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Assessing the recovery of immune proteins from mummified soft tissue versus archaeological bones

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ABSTRACT

Palaeoproteomic research has primarily concentrated on studying human bone, dentine, dental enamel, and calculus. In contrast, mummified soft tissue has not been extensively studied, limiting our understanding of what types of proteins can be recovered from these uncommonly preserved tissues. Here we use a published extraction protocol with an added bead-lysis step to increase protein recovery from mummified human tissues and LC-MS/MS to analyse a new dataset of three individuals from 18th-century Finland. We compare these data to three previously published datasets with samples from different tissue types, time periods, and taphonomic environments. Mummified soft tissue yielded a greater number of human immune proteins when compared to bone samples in general, and in particular when compared to archaeological bone. Overall, this study highlights the potential of soft tissue proteomics combined with more traditional methods for bioarchaeological research of disease and human-pathogen interactions.

1. Introduction

Proteomics has become a powerful tool in archaeology, with several studies demonstrating its potential for answering questions of species identification, diet, and overall health (Hendy, 2021; Sawafuji et al., 2017; Wilkin et al., 2023). While proteins offer increased durability over DNA for taxonomic identification, they can also provide additional information about functionality and tissue specificity. Recently, palaeoproteomics has been successfully applied to human and animal bones (Buckley and Wadsworth, 2014; Procopio et al., 2021; Wadsworth et al., 2017), ancient eggshell (Demarchi et al., 2020b), human dental calculus (Jersie-Christensen et al., 2018; Tang et al., 2023; Ventresca Miller et al., 2023; Wilkin et al., 2020, 2021b), dental enamel (Buonasera et al., 2024; Shaw et al., 2023), mummified tissues (Demarchi et al., 2020a; Maixner et al., 2013, 2018), and ceramic and metallic vessel residues (Evans et al., 2023; Hendy et al., 2018; Wilkin et al., 2023, 2024a).

Most proteomic studies of mummified tissue and skeletal material have concentrated on proteins recovered from surface tissues, such as skin, hair, and oral swabs (Corthals et al., 2012; Fresnais et al., 2015; Maixner et al., 2013; Mikšík et al., 2016), tissue-specific proteins (Barberis et al., 2022; Fresnais et al., 2015; Hendy, 2021; Maixner et al., 2018; Morton-Hayward et al., 2025), and the preservation and relative protein yield of skeletal samples (Buckley and Wadsworth, 2014; Ntasi et al., 2022; Procopio et al., 2018, 2021) with several other studies using protein analysis to study the health of past individuals (Buonasera et al., 2024; Corthals et al., 2012; Loufouma Mbouaka et al., 2021; Schmidt-Schultz and Schultz, 2015; Shaw et al., 2023; Wilkin et al., 2024b). Methodological research has focused on the development of minimally invasive sampling methods (Barberis et al., 2022; Demarchi et al., 2020a; Kontopoulos et al., 2020; Multari et al., 2022; White et al., 2023) and optimisation of multi-enzymatic digestion protocols (Fagernäs et al., 2024; Lanigan et al., 2020; Wilkin et al., 2024b). Here, as research on

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human health and pathogen interactions is becoming increasingly common within the field, we focus on host immune-related proteins. Palaeoproteomics offers promising new avenues to study human health in tandem with pathogen DNA studies, or in cases where DNA or RNA are no longer preserved. Immunoproteins have been studied broadly within the modern medical literature (Alves et al., 2011; Deng et al., 2025; Dumitriu et al., 2023; Kliuchnikova et al., 2023; Uhlén et al., 2015), but questions about the preservation of different proteins, the effects of taphonomic factors and the difficulties in identifying specific organs in mummified tissues still require further research to understand how the preserved immune proteomes of ancient individuals can be interpreted.

This study compares differences in the recoverable human proteome from bone and mummified soft tissue. We compare proteomic data from three previously published skeletal studies (Mickleburgh et al., 2021; Ntasi et al., 2022; Sawafuji et al., 2017) against a new dataset of proteins recovered from three individuals buried in the crypt of Seili Church in south-western Finland in the late 18th century. These four datasets include individuals from various time periods, different sampled tissue types, and different taphonomic contexts. Two of the compared studies are archaeological (Ntasi et al., 2022; Sawafuji et al., 2017) from the 1st century CE and late 17th century CE, respectively, while one (Mickleburgh et al., 2021) is a forensic study. We purposefully selected Mickleburgh et al. (2021) as a comparison to represent the human bone proteome shortly after death. By adding a bead-lysis step to a published protocol for archaeological samples, we were able to recover a diverse range of proteins from a broad proteome from the mummified Seili individuals, even when compared to a dataset from relatively "young" bone samples from the forensic case study.

In the initial stages of the study, it was expected that the proteomes of bone and soft tissue would differ: bone samples were expected to contain more proteins relevant to skeletal structure, such as collagens and biglycans, whereas soft tissue samples were expected to show skin and muscle proteins, such as myosins, titin, and keratins. Even though no species-specific bacterial proteins were recovered, we still expected to recover immune proteins, based on earlier studies (Corthals et al., 2012; Jones et al., 2016; White et al., 2023; Wilkin et al., 2024b).

2. Materials and methods

2.1. Articles chosen for the meta-analysis

The studies we compared our newly produced data to were chosen due to their shared methodologies (eg. extraction protocols, LC-MS/MS instruments) and comparative material (bone). While an effort was made to find studies as close to our methodology and instruments as possible, some of the machinery differs, as technology continues to improve. The Exploris 480 used in this study is more sensitive than the Q-Exactive used in comparison studies. This may in part have contributed to the higher protein yield from our samples. Bone, as the most widely studied human tissue in archaeological studies, serves as a baseline for our comparison of recoverable proteins. Two systematic studies of the bone proteome (Sawafuji et al., 2017; Ntasi et al., 2022) sampled ribs from eight archaeological individuals, from the Japanese Edo period site of Hitosubashi (17th century CE) and from Roman Baia Scalandrone, Italy (1st century CE). The first study (Sawafuji et al., 2017) is a pioneering examination of the skeletal proteome of archaeological human remains. The paper presents an analysis of six adults and two children, with a special focus on differences in human proteins related to age and health. Ntasi et al. (2022) compare proteome preservation in victims of the eruption of Vesuvius in 79 CE. In addition to the remains of those from Pompeii and Herculaneum, they used three individuals from the coeval Baia Scalandrone site that were not affected by the volcanic eruption as a reference control. To increase the comparability between samples, we used the latter individuals, unaffected by the eruption. Sample 295 (adult male individual) from Sawafuji et al. (2017) and Sample BSC180 (adult male individual) from Ntasi et al. (2022) data had to be excluded, due to corrupted raw files, meaning only seven samples from Sawafuji et al. (2017) and two from Ntasi et al. (2022) are included in this comparison. Mickleburgh et al. (2021) analysed the bone proteome of four elderly body donors before burial as well as over two years after, when the bodies had been skeletonized. Two of the four individuals were buried in the ground instead of more traditional forensic circumstances (i.e., open pit burials). As most archaeological skeletons are from burial contexts, we compared data from the two ground burials to the archaeological data.

