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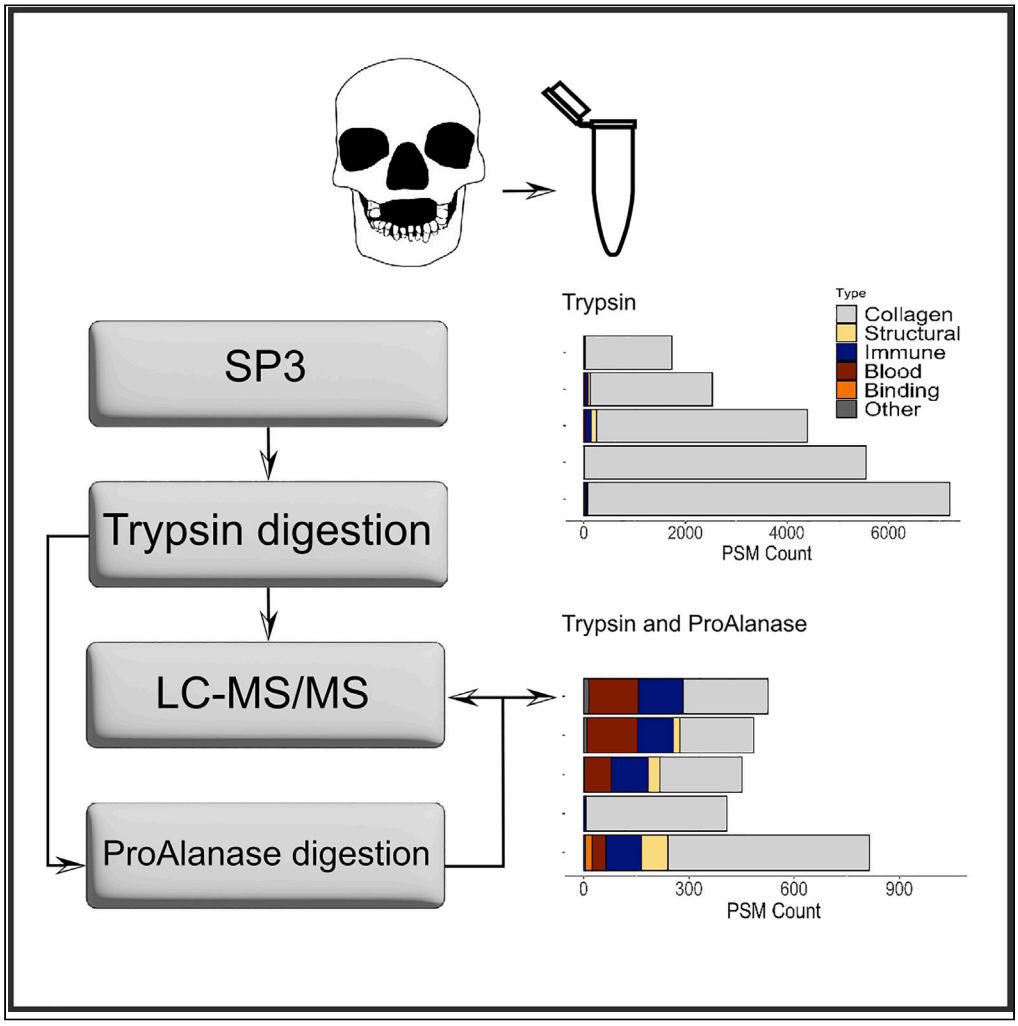

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Highlights

Using a sequential digestion enzyme protocol, we significantly reduced collagen recovery

Collagen reduction exposed more of the proteome

Comparison of the immune proteomes from a leprosarium to a non-leprosarium cemetery

Recovered immune proteomes lend insights into active mycobacterial infections

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Sequential trypsin and ProAlanase digestions unearth immunological protein biomarkers shrouded by skeletal collagen

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SUMMARY

This study investigates the efficacy of proteomic analysis of human remains to identify active infections in the past through the detection of pathogens and the host response to infection. We advance leprosy as a case study due to the sequestering of sufferers in leprosaria and the suggestive skeletal lesions that can result from the disease. Here we present a sequential enzyme extraction protocol, using trypsin followed by ProAlanase, to reduce the abundance of collagen peptides and in so doing increase the detection of non-collagenous proteins. Through our study of five individuals from an 11th to 18th century leprosarium, as well as four from a contemporaneous non-leprosy associated cemetery in Barcelona, we show that samples from 2 out of 5 leprosarium individuals extracted with the sequential digestion methodology contain numerous host immune proteins associated with modern leprosy. In contrast, individuals from the non-leprosy associated cemetery and all samples extracted with a trypsin-only protocol did not. Through this study, we advance a palaeoproteomic methodology to gain insights into the health of archaeological individuals and take a step toward a proteomics-based method to study immune responses in past populations.

INTRODUCTION

When studying health and disease in the past, the most commonly studied materials are archaeological human remains; however, this is often complicated due to the manner in which diseases cause death. Some conditions that kill, such as infectious diseases, tend to do so relatively quickly without leaving physical indicators on the skeleton. Conversely, those that survived pathogenic infections would appear less skeletally healthy. Chronic conditions, such as systemic infections, neoplasms, and metabolic disorders, can affect the skeletal system, but determining the cause of these effects is often challenging. Unrelated diseases such as tuberculosis and brucellosis can leave similar lesions on the skeleton, and others that leave distinctive lesions, such as leprosy, only do so in a small percentage of chronic cases.

In some cases, infections may leave recoverable biomolecular evidence behind in the genetic signatures of the pathogens.[1,2] The value of gene amplification by polymerase chain reaction (PCR) was quickly realized by palaeopathologists and archeologists. Some of the first archaeological targeted PCR studies were aimed at pathogens, notably *Mycobacterium*: *M. tuberculosis*[3–6] and *M. leprae*.[7,8] However, other researchers suggested that PCR techniques are inappropriate for damaged and fragmented DNA that is typically extracted from archaeological bone,[9] and further that identification of pathogens from the past was complicated by contamination, microbial relatedness, low abundance, and a lack of validation.[10–12] The introduction of next generation sequencing (NGS) allowed for a new era of aDNA and pathogen

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identification from archaeological human remains, including the identification of the causative agent of the black plague as *Yersinia pestis*,[13] and the exploration the evolution of leprosy at the genome-wide level.[1] Unlike earlier PCR studies, NGS allows for the possible recovery of whole genomes, with mapping coverage, and the ability to look at damage-patterns (i.e., deamination), allowing for better authentication. This NGS 'revolution' has allowed for better identification of true positive pathogenic agents in archaeological bone.[14]

Unlike DNA, proteomics allows for the characterization of biological processes, both from pathogens and the host. The host immune response and function-specific proteins are potentially powerful tools for understanding infections in the past. These lines of evidence can be suggestive of an active infection, instead of just the presence of pathogen genetic material. Some of the earliest studies of protein preservation focused on non-collagenous proteins (NCPs) from archaeological bones[15–18] using electrophoretic and immunoblot techniques. Other palaeoimmunology studies used clinical immunological techniques; such as ELISA and rapid diagnostic tests, to find antigenic evidence of pathogens, including treponemal disease,[19] malaria,[20,21] *Yersinia pestis*[22] from archaeological soft and skeletal tissues.

Proteins from archaeological osseous tissues are highly unlikely to be in their native state, may be fragmented, and the loss of key side chains or epitopes is probable. Therefore the use of immunoassays is problematic, especially given possible cross-reactivity of breakdown products from the sample tissues and from the burial environment.[23] Advances in tandem mass spectrometry (LC-MS/MS) studies allowed the nascent field of proteomics-based palaeoimmunology to move away from single proteins to shotgun metaproteomics, allowing for a whole suite of proteins to be recovered and authenticated from relatively ancient bones.[24] Early metaproteomic studies recovered a number of immune related proteins, such as leukocyte related proteins, and suggest they are related to active infection at time of death.[25–27] Dental calculus also has been shown to preserve immunological proteins related to both the adaptive and innate immune system,[28,29] and thousands of bacterial proteins, including virulence factors from periopathogens.[28,29] So far, convincing evidence of mycobacterial proteins from skeletal materials has proven elusive,[26] but they have been recovered from the dental calculus of an individual with leprosy from medieval Trondheim, Norway.[30] The proteome preserved in osseous lesions, or more widely in the skeleton and teeth, is not well understood and has been underexplored in ancient remains, and issues with the overabundance of collagen limit NCP recovery. Consequently there is an "unknown" ancient proteome[31] waiting for methodological improvements.

