STAR: Science & Technology of Archaeological Research

ISSN: (Print) 2054-8923 (Online) Journal homepage: http://www.tandfonline.com/loi/ysta20

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To cite this article: Ross Kendall, Jessica Hendy, Matthew J. Collins, Andrew R. Millard & Rebecca L. Gowland (2016) Poor preservation of antibodies in archaeological human bone and dentine, STAR: Science & Technology of Archaeological Research, 2:1, 15-24

To link to this article: http://dx.doi.org/10.1080/20548923.2015.1133117

Science and Technology of Archaeological Research

Research Paper

Poor preservation of antibodies in archaeological human bone and dentine

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Abstract The growth of proteomics-based methods in archaeology prompted an investigation of the survival of non-collagenous proteins, specifically immunoglobulin G (IgG), in archaeological human bone and dentine. Over a decade ago reports were published on extracted, immunoreactive archaeological IgG, and the variable yields of IgG molecules detected by Western blots of 1D and 2D SDS-PAGE gels. If IgG can indeed be recovered from archaeological skeletal material, it offers remarkable opportunities for exploring the history of disease - for example in applying functional anti-malarial IgGs to study past patterns of malaria. More recently, the field has seen a move away from immunological approaches and towards the use of shotgun proteomics via mass spectrometry. Using previously published techniques, this study attempted to extract and characterize archaeological IgG proteins. In only one extraction method were immunoglobulin derived peptides identified, and these displayed extensive evidence of degradation. The failure to extract immunoglobulins by all but one method, along with observed patterns of protein degradation, suggests that IgG may be an unsuitable target for detecting disease-associated antigens. This research highlights the importance of revisiting previously 'successful' biomolecular methodologies using emerging technologies.

Key words Antibodies; immunoglobulin G; proteomics; protein extraction

Received 18 March 2015; **accepted** 07 July 2015

Statement of significance

The extraction of functional antibodies from archaeological skeletal material offers a unique and exciting avenue of investigating disease presence in past populations. Previously published work has suggested that non-collagenous proteins, including immunoglobulin G (IgG), should survive intact in well-preserved skeletal material. However, little confirmatory research specifically addressing the survival of IgG has been conducted. This research assesses the efficacy of three published techniques for extracting archaeological IgGs. Significantly, only one extraction method yielded immunoglobulin derived peptides, with high-resolution proteomic analysis revealing evidence of extensive degradation. These findings suggest that IgG survival is likely independent of bone preservation, and that current methodological constraints prevent the utilization of extracted archaeological IgGs in biomolecular palaeopathology. Furthermore, the research highlights the importance of evaluating the efficacy of previously 'successful' protein extraction techniques utilizing new technologies.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaíno et al. 2014) via the PRIDE partner repository with the dataset identifier PXD002295.

Introduction

Proteins are increasingly used as a powerful research tool for understanding ancient diseases, diets, and phylogenies (Cappellini, Collins, and Gilbert 2014; Warinner et al. 2014b; Welker et al. 2015). New high-resolution mass spectrometric technology is now allowing access to archaeological proteins of low abundance preserved in a range of materials (e.g., Cappellini et al. 2012; Wadsworth and Buckley 2014; Warinner et al. 2014a; Warinner et al. 2014b). Early archaeological bone protein research (Gürtler et al. 1981; Hedges and Wallace 1978) isolated collagen, confirming it as the dominant protein in archaeological samples. More recent research has confirmed the longevity and stability of collagen in bone approximately 1.5 million years old (Buckley and Collins 2011). Bone

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10.1080/20548923.2015.1133117

and dentine also contain a number of non-collagenous proteins (NCPs), and it has been argued that the high affinity of some of these (e.g. osteocalcin, matrix gla protein) for bone mineral (bioapatite) may offer a degree of protection from diagenesis following death and inhumation (Collins et al. 2000; Freundorfer, Grupe, and Wieckmann 1995; Grupe and Turban-Just 1996; Masters 1987; Nielsen-Marsh and Hedges 2000; Smith et al. 2005; Wiechmann, Brandt, and Grupe 1999; but see Buckley et al. 2008). Other studies have reported apparent success in detecting NCPs in archaeological bone (e.g., Brandt, Wiechmann and Grupe 2000; Cattaneo et al. 1992; Schmidt-Schultz and Schultz 2004; Wadsworth and Buckley 2014; Wiechmann, Brandt, and Grupe 1999), including bone that has undergone post excavation treatment and museum curation (Tuross 1991), and human dentine and dental calculus (Warinner et al. 2014a).

Immunoglobulin antibodies represent an important target NCP group for biomolecular paleopathological investigation, offering enormous potential in the detection and characterization of past disease, either through the confirmation of a suspected diagnosis, or the identification of latent conditions. Immunoglobulin G (IgG) antibodies are the most abundant antibody class, accounting for approximately 75% of serum immunoglobulins (Nezlin 1998). They are large molecules (approximately 150 kDa) consisting of a classic Y-shaped paired heavy and light chain structure connected by disulfide bonds, and are mostly active in the adaptive immune response (Janeway 2001). IgG titers become elevated during periods of infection and disease-specific IgGs have been clinically shown to circulate long after infection and associated pathogenic molecules have been cleared from the body. Wipasa et al. (2010), for example, demonstrated long-term stable anti-malarial IgG titer in patients in the absence of reinfection. Thus, the presence of circulating disease-specific IgGs may be used to infer past infections, long after the patient has recovered. The high affinity of IgG for bioapatite and its tendency to concentrate within bone mineral (Nakagawa et al. 2010; Omelyaneko et al. 2013) should, theoretically, provide increased protection against diagenetic factors, thus enhancing the possibility of IgG surviving in a functional state. Consequently, assuming that antibodies are indeed retained in the bone, they represent a more stable target for immunological analysis than pathogenic molecules, which are, by nature, transitory.

Targeting ancient antibodies as markers of disease, however, makes a number of assumptions: that IgG is concentrated in sufficient quantity in skeletal material; that they can then be successfully extracted from this reservoir; and that they are sufficiently well preserved to remain immunoreactive. Considering their great potential for enhancing palaeopathology, surprisingly little research has concentrated on archaeological IgG. Cattaneo et al. (1992) concluded that the protein represents a poor choice for biomolecular analysis following attempts to detect IgG using ELISA. Since then, there have been at least two independent reports of successful extraction of immunoreactive and well-preserved IgGs from archaeological human bone, assessed by ELISA (Kolman et al. 1999) and SDS-PAGE/Western Blot (Schmidt-Schultz and Schultz 2004), respectively. However, in two recent shotgun proteomics analyses of prehistoric bone (Buckley and Wadsworth 2014; Cappellini et al. 2012), only one found highly degraded IgG (in a 43000 year old Mammoth sample from the Siberian permafrost; Cappellini et al. 2012). This dissonance of evidence prompted an investigation specifically targeting archaeological IgG. The primary aim of this study is to assess the survival of IgG in archaeological human bone and dentine using shotgun proteomic analysis based on published extraction and characterization techniques (Cappellini et al. 2013; Jiang et al. 2007; Schmidt-Schultz and Schultz 2004; Warinner et al. 2014a).