2.2. Seili

Proteins were extracted from three of the individuals interred in the crypt of Seili Church. Seili is an island located approximately 30 km south of Turku, Finland (Fig. 1). The parish church was built in 1733, and the crypt underneath the church floors has been used for at least seven burials during the late 18th century. Three individuals were sampled during fieldwork for protein analysis. The results of the archaeological fieldwork have been published in Moilanen and Paasikivi (2023).

While the Seili crypt is not an undisturbed context, it has preserved the human remains in relatively stable conditions. One of the individuals sampled (burial 1) was naturally mummified from the waist up, and another (burial 2) still had soft tissue attached to the skull (see Supplementary Material 3; Fig. 6). The Seili tissue samples consist of bone from each individual (1-ZH1658, 2-ZH1672, 3-ZH1676), mummified tissue from two individuals (1-ZH1659, 1-ZH1670, 2-ZH1673), and coffin bedding and presumed soft tissue (1-ZH1656 and 1-ZH1657) from one individual (Table 1). Samples 1-ZH1659 and 1-ZH1657 consisted of organic material, which in the field was presumed to be either plant material from the coffin bedding or decomposed human soft tissue.

The context of the crypt is complex, and due to the inconsistencies between archaeological, historical, and osteological evidence, it is difficult to determine the identities and causes of death of those buried there (Moilanen and Paasikivi, 2023). According to historical records, three males, three females, and one female infant were buried in the crypt. Based on osteological analysis and sex estimation, burials 1 and 2 are male, while the sex of the individual in burial 3 could not be reliably assessed (Liira, 2021). As the research permit did not allow us to remove any of the remains from the crypt, the osteological analysis relied heavily on photographs of the burials. Most of these photographs were taken from a distance in dim light. Two of the males buried in the crypt have been listed in the church records as having died of pulmonary disease *(lungsot)*, a term often used for tuberculosis (TB), which was very common in Finland at the time (Vuorinen, 2006, pp. 72, 74, 116–121). No signs of TB were found in the osteological analysis of the remains. The third male has been listed as having died of edema. While identifying these individuals with certainty is not possible, we examined immune protein profiles to see whether there were signs of any of these individuals having had an active mycobacterial infection.

Each Seili individual was sampled *in situ*, with bone, soft tissue, and coffin bedding taken where possible. The tissue samples were subsampled (~10 mg for each) in 2 mL Eppendorf tubes in the aDNA laboratory of the University of Zurich's Institute of Evolutionary Medicine (IEM). Protein extraction took place in the dedicated ancient protein lab following an SP3 protocol for archaeological samples (Cleland, 2018; Hughes et al., 2019; Wilkin et al., 2021a) at the IEM, where samples were denatured, reduced, alkylated with 200 μL 6M GuHCl and 30 μL 30 mM TCEP/CAA and heated for 10 min at 99 °C. After heating, sterile glass beads were added to each tube and placed on the tissue-lyser (Qiagen TissueLyser LT) for 5 min at top speed (50 Hz). Samples were then removed from the beads and transferred to new tubes, where 20 μL magnetic beads (Sera-Mag SpeedBeads, 20 μg/μL; 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

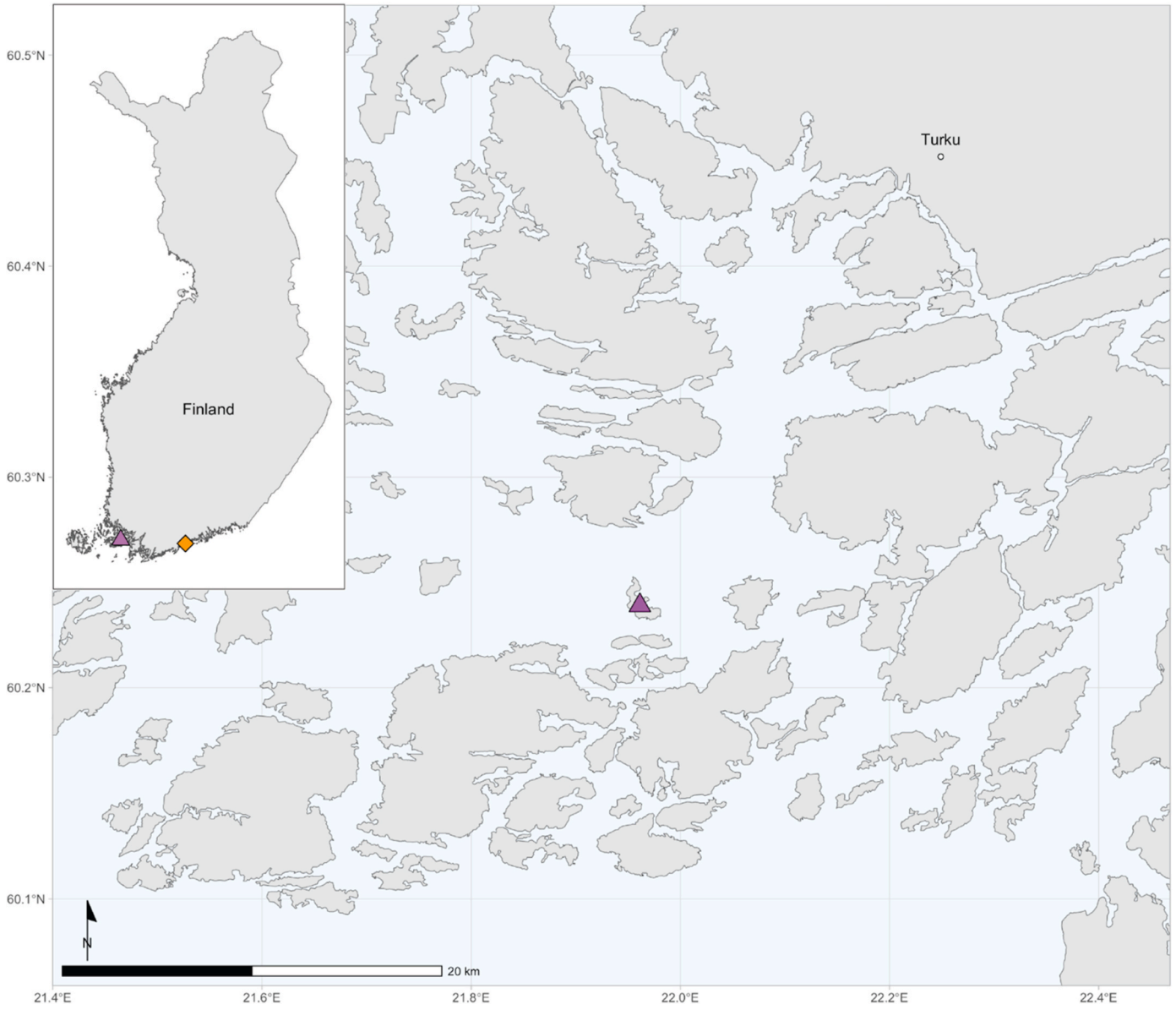

Fig. 1. A.) Map of Seili (purple triangle) within the Archipelago Sea, and Helsinki marked with an orange diamond (top left). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Seili samples. For a full sample description, see Supplementary Table 1 (ST1).