Leprosy (Hansen's disease) is a chronic disease that, in some cases, affects the skeleton with lesions that are characteristic of *M. leprae* infections. Leprosy may result in severe nerve impairment and hypoesthesia, resulting in damage to epithelial tissues, secondary infections and loss of the extremities of the hands and feet, and blindness.[32] To this day, it is still an endemic and often debilitating disease in developing countries with tropical climates.[33] In skeletal remains, these lesions can affect the pyriform aperture, resulting in osseous destruction to the nasal cavity and loss of the maxillary bone supporting the upper teeth.[34] Osteomyelitis arising from denervation of the hands and feet can lead to distal and distinct concentric bone loss that progressively and profoundly affects the extremities,[35] and may result in loss of function, and especially mobility due to "Charcot's foot." These lesions collectively are highly indicative of severe cases of leprosy,[36] but are by no means ubiquitous, and further remarks on the complicated nature of *M. leprae* pathology can be found in existing literature.[37–39]

The osteologically distinct characteristics of leprosy in these severe cases and the nature of the disease makes it a prime candidate for palaeopathological and biomolecular studies of disease.[37] Furthermore, leprosaria, where individuals that were afflicted by the condition were hospitalized, lived, and died, provide a unique opportunity to explore this disease and what can be retrieved archaeologically. The pathogenic bacterium that causes leprosy, *Mycobacterium leprae*, can be molecularly detected from skeletal remains, confirming infection in past individuals. The high taxonomic resolution that DNA studies provide allows for the creation of phylogenetic trees to trace the spread and evolution of archaeologically endemic diseases.[1,30,40] In addition to DNA, ancient proteomics may offer a new avenue in understanding past health by contributing insights into immune responses. Studies of contemporary leprosy have identified numerous proteins from patients and household contacts,[39,41,42] providing a framework for classifying a leprosy-associated immune response in past people. We propose that the recovery of leprosy-related immune proteins from past individuals can lend insights into past disease states, as well as triage ideal samples for pathogen DNA recovery.

When using proteomics to investigate questions of health in archaeological individuals, human skeletal remains, including bones and teeth, are the most commonly accessible material. Tooth enamel has a limited proteome and is not conducive to the recovery of immune/inflammatory proteins,[43] however, both bone and dentine, and associated tooth pulp have a much richer proteome that includes blood-borne immune/inflammatory markers.[24,44] In looking for leprosy-associated proteins from the human host, the ultimate goal is to recover those associated with the different forms of leprosy: lepromatous/multibacillary (high humoral response), tuberculoid/paucibacillary (high cellular response), and borderline cases (in between lepromatous and tuberculoid); as discernible by the two types immune response: Type 1/reversal reaction/RR and Type 2/erythema nodosum leprosum/ENL (Table 1). While modern studies provide us with a framework of expected leprosy-associated proteins, we must be mindful that contemporary study samples are generally taken from saliva, blood, or skin lesions, which are not equivalent to archaeological skeletal remains.

When identifying proteins via LC-MS/MS in data-dependent acquisition (DDA) mode, the most intense peptides are preferentially selected within a range of mass-to-charge (m/z) signal intensity for further fragmentation. Consequently, low abundance peptide(s) with a similar m/z as a high abundance peptide(s) may be missed and overlooked i.e., "swamped out" or "masked". Specifically, for archaeological individuals, bones and teeth (dentine) proteomes consist of up to 90% collagen.[61–64] Additionally, keratins from modern human hair and skin can be introduced through handling during excavation, examination, and curation, which can further mask non-collagenous proteins (NCPs).

Table 1. Table of proteins associated with the two types of immune responses to leprosy

Protein	Type 1/RR	Type 2/ENL	Citation
Anti PGL-1	x	x	Gurung et al.[45]
IFN-y	x		Rangaka et al.[46]
IL-1, IL-10	x	x	van Hooij et al.[47]; van Hooij et al.[48]; Geluk et al.[49]
IL-2	x		Rangaka et al.[46]
IL-3, IL-4, IL-5		x	Rangaka et al.[46]
TNF-alpha and beta	x		Rangaka et al.[46]
CCL2, CCL4	x	x	van Hooij et al.[48]; Geluk et al.[49]
IgA, IgG, IgM	x	x	van Hooij et al.[50]; van Hooij et al.[47]; Gupta et al.[51]; van Hooij et al.[52]; Lima et al.[53]
S100-A12	x	x	van Hooij et al.[47]; van Hooij et al.[48]; van Hooij et al.[52]
Complement C1q, C3, C4, C5, C8, C9		x	Bahia El Idrissi et al.[54]; van Hooij et al.[47]
Haptoglobin (alpha 2 chain)		x	Gupta et al.[51]
IGF-1, OASL, **Fibrinogen, CRP**	x	x	van Hooij et al.[47]; van Hooij et al.[48]; Rodrigues et al.[55]; de Toledo-Pinto et al.[56]
MMP2, MMP9, **IHRP**		x	Gautam et al.[57]; Oliveira et al.[58]; Teles et al.[59]; Chegou et al.[60]

Proteins in bold were recovered in this study.

A logical strategy to unmask these lower abundance NCPs within the dentine and bone proteomes is to selectively reduce overall collagen content/detection. Achieving this goal has the potential to mine deeper into the proteome for more informative NCPs that may include proteins involved with bone turnover and regulation, but also extracellular matrix, immune-related, and non-host proteins.[65–68] Trypsin, the most commonly used protease, efficiently cleaves collagen resulting in an overabundance of collagen peptides in the overall proteome, which can reduce the detection of lower abundance, and potentially more informative NCPs. In order to reduce this overabundance of collagen and expose additional proteins, we incorporated a multi-enzyme approach by adding a second round of digestion with a recently developed digestive enzyme, ProAlanase, subsequent to trypsin digestion (Figure 1). ProAlanase cleaves after proline, hydroxyproline and alanine residues, and as collagen sequences are incredibly proline-rich, this enzyme has been shown to greatly reduce collagen recovery and masking.[65]

RESULTS

Sampling and *M. leprae* screening of non-leprosarium individuals

In order to assess immune responses to leprosy in skeletal remains, we employed a systematic methodological approach to explore the proteomes from bone and teeth from five individuals interred at the High Medieval Period (12th-13th Century) Cemetery at the Sant Llàtzer leprosarium in Barcelona, Spain (Figure 2).[2,69] During this period, not all individuals living within leprosaria are guaranteed to have had *M. leprae* infections, however, we assume that most of those buried within the leprosarium were likely leprosy sufferers (See Supplementary Data for detailed information on each cemetery and individual; Figures S1–S3). We compare these with four individuals from a neighboring and contemporaneous non-leprosy associated cemetery, Caputxes 1, of the Santa Maria del Mar church, archaeologically dated to the 14th-15th centuries (Figure 2; Table S1). While the cause of the mortality outside of the leprosarium is impossible to discern, we focused on

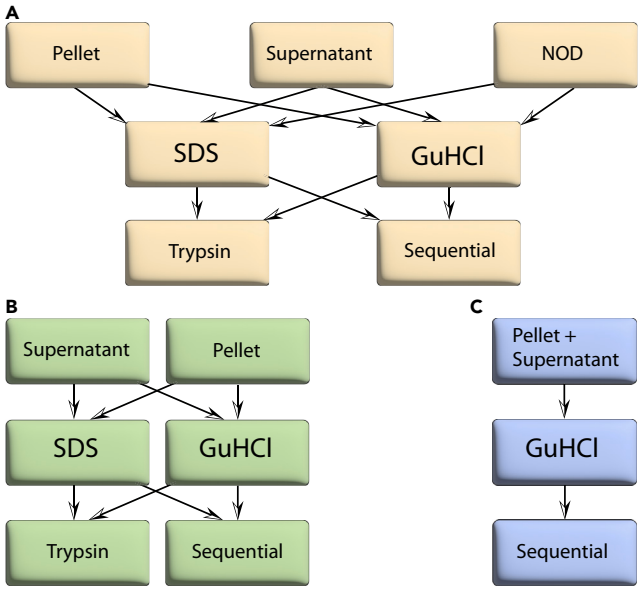

Figure 1. Experimental design

Diagram of three experiments.

(A) Overview of protein extraction performed on leprosarium skeletal samples using sodium dodecyl sulfate (SDS) and guanidine hydrochloride (GuHCl) based protocols with both trypsin and sequential trypsin then ProAlanase digestions; NOD = No demineralization.

(B) Overview of SDS- and GuHCl-based extraction protocols along with 'sequential' trypsin then ProAlanase digestion.

(C) "Negative" control samples with GuHCl protocol with sequential trypsin then ProAlanase digestion.

individuals with no apparent skeletal lesions. In our aim to identify possible immune proteins that are representative of active leprosy infections, we consider these individuals to be our negative controls, despite being aware that they may have succumbed to other infections, and/or diseases that do not leave traces on the skeleton.

M. leprae genomes recovered from leprosarium individuals, but not from non-leprosarium individuals

Individuals from the leprosarium were previously analyzed and published by Pfrengle et al. for the presence of *M. leprae*.[2] In that study, samples which were positively enriched for three leprosy genes (RLEP, GyrA and proS) were later used for genome wide enrichment. If it was possible to reconstruct the genome of a sample at minimum 60% 1-fold coverage the sample was defined as positive for leprosy and usable for genome-wide analysis. Metagenomic screening for *M. leprae* was conducted for bone and dentine samples from every non-leprosarium individual. In each case, detection of *M. leprae* was negative (Table S2). While there were some overall matches to the genus *Mycobacterium*, none of these were specific to *M. leprae*, but rather matched to many strains, including those commonly found in the soil environment. No proteins were identified as deriving exclusively from *M. leprae* in any study individuals.