Methods

Site selection and sample preparation

To provide a focus for our study we targeted archaeological individuals from two different environments where they are likely to have been exposed to malaria. Malaria is a debilitating disease which may lead to mortality, particularly if comorbid with other conditions (Dobson 1997). Infected individuals have been shown to exhibit long-term elevated levels of circulating IgG (Wipasa et al. 2010).

A recent study (Gowland and Western 2012) has proposed that *Plasmodium vivax* may have been a common pathogen in past British marshland populations. We selected UK sites associated with the historically recorded presence of malaria vector *Anopheline* species (Nuttall, Cobbett, and Strangeways-Pigg 1901), and within an appropriate distance (approximately 3–12 km, depending upon mosquito diet) of likely *Anopheline* breeding grounds (Kaufmann and Briegel 2004) (Table 1). Furthermore, 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). A number of individuals were sampled from each UK site in order to ensure procurement of well-preserved bone. Preservation of each

Table 1 Cemetery sites selected for investigation. Lincs: Lincolnshire; Cambs: Cambridgeshire.

Site	Period	Number of samples
Hoplands, Sleaford, Lincs	Roman	33
Watersmeet, Huntingdon, Cambs	Roman	20
Castledyke South, Lincs	Anglo-Saxon	51
Edix Hill, Cambs	Anglo-Saxon	25
Highfield Farm, Littleport, Cambs	Anglo-Saxon	66
Orchard Lane, Huntingdon, Cambs	Medieval	18
Hanging Ditch, Manchester	Post-medieval	26
Rupert's Valley, St. Helena	19th-century	2

sample was subsequently characterised histologically by thick section, using the 0–5 categories of the Oxford Histological Index (following Hedges, Millard, and Pike 1995; Millard 2001).

Ribs, phalanges, and cranial fragments were selected, since the removal of these elements 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, Fritsma, and Keohane 2012) and extracellular blood serum proteins (including IgG).

Two samples of historic dentine were also selected. These came from individuals buried on the south Atlantic island of St. Helena from 1840–1872 (Pearson et al. 2011), representing individuals of 19th century West Central African origin who are likely to have been exposed to *Plasmodium falciparum* infection prior to relocation.

IgG extraction

Three published protein extraction methodologies were attempted. The first (henceforth referred to as 'P1') followed a protocol developed by Schmidt-Schultz and Schultz (2004). This three-stage extraction method aims specifically at disrupting the tight interaction between the bone mineral scaffold and NCPs through a process of demineralization and denaturation designed to loosen the mineral scaffold and remove proteins not bound to the bioapatite. The final bone pellet solubilization stage destroys the scaffold, thus releasing remaining mineral-bound NCPs. The original protocol discards supernatants following the first two extraction stages and retains the final supernatant for analysis following bone pellet solubilisation, thereby assuming that NCPs of interest are retained in the bone pellet until the final extraction stage. Slight changes to the published technique were made. Firstly, supernatants were retained from all extraction stages for protein characterization. Secondly, preliminary experiments showed the ubiquitous presence of collagen commonly seen in ancient protein extractions from bone (Cleland, Voegele, and Schweitzer 2012) and which potentially masks low abundance target NCPs (Wiechmann, Brandt, and Grupe 1999). Therefore, despite the warning in the published protocol that protein purification results in NCP loss, an antibody purification technique (see below) was introduced to eliminate collagen and concentrate target IgGs. Seven human bone samples (Table 2) were subjected to this extraction protocol (based on their histological preservation) and subsequent IgG purification (see below).

The second extraction protocol (henceforth referred to as 'P2') was based on a multi-stage protein extraction from fresh bone in preparation for proteomic analysis (Jiang et al. 2007). The P2 extraction followed the published protocol exactly, other than a change from 1.2 M to 0.6M HCl (after Buckley et al. 2009; Cleland, Voegele, and Schweitzer 2012) and addition of the IgG purification stage. Eleven human bone samples were subjected to the P2 extraction and subsequent IgG purification (Table 2). All P1 and P2 bone samples scored 5 on the Oxford Histological Index, displaying excellent histological preservation.

Table 2 Samples selected for IgG extraction. All bone samples scored 5 on the Oxford Histological Index.

Sample	Site	Type	Extraction Protocol
CD120.2	Castledyke	Adult phalanx	P1
CD165.1	Castledyke	Sub-adult rib	P1
HDAP2	Hanging Ditch	Adult phalanx	P1
HDAP3	Hanging Ditch	Adult phalanx	P1
HDAP5	Hanging Ditch	Adult phalanx	P1
HDAP6	Hanging Ditch	Adult phalanx	P1
HDAR4	Hanging Ditch	Adult rib	P1
CD127.2	Castledyke	Adult cranium	P2
EH156.3	Edix Hill	Adult cranium	P2
HDAN5	Hanging Ditch	Adult rib	P2
HDAP5	Hanging Ditch	Adult phalanx	P2
HDAP5	Hanging Ditch	Adult phalanx	P2
HDAP5	Hanging Ditch	Adult phalanx	P2
HDAR2	Hanging Ditch	Adult rib	P2
HDAR9	Hanging Ditch	Adult rib	P2
HP154.1	Hoplands	Adult rib	P2
OL1104.3	Orchard Lane	Adult phalanx	P2
WM2316.1	Watersmeet	Sub-adult rib	P2
SH10806	St. Helena	Adult dentine	P3
SH10809	St. Helena	Sub-adult dentine	P3

A third extraction (P3) was performed on the two St. Helena dentine samples (Table 2) using a modified FASP protocol described by Cappellini et al. (2013) and Warinner et al. (2014a), designed for samples of archaeological bone, dentine, and dental calculus.

IgG purification

Due to the generally low abundance of the target protein even in modern serum samples, antibody purification requires techniques that result in the highest yields. Kolman et al. (1999), for example, extracted IgGs from relatively recent archaeological bone and purified them using HPLC over protein A affinity columns, prior to ELISA against syphilis antigens.

Thiophilic adsorption chromatography (TAC) was developed during the 1980s and has become well established as a cost-effective, gentle affinity resin method of purifying IgG antibodies (Hardouin et al. 2007). Briefly, sulfhydrl-containing ligands are immobilized to form a thiophilic adsorbent gel, which have a high affinity for IgG (Huse, Böhme, and Scholz, 2002). Samples are incubated at a high concentration of lyotrophic salt, before being introduced into the gel. Bound antibodies are then eluted (or desorbed) from the gels using salt-free buffer. TAC should, theoretically, be an optimal method for purifying ancient IgGs (should the required sulfhydryl groups survive intact), since it offers a highly selective environment for retrieval of target proteins, yet avoids the harsh elution conditions associated with, for example,

traditional protein affinity A columns. Of potential importance for ancient samples, thiophilic gel is able to purify fragmented IgGs (Huse, Böhme, and Scholz, 2002). This study represents the first to attempt TAC immunoaffinity with ancient samples.