Univ. Zurich Sample ID	Univ. Turku Sample ID	Burial	Tissue
1-ZH1656	TYA 981:8	1	Coffin bedding/soft tissue
1-ZH1657	TYA 981:12	1	Coffin bedding/soft tissue
1-ZH1658	TYA 981:15	1	Bone, metatarsal
1-ZH1659	TYA 981:18	1	Soft tissue, lower abdomen/ groin
1-ZH1670	TYA 981:19	1	Soft tissue, near the lung and abdominal area
2-ZH1672	TYA 981:22	2	Bone, metatarsal
2-ZH1673	TYA 981:24	2	Soft tissue, from the skull
3-ZH1676	TYA 981:29	3	Bone, metatarsal

protein adherence to the magnetic beads. The tubes were then put on the thermomixer at room temperature (24 °C) at 1000 RPM for 5 min. 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 tube, followed by 2 μL of a trypsin solution (0.2 mg/mL) for enzymatic digestion overnight. Following digestion, samples were acidified to a pH below 2 with 5% TFA and purified with stage tips and dried 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).

Mass spectrometry analysis was performed on an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) equipped with a Nano spray 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 (100A, 5 mm, 180 mm × 20 mm, Waters) followed by a nano-Ease MZ C18 HSS T3 Column (100A, 1.8 mm, 75 mm × 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 an 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 the loading condition for another 10 min.

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 the 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.

2.3. Data handling and analyses

2.3.1. Database search

All MS/MS raw files from Seili and the other included publications were converted to Mascot Generic Files (MGF) using Proteome Discoverer, v. 1.4 (Thermo Fisher Scientific) using the automated rule based converter control (Barkow-Oesterreicher et al., 2013) and searched against a database that included (Swissprot (downloaded 18.05.2021)) Swissprot (downloaded 18.05.2021) with additional proteomes of *M. tuberculosis* and *M. leprae* (downloaded 2021.05.02). Previously published studies were all run on MS/MS orbitrap instruments manufactured by Thermo Scientific (Q-Exactive, etc)(Supplementary Table ST2). In order to assure comparability across all samples, raw data files were acquired from each study, converted to MGF files, and searched via Mascot (Matrix Science v. 2.7.0.1) with the same settings against the same database (Wilkin et al., 2024b). Settings included trypsin as the digestive enzyme, a maximum of three missed cleavages, peptide mass tolerance of 10 ppm with fragment mass tolerance of 0.01 Da, with allowances for one carbon isotopic shift. Modifications included carbamidomethylation of cysteine as fixed, with variable modifications of deamidation of asparagine (N) and glutamine (Q), oxidation of methionine (M) and proline (P). The resulting search data was filtered using a freely available custom R script that excludes all protein IDs supported by fewer than two peptide spectral matches (PSMs), and all PSMs with an expected value of over 0.01 (Hagan, 2018). The resulting protein and peptide identifications were verified using an NCBI BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.2. Data processing

All data processing and statistical tests were conducted using R version 4.3.1 (R Core Team, 2023) within the RStudio environment (Posit team, 2023). Mascot output files were iteratively parsed and combined with custom scripts (https://zenodo.org/records/18849075 doi:10.5281/zenodo.18849075). Briefly, individual MASCOT output files for each sample from each of the four studies were compiled on a per-study basis, filtered for primate-specific peptides, and the records were grouped by protein description. Grouping allowed for different peptides that code for the same protein to be identified programmatically. To reduce the occurrence of spurious identifications, we retained only those proteins that were supported by at least two unique peptide spectral matches ($PSM \geq 2$). The four filtered, per-study files were then combined into a single data frame for downstream analyses. Finally, this data frame was filtered to include only those proteins with known immune or immune-related functions. Due to study, tissue type, and context being partially confounded, we fit two models that explored differences between tissues and between contexts separately in order to aid model fitting.

2.3.3. Analyses

1 *Does the abundance of recovered immune proteins differ between bone and soft tissue?*

Using *PSM* as a proxy, we tested whether tissue type affected immune protein abundance. Specifically, we tested for differences between bone (both archaeological and forensic) and soft tissue. Using the *glmmTMB* package (Brooks et al., 2017), we applied a generalized linear mixed-effect models (GLMMs) to the immune protein data with *PSM* as the response variable, *tissue* as a two-level categorical fixed effect (bone (all) vs soft tissue), and *sample_ID* as random intercepts in order to capture between-sample heterogeneity in *PSM*. Since *PSM* can only take non-zero integer values, we modelled the response distribution of *PSM* using a zero-truncated negative binomial distribution with a log link function.

2 *Does the abundance of recovered immune proteins differ between contexts?*

Using the same methodology and model framework as above, we again fitted a zero-truncated negative binomial GLMM to the data to test whether the abundance of immune proteins differed between contexts. In this case, we used a three-level categorical variable (archaeological bone, forensic bone, archaeological soft tissue) as the fixed effect.

The *emmeans* function (Lenth et al., 2025) was used to calculate the estimated marginal means (EMM) for *PSM* for each pairwise comparison between levels of the fixed effect for both of the retained models separately. EMMs for each comparison were calculated based on their respective models' coefficients (Supplementary Material 2). For both sets of comparisons, the Tukey adjustment of *p*-values was done to correct for multiple testing.

The fit of both models was assessed by comparing the aforementioned "full" models to "reduced" models with no fixed effects using the anova function of the *stats* package. Model residuals were inspected using the *DHARMa* package (Hartig, 2024). In both cases, the full model was retained as it fit the data significantly better than the reduced model. Inspection of model residuals further suggested that both models fit the data reasonably well. Inspection of residuals suggested some unexplained variation in the models, but without further measured predictors, we could not explore this. Additionally, we attempted to fit a model to test for differences in recovered protein abundance from bone between studies. Based on initial inspection of the data, we presumed there would be no difference. While the model did indeed show no difference, the model fit was poor, so no further inference was made from this result (results not shown).

3. Results

When comparing immune protein recovery between bone and soft tissue, we found a 3.61-fold greater abundance of immune proteins recovered from soft tissue compared to bone (Fig. 2a; fold-change ratio = 3.61, $\sigma = 1.06$, z-ratio = 4.391, $p < 0.0001$). There was no difference in immune protein abundance between archaeological bone and forensic bone (see Fig. 2b; fold-change ratio = 1.777, $\sigma = 0.603$, z-ratio = 1.696, $p = 0.2067$), and a minor difference between archaeological soft tissue and forensic bone (see Fig. 2b; fold-change ratio = 0.424, $\sigma = 0.146$, z-ratio = −2.485, $p = 0.0346$). There was a 4.195-fold greater abundance of immune proteins recovered from archaeological soft tissue than from archaeological bone (see Fig. 2b; fold-change ratio = 4.195, $\sigma = 1.070$, z-ratio = 5.641, $p < 0.0001$).

