; Oxidation(P)@13; Oxidation(P)@16		1812.9
VGPPGPSGNAGPPGPPGPAGK	Oxidation(P)@4; Oxidation(P)@13; Oxidation(P)@16		1811.9
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Oxidation(P)@13; Oxidation(P)@15		1561.8

GANGAPGIAGAPGFPGAR	Deamidated(N)@3; Oxidation(P)@6; Oxidation(P)@12; Oxidation(P)@15		1585.8
GESGPSGPAGPTGAR			1296.6
GPPGPMGPPGLAGPPGESGR	Oxidation(P)@3; Oxidation(M)@6; Oxidation(P)@9; Oxidation(P)@15		1847.8
GPSGLAGPKGANGDPGRPGEPGLPGAR	Oxidation(N)@12; Cation:Na(D)@14; Oxidation(P)@15	missed K-G@9	2492.2
DGLNGLPGPIGPPGPR	Deamidated(N)@4; Oxidation(P)@7; Dioxidation(P)@12		1561.8
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@6; Oxidation(P)@12		1343.6
GFSGLDGAK			850.4
GLPGTAGLPGMKGHR	Oxidation(P)@9; Oxidation(M)@11; Formyl(K)@12; Deamidated(R)@15	missed K-G@12	1508.7
GFPGLPGPSGEPGK	Oxidation(P)@3; Oxidation(P)@8		1327.6
GISVPGPMGPSGPR	Oxidation(M)@8	cleaved G-G@N-term	1323.7

Group 13: cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein

Sequence	Modifications	Cleavages	Prec MW
HTLNQIDEVK	Deamidated(N)@4		1196.6
SNFQLEEISR	Deamidated(N)@2; Deamidated(Q)@4	cleaved G-S@N-term	1223.6
HTLNQIDEVK	Deamidated(N)@4		1196.6

Group 14: Dermatopontin

Sequence	Modifications	Cleavages	Prec MW
GATTTFSAVER			1138.6

Group 16: cDNA, FLJ93532, highly similar to *Homo sapiens* osteomodulin

Sequence	Modifications	Cleavages	Prec MW
LLLGYNEISK	Deamidated(N)@6		1149.6
LLLGYNEISK	Deamidated(N)@6		1149.6

Group 17: Collagen alpha-1 (XII) chain

Sequence	Modifications	Cleavages	Prec MW
PLGAPGEDGRPGPPGSIGIR		cleaved G-P@N-term	1898.9

Group 19: Lumican variant (fragment)

Sequence	Modifications	Cleavages	Prec MW
FNALQYLR	Deamidated(N)@2		1024.5