For each P1 and P2 extraction stage, sample supernatants were subjected to thiophilic purification using a Pierce® Thiophilic Adsorption Kit (Thermo Scientific), following the manufacturer-supplied methodology. Polyvinylidene flouride (PVDF) filters were used in sample preparation, since these significantly decrease the incidence of IgG filter-binding compared to cellulose acetate filters (Walsh and Coles 1980). Flow-through fraction and elution absorbance was monitored at 280 nm against pure binding buffer using a CamSpec M330 spectrophotometer.

IgG detection and characterization

SDS-PAGE

Selected samples from the P1 and P2 extractions displaying the highest post-TAC protein concentration (determined by spectrophotometry) were subjected to 1D SDS-PAGE and subsequent gel band proteomic (nanospray LC-MS/MS) analysis, in order to identify and characterize any surviving IgGs. Due to a dearth of published protocols aimed specifically at electrophoretic characterization of ancient IgG, it was necessary to identify, by trial and error, the most suitable SDS-PAGE techniques for this protein. After multiple trials utilizing slightly different precipitation techniques and buffer/gel recipes, it was found that 8% TCA precipitation (after Schmidt-Schultz and Schultz 2004) followed by introduction into a 5% stacking/15% resolving gel resulted in the clearest bands for IgG (positive control) heavy and light chains. This configuration resulted in generally clear bands for both the positive control and resolved ancient proteins. The addition of the IgG positive control proved useful in determining the ideal polyacrylamide gel concentrations, at least in terms of modern IgGs.

Proteins were separated in a Bio-Rad Mini PROTEAN® II Electrophoresis Cell. 500 µl of each sample was precipitated overnight in an equal volume 8% TCA at 4°C. Each sample was centrifuged for 15 minutes at 10,000 g, and washed twice in 300 µl cold acetone, before being centrifuged for 5 minutes at 10,000 g. The supernatant was removed and the pellet air dried for a maximum of 10 minutes. Pellets were resuspended in a 200 µl sample buffer (200 µl 0.5M Tris; 1 g sucrose; 490 mg bicine; 250 µl 2-mercaptoethanol; 150 mg SDS; trace Bromophenol blue; 2.4 ml dH20, pH 6.8), boiled for three minutes at 95°C (following the observed stronger protein bands using this technique compared to Wiechmann, Brandt, and Grupe 1999) and allowed to return to room temperature.

A 15% polyacrylamide resolving gel (1.25 ml 3M Tris; 1.25 ml 0.8% SDS; 5 ml 30% acrylamide; 50 µl 10% APS; 20 µl TEMED; 2.43 ml dH20; pH 8.8) with 5% stacking gel (625 µl 1M Tris; 625 µl 0.8% SDS; 600 µl 30% acrylamide; 25 µl 10% APS; 20 µl TEMED; 3.1 ml dH20, pH 6.8) was placed into running buffer (16 ml 1M Tris; 2.64 g bicine; 8 ml 10% SDS; 776 ml dH20, pH 8.3). 20 µl of each sample was pipetted into the sample wells and electrophoresed at 125 volts until samples reached the end of the gels. Gels were fixed overnight in 100 ml 40% ethanol, 10% acetic acid and washed twice for 10 minutes in 100 ml dH20. Protein bands were visualized by staining overnight with Colloidal Coomassie Brilliant Blue G (Sigma-Aldrich®) (Figs. 1 and 2) using a working solution of four parts dye stock solution to one part methanol. Gels were repeatedly washed in 1% acetic acid until the background became clear, and were then 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 proteomic analysis. Where necessary, gels were silver stained using the ProteoSilver™ Plus Silver Staining Kit (Sigma-Aldrich®) following the manufacturer-supplied instructions. Reserved bands were chosen and excised based on molecular weights closely corresponding to IgG heavy chains and light chains (approximately 50 kDa and 25 kDa, respectively) and were analyzed by nanospray liquid chromotography-mass spectrometry (nLC-MS/MS), in the Department of Biological and Biomedical Sciences, Durham University.

Proteomic analysis of P1 and P2 samples

Excised gel bands (Figures 1 and 2) and two in-solution post-TAC eluted samples, CD120.2 and EH156.3 (the latter two samples displaying excellent histological preservation and high post-TAC protein concentration), were chosen for proteomic analysis (nLC-MS/MS). Tryptic digestion of samples was performed using a ProGest robot (Genomic Solutions). 15 µl of

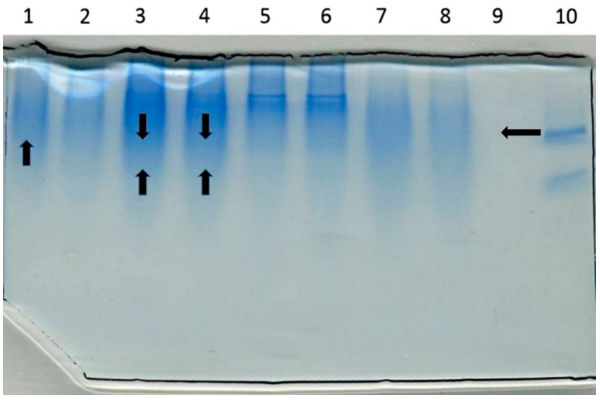

Figure 1 Example of P1 post-TAC Colloidal Coomassie stained gel. Lanes 1&2 – EH198.1; 3&4 – CD120.2; 5&6 – HDAP5; 7&8 – Castledyke 165.1; 9 - blank; 10 - IgG positive control. Horizontal arrow indicates expected bands for IgG heavy chains. Vertical arrows indicate bands excised for proteomic analysis (Table 4).