In total, 68 proteins with immunological functions were found across all datasets (Supplementary Table ST3). While immunoproteins were

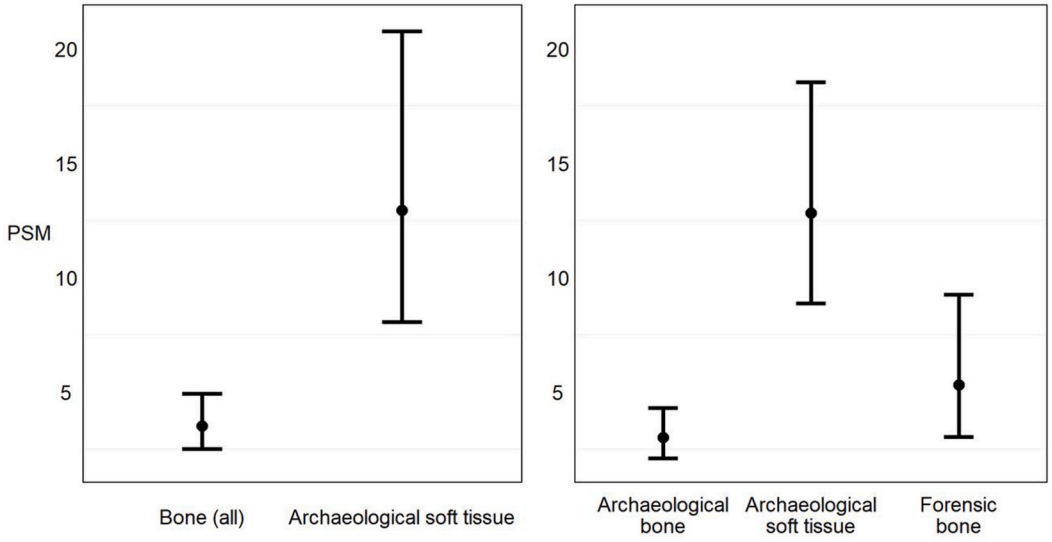

Fig. 2. Differences in the estimated marginal means (EMM) for immune protein PSM (Peptide Spectral Match) between (a) tissue types and (b) contexts. EMMs calculated from the model coefficients of zero-truncated negative binomial GLMMs using emmeans (see Supplementary Material 2 (Lenth et al., 2025)). Error bars represent 95% confidence intervals. Archaeological bone included altogether ten samples (Seili, n=3; Sawafuji, n=7), Forensic bone included 8 samples, all from Mickleburgh et al. All soft tissue (n=3) were from Seili. Two samples containing both coffin bedding and possible human soft tissue were excluded due to this uncertainty. No immune proteins were recovered from the Ntasi study samples.

found in both bone and soft tissue samples, their increased abundance in soft tissue may be explained by their prevalence in blood and other bodily fluids, which can be preserved within mummified tissues (Khan et al., 2021; Maier-Begandt et al., 2024). Forty-eight of these immunoproteins were present in the Seili individuals, with 33 present in a single soft tissue sample taken near the anterior end of the sixth rib bone (1-ZH1670) from a partially mummified individual. The other soft tissue sample from the same individual, taken from the lower

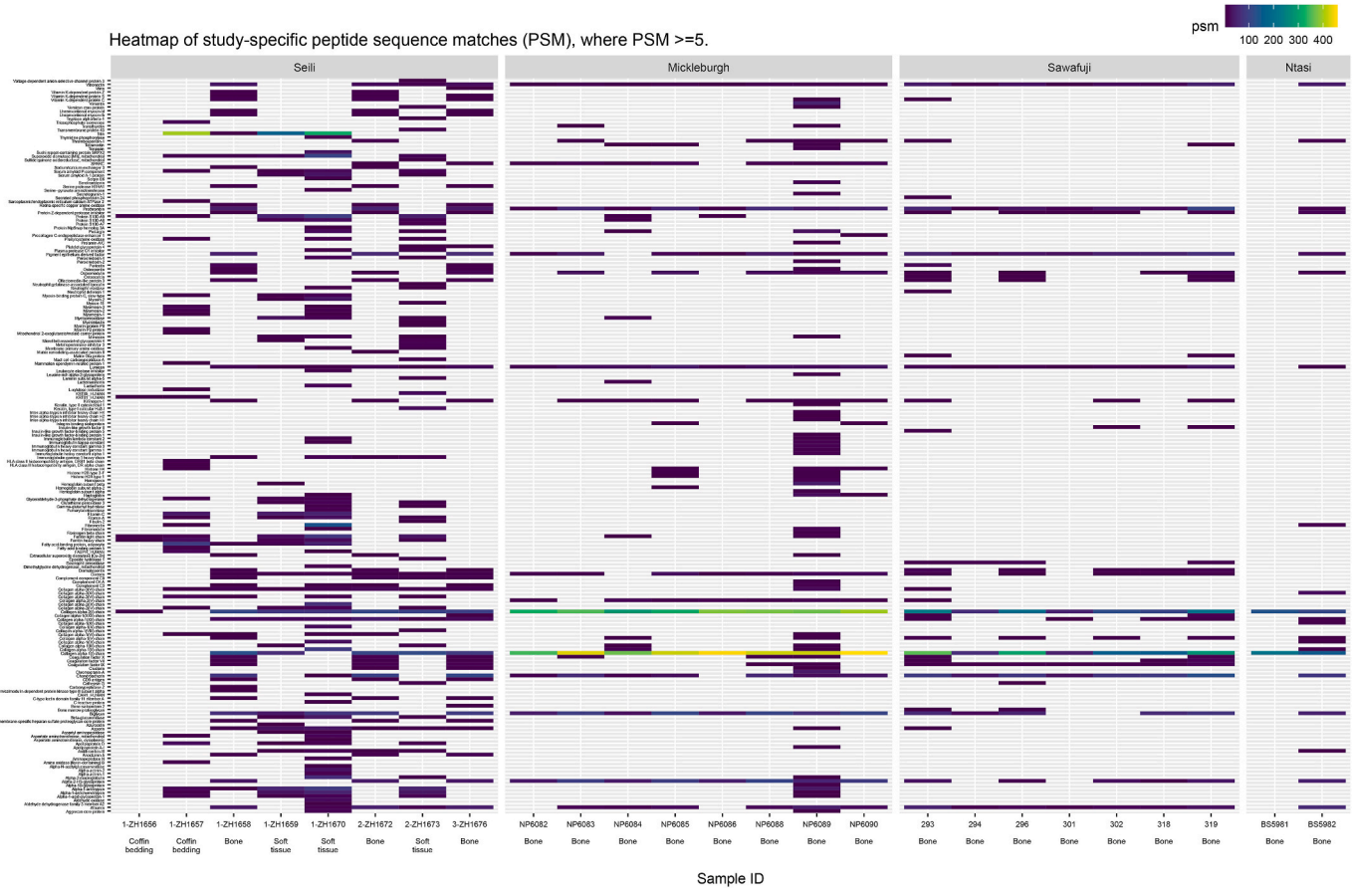

Fig. 3. The abundance of human proteins in each dataset, where PSM (Peptide Spectral Match) of each protein is > 5. The colours in the heatmap go from blue (the lowest) to yellow (the highest). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

abdomen/groin area (sample 1-ZH1659), contained fewer immunologically related proteins (n = 15). Thirty-five immunoproteins were detected in the soft tissue sample of a different individual (2-ZH1673). Similar immunoproteins (cathepsin G, defensin alpha 1, eosinophil peroxidase, ferritin, fibrosin, myeloperoxidase precursor, proteinase 3 and protein S100-A8) were identified in a 2016 study conducted on skin samples from Egyptian mummies (Jones et al., 2016). However, both the sample 2-ZH1673 and the studied tissue in Jones et al. (2016) are surface-level tissues, and immune proteins are a common and major constituent of the human sweat proteome (Yu et al., 2017).