More non-collagenous proteins recovered with trypsin/ProAlanase than trypsin alone

A total of 72 proteins (collagenous and NCPs) were identified in both the trypsin and the sequential trypsin then ProAlanase fractions. However, the collagen peptide spectral matches (PSM) counts were much higher in the trypsin-only extractions (Figure 3). Overall, in addition to collagen, proteins with structural, binding, and immune-related functions were recovered, as well as those found in blood. Immune-related human proteins (n = 18) recovered include: alpha-2-HS-glycoprotein; antithrombin-III; alpha-1-antitrypsin; alpha-2-macroglobulin; clusterin; complement C3; complement C9; complement factor B; IgG 1 heavy chain; Ig light chain constant 2; kininogen-1; osteopontin; pigment epithelium-derived factor; protein S100-A7; prothrombin; lysozyme-C; thrombospondin; and vitronectin. Although these immune proteins were recovered from both fractions, those in the ProAlanase fraction were found at higher PSM counts, indicating increased detection of these proteins (Figure 3).

In the trypsin only extracts, 48 identified human proteins were identified. Proteins were primarily either structural or related to binding or immune functions. For the immune proteins (n = 9) specifically, amyloid-beta A4; arginase-1; cathepsin; complement C8 beta and gamma chains; heat shock proteins beta-1; IgG-1 chain C; Ig kappa and Ig lambda constant regions; and retinoic acid receptor protein were recovered (Figure 4).

The sequential trypsin then ProAlanase digestion revealed an additional 42 proteins that were not recovered with trypsin alone, validating the hypothesis that collagens mask NCPs. These included a number of proteins with functions related to the immune system or binding roles. These extracts contained a number of immune-related proteins (n = 24) including: alpha-1-antichymotrypsin; C-reactive protein; CD9 antigen; complement C1q subcomponent; complement C4-A; complement factor H; eIF-2-alpha kinase; fibrinogen alpha chain; fibrinogen beta chain;

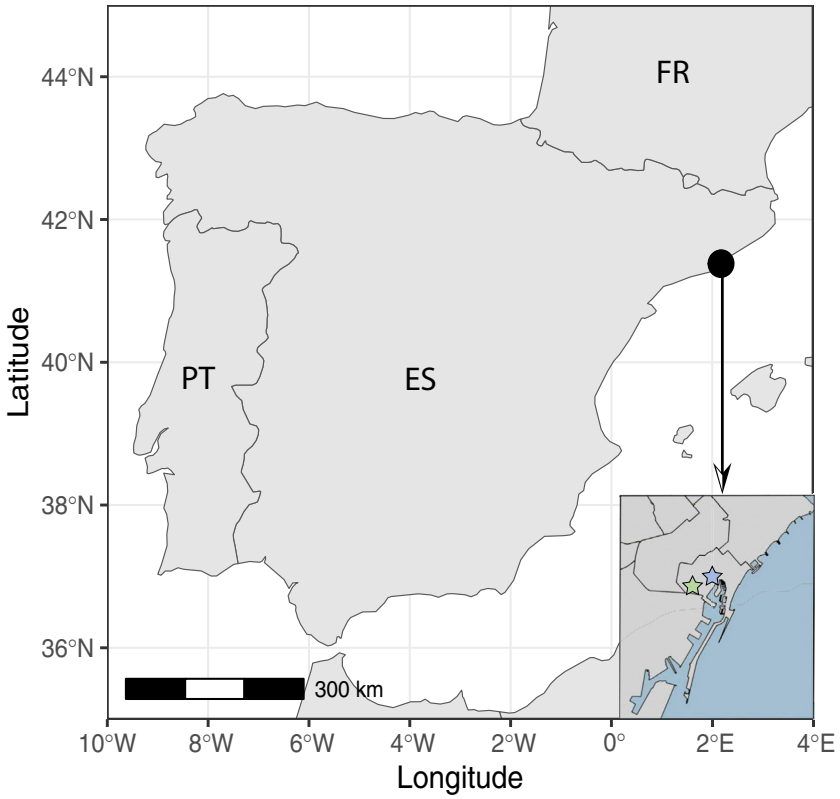

Figure 2. Site location

Map of study sites in Barcelona, green star is the Sant Llàtzer leprosarium, and the blue star is Santa Maria del Mar church.

fibrinogen gamma chain; haptoglobin; heat shock protein; hemopexin; histidine-rich glycoprotein; IgA heavy constant 1; IgG heavy constant 2; IgG heavy constant 3; Ig kappa light chain; Ig kappa variables 1–27, 1–6, and 3D-20; Ig lambda constant; IgM heavy chain; and inter-alpha-trypsin inhibitor heavy chain H4. Overall, the sequential trypsin and ProAlanase digests resulted in the highest number of immune-related proteins (n = 32) (Figure 4;Tables S5A–S5E).

Statistically, the reduction in collagen proteins from the trypsin alone compared to the sequential trypsin/ProAlanase digests is significant. We compared the number of collagen proteins recovered from each sample from Table S2, Proteins (Paired t test result, two-tailed: $p < 0.0001$, 95% confidence interval). Similar results were observed from the decrease in collagen PSMs recovered between both digestion methods by comparing the data from Table S2, PSMs (Paired t test, two-tailed: $p < 0.0001$, 95% confidence interval), and again with the comparison of immune protein PSMs compared to the overall PSMs recovered, also taken from Table S2, PSMs (Chi-square, with Yates correction due to large PSM counts: $p < 0.0001$).

The following results are taken from the proteins recovered from individuals from within the leprosarium cemetery, and the non-leprosarium cemetery. As our experiment was focused on the recovery of immune-related proteins, and more NCPs were identified using the sequential digestion method using both trypsin and ProAlanase, the subsequent results sections include only the proteins recovered from dually digested samples. Also, keratins were excluded from subsequent analysis as it seems difficult to isolate any endogenous signal from the widespread contamination by this protein family seen in blank samples.

Bone and dentine samples contain similar immune proteins

The samples taken from bone and tooth dentine, including those from both the leprosarium and non-leprosarium individuals, contained 72 proteins in common, including primarily structural, blood, and immune-related proteins. 'Structural proteins' included collagens, as well as biglycan, decorin, dermatopontin, desmoplakin, fibronectin, filaggrin-2, hornerin, lumican, matrix metalloproteinase-20, osteomodulin, procollagen C-endopeptidase, protein-glutamine gamma-glutamyltransferase E, and osteomodulin. 'Blood proteins' consisted of coagulation factor VII, IX, and X; vitamin K-dependent proteins C and S; protein Z-dependent protease inhibitor, and serum albumin. Overall, 26 immune-related proteins were recovered in both tissues, including: alpha-1-antitrypsin; antithrombin-3; alpha-2-HS-glycoprotein; alpha-2-macroglobulin; complement C3, C4-A, and C9; dermcidin; eIF-2-alpha kinase; fibrinogen beta and gamma chains; haptoglobin; hemopexin; histidine-rich glycoprotein; IgG1 heavy chain; IgA constant 1; IgG heavy constant 2; IgG heavy constant 3; Ig kappa light chain; Ig lambda constant 2 and 7; IgM heavy chain; kininogen-1; pigment epithelium-derived factor; prothrombin; lysozyme C, thrombospondin, and vitronectin.

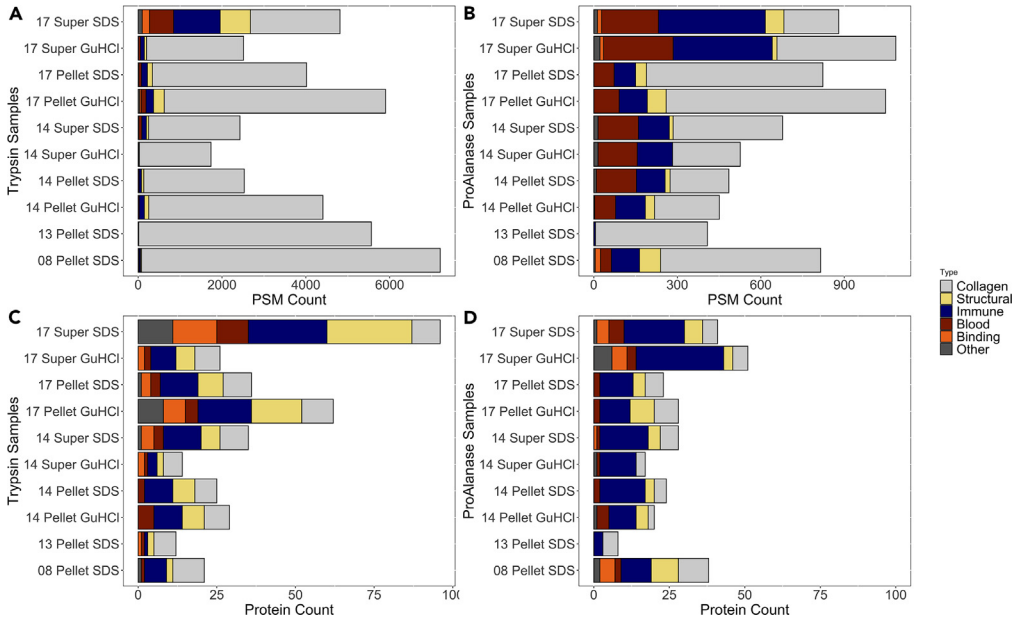

Figure 3. Collagen reduction bar chart

Bar charts showing the number of human proteins and PSMs per sample separated by protein classification as collagenous, structural, immune, blood, or "other" functions.