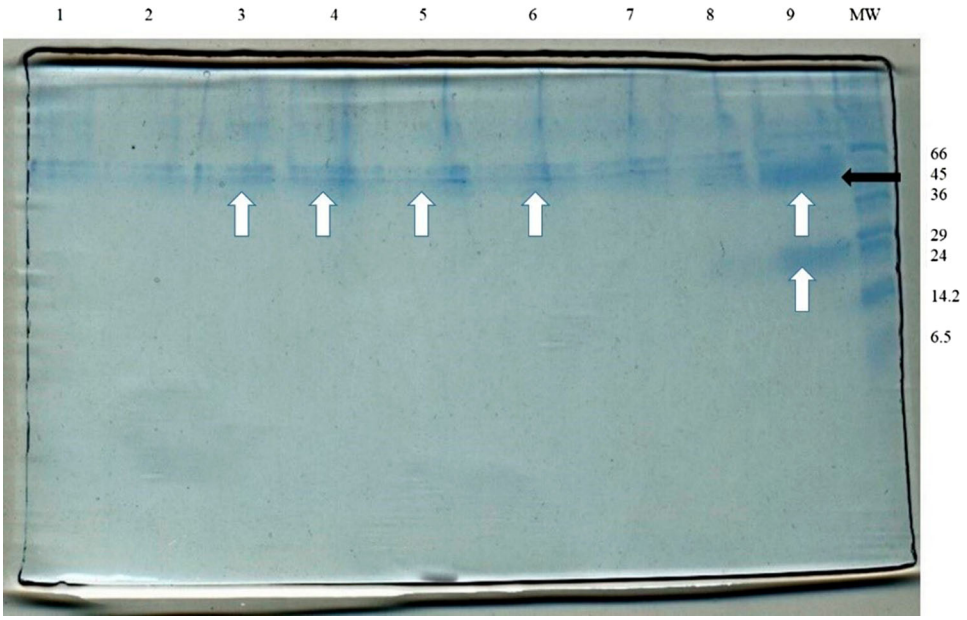

Figure 2 Example of P2 post-TAC Colloidal Coomassie stained gel. Lane 1 – WM2316.1; 2 – CD127.2; 3&4 – LP3845.1; 5&6 – HDAN5; 7 – HDAR3; 8&9 - IgG positive control. MW = molecular weight marker (kDa). Black arrow indicates expected bands for IgG heavy chains. White arrows indicate bands excised for proteomic analysis (Table 5).

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

MS-MS data were acquired using 1 second survey scan and 3×3 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 cycled every 10 seconds between a 1.0 second survey scan (MS peptide parent ion mass) and 3×3.0 second MS-MS scans (3 peptides fragmented). Protein Pilot 2.0.1 (AB Sciex) was used to interrogate the Swissprot or human Trembl sequence databases (both accessed in September 2013) with the acquired mass data. Protein Pilot uses a probability-based (Paragon) algorithm to rank peptide-spectrum and provide protein scores, thus giving an indication of protein match confidence. The threshold for a protein identification is usually either 1.3 (one peptide at 95% confidence) or 2.0 (one peptide at 99% confidence). Additional database searching was performed using Mascot Matrix Science™, version 2.4.01, against all available sequences in SwissProt (accessed March 2015). These searches were performed against a decoy database to generate false discovery rates. Peptide tolerance was 10 ppm, and with a semi-tryptic search with up to two missed cleavages. MS/MS ion tolerance was set to 0.1 Da. Based on previous observations of ancient proteome modification (Cappellini et al., 2012), the post-translational modifications were set as carbamidomethylation (fixed modification) and acetyl (protein N-term), deamidated (NQ), glutamine to pyroglutamate, methionine oxidation and hydroxylation of proline (variable modifications).

Proteomic analysis of P3 (St. Helena) samples

MS/MS analysis on tryptic peptides extracted from the St. Helena samples, using the FASP based approach, was performed using a Q-Exactive at the Mass Spectrometry Laboratory of the Target Discovery Institute, Oxford. Q-Exactive analysis was performed after UPLC separation on an EASY-Spray column (50 cm×75 µm ID, PepMap RSLC C18, 2µm) connected to a Dionex Ultimate 3000 nUPLC (all Thermo Scientific®) using a gradient of 2–40% Acetonitrile in 0.1% Formic Acid and a flow rate of 250 nl/min @40°C. MS spectra were acquired at a resolution of 70000 @200 m/z using an ion target of 3E6 between 380 and 1800m/z. MS/MS spectra of up to f15 precursor masses at a signal threshold of 1E5 counts and a dynamic exclusion for 7 seconds were acquired at a resolution of 17500 using an ion target of 1E5 and a maximal injection time of 50 ms. Precursor masses were isolated with an isolation window of 1.6 Da and fragmented with 28% normalized collision energy. Raw MS/MS spectra were converted to searchable Mascot generic format using Proteowizard version 3.0.6839 using the 200 most intense peaks in each MS/MS spectrum. MS/MS ion

database searching was performed using Mascot against all available sequences in UniProt (accessed November 2014). Searches were performed against a decoy database to generate false discovery rates. Peptide tolerance was 10 ppm, and with a semi-tryptic search with up to two missed cleavages. MS/MS ion tolerance was set to 0.07 Da, with the same fixed and variable modifications as above. Mascot searches were filtered using an FDR of 2%, and an ion score cut-off of 25. BLAST was used to verify matches to immunoglobulins.

Results and discussion

IgG was not positively identified in any analysed samples from extractions based on Schmidt-Schultz and Schultz (2004) or Jiang et al. (2007), in searches using Protein Pilot or Mascot. Using a FASP-based approach on samples of ancient dentine, peptides derived from immunoglobulins were identified, although these peptide fragments indicate post-depositional degradation (Table 7). Despite the attempts to extract, purify, detect and characterize IgG, the detection of only highly degraded immunoglobulin using a single extraction methodology suggests that this protein would be unlikely to survive over archaeological time scales, let alone retain immunoreactive functionality, and thus is an unsuitable candidate for detecting antigens. Some of the factors that may have influenced this detection, as well as some suggested future directions are discussed below.

Past studies attempting to extract archaeological IgGs have tested a variety of skeletal elements (Table 3). Some 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 hematopoietic vertebral bodies in the anticipation that these would contain higher concentrations of IgG. Interestingly, none have tested dentine. Upon consideration of the results of this study, it would seem prudent that future IgG extractions should perhaps concentrate on dentine, with precursory histological analysis to characterise preservation.

One of the most significant factors to overcome in the identification of NCPs is the masking of many of these proteins by collagen, invariably released during extraction. Insufficient detail in the published P1 protocol (Schmidt-Schultz and Schultz 2004) meant that it is unclear how this was overcome. Degraded collagen recovered from all extractions in our study served to mask proteins of lower concentration. Thus, it was impossible to evaluate the efficacy of the Schmidt-Schultz and Schultz protocol. We were also unable to replicate their exceptionally clean SDS-PAGE gels. It is possible that alternative methods of bone demineralization, and solubilization may influence the extraction of NCPs over collagens (Cleland, Voegele, and Schweitzer 2012). However, despite the approaches

Table 3 Comparison of published techniques reported to have extracted and detected archaeological IgG.

