The application of proteomics techniques to the study of ancient human tissues

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Introduction

The taphonomic fragmentation of bone specimens is a factor that affects many archaeological assemblages, with high proportions of morphologically-unidentifiable remains frequently assumed uninformative. Molecular methods for objective species identification have existed for several decades, the most notable of these being DNA analysis. However, DNA analyses in warm environments, such as in the tropics, has long known to be a major detrimental factor resulting in poor success rates and often at high cost. Protein analyses on the other hand are proving to be a low-cost alternative to identifying the species of remains at archaeological and palaeontological sites. In this presentation we emphasize the potential of collagen fingerprinting using methods called **ZooMS** (**Zoo**archaeology by **M**ass **S**pectrometry) for distinguishing between human and animal bone fragments in application to archaeological sites ranging from the Upper Palaeolithic site of Pin Hole cave (UK) to the Etruscan site of Poggio Civitate dating to ~600 BCE. Here, the neonatal bones are often fragmented, making them difficult to identify and to distinguish from the fragmented bones of other taxa, such as piglets.

More advanced proteome sequencing methods have also been used to study the potential causes for variations in nitrogen stable isotopes observed between different tissues such as skin and bone recovered from remains of the same individual (Finucane 2007; Fig. 1).

It is thought that such variations are simply due to the differences in turnover between each tissue, but if considered solely as collagen-dominated tissues the source of such enrichment remains unclear.

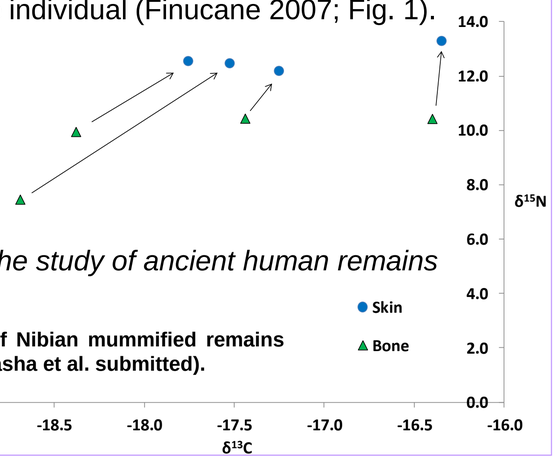


Figure 1 – Stable isotope variation between tissue types of Nibian mummified remains (AD 550-800) from the island of Kulubnarti (modified from Basha et al. submitted).

Aims: to evaluate the application of proteomics to the study of ancient human remains

Methods

ZooMS Collagen Fingerprinting

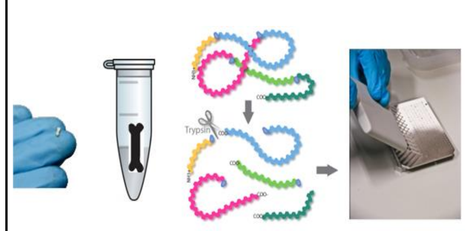
For hundreds of faunal remains from a range of vertebrate fauna collected in the Neotropics, collagen extraction was carried out following previous methods (Buckley et al. 2009). In brief, decalcification with 0.6 M hydrochloric acid (HCl) for ~18 hours (overnight), and centrifuged at 14,000 rpm for 5 min. The supernatant was then discarded, whilst the acid-insoluble residue was gelatinised with 50 mM ammonium bicarbonate (ABC) for 3 hours and then digested with 2 µg sequencing grade trypsin (Promega, UK) at 37ºC overnight. The tryptic digests were then cleaned using C18 ziptips, eluting with 50% acetonitrile/0.1% trifluoroacetic acid (TFA), dried down and resuspended with 10 µL 0.1% (TFA). 1 µL of each sample was then spotted onto a Bruker 384 well Matrix Assisted Laser Desorption Ionization (MALDI) target plate and co-crystalised with 1 µL alpha-cyano hydroxycinnamic acid prior to MALDI analysis. MALDI spectra were acquired on a Bruker Ultraflex II with a Time of Flight (ToF) mass analyser, over an *m/z* range of 700-3700 using up to 2000 laser acquisitions.

In-depth Proteomics

Decalcified samples were extracted with 6 M Guanidine hydrochloride/5 mM Tris-HCl for 18 hours. The solubilised proteins were then applied to a 10 kDa ultrafilter (Vivaspin, UK) and centrifuged, two volumes of 50 mM ABC were also passed through. Once both volumes had filtered through, a further 200 µL ABC was added to the filter, mixed and recovered. This was then incubated with 10 µL 100 mM dithiothreitol (DTT) for 10 min at 60ºC, and once cooled, 40 µL of iodoacetamide was then added to each sample and then stored in the dark at room temperature for 45 min. A further 10 µL 100 mM DTT was added to quench the reaction and the sample digested overnight with analysed by LC–MS/MS using an UltiMate® 3000 Rapid Separation LC coupled to an Orbitrap Elite mass spectrometer. Peptides in the sample were separated on a 75 mm × 250 µm i.d. 1.7 µM Ethylene Bridged Hybrid C18 analytical column using a gradient from 92% A (0.1% formic acid in water) and 8% B (0.1% formic acid in acetonitrile) to 33% B in 44 min at a flow rate of 300 nL min^{-1}. For preliminary quantitative investigations, Progenesis was used for alignments and analysis of combined datasets.