Frames A and B show the overall PSM count recovered from the trypsin and trypsin>ProAlanase digests (X axis values are different in charts (A and B). Frames C and D show the overall unique human protein count from the same samples (Tables S3A and S3B).

The bone samples contained 16 proteins that were not found in the dentine samples, which consisted of proteins associated with mechanical, structural, and immune-related functions. Annexin-2 and collagen alpha-1(XII) were the recovered structural proteins, as well as myosin binding proteins (2, 6, 7, and 13) and alpha-actinin 3. Osteopontin, a protein involved in bone remodeling and immune modulation, was recovered along with other immune-related proteins including: C-reactive protein; CD9 antigen; complement C1q; and fibrinogen alpha chain.

Dentine samples included 71 proteins not recovered from the bone samples, including blood, structural, binding, transport, and immune proteins. Nineteen structural proteins were recovered, including fibromodulin, filaggrin, fibronectin, and periostin. Transport proteins, which were not identified in any of the bone samples, include apolipoproteins A-I, A-IV, and B-100, as well as ceruloplasmin, lipocalin and transthyretin. The 16 immune-related proteins consisted of alpha-1-chymotrypsin; amyloid-beta-A4 protein; arginase-1; cathepsin D, complement C8 beta and gamma chains; complement factors B and H; Ig kappa constant region, Ig kappa variables 1–27, 1–6, and 3D-20, inter-alpha-trypsin inhibitor heavy chain H4; protein S100A7; and retinoic acid receptor responder protein 2. We also recovered heat shock proteins beta-1 and HSP 90, which are expressed in response to stress (Figures 5 and 6; Table S7).

The number of different proteins recovered from the dentine sample is significantly higher than the bone samples. When comparing the number of proteins specific to each tissue against the number of total proteins recovered from each tissue in a Fisher's exact contingency table, we found the differences to be significant ($p = 0.0005$, Table S5B), with most of the additional proteins recovered from the dental samples having binding, structural and transport functions. However, when investigating immune proteins by comparing the total recovered immune proteins to the total overall proteins in the same test, the difference was not significant ($p = 0.1369$, Table S5B). We find that when exploring the proteome of archaeological individuals, both bone and dentine samples offer similar insights into the immune response.

Pellet and supernatant fractions contain similar number of immune-related proteins

When comparing the identified proteins from the supernatant, pellet, and non-demineralized samples, we found 59 proteins shared across all three fractions. Between all, structural, binding, blood, and immune proteins were shared. Of the structural proteins, 11 were non-collagenous. For the immune proteins, IgG (gamma 1-heavy chain and heavy constant 2), IgA heavy constant 1, and Ig kappa light chain peptides were identified, as well as complement C3, C4A, C9; and 12 additional proteins with immune-related functions. Proteins shared between the pellet and supernatant fractions, but not in the non-demineralized samples, included seven proteins that include calmodulin-like proteins 5; filaggrin-2; and hornerin, but no immune proteins. Twenty-five proteins overlapped between the non-demineralized samples and the pellet fractions, and while no collagen proteins were shared between of these fractions, seven non-collagenous structural proteins were recovered in both. The six proteins with immune functions include eIF-2-alpha kinase; Ig kappa constant and Ig lambda constant 2, but also complement C8 and complement factor B. The non-demineralized fraction and supernatant only share three proteins, one of which is immune related, Ig kappa constant 7 (Figure 5; Table S5C).

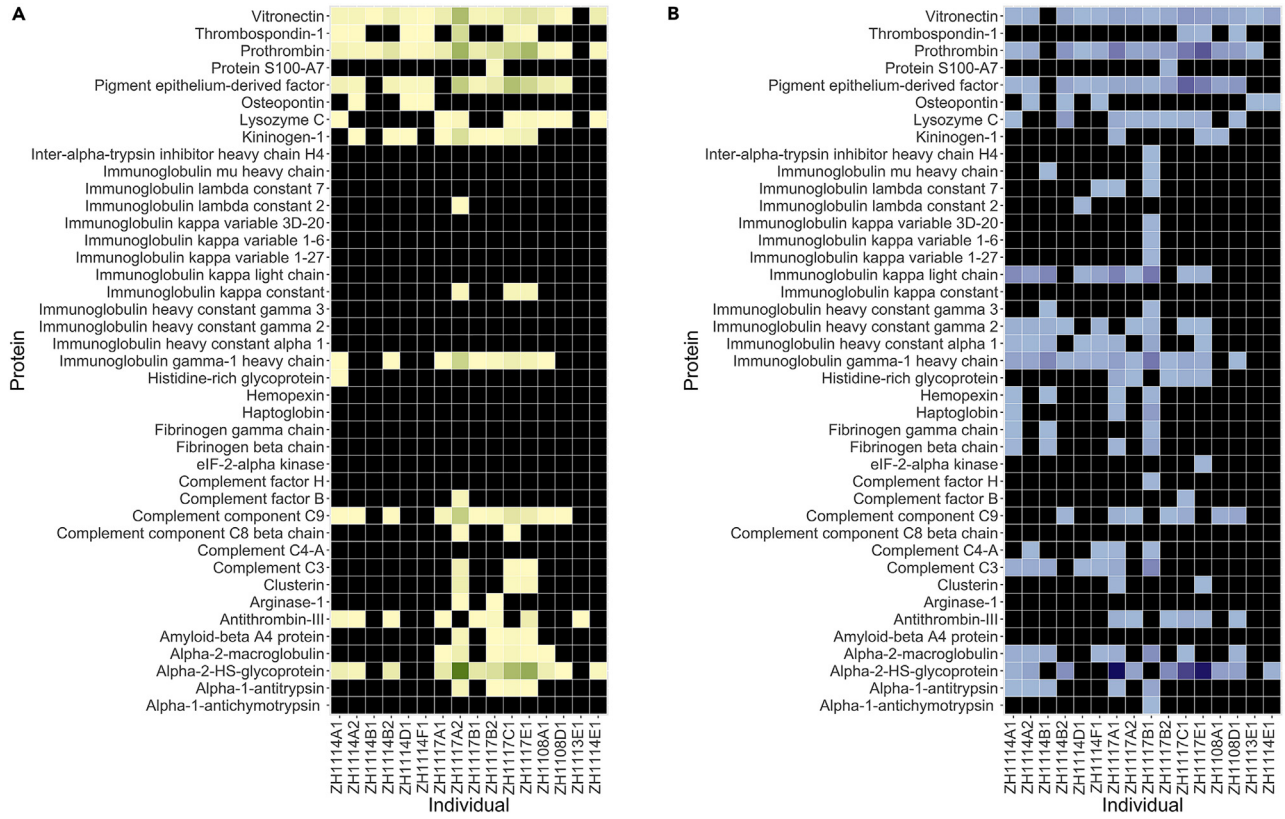

Figure 4. Heatmap of immune protein PSMs

Immune protein recovery comparison between trypsin only and the sequential trypsin (A) then ProAlanase (B) digestions.

Gradient is shown from low (lighter hue) to high (darker hue) PSM recovery, with black squares representing instances of zero recovered PSMs (Tables S4A and S4B).

The pellet fractions contained 37 proteins that were not found in either the supernatant or non-demineralized extractions, and included primarily structural (14), binding (7), and immune (8) proteins. Of the structural proteins, five were collagen-specific with nine other NCPs. Specifically, arginase-1; C-reactive protein.; complement C1q; fibrinogen alpha chain; heat shock proteins beta-1; and protein S100-A7 were recovered with immune-related functions (Figure 5; Table S5C).

Both the pellet and supernatant fractions contained significantly more total unique proteins than the non-demineralized samples when compared in a two tailed Fisher's exact contingency test (pellet vs. non-demineralized $p = 0.0001$, and supernatant vs. non-demineralized $p = 0.0001$, respectively; Table S5C), and had a similar result when comparing only the immune related proteins recovered between the fractions in the same way (pellet vs. non-demineralized $p = 0.0187$, and supernatant vs. non-demineralized $p = 0.0014$). Comparisons with the same statistical method found that the number of unique total proteins and unique immune proteins from the pellet and supernatant fractions were not significant from one another ($p = 0.100$, and $p = 0.458$). However, a notable observation is that the supernatant contained a higher number of immunoglobulin proteins, while the pellet contained more proteins from the complement system.