	Cattaneo et al. (1992)	Kolman et al. (1999)	Schmidt-Schultz and Schultz (2004)	Cappellini et al. (2012)
Elements tested	Vertebral body	Femur	Long bones/cranium	Femur
Sample size	10g	15g	1g	75mg
Grinding	Mechanical	Mechanical	Mechanical under nitrogen	Hand powdered
Low temperature	Partly	Yes	Yes	Partly
Demineralization	EDTA	EDTA	EDTA	EDTA
Solublization	No	No	Guanidine-HCl; sonication	Ammonium bicarbonate
Dialysis	Yes	Yes	No	No
Purification	Cellulose filter	Filtration; HPLC; protein A	No	C-18 stage tips
Characterization	ELISA	ELISA	SDS-PAGE/Western blot	nLC-MS/MS

Table 4 Results of proteomic analysis of P1 gel bands shown in Figure 1. EH – Edix Hill; CD – Castledyke.

Sample	Protein	Average Sequence Coverage (%)
1 – EH198.1	Collagen alpha-1 and 2 [Homo sapiens]	31.8
	Keratin [Homo sapiens]	29.1
3 upper – CD120.2	Collagen alpha-1 and 2 [Homo sapiens]	52.2
	Keratin [Homo sapiens]	10.7
3 lower – CD120.2	Collagen alpha-1 and 2 [Homo sapiens]	67.7
	Keratin [Homo sapiens]	15.1
4 upper – CD120.2	Collagen alpha-1 and 2 [Homo sapiens]	54.4
	Keratin [Homo sapiens]	12.4
4 lower – CD120.2	Collagen alpha-2 [Homo sapiens]	32.7
	Keratin [Homo sapiens]	16.3

attempted, soluble collagen was present in all of the extractions, and revealed itself as a smear in all of the gels (e.g., Figure 1).

IgG purification and detection

Despite TAC purification designed to both remove contaminants and high abundance proteins from samples, and its potential for isolating fragmented antibodies, IgG-related peptides were not identified in any P1 or P2 sample. As suggested by Schmidt-Schultz and Schultz (2004), the introduction of a purification step may increase the opportunity for loss of target proteins, particularly if they have a tendency to adsorb to any of the equipment or filters. However, they fail to provide a strategy for reducing the masking effect of high abundance proteins.

Proteomic analysis of SDS-PAGE gel bands (Figures 1 and 2; Tables 4 and 5) failed to detect any NCPs, revealing only collagen and exogenous keratin. It is unlikely that SDS-PAGE gel bands contained sufficient concentrations for protein precipitation. Following Schmidt-Schultz and Schultz's (2004) reported detection of ancient IgGs (heavy chains) at a molecular weight approximating 55–60 kDa, it was assumed that analysis of bands around this weight range would produce positive results for IgG. However, considering

Table 5 Results of nLC-MS/MS analysis of P2 gel bands shown in Figure 2.

Gel band/Sample	Name	Average Sequence Coverage (%)
3/Littleport (LP)3845.1	Keratin [Homo sapiens]	6.3
4/Littleport (LP)3845.1	Keratin [Homo sapiens]	12
5/Hanging Ditch (HD)AN5	Keratin [Homo sapiens]	12
6/Hanging Ditch (HD)AN5	No result	/
9 upper (IgG positive control heavy chain)	IgG heavy chain[Homo sapiens]	19.3
	Keratin [Homo sapiens]	9.1
9 lower (IgG positive control light chain)	IgG light chain [Homo sapiens]	20.5

Table 6 Extracted endogenous human NCPs for P1 (CD120.2) and P2 (EH156.3) post-TAC samples. Protein scores determined by the Paragon Algorithm.

	Extraction: P1			
	Sample: Castledyke (CD)120.2			
Protein	Sequence Coverage (%)	Protein Score	Sequence (number of spectra)	Modifications
Terminal uridylyltransferase 4	6.5	15	MDDFQLKGIVEEKFVK(1)	Oxidation(M); Lys->Allysine
Ankyrin repeat and SOCS box protein 18	9.2	13	GAHVDARNGRGETALSAACGAAR (1)	
	Extraction: P2			
	Sample: Edix Hill (EH)156.3			
	Sequence Coverage (%)	**Protein Score**	**Sequence (number of spectra)**	
Vitronectin	12.1	16	DVWGIEGPIDAAFTR(1)	
		16	RVDTVDPPYPR(1)	
		16	FEDGVLDPDYPR(1)	
Chondroadherin	19.8	17	FSDGAFLGVTTLK(1)	
		14	SIPDNAFQSFGR(1)	Deamidated(N); Deamidated(Q)
Pigment epithelium-derived factor	24.6	18	DTDTGALLFIGK(3)	
		16	LAAAVSNFGYDLYR(2)	Deamidated(N)
		16	TSLEDFYLDEER(1)	
Biglycan	20.1	13	LGLGHNQIR(1)	Deamidated(N)
		16	PVPYWEVQPATFR(1)	
		14	VPSGLPDLK(1)	
Prothrombin	17.9	15	ELLESYIDGR(1)	
Protein argonaute-4	7.9	15	RPGLGTVGKPIR(1)	Deamidated(R); Oxidation(P)
Protein AHNAK2	11.1	13	GLQEDAPGRQGSAGR(1)	Deamidated(Q)
Alpha-2-HS-glycoprotein	14.4	17	HTLNQIDEVK(1)	Deamidated(N)
Osteomodulin	4.3	14	LLLGYNEISK(1)	Deamidated(N)
Lumican	10.9	13	FNALQYLR(1)	Deamidated(N)
Matrix Gla protein	36.9	18	RNANTFISPQQR(1)	Deamidated(N); Deamidated(N)
		14	YAMVYGYNAAYNR(1)	Deamidated(N); Deamidated(N)
		17	YAMVYGYNAAYNR(1)	Oxidation(M); Deamidated(N); Deamidated(N)

Table 7 Summary of Ig peptides identified in two St. Helena samples of ancient dentine. *indicates a unique match to IgG. See Figure 3 for Ig kappa chain C region spectra from sample 10809.