Research into immunoprotein reactions in TB patients has indicated the importance of neutrophil proteins (Eum et al., 2010; Moideen et al., 2018), which were only present in the soft tissue sample 1-ZH1670 in small (<10 *PSM*) quantities, and eosinophil peroxidase (Borelli et al., 2003; Hendy et al., 2016), which in Seili was only found in the soft tissue sample 2-ZH1673. Among the comparison datasets, Sawafuji et al. was

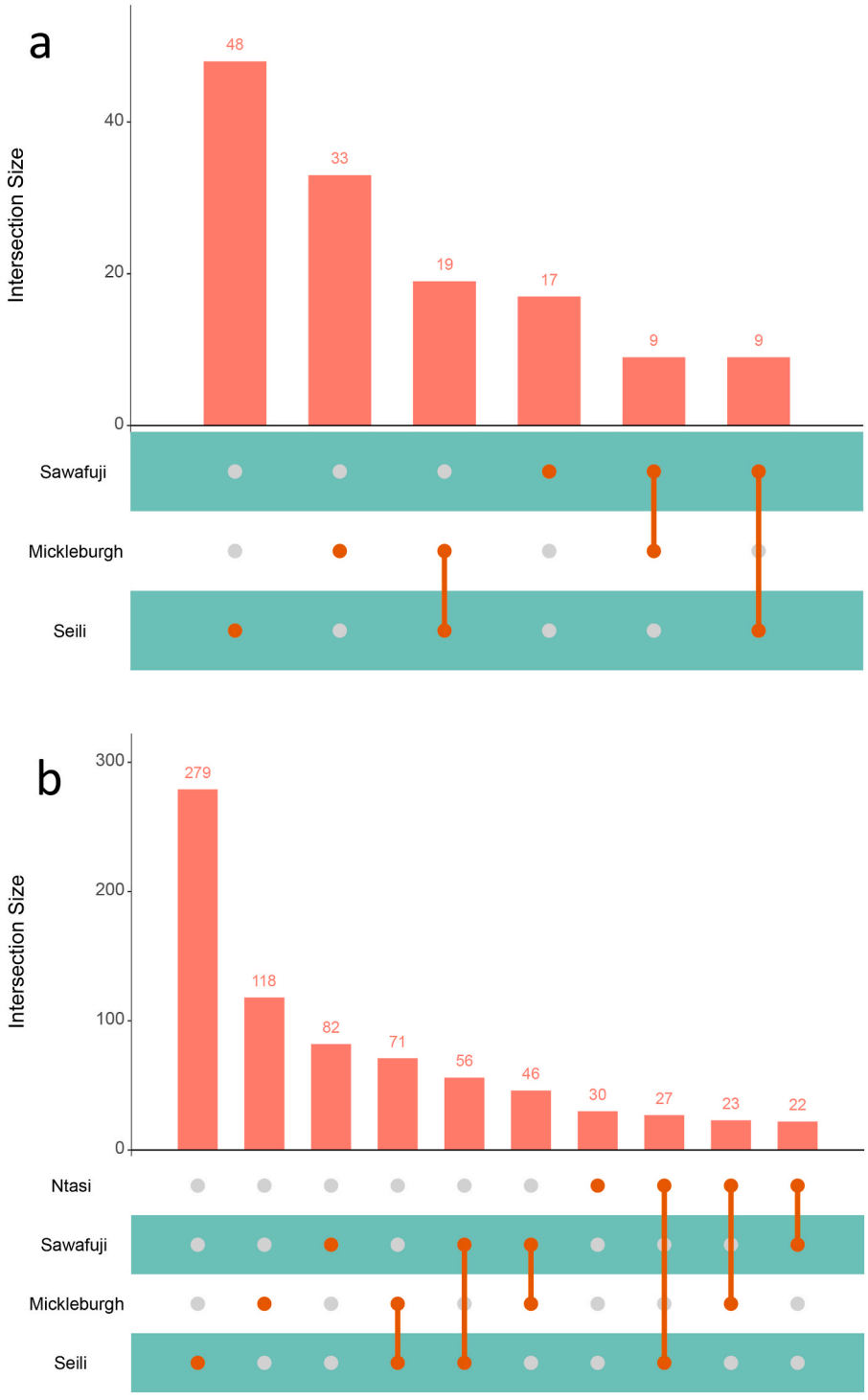

Fig. 4. Upset plots showing the shared and unique recovered (a) immune proteins and (b) all protein types across the four datasets in this study. Ntasi et al. did not include any immune proteins. Each protein included is supported by at least 2 PSMs (Peptide Spectral Match), and each PSM has an e-value under 0.01. Seili (n=8) samples include archaeological bone and archaeological soft tissue, Sawafuji (n=7) and Ntasi (n=2) samples include archaeological bone samples, and Mickleburgh (n=8) includes forensic bone samples. For a list of all immune proteins recovered, see Supplementary Table 3 (ST3).

the only dataset that contained eosinophil peroxidase, with several samples having >10 *PSM*. Low levels of neutrophil elastase and neutrophil gelatinase-associated lipocalin (NGAL) are present in Seili samples 1-ZH1670 and 2-ZH1673, with <10 PSMs in each sample (Supplementary Table ST3). The presence of all immune-related proteins is highest in the Seili soft tissue samples, except for sample NP13-14-15 from Mickleburgh et al. The qualitative assessment of Fig. 3 indicates that the abundance of all proteins recovered from the Seili samples exceeds that reported in other studies. However, the greater protein abundance in Seili could be due to sampling both soft tissue and bone, whereas the other studies only sampled bone. While our search included all bacterial proteins contained within the SwissProt database, as well as in-depth proteomes of *M. leprae* and *M. tuberculosis* downloaded from TrEMBL, we did not recover any peptides specific to either species.