SDS and GuHCl protocols result in similar overall and immune protein recovery

In both the sodium dodecyl sulfate (SDS) and guanidine hydrochloride (GuHCl) denatured extractions there were 98 shared proteins, which included primarily binding (12), blood (8), structural (34), and immune (31) proteins. About one-third of the structural proteins (11/34) were collagens, and the recovered structural NCPs contained biglycan; caspase-14; chondroadherin; fibromodulin; fibronectin; lumican; matrix metalloproteinase-20; mimecan; osteomodulin; periostin; procollagen C-endopeptidase enhancer; and tetranectin. The range of immune-related proteins featured alpha-1-antitrypsin; alpha-2-macroglobulin; CD9 antigen; complement C3, C4-A, and C9, eIF-2-alpha kinase; fibrinogen beta and gamma; haptoglobin; histidine-rich glycoprotein; thrombospondin, clusterin, prothrombin, pigment epithelium-derived factor; vitronectin, osteopontin, as well as multiple immunoglobulins, including, IgG-1 and 2, IgA, Ig kappa constant and light chains, and Ig lambda constant.

In the SDS, but not the GuHCl fraction, there were 35 individual proteins that consist primarily of binding (10), structural (10), and immune (6) proteins. Of the eight immune proteins, we identified complement C1q, complement C8 (beta and gamma chains), complement factor B,

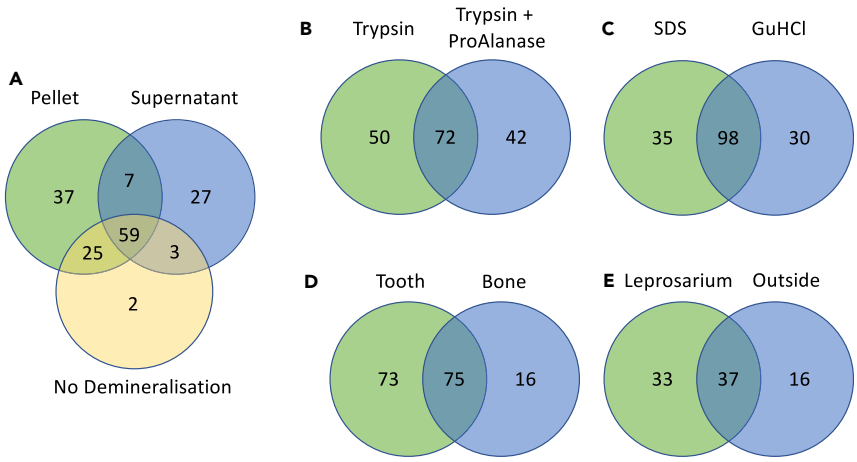

Figure 5. Comparative results between experiments

Venn diagrams showing the amount of protein identifications per extract type.

Protein ID counts for: (A) the pellet, supernatant, and no demineralization conditions; (B) trypsin digestion alone versus trypsin and subsequent ProAlanase digestion; (C) denaturation agent SDS and GuHCl; (D) bone and tooth extracts; (E) individuals buried within the leprosarium and the non-leprosarium cemetery who are assumed to have died from non-leprosy related causes (includes only extracts using a GuHCl and sequential digestion protocol). For all values, Tables S5A–S5D, and S5F.

eIF-2-alpha kinase, fibrinogen alpha chain, IgG gamma-1 chain C region, Ig lambda constant 2. Of the binding proteins, four different myosin proteins (2, 6, 7, and 13) were recovered.

In the GuHCl fraction there were 30 specific proteins recovered. Similar to the SDS extractions there were primarily binding, structural, and immune proteins. We recovered C-reactive protein in one sample (UF_700/ZH1114F), which has been strongly linked to infectious diseases, including leprosy, in modern, living patients.[70] We also recovered 12 additional immune-related proteins including, inter-alpha-trypsin inhibitor, protein S100-A7, and three Ig kappa variable region proteins (1–27, 1–6, and 3–20); IgG-3; and IgM (Figure 5; Table S5D).

When comparing the total and unique proteins recovered from SDS and GuHCl extractions using a two-tailed Fisher's exact test, the results were not significant ($p = 0.7817$). Similarly, the same comparison of immune-related proteins was also not significant ($p = 0.3113$). While the immune proteins recovered from both extraction types included complement ($n = 3$) and immunoglobulins ($n = 6$), the SDS fraction contained a further four complement proteins, and the GuHCl fraction contained five additional immunoglobulin proteins.

More immune proteins recovered from leprosarium individuals

Between the leprosarium and non-leprosarium individuals, when comparing all leprosarium extracts to the non-leprosarium extracts, there were 43 proteins shared. Almost half of these were structural, such as collagens, keratins, osteomodulin, and periostin. Two binding, six blood, and ten immune-related proteins were also found. Of the immune-related proteins alpha-2-HS-glycoprotein, antithrombin-III, CD9 antigen, C9, IgM, IgG, prothrombin, vitronectin, kininogen, and prothrombin were recovered. While we expected to recover peptides specific to the pathogen *M. leprae*, these have remained elusive in all samples.

The leprosarium individuals' samples contained 119 specific proteins not found in those individuals buried in non-leprosy-associated contexts. Proteins included binding (26), blood (3), structural (27), transport (7), and immune-related (40) proteins (Table S7). Of the immune proteins specifically, the leprosarium samples contained inter-alpha-trypsin inhibitor heavy chain H4; alpha-1-antitrypsin; histidine-rich glycoprotein; alpha-2-macroglobulin; amyloid-beta A4; arginase-1; C-reactive protein; cathepsin D; clusterin; eIF-2-alpha kinase; haptoglobin; heat shock protein beta-1 and HSP 90-beta; osteopontin; thrombospondin; six complement system proteins (C3, C4-A, C8 beta, C8 gamma, factor B); three fibrinogens (alpha, beta, and gamma chains); and 10 immunoglobulins: IgA heavy chain constant region 1, IgG heavy constant 2 and 3, Ig kappa chain constant region, Ig kappa light chain, Ig kappa variable (1–27, 1–6, and 2D-20), and Ig lambda constant 2 and 7. Most of the immune proteins were recovered from the two specific individuals (ZH1114, ZH1117). They may have been suffering from active leprosy infection with a type 2 immune reaction (erythema nodosum leprosum), painful inflammation seen mostly in individuals who have lepromatous leprosy[41,54,57,71,72] (Figures 5 and 6; Table S5E).

However, as there were far fewer non-leprosarium extracts ($n = 8$) than overall leprosarium extracts ($n = 56$), we also conducted a subsequent comparison between the samples from the non-leprosarium extracted with the final protocol against the leprosarium samples using the same protocol ($n = 6$). When comparing the proteins with immune related function to the total proteins unique to the leprosarium vs. non-leprosarium, we found that the difference was significant (two-tailed Fisher's exact test, $p = 0.0276$). In addition to those that are specific to each, we found that 37 proteins were shared between both cemeteries, and of these, seven have immune-related functions, such as CD9 antigen, C9, IgG, prothrombin, vitronectin, kininogen, and alpha-2-HS-glycoprotein. Within the extracts from the leprosarium individuals, we also found 33 unique proteins, including 17 with immune functions: alpha-antitrypsin, alpha-2-macroglobulin, amyloid-beta A4 protein,

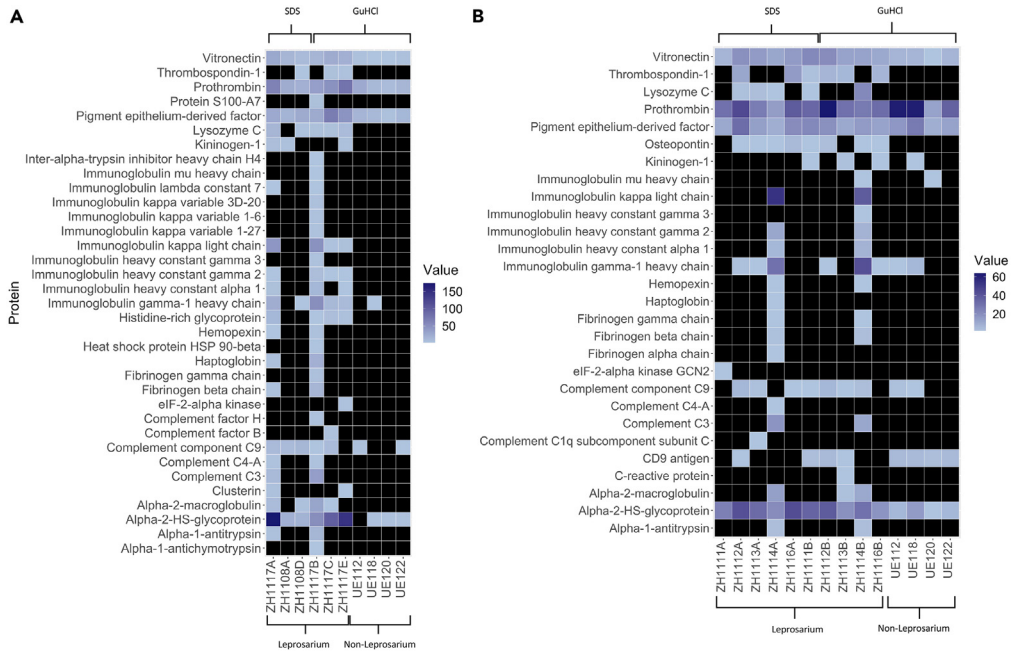

Figure 6. Protein recovery comparison between leprosarium and non-leprosarium associated cemetery

Immune-related protein identifications from leprosarium and control group individuals from (A) dentine samples; and (B) bone samples. Samples from the leprosarium include PSMs from both supernatant and pellet fractions of the sequential trypsin and ProAlanase digestion. Extractions from the non-leprosarium cemetery individuals were extracted with both supernatant and pellet fractions combined, and also with trypsin then ProAlanase sequential digestion.

