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The impact of maceration on the 'Osteo-ome'; a pilot investigation

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ABSTRACT

The bone proteome, *i.e.,* the 'osteo-ome', is a rich source of information for forensic studies. There have been advances in the study of biomolecule biomarkers for age-at-death (AAD) and post-mortem interval (PMI) estimations, by looking at changes in protein abundance and post-translational modifications (PTMs) at the peptide level. However, the extent to which other post-mortem factors alter the proteome, including 'maceration' procedures adopted in human taphonomy facilities (HTFs) to clean bones for osteological collections, is poorly understood. This pilot study aimed to characterise the impact of these 'cleaning' methods for de-fleshing skeletons on bone biomolecules, and therefore, what further impact this may have on putative biomarkers in future investigations. Three specific maceration procedures, varying in submersion time (one week or two days) and water temperature (55 °C or 87 °C) were conducted on six bovid tibiae from three individual bovines; the proteome of fresh and macerated bones of each individual was compared. The maceration at 87 °C for two days had the greatest proteomic impact, decreasing protein relative abundances and inducing specific PTMs. Overall, these results suggest that routinely-employed maceration procedures are harsh, variable and potentially threaten the viability of discovering new forensic biomarkers in macerated skeletal remains.

Significance: For the first time, the application of bone proteomics in understanding maceration procedures was conducted to help address the risks for experimental confounding associated with this post-mortem cleaning technique. This pilot study demonstrates that recent advances in biomarker discovery for post-mortem interval and age-at-death estimation using bone proteomics has potential for confounding by differing and destructive bone-cleaning methods.

1. Introduction

In recent years, the survival and changes of biomolecules in bones have been investigated in forensic contexts for age-at-death (AAD) and post-mortem interval (PMI) estimations. However, understanding how molecular persistence and degradation are modulated by intrinsic (*i.e.*, belonging to the body) and extrinsic (*i.e.*, taphonomic) variables is crucial for the future application of these methods to routine casework. Amongst biomolecules, proteins are acquiring a significant role in this field; specifically, variations in the bone proteome and subsequently at peptide level (*via* oxidation and deamidation based post-translational modifications, PTMs) have been shown to be correlated with either PMI and/or AAD. Therefore, any post-recovery processing procedure on bones that is able to impact the biomolecules present within the skeletal remains should be avoided or minimised, to allow the implementation of biomolecular studies for research and for practical forensic applications.

Processing procedures (such as removal of the soft-tissue) has been a wide-spread, albeit non-standardised practice in human taphonomy facilities (HTFs) following their forensic anthropological investigations into the decomposition process of human remains. Moreover, in forensic laboratories, remains from forensic caseworks are received and macerated prior to genetic identity confirmation of the individual [1,2]. This soft-tissue decomposition of a carcass or corpse to clean the skeletal remains is known as 'maceration' [3–5]; temperature and reagent use in protocols are often personalised to the state of the skeletal remains (mouldy, greasy, or partially fleshed for example), meaning there is an inconsistency that might impact the molecular state of the remains. Specifically, the impact of maceration on the bone proteome has not been investigated, and consequently, the extent of changes and their impact is unknown.

There is scant literature discussing in detail how this is an issue in HTFs and skeletal collections. The unintended impact of extreme

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maceration conditions on skeletal collections was reported after using transmission electron microscopy (TEM) to compare skeletal remains of the Hamann-Todd osteoarchaeological collection to modern human samples heated to high temperatures [6]; the surface damage in both cohorts was extensive and similar. Studies on the maceration of bones for cleaning compared several different protocols for aesthetic effectiveness, focussing on the observation of unobscured surfaces and the minimisation of the potential for harm to the researcher, rather than biomolecule preservation [4,7,8]. Damage to bone proteins and post-translational modifications to peptides from elevated temperatures and/or extreme pH conditions have been recorded previously, but not in the maceration context [9].

Impacts on DNA from maceration have previously been investigated [10–16]; DNA integrity was maximised in autopsied bones that were defleshed post-mortem with an enzymatic detergent at a lower temperature (55 °C) compared to boiling [17]. However, in a much larger study that involved different maceration protocols (plain water, enzyme detergent, bleach, ammonia, dermestid beetles and manual removal), it was found that bleach, ammonia and enzymes severely impact DNA yield [12]. There is little exploration on the impact of maceration aids such as cleaning detergents alone on proteins in different tissue samples; the effect of treatments involving detergents on the proteome of blood stains has been investigated and revealed a lower mg/mL average protein quantity compared to plain water being used or no treatment [18]. Detergents containing bleaching agents, SDS (or other detergent from the same chemical family) are well known for protein denaturation [19,20].

The choice of maceration aid *(i.e.,* increased temperature, use of a biological or a chemical detergent) can greatly affect the biomolecular integrity of skeletal remains. Extreme temperatures could also increase biomolecular damage. Bone collagen is known to exhibit several stages of temperature-dependent denaturation, based on the type of bone itself and on its mesostructures (cortical/outer structure or cancellous/inner structure) which may lead to different influencing physical-chemical mechanisms [21]. Various techniques, ranging from Raman spectroscopy, thermogravimetry to transmission electron microscopy (TEM) have been used to investigate heat damage to collagen fibrils [22–24]; Koon investigated this with modern, forensic and archaeological bone, reporting alterations of concern [6]. Both the organic and inorganic components of bone can influence its thermal stability. The presence of the mineral component influences the endothermic transition at increasing temperatures; mineral crystals in the bone surround and integrate with collagen fibrils, so that there is less free space for collagen to collapse and break down into during thermal denaturation at increasing temperatures [25,26]. Also, in older samples (*i.e.* higher biological age), there is likely more mature multivalent cross-linking from older collagen which impacts thermal lability [27]. Overall, biological age, temperature and environmental exposure together will influence the denaturation of the bone proteome; therefore, anything that might disturb the mineral content of the bone (such as experimental decomposition in taphonomic studies at HTFs) could lead to greater thermal damage due to the lack of mineral protection of the collagen component during the severe post-mortem maceration cleaning procedures of the bones, if performed at higher temperatures.

This has been highlighted in previous archaeological studies that have investigated the impact of cooking on bone integrity [28–30]. Bonds between apatite crystals break down which can form larger crystals at the expense of smaller crystals (known as Ostwald ripening), reducing space between components, increasing crystallinity and decreasing collagen content [31–33]. The bone proteome at the amino acid (AA) level is subject to compositional changes and racemisation (which can impact racemisation age estimation techniques) when bones have been subjected to super-physiological temperatures, or even boiled in water [34–40]. Although these studies did not specify thermal tolerances of bone proteins, they established how AA concentrations can change in increasing temperatures [34–40].

Overall, whilst there has been research into thermal stability of AAs, inorganic and organic bone structural components, there is little specific research into the direct biomolecular impact of macerating bones at higher temperatures with/without detergents. Therefore, in this study, three routinely employed maceration protocols from the United States (US) HTFs (Texas State Forensic Anthropology Centre (FACTS) and Southeast Texas Applied Forensic Science facility (STAFS)) were applied in the current pilot investigation. Changes to the bone proteome of bovid skeletal remains (as human proxies) from maceration were characterised and applied to understand the biomolecular impact of bone maceration from a starting point. The aims were to clarify the impact of these three maceration protocols on protein and peptide levels and to gain insight into which proteins may not be suitable biomarkers for subsequent AAD and PMI estimations in future investigations based on their potential thermal instability ranging from the protein level to the AA level based on sequence motif tolerance.

2. Materials and methods

2.1. Sample collection and sub-sample preparation

Six fresh bovid (*Bos taurus*) individual tibiae ($n = 3$ right, $n = 3$ left) were obtained from a local butcher and we de-fleshed the bones by hand using scalpel and tweezers, removing any remaining skin and muscles, periosteum and tendons from the bone surface. The midshaft tibia was chosen for analysis due to previous research conducted on intra-skeletal differences in pig bones [41]. Right tibiae were macerated, whereas left tibiae were kept fresh; sampling was conducted on both fresh and macerated remains as shown in Table 1. Bone powder (approximately 100 mg) was taken from each tibia using a diamond-tipped Dremel in duplicate (A and B samples) by creating transverse parallel lines across the anterior midshaft of each bone. Subsequently, sub-samples of 25 mg for each biological replicate were taken to be used for downstream proteomic analysis.

For the 'macerated' bone samples (T2, T4 and T6), three protocols of interest (Table 1) were chosen based on their frequent use at the HTFs outlined in the introduction. Following the maceration protocols, the bones were removed from the temperature-controlled water baths, wiped carefully and left to dry overnight in fume hoods. Bone powder samples and sub-samples were obtained with the same technique and amount as the fresh samples and stored at room temperature ready for downstream processing. The sample size reflects the scope of this pilot study, which was intended for the qualitative assessment of the impact of maceration on animal skeletal remains as proxies for human samples.