When looking at proteins shared between and unique to each dataset with PSM ≥2, the number of unique proteins in the Seili dataset was 186 (Supplementary Table 4 (ST4)). As was expected due to the tissue types, several of the proteins unique to Seili soft tissue samples are primarily present in muscle tissue (titin, myomesin-1, myomesin-2, myomesin-3, myosin-binding protein C slow-type, and vitrin) or adipose tissue (fatty acid-binding protein 5, fatty acid-binding protein adipocyte, and peripheral myelin protein 2). The presence of muscle proteins is unsurprising, as Seili is the only site that included soft tissue samples. Out of 68 immune proteins across all datasets, 25 were unique to Seili, 10 to Mickleburgh et al., 3 to Sawafuji et al., and no immune proteins were identified in the Ntasi et al. data. There was no statistical difference in the proportion of unique proteins recovered from each study. In the original papers, Mickleburgh et al. report 133 individual proteins, Sawafuji et al. 188, and Ntasi et al. 32 proteins across all their samples. These numbers differ from those in Fig. 4b, as each original study used unique search settings and databases. Mickleburgh et al. and Seili share the most proteins (71) between the two datasets (Fig. 4b) as well as most immune proteins (19) (Fig. 4a).

The recovered proteomes from the soft tissue samples expectedly differed from the bone samples as they included several proteins characteristic of muscle and fat tissue, titin being especially abundant in the sample 1-ZH1659. Bone samples from all datasets included an abundance of collagens and other bone proteins such as biglycan (Fig. 4). Samples 1-ZH1656 and 1-ZH1657 included some coffin bedding but were taken from areas with unrecognizable organic material. In the field, it was suggested that this organic material could be decomposed human tissue or plant material. While 1-ZH1656 showed poor protein recovery, 1-ZH1657 included muscle proteins like titin, myosin binding protein C, myomesin 2, myosin 7, and some immune proteins like immunoglobulins and serpin family proteins (Supplementary Table 3 (ST3)). This shows that in some cases, even decomposed and barely recognizable soft tissue may still yield an analysable proteome.

There were also differences between the soft tissue samples. The sample from burial 2 (2-ZH1673) was a relatively thick piece of tissue still attached to the skull. It contained proteins characteristic of skin and hair, such as keratins, but also muscle proteins like myosin 11. While keratins are often considered a common contaminant from sample handling, they are also the most common type of protein in the outer layers of human skin, and are expected when analysing mummified remains. Furthermore, we also have other proteins from skin, such as collagens (collagen alpha-1 and collagen alpha-2) and collagen-associated proteins like decorin, prolargin, and lumican (Dyring-Andersen et al., 2020), which are present in all three samples (1-ZH1659, 1-ZH1670, and 2-ZH1673) consisting of soft tissue. The highest number of skin proteins was found in sample 2-ZH1673, which also contains metalloproteinase 3 and laminin, the latter of which is most abundant in the dermis (Dyring-Andersen et al., 2020). Collagen IV, which is associated with laminin in the basement membrane (Charonis et al., 1985; Kim et al., 2001; Mak and Mei, 2017), is also found from the sample 2-ZH1673 (PSM 3). This was to be expected, as 2-ZH1673 visibly included skin as well as soft tissue, whereas samples 1-ZH1659 and 1-ZH1670 were drilled from inside a thicker piece of what appeared to be mummified muscle and fat tissue (see Supplementary Material 4).

4. Discussion

Modern proteomics has identified myosin, titin and actin as the most common proteins in the human muscle proteome, whereas collagens, keratins and proteins such as lumican and prolargin are most abundant in modern skin samples (Gonzalez-Freire et al., 2017; Mikesh et al., 2013; Uhlén et al., 2015). Based on modern literature and previous research on mummified individuals, it was expected that the proteome of bone and soft tissue samples would differ, with bone samples having more proteins related to the structural function of bone tissue, such as collagenous proteins, biglycan and osteopontins and soft tissue samples possibly retaining some of the immune and muscle proteins seen also in earlier studies conducted on archaeological mummified tissue (Alves et al., 2011; Corthals et al., 2012; Mickleburgh et al., 2021; White et al., 2023). However, it is important to note that comparing modern proteomes to those recovered from archaeological samples is not straightforward. The degradation of proteins after death - especially in archaeological soft tissue - is not well understood. Some proteins break down very soon after death, while others are more durable. This durability, however, is not necessarily linked to their abundance, and more research is needed to clarify which proteins are present in archaeological samples due to durability rather than abundance (Cleland, 2018; Warinner et al., 2022). This is especially important in regard to soft tissue, where comparisons between modern and archaeological tissue face challenges such as the complexity of the serum proteome in living or recently deceased individuals and the difficulty of identifying archaeological tissues, which are often partially decomposed.

This study demonstrates that archaeological soft tissue is a viable source of ancient proteins, especially for proteins related to immune functions. Forty-eight proteins with immunological functions were found in the Seili samples, and the number of immunoproteins was highest in the three samples consisting of soft tissue (1-ZH1659, 1-ZH1670, 2-ZH1673). These samples belonged to two different individuals, burial 1 and burial 2. With respect to immune proteins, Seili most closely resembles Mickleburgh et al. (2021): these two studies share the most immune proteins and are the only ones where multiple immunoglobulins are present (Fig. 4a and Supplementary Table 3 (ST3)). Only a single hit for immunoglobulin superfamily member 8 was recovered from Sawafuji et al. data (Supplementary Table 3 (ST3)), whereas the nineteen uniquely shared immune proteins between Mickleburgh et al. and Seili (Fig. 4a) include five immunoglobulins (immunoglobulin gamma-1 heavy chain, immunoglobulin heavy constant gamma 2, immunoglobulin kappa constant, immunoglobulin kappa variable 3-20, immunoglobulin lambda constant 2). Several immunoglobulins have relatively short half-lives (Schroeder and Cavacini, 2010), so finding immunoglobulins in two of the youngest datasets may reflect their short-lived presence in the body, as well as denaturation due to taphonomic factors.

Five immunoglobulin proteins (immunoglobulin heavy constant alpha 1, immunoglobulin heavy constant gamma 1, immunoglobulin heavy constant gamma 3, immunoglobulin heavy constant mu, and immunoglobulin lambda constant 6) are present only in the modern forensic dataset of Mickleburgh et al. (2021). The Sawafuji et al. (2017) dataset stood out from the others by including relatively high levels of eosinophil peroxidase, which the authors associated with possible parasitic infections. While eosinophil peroxidase and eosinophil cationic protein were also present in Seili (PSM <4), the Sawafuji et al. (2017) data had multiple samples with eosinophil peroxidase PSM >10. The comparison of neutrophil proteins between Sawafuji et al. (2017) and Seili shows differences; neutrophil defensin 1 (DEFA1) and 2 are present in Sawafuji et al. (2017), but not in Seili, whereas Seili has higher levels

of neutrophil elastase and NGAL. More research is needed to determine whether this difference reflects actual differing health conditions between individuals or arises from taphonomic factors.

All datasets aside from Ntasi et al. (2022) contained immune-related proteins unique to each study. This suggests high variation in the recovery of immunoproteins, possibly due to differences in taphonomic processes, the types of tissues studied, general inflammations not connected to the cause of death, or inter-individual variation in protein preservation. Fig. 5 shows the abundance of immunoproteins in soft tissue samples in comparison to both other datasets, as well as bone and coffin bedding samples from Seili. Seili was the only dataset having more than two immunoproteins with PSM >25 (Fig. 5).