C3, C4-A, histidine-rich glycoprotein, IgA-1, IgG-2, Ig kappa light chain, Ig kappa variable 1–27, Ig kappa variable 1–6, Ig kappa variable 3D-20, inter-alpha-trypsin inhibitor heavy chain H4, S100-A7, lysozyme C, thrombospondin, and osteopontin. In the non-leprosarium individuals, we found 16 unique proteins, with IgM as the only represented immune protein. (See Table S5F for comparison of proteins recovered from leprosarium and non-leprosarium GuHCl extraction samples).

DISCUSSION

Our study was dually focused on both identifying the presence of *M leprae* and immune proteins that suggest active leprosy infection at the time of an individual's death. *M. leprae* DNA appears to be more straightforward to recover than *M. tuberculosis* from ancient skeletal tissues - likely as a consequence of the differential pattern of tissue infection, but this has not been unequivocally demonstrated.[39] We were surprised that given the ability to recover *M. leprae* DNA, detection of *M. leprae* specific proteins was unsuccessful. Nevertheless, our systematic method development resulted in a novel sample preparation workflow (tissue lysis, sequential digestion with trypsin then ProAlanase) capable of enhanced recovery of immune-related proteins. Through the sequential digestion with both trypsin and ProAlanase we were able to significantly reduce collagen PSMs, allowing access to a broader proteome of non-collagenous proteins not recoverable with trypsin digestion alone. Beyond collagen, the newly recoverable proteins included binding, structural, blood coagulation, and immune-related functions (Tables S3A and S3B). We further found that extractions performed with SDS or GuHCl recovered similar overall proteomes, but that GuHCl extractions recovered a slightly higher number of immune proteins. Specifically, in one of the individuals (ZH1117) the GuHCl extractions (ZH1117B1, ZH1117B2) recovered eight additional immune proteins than the concurrent SDS extractions (ZH1117A1, ZH1117A2) (Table S3A).

We found that the dentine sample contained a greater diversity of protein than the bone samples, and this is likely due to the active blood flow within the tooth and the preservation allowed by the dentine's containment within the dental enamel.[73] While the bone samples contained primarily proteins with structural and mechanical functions, the dentine samples contained more proteins found in blood with binding and transport functions. However, while there are differences in the overall proteome of each tissue, we found that both contained similar immune profiles, with neither tissue type performing significantly better in the present study of the immune response. Similarly, the pellet fractions contained more individual proteins than those of the supernatant, however, they both contained a similar number of immune proteins. Importantly, we found that the immune proteins recovered from each fraction differed, and would suggest including both the supernatant and pellet fractions when exploring the immune proteome.

We discovered striking differences in the recovered immune proteins of the individuals from the leprosarium and those who were buried in the non-leprosy associated cemetery. While two leprosarium individuals' samples contained immune proteins associated with leprosy, the

recovered proteomes from the leprosarium cohort were extremely variable, forcing us to consider differences between chronic and acute leprosy infections, varied immune responses, and that healthy people or those with other illnesses beyond leprosy could have also been living within the leprosarium. It also raises questions related to differential biomolecular preservation of immune proteins between individuals. Nevertheless, this approach opens a door to investigating acute and chronic immune responses and inflammation in past populations.

Of the immune-related proteins we recovered, some have been linked to leprosy in modern patients responding to active infections. Specifically, in the individuals from the leprosarium, we recovered complement C1q, C3, C4A, and C9. Clusterin and complement factor B were found to be present in the blood serum of leprosy patients from Bangladesh[54] and da Silva and colleagues[72] found that complement C4 and inter-alpha-trypsin inhibitor heavy chain (IHRP) were found in the blood serum of leprosy patients and not in the control group. While we found that C9 was equally present in individuals from within and outside of the leprosarium, we did see increased PSMs of C3 and C4A in two individuals (ZH1114, ZH1117); as well as complement factor B and H, as well as IHRP in one of the same individuals (ZH1117). Proteins of the complement system are highly linked to the innate response to leprosy, but also to many other unrelated infections,[54,72] and includes the fusion of multiple complement protein fractions to form membrane attack complexes that defend against the pathogen. However, the complement response can often become "overactivated" during leprosy infection, resulting in extensive nerve and skin damage in the patient.[41,74]

Fibrinogens have also been identified in modern leprosy patients, particularly prior to receiving multi-drug treatment. As a part of the acute inflammatory response that plays an active role in blood clotting to stop bleeding, fibrinogen expression increases in response to both acute and chronic inflammation and has been shown to increase in multibacillary (MB) leprosy patients. Fibrinogen is a glycoprotein complex composed of alpha, beta, and gamma chains, and we recovered all three chains in two of the leprosarium individuals with strong immune responses (ZH1114, ZH1117). The presence of all three chains in these specific individuals suggests that they were suffering from active symptoms of leprosy at their time of death.

Immunoglobulins are linked to modern leprosy infections as well as varied other types of immune responses. IgM, IgA, and IgG have all been linked to both the reversal reaction and erythema nodosum leprosum reactions in patients with MB leprosy[47,48,50,71]; however, without determining the antigen specificity of the recovered immunoglobulins, which is beyond the scope of this study, we are unable to utilize them diagnostically. Other proteins associated with an increased expression during leprosy infections and immune responses are haptoglobin[51,75]; C-reactive protein,[41,48] and Apolipoprotein A-1.[48] We found that extracts from two individuals (ZH1114, ZH1117) buried within the leprosarium demonstrated the strongest identified immune response suggestive of active leprosy. Both individuals, ZH1114 (bone) and ZH1117 (tooth), contained multiple immune-related proteins that have been linked to leprosy. The presence of complement component proteins, haptoglobin, immunoglobulins, and all three fibrinogen chains suggest that their immune systems could have been actively working to combat the disease or leprosy-associated nerve damage.

Taking cues from modern leprosy studies, there are some proteins that we hoped to find, such as d-dimers and cytokines; however, they were not recovered from the archaeological samples. D-dimers are produced when a fibrinogen complex is broken into its requisite three chains upon activation of the inflammatory response. However, as the total sequence for the d-dimer is a combination of fragments of the alpha, beta, and gamma chains connected by disulfide bonds, following reduction, alkylation, and digestion the peptides would likely be identified as one of the three (alpha, beta, gamma) chains on the LC-MS/MS. Cytokines represent a broad class of potent signaling molecules (pro-inflammatory or anti-inflammatory) that function to direct the growth, differentiation, and infiltration of immune cells to tailor the immune response to the pathogen. Analysis of cytokines (presence/absence and concentration) provides powerful information on the nature of the pathogen that elicited the immune response. Despite high local concentrations at immunological synapses, the circulating serum levels are often vanishingly low. The low circulating concentration is compounded by short half-life e.g., the cytokine tumor necrosis factor alpha (TNFα) has a half-life of 15–30 min. Additional confounding factors for the detection of cytokines in archaeological material include the sample type - skeletal tissue does contain a fraction derived from blood, but this is a small component. That combined with diagenesis-induced damage/modification, and limited sample availability, likely contributed to reduced protein recovery.

Interestingly, while ZH1114 and ZH1117 samples have a very similar recovered immune signature, the aDNA samples conducted did not have the same results. Pathogen genome recovery for individual ZH1114 was positive for *M. leprae*, while ZH1117 was not.[4] Also, the samples with similar immune proteins to the "control" samples contain a mixture of positive and negative recovery of *M. leprae* genomes. Interestingly, in the skeletal individual that sample ZH1114 derived from, there were two tested samples, which contained very different proteomes. While each sample was taken from a different portion of the skeleton (ZH1113, right metatarsal; ZH1114 left metatarsal, for details, see Table S1), the difference in immune protein recovery is intriguing. It raises questions of whether the difference in recovered proteins is due to sampling bias i.e., differing amount of cortical vs. compact bone, proximity to lesions, or differential preservation, possibly due to taphonomic changes that occur in the years after death and internment. Proteome preservation of archaeological samples experience differential diagenesis rates affected by factors such as deposition time and the burial environment, and we suggest that future studies should include this aspect as a part of the experimental design. It is likely therefore that palaeoimmunology studies using proteomics will be limited to the detection of more abundant immune/inflammation proteins such as antibodies, those in the complement system, and fibrinogens. Nevertheless, detection of immune proteins has the potential to be highly informative in the study of disease burden in past populations.