2.2. Protein extraction

Once the 12 sub-samples were obtained from the prepared six bovine bones ($n = 3$ fresh, $n = 3$ macerated), they were subjected to bone protein extraction following Procopio and Buckley [9]. Briefly, 1 mL of 10 *v*/v % formic acid (Sigma Aldrich, U.K.) was added to 25 mg of each bone powder sample; samples were then vortexed and incubated for 6 h at 4 °C. Samples were then centrifuged at maximum speed for 1 min, and the supernatant (acid soluble fraction) removed and frozen. Subsequently, 500 μL of 6 M guanidine hydrochloride/100 mM TRIS buffer (pH 7.4, Sigma-Aldrich, U.K.) was added to each sample pellet for an 18-h overnight incubation at 4 °C. Pellets were discarded following maximum speed centrifugation for 1 min; the supernatant (500 μL, acid insoluble fraction) from each sample was transferred to a 10 K molecular weight cut off filter tube (Vivaspin 500 polyethersulfone, 10 kDa, Sartorius, Germany) and centrifuged for 20 min. Two washes with 500 μL of 50 mM ammonium acetate (Scientific Laboratory Supplies, U.K.) followed, with 20-min centrifugation sessions at maximum speed after each wash. Remaining samples were collected in 50 mM ammonium acetate (up to 100 μL) and transferred to fresh Eppendorfs for reduction and alkylation steps. Samples were reduced with DTT (Fluorochem, U.

Table 1
Description of how the bones were sampled (location), which side, how many replicates per sample (A and B), and the further protocol details. These protocols adapted from human taphonomy facilities to be replicated with bovid bone powder samples.

Sample	Macerated or fresh	Skeletal element/ subsample location	Bovid individual/side	Protocol number	Protocol detail
T1A/B	Fresh	Tibia/Mid-shaft	1/Left	Fresh comparison to Protocol 1 (unmacerated)	–
T2A/B	Macerated	Tibia/Mid-shaft	1/Right	Protocol 1	Specimens were macerated for 1 week at 55 degrees centigrade in a covered water bath with dish detergent; detergent: deionised water (1:3% *V*/V)
T3A/B	Fresh	Tibia/Mid-shaft	2/Left	Fresh comparison to Protocol 2 (unmacerated)	–
T4A/B	Macerated	Tibia/Mid-shaft	2/Right	Protocol 2	Specimens were macerated for 2 days at 55 degrees centigrade in a covered waterbath with dish detergent; detergent: deionised water (1:3% V/V)
T5A/B	Fresh	Tibia/Mid-shaft	3/Left	Fresh comparison to Protocol 3 (unmacerated)	–
T6A/B	Macerated	Tibia/Mid-shaft	3/Right	Protocol 3	Specimens were macerated for 2 days at 87 degrees centigrade in a covered waterbath with laundry detergent: 2 oz. detergent/15 L

K.) for 40 min at room temperature, then alkylated with iodoacetamide (Sigma-Aldrich, U.K.) for 45 min at room temperature in the dark. DTT was added again immediately after this step to quench the alkylation step. For each sample, 1 μL of Trypsin (Promega, U.K.) was added to the samples and left for 5-h at 37 °C. 12 μL of TFA (Fluorochem, U.K.) at 1 *v*/v % was added to each sample to stop the digestion, ready for the zip-tipping stage. Each OMIX C18 Zip-tip (Agilent Technologies, U.S.A.) column was first conditioned with 100 μL of ACN (ThermoFisher Scientific, U.K.) twice, then washed with 0.1 *v*/v % TFA twice, to allow the samples to be resuspended in the zip-tips. Tips were then washed again with 0.1 *v*/v % TFA twice, resuspended and then released in the ACN solvent. Samples were dried at room-temperature ready to undergo sample pre-processing for the Orbitrap Mass Spectrometer.

2.3. LC/MS-MS analysis

Samples resuspended in 3 v/v % ACN/1 v/v % TFA were analysed by LC − MS/MS using an ultimate 3000 Rapid Separation LC (RSLC) nano LC system (Thermo Corporation, Sunnyvale, CA, USA) coupled to an Exploris 480 Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Peptides were separated on an EASY-Spray reverse phase LC Column (500 mm length, 75 μm diameter (i.d.), 2 μm particles, 100 Å pore size, Thermo Fisher Scientific, Waltham, MA, USA) using a gradient from 96 *v*/v % A (0.1 v/v % FA in 3 v/v % DMSO) and 2 v/v % B (0.1 v/v % FA in 80 v/v % ACN 3 v/v % DMSO) to 8 v/v %, 30 v/v %, and 50% B at 14, 50, and 60 min, respectively, at a flow rate of 250 nL/min. Acclaim PepMap 100 C18 LC Column (5 mm length, 1 mm diameter, i.d., 5 μm particles, 100 Å pore size, Thermo Fisher Scientific) was used as trap column at a flow rate of 10 μL min − 1 maintained at 45 °C. The LC separation was followed by a cleaning cycle with an additional 15 min of column equilibration time. Then, peptide ions were analysed in the full scan MS scanning mode at 60,000 MS resolution with an automatic gain control (AGC) of 3E 106, injection time of 200 ms, and scan range of 375–1400 *m/z*. The top 20 most abundant ions were selected for data-dependent MS/MS analysis with a normalised collision energy level of 30 performed at 17,500 MS resolution with an AGC of 1 Å ~ 105 and maximum injection time of 100 ms. The isolation window was set to 2.0 *m/z*, with an underfilled ratio of 0.4%, dynamic exclusion was employed; dynamic exclusion allows for the selection of increasingly lower abundance precursors through the application of rolling dynamic exclusion m/z lists. A dynamic exclusion of 25 s was employed: thus, after being selected for MS/MS, a precursor was excluded for MS/MS selection for 25 s after this point. All ions were still detected in MS full scan mode to allow peak integration for relative quantification. The MS/MS spectra were used to positively identify the peak of interest.

2.4. Data acquisition and Bioinformatic analysis with Progenesis

Progenesis Qi for Proteomics (Nonlinear Dynamics, Newcastle, UK) was used to group the mass spectra data using the between-subject design scheme in order to compare samples; these groups were Macerated *versus* Fresh Samples ("MF"), Macerated samples only ("M"), and Fresh samples only ("F"). Mass spectra were searched against the SwissProt database for *Bos taurus* using the Mascot search engine to match to primary protein sequences. The variable modifications were deamidation (of asparagine and glutamine) and oxidation (lysine, methionine and proline), the fixed modification was carbamidomethyl modification. Deamidation and Oxidation modifications were chosen based on previous experiments highlighting their relevance in forensic proteomic analyses [42].

The mass tolerances for precursor and fragmented ions were respectively 5 ppm and 10 ppm. Trypsin was set as the enzyme with two missed cleavages allowed. Peptide ions were excluded from the analysis based on the Mascot evaluation of the peptide score distribution for the .mgf file from Progenesis (FDR = 0.05). Proteins with a unique peptide count of <2 were excluded.

The relative abundance of specific proteins were measured through area under the curve (AUC) calculated by Progenesis; summing the areas below the scan lines within the isotope boundaries allowed downstream analyses to be based on ion intensities. Thus, abundances of overlapping peptide ions could be separated, followed by the use of Hi-N to provide the protein abundance.

2.5. Post-workflow processing

The output data from Progenesis QI underwent several filtration and rearrangement stages to aggregate the data in the desired format (Fig. 1). Stages 1–2 were a simple data clean-up and removal of specific peptides belonging to an exclusion group of collagenous proteins, as the target of this study were non-collagenous proteins.