Proteins associated with TB, such as alpha-1-acid-glycoprotein (Martìnez Cordero et al., 2008; Mateos et al., 2020), alpha-2-macroglobulin (Bapat et al., 2015), CRP (Mateos et al., 2020), eosinophil cationic protein (Bystrom et al., 2011; Moideen et al., 2018), haptoglobin (Bapat et al., 2015; Mateos et al., 2020), neutrophil elastase (Alcantara et al., 2023; Eum et al., 2010; Moideen et al., 2018), NGAL (Romejko et al., 2023), protein S100-A8 (Gopal et al., 2013; Ren et al., 2024), protein S100-A9 (Gopal et al., 2013), SERPIN A3 (Jin et al., 2022) serum amyloid A-1 (Kawka et al., 2021) and serum amyloid P-component protein (Mateos et al., 2020) were all present in the sample 1-ZH1670, which was taken near the lungs of the burial 1. While all these proteins can be linked to active TB in modern patients, they are also present in many other infections, with some also present in healthy individuals, and are not specific to TB (Bapat et al., 2015; Bystrom et al., 2011; Ren et al., 2024; Song et al., 2014). Similarly, proteins such as DEFA1 and complement component C9 (C9) have also been linked to TB (Mateos et al., 2020). DEFA1 was not present in any of the Seili samples, whereas C9 could be found in the bone sample of burial 1, but not in the soft tissue samples (Supplementary Table 3 (ST3)). In the second individual from Seili, C9 is only present in the soft tissue (2-ZH1673).

A 2014 study found that the level of alpha-1-antitrypsin (AAT), one of the most abundant immune-related proteins in burial 1, was 4.4 times higher in modern TB patients (Song et al., 2014). While the baseline levels of proteins cannot be determined within archaeological individuals, it is possible that the high recovery (see Table 2) of AAT could indicate its abundance at the time of death. Sample 1-ZH1670 also included several proteins which have in general a strong role in the human immune system, such as complement system protein C3 (Sim and Tsiftsoglou, 2004), several immunoglobulins (IGG, IGKC, IGKV3-20, IGLC2) (Schroeder and Cavacini, 2010), myeloperoxidase (MPO)

Table 2
Proteins associated with TB and the PSM count from each soft tissue sample from Seili.

Protein	1-ZH1659	1-ZH1670	2-ZH1673
alpha-1-acid-glycoprotein	9	18	19
alpha-1-antitrypsin	54	85	51
alpha-2-macroglobulin	0	67	8
CRP	0	6	3
complement component C9	0	0	12
eosinophil cationic protein	0	3	4
haptoglobin	0	5	0
neutrophil defensin 1	0	0	0
NGAL	0	4	5
protein S100-A8	15	26	16
protein S100-A9	54	95	45
SERPIN A3	30	53	34
serum amyloid A-1	16	33	8
serum amyloid P-component protein	8	23	20

Proteins associated with TB and the PSM count from each soft tissue sample from Seili.

(Arnhold and Flemmig, 2010), plasma protease C1 inhibitor (Davis et al., 2008), serpin B1 and serpin B6 (Janciauskiene et al., 2024). AAT was not present in Sawafuji et al. or Ntasi et al., and only one sample (NP13-14-15) in Mickleburgh et al. had more than >2 PSM of AAT (Supplementary Table 3 (ST3)). Recently published Protein Phenome Atlas also links SERPINA3, AAT, IGLC2 and MPO to several respiratory issues in modern patients (Deng et al., 2025).

We recovered several proteins that are also considered acute-phase proteins (APP), such as neutrophil proteins (Supplementary Table 3 (ST3)). The abundance of APPs rises rapidly in response to an infection and lowers as the acute phase of the infection passes (Jain et al., 2011; Mantovani and Garlanda, 2023). Neutrophil proteins such as neutrophil elastase and NGAL are considered markers for acute infections in living individuals, as they are relatively short-lived APPs (Faurschou and Borregaard, 2003; Hidalgo et al., 2019). CRP also has a short half-life of only approximately 19 h in plasma (Pepys and Hirschfield, 2003). While neutrophils and CRP occur in a number of different conditions, both acute and chronic, and cannot offer a differential diagnosis, we suggest that looking into such short-lived APPs might be beneficial in palaeoproteomics, as they may indicate health issues immediately prior to death. In general, accounting for the different cellular turnover rates and remodelling across tissue types may also be beneficial when interpreting which proteins may have been present near or during the time of death

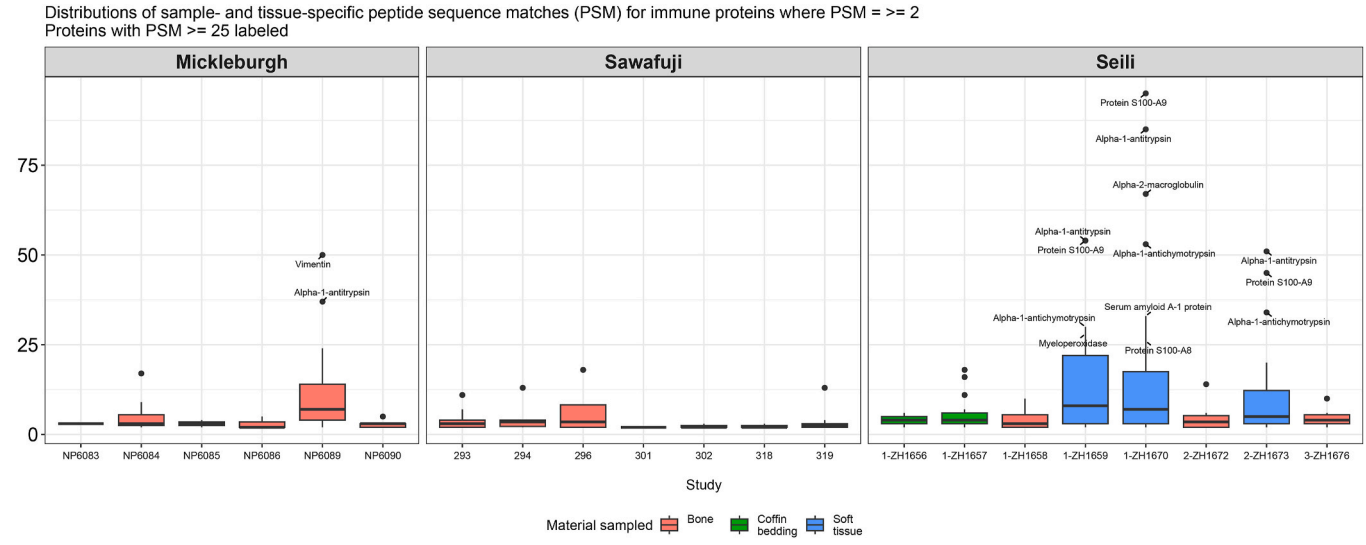

Fig. 5. Box plot showing the abundance of immune proteins in each sample and tissue type. No immune proteins were recovered from Ntasi et al. (2022). PSM (Peptide Spectral Match) was used as a proxy for protein abundance, where PSM≥2. Proteins with PSM≥25 are labelled.

of an individual. While the human bone tissue remodelling rate is generally 5-15% per year, certain plasma cells and the proteins associated with them are very short-lived (Sender and Milo, 2021). While plasma proteins can also be recovered from bone, our results indicate that the recovery of short-lived immune proteins is more likely when sampling mummified tissue. However, more research is needed to understand how common the presence of APPs is in archaeological soft tissue. Unknown differences in individual baselines for immune proteins and variation in protein recoverability from different tissue types should also be considered when interpreting recovered immune-related proteins: many immune proteins are present in low levels in healthy individuals. Therefore, more research into what normal immune protein baselines from archaeological tissue look like is crucial for determining when levels of APPs should be considered unusual in historic or archaeological individuals.