The fact that individual ZH1117 was buried in the leprosarium and contained numerous leprosy-associated proteins, yet did not have any observable characteristic bone lesions associated with leprosy or evidence of DNA of *M. leprae*, is interesting, yet not entirely surprising. While the legs of this individual were not recovered with the skeletal remains, there were no leprosy-associated lesions on the maxilla or the hands. Importantly, not all individuals with leprosy have severe cases that result in the loss to the digits or damage to the face. The variations in the clinical manifestations of leprosy are strongly linked to the immune status of the patient and cover a spectrum.[76] At one extreme

of the spectrum, the patient exhibits high but inefficient humoral response (Th2) to *M. leprae*, which leads to a multibacillary (often more infectious) highly infective form of the disease (lepromatous leprosy). This form results in symmetrical lesions on the limbs and the characteristic damage in the nasal area and the palate known as rhinomaxillary syndrome.[34] At the other extreme of the spectrum, patients do exhibit a high cellular immune response (Th1), and the disease manifestation is less severe, notably asymmetrical on the limbs, and does not involve the rhinomaxillary area. The individual ZH1117 could have had skeletal lesions only on the lower extremities, or a case of leprosy that did not visibly affect the skeleton. Our findings suggest that even in individuals with scant skeletal remains, immune-related protein recovery may be able to suggest which individuals were likely afflicted with leprosy, or other types of infections, in the past.

Conclusion

This study demonstrates how the sequential use of trypsin and ProAlanase digestion can further enhance the proteome recovery from human skeletal remains, as we were able to identify immune proteins that would have remained invisible with trypsin digestion alone. While this study was limited to five leprosarium individuals and four non-leprosarium individuals, it represents an important methodological step toward understanding ancient immune responses and active infections in the past. For future studies focused on immune protein recovery, we recommend sampling from either bone lesions or tooth dentine, as both appear to contain numerous immune proteins. However, this may vary greatly depending on the disease or illness under study. Depending on which proteins are targeted for recovery, we recommend testing extraction methods to discern which is more effective, as well as to identify which additional proteins become detectable with specific protocols and collect several samples from each individual. Additional work on preservational differences between regions and individuals should be studied with larger sample sizes in order to differentiate which immune-related proteins consistently persist over time in archaeological individuals. More extensive experiments will be essential to confirm the efficacy of this approach, and we hope that future studies will continue to contribute to our understanding of palaeoimmunology and ancient proteomics.

Limitations of the study

The small sample size of individuals from a leprosarium and non-leprosy associated cemetery is a limitation of this study. As of now, while many studies are focused on the differences in collagen proteins from extinct and extant species, there is no published standard for the immune-related proteome of archaeological bone. Ideally, to better support the initial findings from this paper, the study of the proteome should include additional samples from an increased number of individuals. A baseline of recoverable immune proteins from historic or ancient individuals will be critical, as many people in the past without serious diseases would still have suffered from low-level infections and immune responses to minor injuries. Additionally, this study includes individuals from two neighboring, yet distinct burial environments, and as biomolecular preservation is variable between and within sites, proteome preservation is also expected to be variable. In order to pinpoint specific and widely recoverable protein biomarkers for leprosy, it will be essential to understand which immune-related proteins are recovered in individuals with and without pathogenic diseases, as well as from remains from a large range of preservational states.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109663.

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AUTHOR CONTRIBUTIONS

S.W., V.J.S., F.R., M.J.C., and A.J.T. formulated the study. N.M., S.P., and M.F.C. collected samples and conducted pathological analysis. S.W. completed the proteomics lab work with experimental design input from A.J.T., A.D., and L.K.; proteomics data analysis was completed by S.W. DNA extractions and data analysis were conducted by S.W., K.M., and D.S. Contextualization of results was conducted by S.W., M.S., C.A., P.S., and V.J.S. Funding for this study was acquired by V.J.S., M.J.C., F.R., and S.W.; project administration was carried out by V.J.S., M.J.C., and S.W.; and resources were provided by N.M., M.F.C., and S.P. The manuscript was written by S.W., L.T.L., and A.J.T. with input from all co-authors.

DECLARATION OF INTERESTS

We have no conflicting interest to declare.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
ProAlanase, Mass Spec Grade, 15μg	Promega	VA217A
Deposited data		
LC-MS/MS data, including raw, mgf, and mzid files for all samples, positive controls, and extraction blanks are available under accession PXD040419	This paper	proteomeXchange.org
Software and algorithms		
Mascot, Matrix Science version 2.7.0.1	Perkins et al.[77]	Matrixscience.com

RESOURCE AVAILABILITY

Lead contact

Further information or requests for resources should be directed to the lead contact, Shevan Wilkin, shevan.wilkin@iem.uzh.ch.

Materials availability

This study did not generate any new unique reagents, but did involve the use of a recently created enzyme, ProAlanase, which is available through Promega, ProAlanase (Mass spec grade), catalogue number VA2161.

Data and code availability

Protein data files (.raw, .mgf, .mzml, and .dat) have been deposited at proteomeXchange and are publicly available as of the date of publication. Accession number (PXD040419) are also listed in the key resources table. Genomes are available at EMBL-EBI (part of the European Molecular Biology Laboratory), in accession PRJEB60522, which is publicly available. This paper does not report original code. However, resulting protein result csv files were further filtered using MS-MARGE, which is freely available at https://bitbucket.org/rwhagan/ms-marge/src/master/.[78] Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

METHOD DETAILS

Methods A: Sample collection

Leprosarium and non-Leprosarium individual sample selection

In selecting the individuals from the leprosarium, we specifically chose those that were previously screened for *Mycobacterium leprae* genomes[4] including those that gave both positive and negative results. Most of the selected individuals did not have clearly visible lesions, or were too incomplete to carry out a differential diagnosis, although they did show at least one lesion that could be relatable to leprosy (UF_101/ZH1108, UF_800/ZH1111/ZH1112, UF_700/ZH1113/ZH1114, and UF_802/ZH1116). We also included one individual without leprotic lesions (UF 102/ZH1117). All skeletons were archaeologically dated to the High Mediaeval period (from the 11th century to the 13th century), and two of them (UF_101 and UF_700) were radiocarbon dated to the same period.[4]

In order to properly assess immune response in individuals buried within the Sant Llàtzer leprosarium, we extracted proteins from both bone and teeth from four individuals from the same region and time period that were not living or buried within the leprosarium community (UE112_B, UE112_T, UE118_B, UE119_T, UE120_B, UE120_T, UE122_B, UE122_T). We selected four individuals with no skeletal lesions from another Barcelona site, Caputxes 1, corresponding to the necropolis of Santa Maria del Mar church. The skeletons were archaeologically dated to the 14th-15th centuries. While the cause of the mortality is impossible to discern, we focused on individuals with no apparent skeletal lesions. In our aim to identify possible immune proteins that are representative of active leprosy infections, we consider these individuals to be our negative control despite being aware that they may have succumbed to other more malignant infections or diseases that do not leave traces on the skeleton. See supplemental information for listing of all individuals, presence/absence of leprotic lesions, *M. leprae* genome recovery, and leprosy-associated protein immune response.

All teeth were cleaned with 7% bleach and afterwards rinsed with DNA-free water in order to reduce contamination potential. Both teeth and bones were UV treated twice for 30 minutes, each time from a different angle. As to conduct internal sampling of the dentine, each tooth was sectioned at the cemento-enamel junction separating the root from the crown using a fretsaw. Dental powder was collected from each (between 50 and 100 mg for aDNA, and between 10-20 mg for proteins) by using a dental drill to remove the dentine. For bone samples, skeletal elements were prepared by removing 1 - 2 mm of the bone surface using drill bits (New Technology Instruments) to remove the layer

with closest contact to the soil. Subsequently, they were drilled to collect bone powder (between 50 and 100 mg for aDNA, and between 10-20 mg for proteins). All samples were collected in 2 mL Eppendorf DNA lo-bind tubes. Each bone or dentine sample was split into two for protein and aDNA analysis.

Methods B: Protein extractions

Leprosarium individuals: bone and dentine powder were sampled at the same time as DNA sampling, and powdered samples were taken from each individual bone or tooth in a DNA clean laboratory at the Institute of Evolutionary Medicine. Powdered samples were stored in 1.5 mL sterile Eppendorf tubes until protein extraction. Bone samples were taken from lesion sites, and tooth samples were drilled from dentine. See Tables S1 and S2 for information on site, sample museum ID, lab ID, skeletal element, sample weight, pathogen screening, and proteomic results. Samples were either demineralised in EDTA or were taken straight to extraction with either SDS or GuHCl, see Table S8 for the complete trial list and details.

The final and optimised protocol is listed here, with all experimental protocols described in the supplement (see Table S8). Powdered bone and dentine samples from leprosarium individuals were split into subsamples containing between 3-6 mg each. Samples were demineralised with 300 μL EDTA (0.5 M, pH 8) for 5 days. Following demineralization, samples were centrifuged at 20,000 RCF for 10 minutes and 200 μL of the supernatant was removed and stored. Samples were then denatured, reduced, and alkylated with 6M GuHCl, 10 mM TCEP/CAA, and heated for 10 minutes at 99°C. Following heating, sterile glass beads were added to each sample and they were placed on the tissue-lyser (Qiagen TissueLyser LT) for five minutes at top speed. Samples were then removed from the beads and transferred to new tubes, where magnetic beads (Sera-Mag SpeedBeads 50:50 Hydrophobic:Hydrophilic) were added to collect proteinaceous material. Ethanol was added to each sample (350 μL, or the total starting volume) to increase protein adherence to the magnetic beads. The tubes were then put on the thermomixer at room temperature (24°C) at 1000 RPM for five minutes. Samples with beads were placed on a magnetic rack, and the beads were cleaned three times with 80% ethanol. After the final rinse, all of the ethanol was removed by pipetting and 100 μL of 50 mM ammonium bicarbonate was added to each, followed with 2 μL of a trypsin solution (0.2 μg/μL). Samples were first digested with trypsin overnight (~18 hours). Following the initial digestion, samples were acidified with 5% TFA and evaporated down. After drying, samples were rehydrated with 20 μL MS buffer (3% ACN, 0.1% FA), and 2 μL of each sample was analysed on the LC-MS/MS (Waters, Thermo Exploris). The remaining sample was further digested with ProAlanase for 2 hours at 37°C, and samples were analysed on the LC-MS/MS again. See full protocol for each experiment in the Supplementary methods data.