Sample	Protein	Sequence (number of spectra)	Mascot score	Modifications
10806	Ig gamma-1 chain C region	K.FNWYVDGVEVH.N* (1)	45	
		K.FNWYVDGVEVHNAK.T* (1)	28	Deamidated (NQ)
		K.FNWYVDGVEVHNAK.T* (3)	54	Deamidated (NQ)
		K.FNWYVDGVEVHNAK.T* (2)	34	2 Deamidated (NQ)
		K.GFYPSDIAVEWESNGQPENNYK.T* (2)	52	3 Deamidated (NQ)
		K.GFYPSDIAVEWESNGQPENNYK.T* (1)	51	3 Deamidated (NQ)
	Ig lambda-1 chain C regions	Y.LSLTPEQWK.S (1)	36	
		Y.LSLTPEQWK.S (1)	52	Deamidated (NQ)
		S.SYLSLTPEQWK.S (1)	41	
10809	Ig kappa chain C region	-.TVAAPSVFIFPPSDEQLK.S* (1)	55	Deamidated (NQ)
	Ig gamma-1 chain C region	K.ALPAPIEK.T* (1)	30	
		R.VVSVLTVLHQD.W* (1)	44	Deamidated (NQ)
		R.EPQVYTLPPSR.D* (1)	29	Deamidated (NQ)

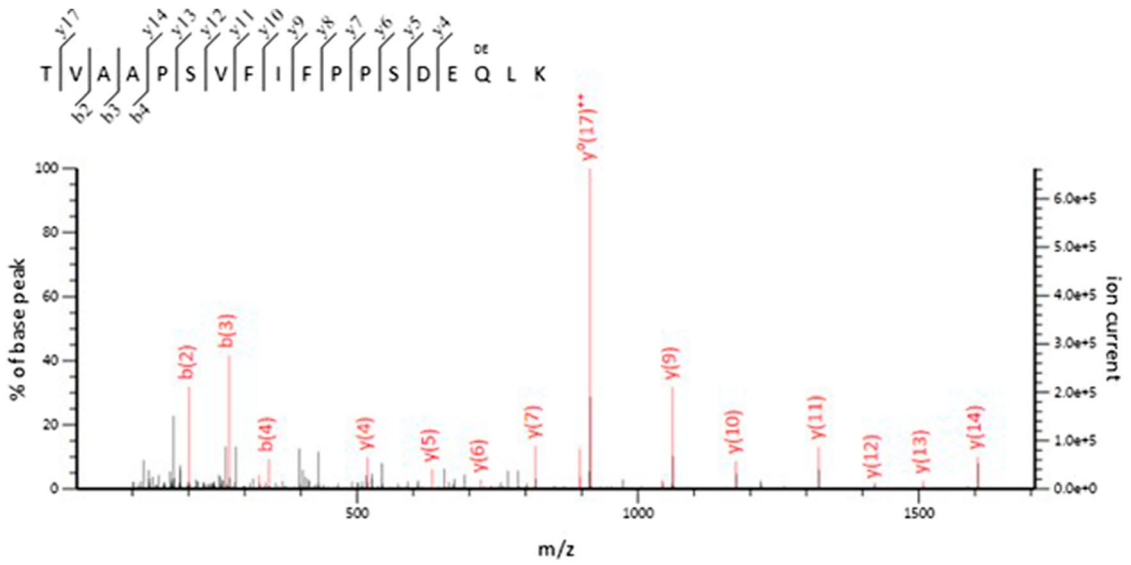

Figure 3 Mass spectrum from St. Helena sample 10809, Ig kappa chain C region (Table 7).

the degraded state of IgGs detected in the St. Helena samples, it is unlikely that any extracted IgGs would survive at the discrete molecular weights reported by Schmidt-Schultz and Schultz (2004) in their SDS-PAGE gels.

Proteomics (nLC-MS/MS)

Five NCPs (identified based on more than one peptide) were detected using MS/MS in the P2 (EH156.3) post-TAC in-solution eluted sample (Table 6), not including IgG. The range of post-TAC NCPs extracted and characterized in this study (Table 6) are a very small fraction of the whole extracted proteomes. 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 (Mansson et al. 2001; Nikitovic et al. 2008; Schvartz, Seger, and Shaltiel 1999; Wiberg et al. 2002).

Of the sequences obtained from IgG (e.g., Figure 3) in the St. Helena sample, many displayed significant post-translational modifications (Table 7). These peptides display evidence of degradation, in the form of both non-tryptic cleavage and deamidation. Deamidation of glutamine and asparagine, post-translational modifications indicative of protein degradation (Doorn et al. 2012), was observed in six of nine peptides. The two St. Helena samples yielded 87 proteins of human origin, of which 74 were NCPs. The extraction of this relatively rich proteome suggests that these samples are reasonably well preserved, and that the degradation of IgG is not due solely to poor overall sample preservation. The detection of highly degraded IgG in these relatively recent (19th century) samples may be indicative of the poor longevity of the protein in archaeological remains. Amino acid modification of the variable light chain region (the antigen-antibody reaction site of the IgG molecule)

may also limit the immunoreactivity of recovered immunoglobulins.

We agree with other studies (e.g., Cappellini et al. 2012; Wadsworth and Buckley 2014) in suggesting that sample age and burial location are likely to be important factors in the survival of NCPs, including IgG. However, given the novelty of using shotgun proteomics for detecting NCPs, we do not yet fully understand the nature and extent of this preservation, especially across multiple extraction methods and instrumentation. For this study, it should be noted that making an accurate comparison of proteome preservation between St Helena and other sites is difficult given that i) a different mineralized tissue (i.e. dentine) was utilized, and ii) a different extraction procedure and mass spectrometer was utilized. Our Anglo-Saxon proteomes were similar to those reported by Buckley and Wadsworth (2014), supporting the suggestion that certain proteins (e.g., biglycan and pigment epithelium derived factor) may preferentially survive. Further research is, however, necessary. Perhaps in the case of IgG, it would be useful to perform sequential, high-resolution monitoring of IgG degradation in modern bone and dentine samples exposed to different burial conditions.

Conclusion

Evaluation of published protein extraction protocols (Jiang at al. 2007; Schmidt-Schultz and Schultz 2004) bone failed to yield antibodies from archaeological bone displaying excellent histological preservation. MS/MS analysis of TAC elutions demonstrated non-specific binding of collagen and a small quantity of endogenous NCPs. The more sensitive modified FASP approach detected IgG in historic dentine, but revealed extensive degradation, despite the relatively rich proteome of these samples. This, combined with the failure to extract IgG from relatively well preserved bone samples, suggests that the poor preservation of immunoglobulins is not necessarily related to the overall structural and biomolecular preservation of these particular samples. It also calls into question the hypothesis that IgG may be preferentially protected from diagenetic factors due to its affinity for bioapatite.

It is evident that survival of IgG is not universal. Indeed, given the low levels of IgG detected, and the evidence from this and other studies of extensive hydrolysis and deamidation, we would caution future researchers that approaches intending to use well-preserved IgG to confirm the presence of disease, may not meet with the success reported over a decade ago (e.g., Kolman et al. 1999). Given the evidence of non-tryptic peptide cleavage and deamidation, even in samples with rich proteomes, the challenge remains the extraction, detection, and the ultimate utilization of an incredibly elusive biomolecule that offers such high potential for paleopathology. This research also highlights the importance of revisiting previously 'successful' biomolecular methodologies using the latest technologies in order to further assess their efficacy.