In stage 3, the PTM ratios were measured on peptides that show both modified and non-modified states (example shown in [42]); such as deamidated asparagine (N), deamidated glutamine (Q), oxidated proline (P), oxidated lysine (K) and oxidated methionine (M). For these peptides, the modification ratios were calculated as below:

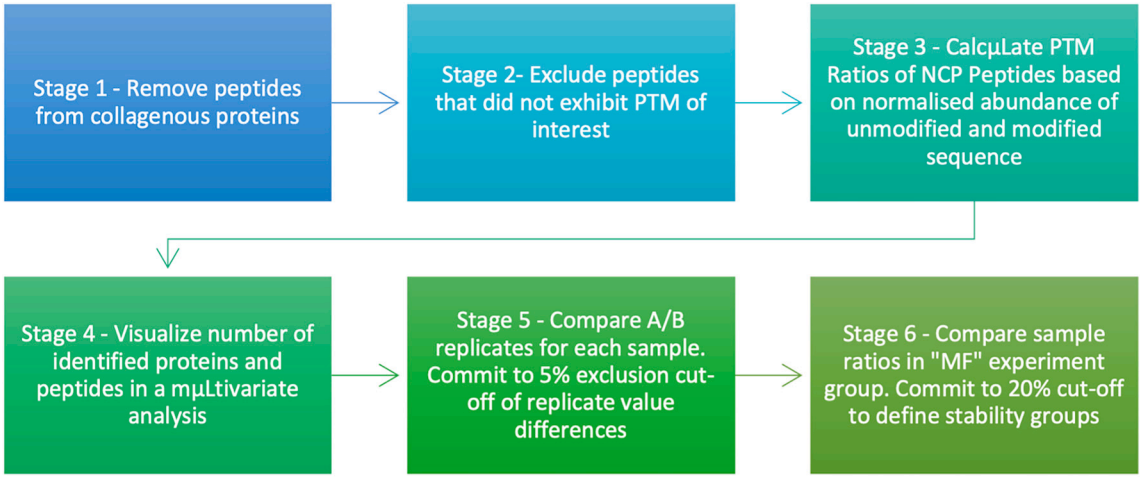

Fig. 1. Summary flow chart of proteomics data analysis following Progenesis QI workflow.

$$Modification\ Ratio\ [\%] = \frac{\text{Total relative abundance of deamidated or oxidated states}}{\text{Total relative abundance of the peptide}}\ x\ 100$$

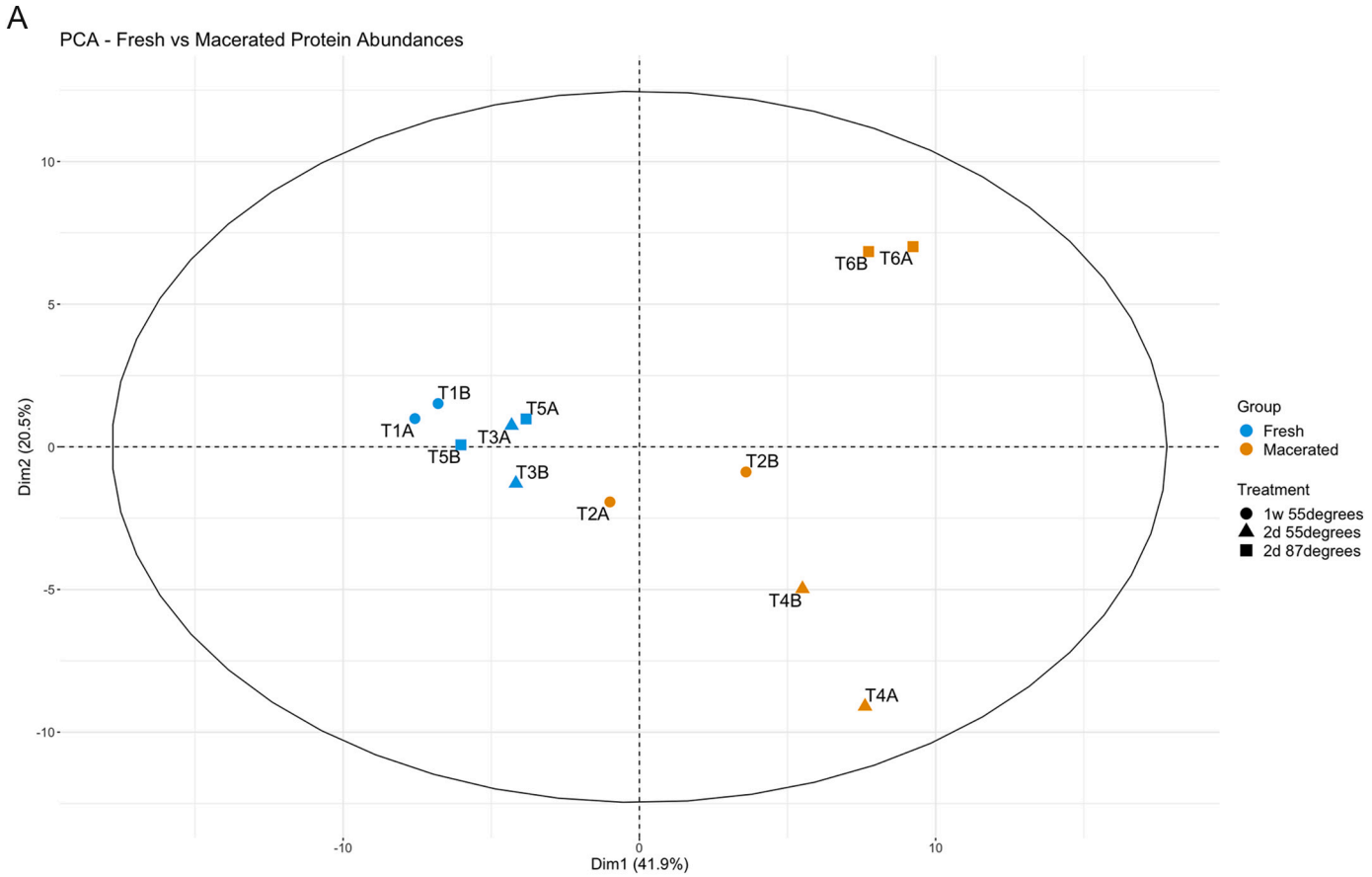

Fig. 2. (A) Principal Component Analysis (PCA) of 97 proteins found within the Macerated *versus* Fresh experiment subgroup for each of the bovid skeletal tibiae specimens. Axes 1 and 2 explain 62.4% of the variance.
(B) Heatmap with Euclidean hierarchical clustering between experiment 1 subgroups 'Fresh' and 'Macerated' with additional annotations specifying the maceration protocols for each macerated sample and associated fresh sample. The larger differences are driven by the maceration protocol as opposed to the individual. Further labels are for the individual Bovids; B1 = Bovid 1, B2 = Bovid 2 and B3 = Bovid 3.

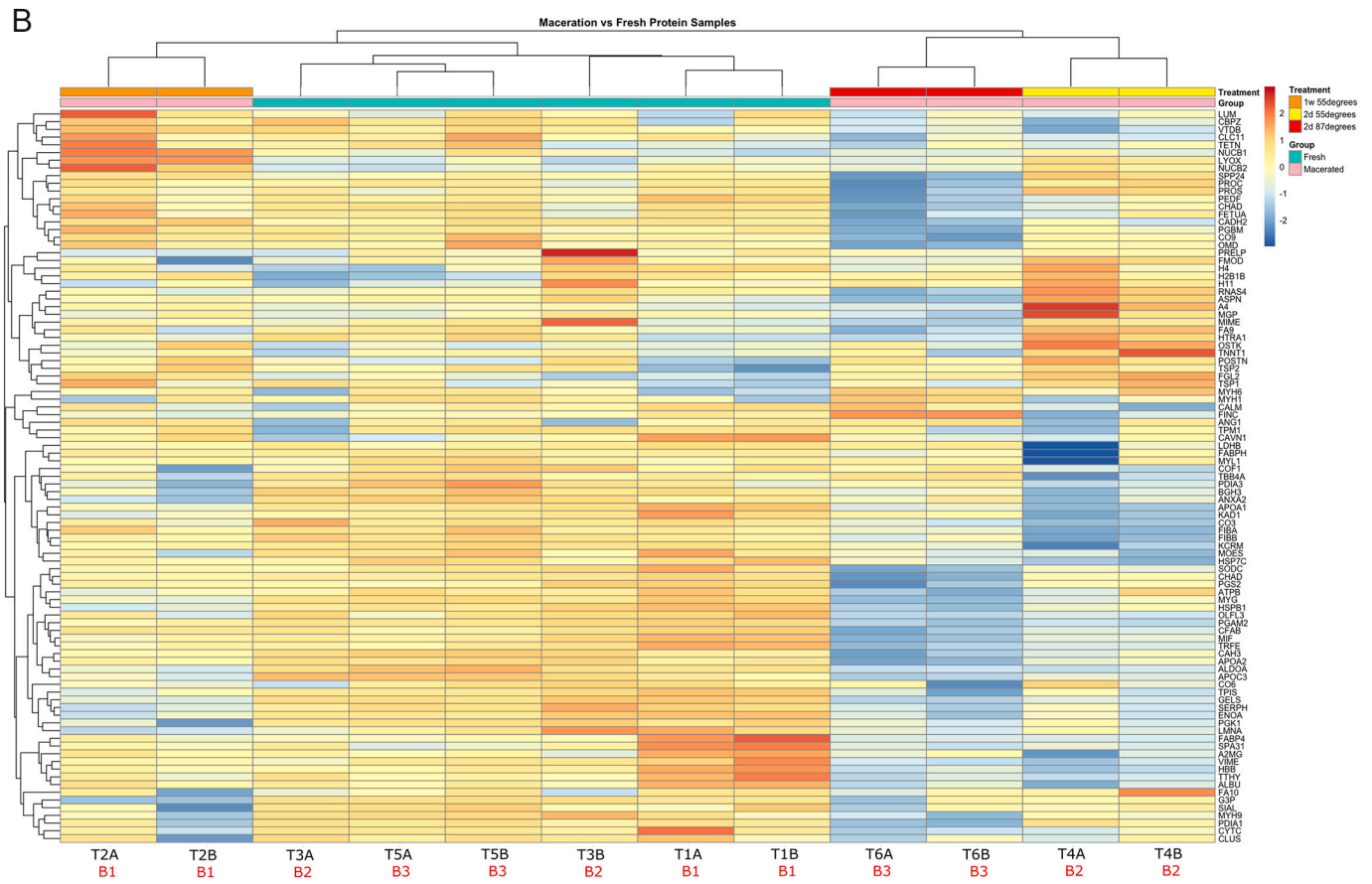

Fig. 2. (*continued*).