Methods C: LC-MS/MS analysis

Mass spectrometry analysis was performed on an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) equipped with a Nanospray Flex Ion Source (Thermo Fisher Scientific) and coupled to an M-Class UPLC (Waters). Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% acetonitrile for channel B. Column temperature was 50°C. For each sample 2 μL of peptides were loaded on a commercial nanoEase MZ Symmetry C18 Trap Column (100Å, 5 μm, 180 μm x 20 mm, Waters) followed by a nanoEase MZ C18 HSS T3 Column (100Å, 1.8 μm, 75 μm x 250 mm, Waters). The peptides were eluted at a flow rate of 300 nL/min. After a 3 min initial hold at 5% B, a gradient from 5 to 22 % B in 90 min and 5 to 35% B in additional 10 min was applied. The column was cleaned after the run by increasing to 95 % B and holding 95 % B for 10 min prior to re-establishing loading condition for another 10 minutes.

The mass spectrometer was operated in data-dependent mode (DDA) with a maximum cycle time of 3 s, using Xcalibur, with spray voltage set to 2.2 kV, funnel RF level at 40 %, heated capillary temperature at 275°C, and Advanced Peak Determination (APD) on. Full-scan MS spectra (350−1,200 m/z) were acquired at a resolution of 120,000 at 200 m/z after accumulation to a target value of 3,000,000 or for a maximum injection time of 45 ms. Precursors with an intensity above 5,000 were selected for MS/MS. Ions were isolated using a quadrupole mass filter with a 1.2 m/z isolation window and fragmented by higher-energy collisional dissociation (HCD) using a normalised collision energy of 30 %. HCD spectra were acquired at a resolution of 30,000 and maximum injection time was set to Auto. The automatic gain control (AGC) was set to 100,000 ions. Charge state screening was enabled such that singly, unassigned and charge states higher than six were rejected. Precursor masses previously selected for MS/MS measurement were excluded from further selection for 20 s, and the exclusion window was set at 10 ppm. The samples were acquired using internal lock mass calibration on m/z 371.1012 and 445.1200.

Methods D: Protein data analysis

LC-MS/MS data was searched against Mascot (MatrixScience version 2.7.0.2) using Swissprot and all sequences from the proteomes of M. leprae and M. tuberculosis from UniProt (downloaded 2021.05.02). For trypsin digested samples, trypsin was selected as the digestion enzyme, and trypsin/ProAlanase was used for sequentially digested samples. Mass values allowed for an isotopic carbon shift, with peptide mass tolerance set at ±10 ppm, and fragment mass tolerance was set at ±0.01 Da. Up to three missed cleavages were allowed, and the instrument type was set as "Q-Exactive". Carbamidomethylation of cysteine was selected as a fixed modification, with deamidation of asparagine (N) and glutamine (Q), oxidation of methionine (M), and hydroxylation of proline (P) set as variable modifications.

Methods E: DNA

aDNA extraction, library prep, and shotgun sequencing

One DNA extraction per specimen was performed in the clean room facilities of the Institute of Evolutionary Medicine in Zurich, using the standard ancient DNA extraction protocol as described by Dabney et al.[79] An extraction blank was included to check for potential contaminations.

Preparation of indexed double-stranded libraries and shotgun sequencing

Each double-stranded library was prepared according to the modified protocol of Meyer and Kircher[80] by using a 20 μl extract of each sample. As a negative control a library blank (LB) was included and the Solexa adapter mix was prepared. Since aDNA is highly fragmented, the fragmentation step was skipped, and blunt-end repair was performed directly. A master mix (MM) containing 1 x NEB Buffer, 100 μM dNTP mix, 0.8 mg/ml BSA, 1 mM ATP, 0.4 U/μl T4 polynucleotide kinase, 0.024 U/μl T4 polymerase and ultrapure water was added to the samples, for total reaction volumes of 50 μl. Next, samples were incubated for 15 min at 15°C, and then again for 15 min at 25°C. A MinElute PCR Purification Kit (Qiagen, Switzerland) was used to purify the DNA as described in the Kit protocol. Following this, the DNA was eluted using 18 μl TET. Universal adapters were attached with 22 μl MM (1 x Quick Ligase Buffer, 250 nM Solexa Adapter Mix, 0.125 U/μl Quick Ligase) was added to each sample. After a 20 min incubation at room temperature (RT), the samples were purified again with a MinElute PCR Purification Kit (Qiagen, Switzerland). Following this purification step, DNA was eluted with 20 μl TET. The fill-in MM (1 x Isothermopol buffer, 125 nM dNTP mix, 0.4 U/μl Bst polymerase 2.0, ultrapure water) was prepared and added to the purified DNA, reaching a total reaction volume of 40 μl per sample. After a subsequent incubation of 20 min at 37°C, and then at 20 min at 80°C, 1:10 and 1:100 dilutions were prepared for quantification in the PCR laboratory as described in the publication of Meyer and Kircher.[80] During indexing, the samples were split into 4 reactions of 9.25 μl. For each reaction a mixture of 86.75 μl MM (1 x Pfu Turbo Buffer, 250 μM, 0.15 ng/ml BSA, 10 μM P7 primer, 10 μM P5 primer, 25 U/μl Pfu Turbo DNA polymerase (Agilent), ultrapure water) was prepared. All libraries were then transferred to the modern laboratory, also at the University of Zurich, and were placed in a thermocycler (LabGene Scientific). The indexing PCR thermal profile begins with a 12-min denaturation at 98°C, is followed by 10 cycles of 30 sec at 98°C, 30 sec at 58°C and 60 sec at 72°C, and ends with a final elongation step of 10 sec at 72°C. After indexing, the splits of each sample were combined and purified with a MinElute PCR Purification Kit (Qiagen, Switzerland). Finally, the DNA was eluted with 50 μl TET. 1 μl of the indexed libraries were used to produce 1:100 and 1:1000 dilutions to perform a second quantification. To ensure efficient amplification, all libraries were split into a set of 4 reactions. Each reaction contained 5 μl indexed libraries and 95 μl MM (1 x Herculase II buffer, 250 μM dNTP mix, 0.4 μM IS5 and 0.4 μM IS6, 10μM Herculase II Fusion DNA polymerase, ultrapure water). The thermocycler (LabGene Scientific) was programmed with following thermal profile for amplification: 95°C for 2 min; 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec (number of cycles depending on the results of the second library quantification); 72°C for 5 min. Afterwards, the 4 reactions were combined and were purified with a MinElute PCR Purification Kit (Qiagen, Switzerland). The concentration measurements were performed with a tape station, and all libraries were pooled in equimolar concentration and shotgun sequencing on NextSeq500 platforms (Illumina) at the Functional Genomic Centre at the University of Zurich in Switzerland.

Methods F: DNA data processing

De-multiplexed sequencing reads were processed using the Efficient Ancient Genome Reconstruction (EAGER) pipeline version v.1.92.55.[81] The sequencing quality was assessed with FastQC version 0.11.5.[82] AdapterRemoval v.2.2.1a[83] was used to perform adapter clipping, quality trimming, length filtering (<30bp) and merging forward and the reverse reads if there was an overlap in at least 11 positions. The data was mapped against the M. leprae reference genome (NC_002677) with a minimum quality score of 37 (-q) and stringency parameter 0.01 (-n) using BWA v.0.7.17.[84] Read duplicates were excluded with MarkDuplicates version 2.2.1a[85] and DamageProfiler v.3.9[86] was used to calculate the damage patterns of the reads mapping to the reference genome.

After running the eager pipeline we get results of the amount of mapping reads against the reference genome. Furthermore, for the SG-data and the gene-enrichment (RLEP, GyrA and proS) the mapping of the reads were additionally visually checked by IGV. Also, the calculated damage of the DNA was visualised by a graph. Damage profiles of the aDNA for the samples included in this study, were previously published in the supplemental information in the 2021 paper of Pfrengle and colleagues.[2]

To check the preservation of the human DNA, the processed sequencing reads were also mapped against the human genome (RefSeq ID GCF_000001405.40) with the same pipeline and parameter as explained above. To check if any recovered reads matched any regions that are specific to *M. leprae*, we mapped all sample data against a compiled database of the RLEP sequences from NCBI (See Table S8).[2,87,88]