Acknowledgements

The authors would like to thank the British Association of Biological Anthropology and Osteoarchaeology (BABAO) for part funding of the TAC kit, to Quinton Carroll, Rose Nicholson, Tom Lane, and Antony Lee for allowing access to skeletal samples, and to Judy Watson and Andrew Pearson for the collection of samples from St. Helena. JH was supported by Marie Curie grant EUROTAST FP7 PEOPLE-2010 MC IT. We also thank Roman Fischer of the Mass Spectrometry Laboratory of the Target Discovery Institute for MS/MS analysis of the St. Helena samples. Thanks also to Richard Allen, Joanne Robson, William Simon, and Adrian Brown of Durham University for their laboratory expertise and kind assistance throughout.

Conflicts of interest

The authors confirm that there are no conflicts of interest.

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References

Brandt, E., I. Wiechmann, and G. Grupe. 2000. "Possibilities of extraction and characterization of ancient plasma proteins in archaeological bones." *Anthropologischer Anzeiger* 58: 85–91.

Buckley, M., C. Anderung, K. Penkman, B.J. Raney, A. Götherström, J. Thomas-Oates, and M.J. Collins. 2008. "Comparing the survival of osteocalcin and mtDNA in archaeological bone from four European sites." *Journal of Archaeological Science* 35: 1756–64.

Buckley, M., Collins, M., Thomas-Oates, J., and J.C. Wilson. 2009. "Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry." *Rapid Communcations in Mass Spectrometry* 23: 3843–54.

Buckley, M., and M.J. Collins. 2011. "Collagen survival and its use for species identification in Holocene-lower Pleistocene bone fragments from British archaeological and paleontological sites." *Antiqua* 1: e1.

Buckley, M., and C. Wadsworth. 2014. "Proteome degradation in ancient bone: Diagenesis and phylogenetic potential." *Palaeogeography, Palaeoclimatology, Palaeoecology* 416: 69–79.

Cappellini, E., L.J. Jensen, D. Szklarczyk, A. Ginolhac, R.A.R. da Fonseca, T. W. Stafford, S.R. Holen, M.J. Collins, L. Orlando, E. Willerslev, M.T.P. Gilbert, and J.V. Olsen. 2012. "Proteomic analysis of a Pleistocene mammoth femur reveals more than one hundred ancient bone proteins." *Journal of Proteome Research* 11: 917–26.

Cappellini, E., A. Gentry, E. Palkoupoulou, Y. Ishida, D. Cram, A. Roos, M. Watson, U.S. Johansson, B. Fernholm, P. Agnelli, F. Barbagli, D.T.J. Littlewood, C.D. Kelstup, J.V. Olsen, A.M. Lister, A.L. Roca, L. Dalén, M.T.P. Gilbert. 2013, "Resolution of the type material of the Asian elephant, *Elephas maximus* Linnaeus, 1758 (Proboscidae, Elephantidae)." *Zoological Journal of the Linnean Society* 170(1): 222–232.