Stage 4 consisted of data visualisation, prior to any further processing and filtration, to outline the breadth of the identified proteins and peptides in their experimental groups of interest. Within this, the abundance of the identified proteins of the fresh specimen samples were normalised against the most abundant protein (COL1A1) shared across the samples; this is to establish if there is any inter-individual variability between the samples that may contribute towards differences in the experiment.

Stage 5 involved the first 'screening' process conducted on the identified peptides following the determination of their modification ratios. Specifically, this involved testing the intra-skeletal stability of peptides by comparing the peptide ratios between the biological replicates (*i.e.,* A and B) of each bone in the experiment groups (Tables S1–4). An exclusion 'cut-off' was determined (+/− 5%), where those peptides that were found to have a greater difference between their biological replicates would be removed. Thus, remaining candidates were deemed to be unaffected by maceration in the context of intra-skeletal variability. Lastly, in stage 6, peptide modifications were investigated in these remaining peptides (those with low intra-skeletal variability). Empirically determined cut-offs were used to group peptides of the "MF" experiment group into "stable" and "unstable" groups, dependent on the extent of changes induced by maceration observed. Each empirical threshold chosen on the basis of the observed distribution of the peptide percentage changes is described in the text below.

3. Results

3.1. Multivariate analysis of protein abundance and peptide PTM ratios

The 12 LC-MS/MS runs overall allowed for the identification of 108 proteins and 14,696 spectra after applying the refinements and filtration steps detailed in Materials and Methods. The three Progenesis experiment groupings established for the study ("MF", "M" and "F", see Materials and Methods) have been applied to the calculated protein relative abundance data and has been visualized through multivariate data analyses (Fig. 2A-B, Figs. S1–4). Principal component analysis (PCA) for protein abundance of group MF differentiates the macerated and fresh groups (Fig. 2A), with macerated bone samples showing greater variability compared to fresh; 97 proteins were identified for the bovid skeletal tibiae specimens in this experiment group. The heatmap shown in Fig. 2B establishes that fresh bone can be distinguished from macerated by its proteome. Within the macerated samples, treatment type additionally distinguishes macerated samples (Fig. 2A-B, Figs. S1–4). The PCA plot in Fig. 2A shows clear separation between fresh specimens and specimens treated with maceration protocols along the first principal component, however the heatmaps in Fig. 2B shows evidence of heterogeneity between specimens in their response to maceration; samples 2A/B were more similar to fresh specimens than macerated specimens from samples 1 and 3.

Proteins in the macerated samples have in general a lower relative abundance than in the fresh samples; the greatest variation in abundance and lowest relative abundance compared to fresh samples can be observed in the protocol #3 (2 days at 87 °C) group. To take into account inter-individual variability between the protein abundances, all protein abundances were normalised against COL1A1 (the most abundant protein amongst all the samples). After normalisation, the results did not change, meaning that the observed changes in protein abundances were driven by maceration-dependent processes and were greater than the existing inter-individual variability (Figs. S5–7).

PCA was then performed using the calculated PTM ratios for each identified peptide in the experiment groups; 55 uniquely modified peptides were found for the bovid skeletal tibiae specimens in the "MF"

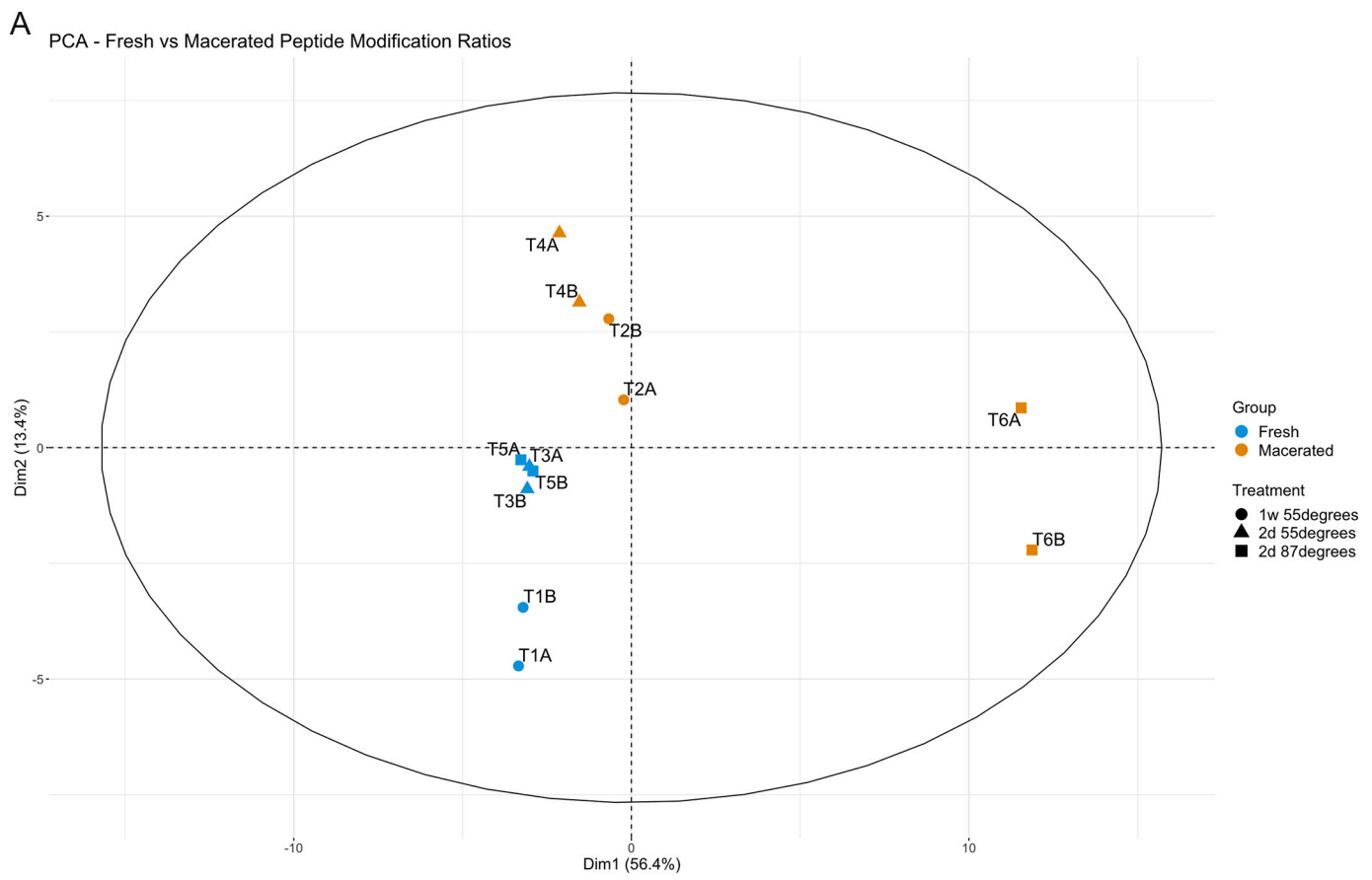

Fig. 3. (A) Principal Component Analysis of 55 peptides found within the Macerated *versus* Fresh experiment subgroup for each of the bovid skeletal tibiae specimens that have had PTM to a specific amino acid residue. Axes 1 and 2 explain 69.8% of the variance.
(B) Heatmap with Euclidean hierarchical clustering between experiment 1 subgroups 'Fresh' and 'Macerated' with additional annotations specifying the maceration protocols for each macerated sample and associated fresh sample. The larger differences are driven by the maceration protocol as opposed to the individual. Further labels are for the individual Bovids; B1 = Bovid 1, B2 = Bovid 2 and B3 = Bovid 3. The units are percentage (%) Deamidated residues are highlighted green and Oxidated residues are highlighted purple on the right labelled y-axis of the heatmap for the peptide names. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiment group (Fig. 3A and Figs. S8–9). Separation between the macerated and fresh groups are still clear at PTMs level (Fig. 3B) and this is reflected in the generated heatmap (Fig. 3B and Figs. S10–11) specific to protocol #3 (2 days at 87 °C). Greater inter-sample variability can be seen in the macerated samples; intra-individual variations observed at peptide level are smaller than those identified between fresh and macerated samples.