Several studies have shown that the proteomic profile varies between different bones sampled from the same individual (Mickleburgh et al., 2021; Procopio et al., 2017; Sawafuji et al., 2017). Furthermore, sampling more highly vascularized bones such as ribs, vertebrae, or os coxae will yield a broader proteome due to greater blood circulation within the tissue during life (compared to more dense, less vascularized peripheral bones) (Mickleburgh et al., 2021; Sawafuji et al., 2017). In the case of Seili, this was not possible due to our sampling permit being limited to only sampling loose metacarpals and metatarsals, as well as the challenging working conditions inside the crypt. Systematic sampling of bones similar to those used in the comparison studies would have made the results more comparable and possibly have improved the protein recovery of the Seili samples.

There are likely several reasons for the excellent protein preservation in the Seili individuals. The crypt has been a relatively stable environment, making it possible for the mummified tissue to survive. Compared to most archaeological research, Seili is also a relatively young site, as the remains are only around 200 years old. The sites in Ntasi, Sawafuji, and Mickleburgh also represent ground burials, which subjects the remains to more bacterial and environmental agents than a crypt environment. Somewhat tautologically, the absence of soft tissue in the four previously published studies also excludes many soft tissue-related proteins from the results. However, preservation of similar muscle proteins is also known from older mummies, such as the mummified remains of an Egyptian woman from c. 660 BCE (White et al., 2023). The extraction method of the Seili samples also likely contributed to the increased protein recovery. By utilising mechanical tissue disruption, we suggest that additional proteome recovery is possible, but this requires further methodological research.

While we suggest that recovering several immunoproteins, especially those with a short lifespan, which tend to decrease significantly with longer post-mortem intervals, could be a sign of their abundance at the time of death, it is still not conclusive evidence for the presence of any specific disease process. While there are known proteomic biomarkers for certain health conditions (Schmidt-Schultz and Schultz, 2015; Wilkin et al., 2024b), all proteins present in these four datasets are connected to wider immunological reactions within the body, and several of these proteins have multiple functions beyond the immune response. Many proteins involved in immune reactions can also be present in healthy individuals, and are especially difficult to interpret when it comes to ancient individuals. Most of the human past has involved living in conditions that predisposed us to minor injuries, low-level chronic metabolic stress, and common infections that have not necessarily been major health crises, but could be reflected in the proteome at the time of death.

We find that in addition to their similarities in regards to immunoproteins, the overall protein abundance in Seili is also most comparable to that of modern forensic samples (Fig. 3). Two hundred and seventy-nine individual proteins with a PSM $\geq$2 were recovered from the eight samples analysed from the three Seili individuals, in comparison to 118 proteins from the modern dataset of Mickleburgh et al., which consisted of eight samples from two individuals. All other datasets had <85 individual proteins across all samples (Supplementary Tables 5–8 (ST5-ST8)). In addition to Seili having proteins present only in soft tissue, this resemblance could be due to the relatively young age of the archaeological site, as well as the protective environment within the crypt. The bone samples in the Sawafuji et al. and Ntasi et al. datasets were subjected to the harsher taphonomic environment of ground burials. This suggests that burials in crypts and beneath church floors could offer new ways to utilize ancient proteins to study past health beyond the bone proteome.

5. Conclusions

Our findings suggest that mummified tissue is preferable to bone when considering the recoverability of immune proteins. While this result is not unexpected, as many immune proteins are present in the blood and other bodily fluids and are more likely to be preserved in soft tissues, it has not been previously reported in archaeological remains. Mummified tissue is recovered less frequently than bone in archaeological contexts, but it should be considered for proteomic analysis when possible, especially in cases where ancient DNA or other destructive methods are already planned. While we suggest mummified tissue is preferable in order to recover immune proteins, we encourage sampling several tissues when possible, to gain as comprehensive a view on the individual's proteome as possible.

We did not find any conclusive evidence of TB, but we were able to recover several immune proteins, some with quite high PSM counts even when compared to modern forensic samples. While these immunoproteins were not specific only to TB, they may potentially indicate inflammatory activity associated with the disease. However, further research into mummified tissues is needed to determine whether these results truly reflect an ongoing infection at the time of death or are the result of individual variation in immune protein levels.

Despite the challenges in comparing datasets from very different archaeological contexts and time periods, the application of new data analysis pipelines on previously published data may yield interesting information and further our understanding of the preservation and recovery of ancient proteins. While our study is limited by the lack of systematic sampling due to the research permit and field conditions in Seili, as well as the limited comparison of tissue extraction methods to fully evaluate the effects of the tissue lysis step, we were able to demonstrate the significance of soft tissue analysis in the context of immunoprotein research. In the future, the ongoing analysis of ancient DNA and isotope data from Seili will also further contribute to our interpretation of this complex archaeological site. As the body of ancient proteomic research is still relatively small, the results in this study add to the growing corpus of knowledge on the immune proteomes of archaeological individuals. While additional research with larger datasets will be crucial to expanding our understanding of past health, we present a framework that can be replicated, adapted, and further developed in the future.

Reproducible results

The Associate Editor for Reproducibility could download all materials and was able to reproduce the results presented by the authors.

CRediT authorship contribution statement

Sofia Paasikivi: Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. **Ronan James O'Sullivan:** Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Ulla Nordfors:** Investigation, Supervision, Writing – original draft, Writing – review & editing. **Anne-Mari Liira:** Investigation, Writing – review & editing. **Liam Thomas Lanigan:** Conceptualization, Writing – original draft,

Writing – review & editing. **Verena J. Schuenemann:** Funding acquisition, Supervision. **Shevan Wilkin:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2026.106589.

Data availability statement

The data (.raw, .mgf, and mzid files) that support the findings of this study are openly available through MassIVE (https://massive.ucsd.edu/) in dataset: MSV000098445; password: FinlandProteins. Filtered Mascot output files as well as the custom R scripts used in this study are available at https://zenodo.org/records/18849075 https://doi.org/10.5281/zenodo.18849075. Supplementary Material 4 includes the filtered.csv files for each sample in this study.

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