Cappellini, E., M.J. Collins, and M.T.P. Gilbert. 2014. "Biochemistry. Unlocking ancient protein palimpsests." *Science* 343: 1320–22.
Cattaneo, C., K. Gelsthorpe, P. Phillips, and R.J. Sokol. 1992. "Detection of blood proteins in ancient human bone using ELISA: a comparative study of the survival of IgG and albumin." *International Journal of Osteoarchaeology* 2: 103–7.
Cleland, T.P., K. Voegele, and M.H. Schweitzer. 2012. "Empirical evaluation of bone extraction protocols." *PLoS One* 7: e31443.
Collins, M.J., A.M. Gernaey, C.M. Nielsen-Marsh, C. Vermeer, and P. Westbroek. 2000 "Slow rates of degradation of osteocalcin: Green light for fossil bone protein?" *Geology* 28: 1139–42.
Dobson M.J. 1997. *Contours of death and disease in early modern England*. Cambridge: Cambridge University Press.
Doorn, N.L., J. Wilson, H. Hollund, M. Soressi, and M.J. Collins. 2012. "Site-specific deamidation of glutamine: a new marker of bone collagen deterioration." *Rapid Communications in Mass Spectrometry* 26: 2319–27.
Freundorfer, S., G. Grupe, and D. Weickmann. 1995. "Mineral-bound non-collagenous proteins in archaeological human skeletons." *Electrophoresis* 16: 817–9.
Gowland, R.L., and A.G. Western. 2012. "Morbidity in the marshes: Using spatial epidemiology to investigate skeletal evidence for malaria in Anglo-Saxon England (AD 410–1050)." *American Journal of Physical Anthropology* 147: 301–11.
Grupe, G., and S. Turban-Just. 1996. "Serum proteins in archaeological human bone." *International Journal of Osteoarchaeology* 6: 300–8.
Gürtler, L.G., V. Jager, W. Gruber, I. Hillmar, R. Schobloch, P.K. Muller, G. Ziegelmayer. 1981. "Presence of proteins in human bones 200, 1200 and 1500 years of age." *Human Biology* 53: 137–50.
Hardouin, J., Duchateau, M., Canelle, L., Vlieghe, C., Joubert-Caron, R., and M. Caron. 2007. "Thiophilic adsorption revisited." *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* 845: 226–31.
Hedges R.E.M., and C.J.A Wallace. 1978. "The survival of biochemical information in archaeological bone." *Journal of Archaeological Science* 5: 377–86.
Hedges, R.E.M, A.R. Millard, and A.W.G Pike. 1995. "Measurements and relationships of diagenetic alteration of bone from three archaeological sites." *Journal of Archaeological Science* 22: 201–9.
Huse, K., H. Böhme, and G.H. Scholz. 2002. "Purification of antibodies by affinity chromatography." *Journal of Biochemical and Biophysical Methods* 51: 217–231.
Janeway, C.A. 2001. *Immunobiology: the Immune System in Health and Disease, 5th edition*. New York: Garland.
Jiang, X., M. Ye, X. Jiang, G. Liu, S. Feng, L. Cui, and H. Zou. 2007. "Method development of efficient protein extraction in bone tissue for proteome analysis." *Journal of Proteome Research* 6: 2287–94.
Kaufmann, C., and H. Briegel. 2004. "Flight performance of the malaria vectors *Anopheles gambiae* and *Anopheles atroparvus*." *Journal of Vector Ecology* 29: 140–53.
Kolman, C.J., Centurion-Lara, A., Lukehart, S.A., Owsley, D.W., and N. Tuross. 1999. "Identification of Treponema *pallidum* subspecies *pallidum* in a 200-year-old skeletal specimen." *Journal of Infectious Disease* 180: 2060–3.
Mansson, B., C. Wenglen, M. Morgelin, T. Saxne, and D. Heinegard. 2001. "Association of chondroadherin with collagen type II." *Journal of Biological Chemistry* 276: 32883–88.
Masters, P.M. 1987. "Preferential preservation of noncollagenous protein during bone diagenesis: Implications for chronometric and stable isotopic measurements." *Geochimica et Cosmochimica Acta* 51: 3209–14.
Millard, A.R. 2001. "The deterioration of bone." In *Handbook of Archaeological Sciences*, edited by D.R. Brothwell, and A.M. Pollard, 637–49. Chichester: J. Wiley.
Nakagawa, T., Ishihara, T., Yoshida, H., Yoneya, T., Wakamatsu, K., and Kodoya, T. 2010. "Relationship between human IgG structure and retention time in hydroxyapatite chromotography with sodium chloride gradient elution". *Journal of Separation Sciences* 33:2045–2051.
Nezlin, R. 1998. *The Immunoglobulins: Structure and Function*. New York: Academic Press.
Nielsen-Marsh, C.M, and R.E.M. Hedges. 2000. "Patterns of diagenesis in bone I: the effects of site environments." *Journal of Archaeological Science* 27: 1139–50.
Nikitovic, D., P. Katonis, A. Tsatsakis, N.K Karamanos, and G.N. Tzanakakis. 2008. "Lumican, a small leucine-rich proteoglycan." *IUBMB Life* 60: 818–23.
Nuttall, G.H.F., L. Cobbett, and T. Strangeways-Pigg. 1901. "Studies in relation to malaria. I. The geographical distribution of *Anopheles* in relation to the former distribution of ague in England (Two Maps.)." *Journal of Hygiene* 1: 4–44.
Omelyanenko, N., Slutsky, L., and Mironov, S.P. 2013. *Connective Tissue: Histophysiology, Biochemistry, Molecular Biology*. Boca Raton: CRC Press.
Pearson, A.F.P., B. Jeffs, A. Witkin, and H. MacQuarrie. 2011. *Infernal traffic: Excavation of a liberated African graveyard in Rupert's Valley, St. Helena*. York: Council for British Archaeology.
Rodak, B.F., G.A. Fritsma, and E.M. Keohane. 2012. *Hematology: Clinical Principles and Applications, 4th edition*. St. Louis: Elsevier Saunders.
Schmidt-Schultz, T.H., and M. Schultz. 2004. "Bone protects proteins over thousands of years: extraction, analysis, and interpretation of extracellular matrix proteins in archeological skeletal remains." *American Journal of Physical Anthropology* 123: 30–9.
Schvartz, I., D. Seger, and S. Shaltiel. 1999. "Vitronectin." *International Journal of Biochemistry and Cell Biology* 31: 539–44.
Smith, C.I., O. Craig, R. Prigodich, C.M. Nielsen-Marsh, M.M.E. Jans, C. Vermeer, and M.J. Collins. 2005. "Diagenesis and survival of osteocalcin in archaeological bone." *Journal of Archaeological Science* 32: 105–13.
Tuross, N. 1991. "Recovery of bone serum proteins from human skeletal tissue: IgG, osteonectin, and albumin." In *Human Paleopathology: Current Syntheses and Future Options*, edited by D.J. Ortner, and A. C. Aufderheide, 51–4. Washington, D.C: Smithsonian Institution Press.
Vizcaíno, J.A., Deutsch, E.W., Wang, R., Csordas, A., Reisinger, F., Ríos, D., Dianes, J.A., Sun, Z., Farrah, T., Bandeira, N., Binz, P.A., Xenarios, I., Eisenacher, M., Mayer, G., Gatto, L., Campos, A., Chalkley, R.J., Kraus, H.J., Albar, J.P., Martinez-Bartolomé, S., Apweiler, R., Omenn, G.S., Martens, L., Jones, A.R., Hermjakob, H. 2014. "ProteomeXchange Provides Globally Coordinated Proteomics Data Submission and Dissemination." *Nature Biotechnology* 32 (3): 223–26.
Wadsworth, C., and M. Buckley. 2014. "Proteome degradation in fossils: investigating the longevity of protein survival in ancient bone." *Rapid Communications in Mass Spectrometry* 28: 605–15.
Walsh, R.L., and M.E. Coles. 1980. "Binding of IgG and other proteins to microfilters." *Clinical Chemistry* 26: 496–8.
Warinner, C., J.F.M. Rodrigues, R. Vyas, C. Trachsel, N. Shved, J. Grossman, A. Radini, Y. Hancock, R.Y. Tito, S. Fiddyment, C. Speller, J. Hendy, S. Charlton, H.U. Luder, D.C. Salazar-Garcia, E. Eppler, R. Seiler, L.H. Hansen, J.A.S. Castruita, S. Barklow-Oesterreicher, K.Y. Teoh, C.D. Kelsrup, J.V. Olsen, P. Nanni, T. Kawai, E. Willerslev, C. von Mering, C. M. Lewis Jr, M.J. Collins, M.T.P. Gilbert, F. Rühli, and E. Cappellini. 2014a. "Pathogens and host immunity in the ancient oral cavity." *Nature Genetics* 46: 336–44.
Warinner, C., J Hendy, C. Speller, E. Cappellini, R. Fischer, R. Trachsel, J. Arneborg, N. Lynnerup, O.E. Craig, D.M. Swallow, A. Fotakis, R.J. Christensen, J.V. Olsen, A. Liebert, N. Montalva, S. Fiddyment, S. Charlton, M. Mackie, A. Canci, F. Bouwman, M. Rühli, T.P. Gilbert, and M.J Collins. 2014b. "Direct evidence of milk consumption from ancient human dental calculus." *Scientific Reports* 4: 7104.
Welker, F., M.J. Collins, J.A. Thomas, M. Wadsley, S. Brace, E. Cappellini, S.T. Turvey, M. Reguero, J.N. Gelfo, A. Kramarz, J. Burger, J. Thomas-Oates, D.A. Ashford, P.D. Ashton, K. Rowsell, D.M. Porter, B. Kessler, R. Fischer, C. Baessmann, S. Kaspar, J.V. Olsen, P. Kiley, J.A. Elliott, C.D. Kelstrup, V. Mullin, M. Hofreiter, E. Willerslev, J. Hublin, L. Orlando, I. Barnes, and R. D.E. MacPhee. 2015. "Ancient proteins resolve the evolutionary history of Darwin's South American Ungulates." *Nature* doi:10.1038/nature14249.
Wiberg, C., D. Heinegard, C. Wenglen, R. Timpl, and M. Morgelin. 2002. "Biglycan organizes collagen VI into hexagonal-like networks resembling tissue structures." *Journal of Biological Chemistry* 277: 49120–6.
Wiechmann, I., E. Brandt, and G. Grupe. 1999. "State of preservation of polymorphic plasma proteins recovered from ancient human bones." *International Journal of Osteoarchaeology* 9: 383–94.
Wipasa, J., C. Suphavilai, L.C. Okell, J. Cook, P.H. Corran, K. Thaikla, W. Liewsaree, E.M. Riley, and J.C. Hafalla. 2010. "Long-lived antibody and B cell memory responses to the human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*." *PLoS Pathogens* 6: e1000770.