3.2. Intra-skeletal sample variability

The stability of the peptides in this context were determined based on their original intra-skeletal variability between sample replicates as outlined in the methods (Stage 5, Tables S4), 5% empirical threshold used); of the 55 uniquely modified peptides, 35 were excluded from further analyses due to their high intra-skeletal variability (>5% difference between A and B) and 20 were used for the remaining stages of analyses highlighted in Materials and Methods as they did not show intra-skeletal variability (<5% difference between A and B, $n = 20$ all belonging to the "MF" group). The following steps were aimed at investigating the impact of maceration on the percentage differences in PTMs ratios between macerated and fresh bones. Of the excluded 35 peptides, 20 were found to be highly variable between the sample replicates (A and B) only after maceration (Table 2). On the contrary, the other 15 showed high levels of intra-skeletal variation in fresh samples but not after the maceration protocols. The observed distribution of these peptide percentage changes (as outlined in Materials and Methods) determined an empirical cut-off (>20% with increased or decreasing percentage change) used to further group the peptides based on their observed stability (Stage 6).

3.3. Inter-group post-translational modification ratio differences

The estimated "unstable" peptides part of the "MF" experiment group are EEAESTLQSFRQDVDNASLAR, NNFPVLAANSFR, and QVQDQTEKELFESYIEGR (highlighted red in Table 3); they exceeded the established 20% cut-off (visualisation shown in supplementary Figs. S12-S17) and were altered largely in the 2 days 87 °C (protocol #3) group. These peptides had their ratios compared to their relative abundance to investigate whether there was a link between increased or decreased PTM with an increased or decreased abundance.

All three of the selected peptides showed a similar pattern of increased deamidation ratios and decreased relative abundances, showing an inversely proportionate relationship between them. This observed relationship also can be found amongst the majority (14/20; 70%) of peptides, whose inter-group variability did not exceed the cut-off. Other observations to note were the opposite relationship with an increase in abundance and decrease in deamidation/oxidation (5/20; 25% of peptides).

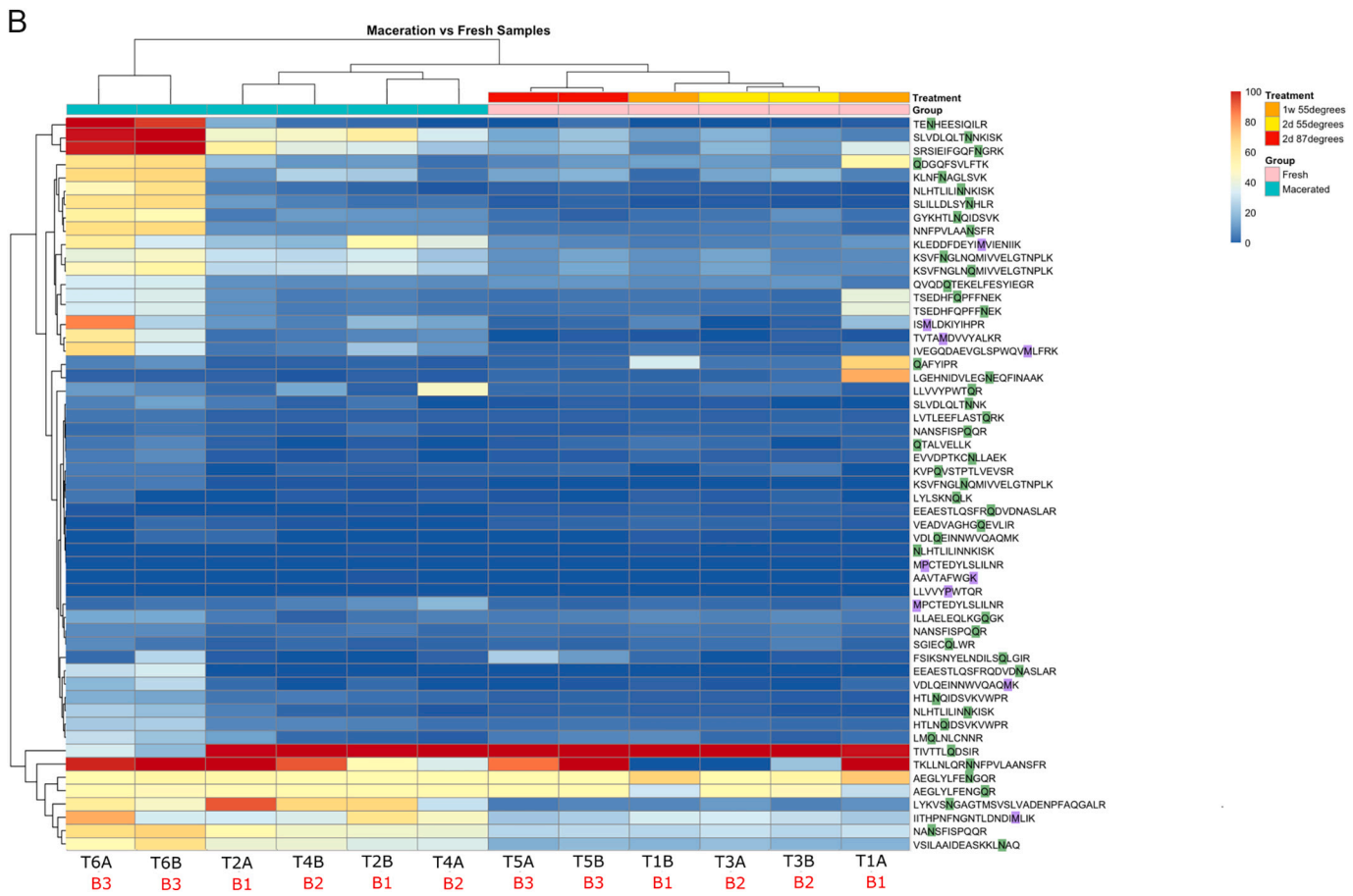

Fig. 3. (*continued*).

Table 2
List of peptides found to be "stable" in fresh specimens, but highly variable in macerated samples between sample replicates. Amino acid residues shown in bold denote the modified amino acid.

Peptides	PTM State	Protein accession
MPCTEDYLSLILNR	Oxidation	ALBU
VSILAAIDEASKKL**N**AQ	Deamidation	APOA1
KLEDDFDEYI**M**VIENIIK	Oxidation	CO3
KLNF**N**AGLSVK	Deamidation	CO9
SLILLDLSY**N**HLR	Deamidation	FMOD
LYKVS**N**GAGTMSVSLVADENPFAQGALR	Deamidation	GELS
TVTA**M**DVVYALKR	Oxidation	H4
LLVVYPWT**Q**R	Deamidation	HBB
SLVDLQLT**N**NK	Deamidation	LUM
NA**N**SFISPQQR	Deamidation	MGP
VEADVAGHG**Q**EVLIR	Deamidation	MYG
LM**Q**LNLCNNR	Deamidation	OMD
VDL**Q**EINNWVQAQMK	Deamidation	PEDF
VDLQEINNWVQAQ**M**K	Oxidation	PEDF
LYLSKN**Q**LK	Deamidation	PGS2
NLHTLILIN**N**KISK	Deamidation	PGS2
NLHTLILI**N**NKISK	Deamidation	PGS2
IVEGQDAEVGLSPWQV**M**LFRK	Oxidation	THRB
TIVTTL**Q**DSIR	Deamidation	TSP1
ILLAELEQLKG**Q**GK	Deamidation	VIME

Lastly, in consideration of inter-individual stability, none of the PTM ratios for the fresh samples (experiment group "F") showed any larger differences between each of the individuals (M and F separate summary tables are shown in supplementary Table S6). This supports the normalisation that was conducted at the protein level previously, therefore, inter-individual variation is minimal and may not contribute towards the observed changes in the macerated samples.

3.4. STRING networks

This study resulted in the characterisation of three main groups of proteins (and related peptides): those that are "unstable" due to intra-skeletal variability potentially as a result of the maceration process (n = 17 proteins and 20 peptides), those which are thermally "unstable" due to the maceration treatment inducing PTMs (n = 3 proteins and 3 peptides), and those that are broadly "stable" both at intra-skeletal and inter-individual level, also after having received different maceration treatments (n = 7 proteins and 17 peptides). These "stable" proteins, therefore, are those that may be considered as "stable" taphonomic biomarkers for future investigations.

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database network analysis [43] was used to visualize protein networks between "stable" and "unstable" proteins when exclusively considering the intra-skeletal variability. To do so, STRING was used to represent the list of proteins whose peptides showed intra-skeletal variations only after the maceration treatments (Table 2) and the list of proteins whose peptides did not show any intra-skeletal variation also after maceration (Table 3). Proteins were then manually arranged to represent groups of proteins that have peptides in both lists (ALBU, HBB, MGP, PGS2, THRB and VIME), proteins that have highly variable peptides in macerated samples only (APOA1; CO3; CO9; FMOD; GELS; H4; LUM; MYG; OMD; PEDF; TSP1); and proteins that have peptides that are low in variability in both fresh and macerated samples (CLC11; FETUA; NUCB1; CHAD) (Fig. 4)

STRING was also used to represent interactions amongst the proteins whose peptides showed either an increase or a reduction >20% in their

Table 3
Macerated *VS* Fresh ("MF") Experiment Group; mean percentage differences between fresh and macerated samples for each peptide in each subgroup. Tables for "M" and "F" separate experiment group are in Supplementary Tables S5–6). The modified amino acid residues are in bold in the peptide sequences. The values in bold are highlighted due to them passing the empirical cut-off threshold selected.

Peptide	PTM State	Protein Accession	1w 55 °C (Protocol #1) Fresh *vs* macerated % change	2d 55 °C (Protocol #2) Fresh *vs* macerated % change	2d 87 °C (Protocol #3) Fresh *vs* macerated % change
KVP**Q**VSTPTLVEVSR	Deamidation	ALBU	1.75	−2.26	3.75
QTALVELLK	Deamidation	ALBU	−2.51	−2.82	4.82
M**P**CTEDYLSLILNR	Oxidation	ALBU	0.02	0.11	0.44
NNFPVLAA**N**SFR	Deamidation	CHAD	6.24	2.97	**57.42**
AEGLYLFE**N**GQR	Deamidation	CLC11	17.61	1.67	−1.40
AEGLYLFENG**Q**R	Deamidation	CLC11	−17.61	−1.67	1.40
HTL**N**QIDSVKVWPR	Deamidation	FETUA	4.12	3.86	13.52
HTLN**Q**IDSVKVWPR	Deamidation	FETUA	4.06	2.86	19.14
EVVDPTKC**N**LLAEK	Deamidation	FETUA	−1.27	−2.72	7.19
AAVTAFWG**K**	Oxidation	HBB	0.32	0.24	0.00
LLVVY**P**WTQR	Oxidation	HBB	0.06	−0.14	−0.25
NANSFISP**Q**QR	Deamidation	MGP	−0.48	−2.93	4.40
NANSFISPQ**Q**R	Deamidation	MGP	1.26	−2.20	3.99
LVTLEEFLAST**Q**RK	Deamidation	NUCB1	−0.46	−1.00	3.70
KSVFNGL**N**QMIVVELGTNPLK	Deamidation	PGS2	0.75	1.06	7.53
NLHTLILINNKISK	Deamidation	PGS2	−0.20	−0.33	−0.55
QVQD**Q**TEKELFESYIEGR	Deamidation	THRB	2.62	−1.51	**20.49**
SGIEC**Q**LWR	Deamidation	THRB	−2.08	−3.30	4.36
EEAESTLQSFR**Q**DVDNASLAR	Deamidation	VIME	−2.83	−2.95	−1.42
EEAESTLQSFRQDVD**N**ASLAR	Deamidation	VIME	0.32	0.24	**32.74**

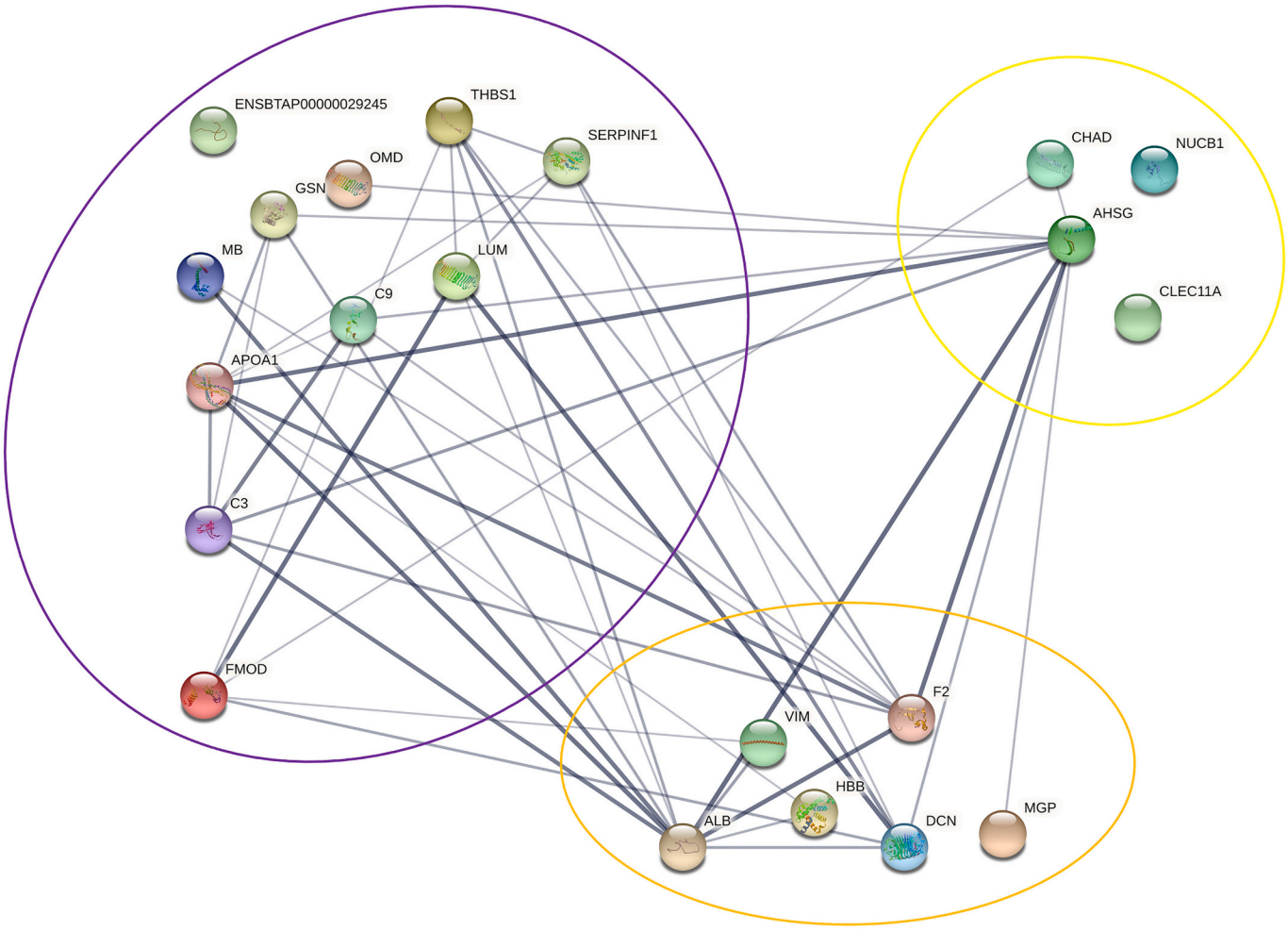

Fig. 4. STRING network analysis identifies three groups of proteins. Group one (purple ellipse) was characterised by peptides that increased in variability between sample replicates after maceration. The ellipses were manually added to highlight the groups; group two (yellow ellipse) was characterised by peptides that did not increase in variability between the sample replicates after maceration. Group three (orange ellipse) proteins exhibited both phenomena. Proteins with strong interactions between each other are shown by the thickness in lines indicating confidence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

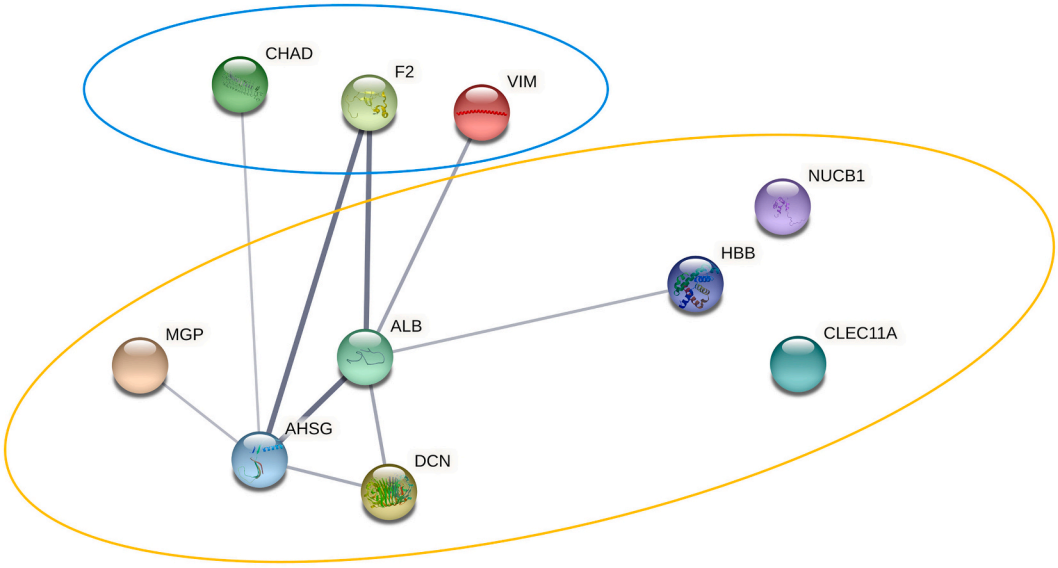

Fig. 5. String network analysis of the group of proteins in Table 2. Group one (blue ellipse) are those that have peptides that have exhibited a greater change in PTM between the fresh and macerated samples. The ellipses were manually added to highlight the groups; group two (orange ellipse) are those that did not exhibit a greater change in PTM between fresh and macerated samples. Proteins with strong interactions between each other are shown by the thickness in lines indicating confidence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Summary table of the identified proteins of the peptides that were processed through the screening methods outlined in methods and results. There are no proteins that were considered "partially stable" for inter-group variability due to the method not allowing for that. The proteins in bold are those that were found to have literature evidence of apparent thermal instability. They are divided into two sections based on the screening context, and grouped based on the stability level.

Screening context	Group 1 – Unstable	Group 2 - Partial Stability	Group 3 - Stable
Stage 5: Intra-skeletal variability	**GELS** **APOA1** **MYG** FMOD LUM OMD TSP-1 PEDF H4 CO3 CO9	**HBB** VIME ALBU MGP THRB PGS2	CHAD CLC11 FETUA NUCB1
Stage 6: Inter-group variability (treatment condition)	VIME THRB CHAD	N/A	NUCB1 MGP PGS2 ALBU THRB FETUA CLC11

PTMs when compared with the non-macerated ones (Stage 6) (Fig. 5). Of these proteins, two groups could be established, those that had peptides that exhibited the >20% increase or reduction, and those who did not.

The proteins of interest summarised from the above analyses can be found in Table 4 which indicate which proteins were considered "stable", "partially stable" and "unstable" for each of the analysis contexts (*i.e.*, intra-skeletal variability and inter-group variability (treatment condition)).

4. Discussion

The primary focus of this study was to investigate the impact of maceration on the bone proteome and its effects on protein abundance and PTMs. Results obtained from studies focused on estimating age-at-death and post-mortem interval by analysing bone proteomics data may be confounded due to differing maceration protocols employed in HTFs, and their effects on potential protein biomarkers. Therefore, it is of paramount importance to understand how proteins and peptides are affected by these procedures, as well as to identify any "stable" proteins that may still assist in the estimation of PMI and AAD when bones are experimentally macerated. This pilot experiment was conducted to investigate the potential impact maceration might have on the skeletal remains that are present in HTF collections for future molecular investigations. Largely, this investigation has shed light on the matter, allowing us to identify which peptides and proteins were at risk of larger changes as a result of the maceration procedures, as well as which ones are robust. Despite the limitations of this study, it has been an informative investigative process that provides a foundation for future investigations in this area.

Prior to any of the filtration conducted on the protein abundances and peptide PTM ratios, the data was visualized through a multivariate supervised analysis, specifically heatmaps and principal component analyses (Figs. 2 and 3). The hypothesis that the maceration procedure had an impact on the bone proteome was first supported by the results of the PCAs, where the differences seen amongst the maceration protocols is clear, specifically for protocol #3 for both the normalised protein abundances and the peptide PTM ratios. This is reflected by the dissimilarity between groups shown in the heatmaps.

The heatmaps gave a more complex but comprehensive look into the relative abundances and ratio changes. It is clear from the heatmaps that maceration induces changes in the proteome of treated bones when compared with non-macerated ones, and this is qualitatively more obvious for the protocol #3, specifically for the proteins outlined above in Table 4 as either being "stable", "partially stable" or "unstable". The fact that maceration induces alteration in the proteome is also reflected in the peptide PTM ratio heatmap, where some effects such as intra-skeletal variability amongst sample replicates can be seen in association with specific maceration treatments.

4.1. Impact of maceration on bone proteins

The proteins identified in this study have been evaluated both in

terms of their intra-skeletal and inter-individual stability prior to any maceration, and in terms of thermal stability by looking at PTMs ratios between different protocols.

Amongst the list of proteins we identified here as being either "unstable", "partially stable" and "stable" (Table 4), there is no literature specifically aimed at addressing their persistence and stability in any maceration study, however, their function and roles can be investigated with regard to their thermal stability. Moreover, with this study it was possible to investigate the potential stability of bone proteins previously identified as AAD/PMI biomarkers.

4.1.1. Intra-skeletal variability

Considering the intra-skeletal variability, between the identified "unstable" proteins (Table 4, Stage 5, Group 1), only gelsolin (GELS), myoglobin (MYG) and apolipoprotein A-I (APOA1) have shown any correlation in the past with thermal instability. GELS is an actin-regulatory protein primarily involved in calcium ion binding and regulation; the calcium concentration needed for the activation of this protein can depend on temperature, however, this is not specific to skeletal remains or a hydrous environment [44]. MYG is highly abundant in skeletal muscle tissue mainly and expressed in several others; it is key in muscular tissue oxygen movement as a metal ion and oxygen binder [45]. Its thermal denaturation has been investigated in culinary sciences with regard to colour change of meats when cooked [46–49]. Higher temperatures and extreme pH can encourage denaturation; structural changes can be reversible up to an approximate temperature of above 75 °C; helices within this protein were found to be largely destroyed above this range (as expected with most proteins), however, this was not investigated in bone specifically [50]. APOA1 is a plasma-based protein that participates in transporting cholesterol from tissues to the liver for excretion. It has been identified as a binder to heat-shock proteins *i.e.,* its established binding function of a protein that has been synthesised or activated in response to stressful conditions such as damage or physiological responses to external stimuli [51].

Regarding the other proteins identified here as being thermally "unstable", it was possible to group them based on either sharing similar functions or belonging to the same family. Thrombospondin-1 (TSP-1) and pigment epithelium-derived factor (PEDF) are part of the glycoprotein family, both have a diverse range of their multi-functional presence in different tissues; they can be found in skeletal remains, however, they can be considered ubiquitous proteins (further information can be found in Table S7). Fibromodulin (FMOD), lumican (LUM) and osteomodulin (OMD) are part of the small leucine-rich proteoglycan (SLRP) family; all of them have key functions in the bone matrix specifically and contribute towards bone formation and remodelling in different roles; however, fibromodulin and lumican can be found in other tissues (Table S7). Histone (H4) and the complement proteins CO3 and CO9 are found in multiple tissues and considered to be ubiquitous also in function, the understanding of why they are "unstable" in this pilot context is not clear based on functions alone (Table S7).

Amongst all the proteins mentioned here, seven have been highlighted in previous investigations because of their connection with either estimation of AAD or of PMI. APOA1 has shown a decrease in abundance with age in male and female rats from 1 week – 1.5 yrs. [52]. CO3 and CO9 complement proteins have exhibited a negative relationship with change in abundance and PMI in human iliac samples [53]. FMOD has shown a negative correlation with PMI, whereas OMD has shown a significant correlation with the type of burial environment in which the cadaver is placed in [54]. However, in terms of PTM ratios, OMD has previously shown a weakly positive increase in deamidation ratio with AAD only [54]. LUM also showed a positive correlation with AAD and its deamidation ratio; both showed no significant change in abundance in the same human skeletal samples [54]. Lastly, in the same study, PEDF exhibited a negative correlation with its abundance and AAD in the human skeletal samples [54].

Between the proteins considered to be "partially stable" due to the presence of some "stable" and some "unstable" peptides (Table 4, Stage 5, Group 2), hemoglobin subunit beta (HBB) has been previously identified as being a thermally labile protein. This is a globular protein that can be found in bone marrow and blood, it mainly transports oxygen from the lung to peripheral tissues as its main role [55]. Bovine hemoglobin (bHb) has been found to withstand temperatures up to 50 °C without irreversible conformation changes [56]. The maceration temperature ranges investigated here are all above 50 °C, therefore, it is possible that the HBB we found in this study may have undergone conformational changes and degradation as a result of the temperature, and this may be the cause of the reduced relative abundance observed in bones treated with protocol #3.

In addition to HBB, also albumin (ALBU), matrix gla protein (MGP), decorin (PGS2) and prothrombin (THRB) were identified here as being "partially stable", despite there being no evidence in the literature to support their potential thermal instability. All of these have key roles in the bone matrix, however, ALBU and THRB are highly abundant proteins also across multiple tissues, despite their key roles in skeletal tissues (Table S7).

These proteins (besides THRB) have been investigated as potential biomarkers for AAD and PMI in taphonomic investigations; for example, there have been several instances where ALBU has been associated with AAD. In serum specific investigations, ALBU has decreased in abundance with chronological age [57,58]. Notably, its abundance in human bones has increased for fresh iliac samples but has decreased over PMI periods ranging from 219 up to 872 dayfor skeletonised iliac samples [53]. This supports the negative correlation between its abundance and PMI found in human skeletal remains sampled from a cemetery[54]. The decrease in abundance with an increasing PMI has also been observed in porcine skeletal remains in further support of this pattern [60]. PGS2 and MGP have additionally shown decreasing abundances with increasing PMI, PGS2 only being found to decrease so far in human specimens, and MGP decreasing in human and porcine remains [53,60]; finally, HBB did decrease in abundance over 6 months PMI range in porcine remains [60].

Finally, we identified a group of "stable" proteins (Table 4, Stage 5, Group 3). None of these proteins have been previously investigated with regards to thermal stability, but they have shown similarities in terms of function and role. Chondroadherin (CHAD) has been identified mostly in cartilage, as well as bone tissue as a major cartilage matrix protein. In mice, C-type lectin domain family 11A (CLC11) has shown evidence of promoting osteogenesis, ossification and mediating carbohydrate binding [61,62]. Nucleobindin-1 (NUCB1) is a major calcium-binding protein with links being a potential modulator of matrix maturation in bone mineralization [63]. Fetuin-A (FETUA) has strong links to ossification, bone mineralization and is largely abundant in the liver, serum and bone [41,64–66].

All of these proteins have been investigated as a suitable potential AAD or PMI biomarker in taphonomic studies; CHAD and CLC11 only have correlated with PMI over several years (1–37) with their abundance, with the former having a weak positive correlation and the latter having a negative correlation [54]. CHAD has also been found in younger ancient bone proteome samples compared to older, however, these are from bone samples that range from 4000 to 1.5 million years old, so the comparison is not applicable here [59]. NUCB1 and FETUA both have shown correlation with AAD and PMI; NUCB1 strictly has exhibited a negative correlation with abundance and PMI in porcine and human skeletal remains [54,41]; Whereas, FETUA in most cases has shown a negative association with AAD in terms of its abundance with one case of it showing an increase in younger porcine skeletal samples [41,53,68–69] in the case of forensic human skeletal samples with a large PMI (1–37 years) and age range (29–85), FETUA has shown negative correlations with its abundance [54].

4.1.2. Inter-group variability (treatment condition)

At this stage, we were interested in proteins that indicate they may

be susceptible to the maceration procedures in this pilot, and specifically how these procedures may have induced a difference in PTMs such as oxidation or deamidation between sample replicates (Table 4, Stage 6, Group 1), as it has been acknowledged that temperature is a risk in optimising the bone protein extraction procedure for inducing such changes in Procopio and Buckley [9]. Three peptides, belonging to the proteins VIME (Vimentin), CHAD and THRB exhibited this larger observed change.

The results showed that the changes they exhibited were inversely correlated with their peptide abundance, meaning as there was an increase in deamidation, there was a decrease in peptide abundance. This is supported by previous studies that outline the deamidation of asparagine and glutamine in bone peptides are an indicator of protein degradation [41,60,70]. This phenomenon has only been investigated in terms of PMI and not directly in the context of post-mortem cleaning methods such as maceration. As it has been established that CHAD is an abundant non-collagenous protein in the bone matrix, and has been investigated as a suitable PMI biomarker, it should now be noted as a maceration risk if used in future taphonomic investigations with knowingly macerated remains.

It should be noted that the samples used in this study are not only non-human, but also fresh. Additionally, the sample size in this pilot is small and therefore restrictive on the data analysis that can be conducted. Normally, human skeletal remains are collected after extended periods of time (*e.g.,* post decomposition) at human taphonomy facilities (HTFs), therefore, the effect of maceration procedures on these remains may be even more exacerbated and should be considered together with bone taphonomic alterations.

The differences between the "unstable" proteins (VIME, CHAD and THRB) based on inter-group variability and "stable" ones (NUCB1, MGP, PGS2, ALBU, THRB, FETUA and CLC11) are largely their functional differences; those in the stable list (Table 4, Stage 6, Group 2) are found in multiple tissues with an array of roles; not all of them are highly abundant in bone and have a key role in it, therefore, the reason behind their stability may not be biased towards a specific tissue or functional role. Their presence in the bone tissue specifically may offer them protection as non-collagenous proteins protected by the mineral component, however, this hypothesis would require validation by investigating how these proteins are impacted by maceration in other tissue types. All these proteins, besides THRB, have been investigated as potential PMI and AAD biomarkers in taphonomic investigations, the implication here is they are considered to be relatively "stable" to the maceration procedures in this pilot, therefore, they may still be characterised in future investigations with the consideration that they may not be overly impacted by maceration procedures.

Understanding why the peptides are behaving as they are might be down to what makes these peptides different to each other, as opposed to what makes their parent protein different to each other in macerated and fresh remains. So far, we have understood that it may not be purely based on functions and roles of the parent proteins for what determines stability; it has been established that the thermal decomposition of a protein can depend on the primary structure (sequential arrangement of amino acids (AAs), secondary structure (polypeptide chain folding) and amino acid composition [71]. Therefore, further investigation into these peptide-level differences is required.

These peptides have been focused on PTMs specifically, largely, there have been differences outlined between the PTMs themselves and their recognised importance in terms of protein degradation; asparagine (N) deamidation has been more useful in terms of molecular clocks for biological ageing in living tissues, whereas glutamine (Q) deamidation has been more useful for more ancient remains [72–74]. This is highlighted here due to the recognised importance of asparagine deamidation being potentially more important in terms of estimating AAD of more modern or forensic remains; therefore, it begs the question on whether this may be more susceptible to other post-mortem influences than glutamine residues. Oxidation of methionine, lysine and proline have not been compared to or investigated in this context; however, the same hypothesis can also be established.

Notable differences between the amino acid residues (AAR) in the peptide sequences were identified for those whose stability was based on intra-skeletal variability. Differences in which AAR was oxidated between the high intra-skeletal variability and low intra-skeletal variability peptides were found; in the former, methionine was only oxidated, in the latter, only lysine and proline were oxidated. There was not a striking difference in deamidation though for these groups. Regardless, this is still something that should be noted and considered when planning future work.

5. Conclusions

We have demonstrated that this pilot investigation has identified a problem with the integrity of the bone proteome after maceration. This study indeed has demonstrated how harmful these cleaning methods used by HTFs and medicolegal centres are on human remains, impacting biomolecular forensic research. Although it was the protocol with the highest temperature (2 days 87 °C) that cause the most dissimilarity to fresh remains, these maceration protocols are only a small sample of the diversity of techniques that are utilised in HTFs and academic taphonomic investigations. The current personalisation of these protocols that are based on how much the bones need to be degreased, changed aesthetically or for efficiency, instead should be replaced by a standardised method that minimises biomolecular degradation.

This pilot has shown that there are proteins (and associated peptides) (APOA1, FMOD LUM, OMD, PEDF, CO9, CO3, CHAD, HBB) that are potential candidates for exclusion as taphonomic biomarkers in skeletal remains obtained from different HTFs due to their risk from maceration based on this pilot; APOA1 and HBB are of specific concern due to their previous experimental history of thermal instability. However, there are proteins that can be considered "stable" (NUCB1, MGP, PGS2, ALBU, THRB, FETUA and CLC11), at least in bovid skeletal remains in these maceration conditions. Although sample size is a limiting factor in this pilot experiment, it provides a foundation for future studies aimed at further characterising protein degradation under different maceration conditions in larger studies.

Future investigations can aim to understand if there actually is a reason that glycoproteins and proteoglycans might be more susceptible to intra-skeletal variability than other protein families, or if the high abundance of a certain NCP in the bone tissue being protected by the mineral component contributes towards its stability in terms of no PTM ratio changes. Additionally, it would be interesting to investigate the differences and similarities between these peptides, and on whether specific AAR are being modified or whether the changes are observed across the entire AA sequence.

Overall, it is paramount to try to standardise maceration procedures at HTFs and medicolegal centres in order to minimize the negative impact of these treatments on biomolecules, to allow the obtainment of reliable biomolecular data from human bones. Currently, maceration procedures considerably limit the possibility of using bones from osteological collections at HTFs for accurate biomarkers discovery, and this comes with huge ethical implications as the molecular information obtainable from donated cadavers is irreversibly compromised by these treatments. Results obtained here suggest that a more standardised approach, which includes maceration only in situations where this cannot be avoided by using lower temperatures and shorter submersion times without detergents, should be preferred than most of the current existing methods. We do suggest that further work at HTFs may establish to which extents a "low" temperature ($\leq$ 55 °C) detergent-free maceration procedure, or any other alternative cleaning method, can both be appropriate for post-mortem bone processing as well as for future biomolecular investigations.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE [75] partner repository with the dataset identifier PXD032295 and https://doi.org/10.6019/PXD032295.

Declaration of Competing Interest

The authors declare that they have no conflicts of interests.

Data availability

Data were uploaded on PRIDE and details for accessing them can be found in the 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.jprot.2022.104754.

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