Results

1) Collagen fingerprinting; identifying human remains



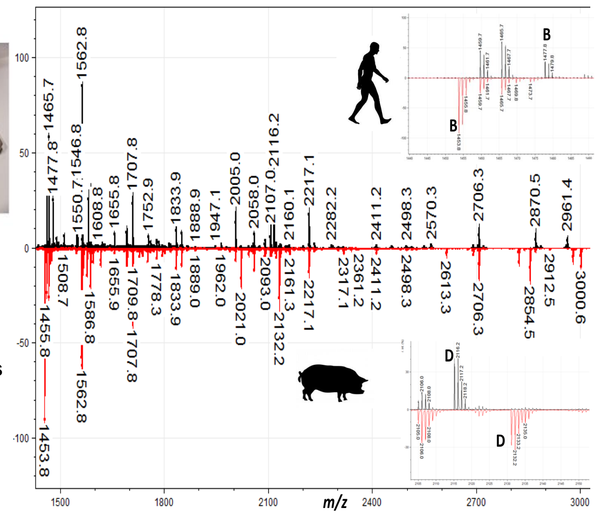


Figure 2 – Workflow and collagen fingerprints from Etruscan samples identified as human and pig using markers B and D from Buckley et al. 2009.

Upper Palaeolithic Pin Hole Cave (UK) – over 11,000 bone fragments were analysed. Many of which (>50%) were clearly microfaunal based on morphology but of uncertain species. Amongst the assemblage many fragments of large mammals and other vertebrate groups (e.g., birds, reptiles, amphibians and fish) identified using fingerprinting but not a single human bone fragment matched.

Etruscan Poggio Civitate (Italy) Only 30 uncertain remains were subject to fingerprinting, of which one could be confidently identified as human from the fingerprinting analyses (Fig. 2). This specimen is one of the three dozen bones of neonatal humans that have been found among the animal remains from this site.

2) Proteomics; differences between skin and bone

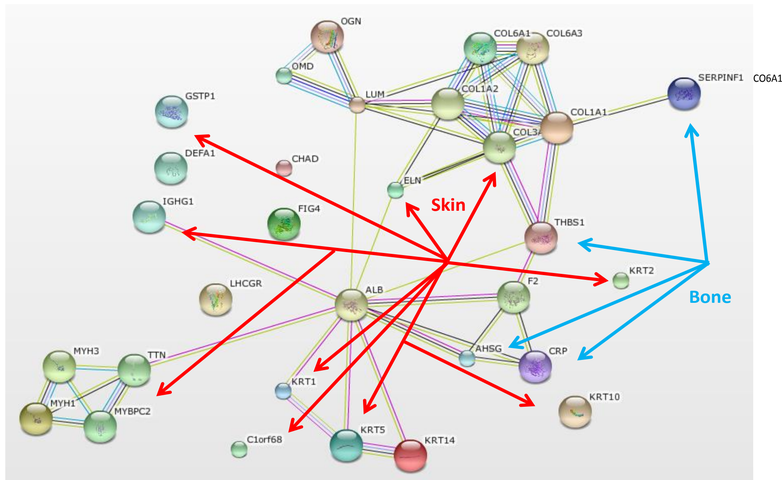


Figure 3 – Network showing functional links between proteins. Arrows indicate proteins more unique to skin (red) or bone (blue).

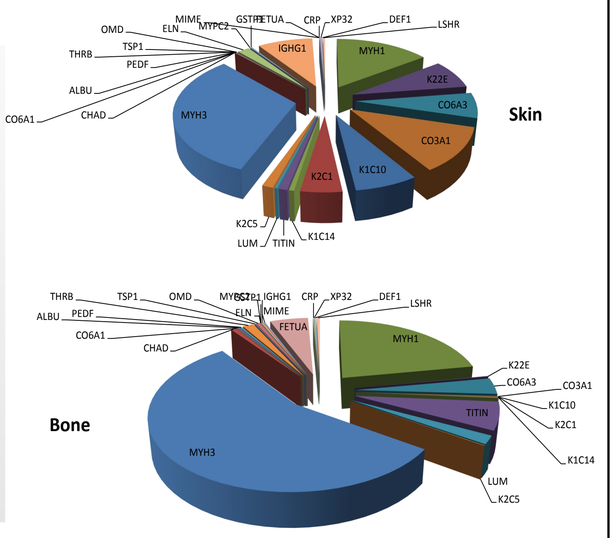


Figure 4 – Pie charts showing relative abundances of proteins in skin and bone (excluding the dominant collagen)

Although the protoemes of the two tissue types were found to be very similar in that they both yielded abundant matches to several collagens (mostly type 1), myosin and minor amounts of several non-collagenous proteins such lumican (Fig. 3) they clearly differed in some components. Skin included substantially more keratins as well as the immunoglobulin protein chain IGHG1 whereas bone had relatively more myosin as well as other serum proteins related to mineral association, e.g., fetuin (Fig. 4).

In order to evaluate the potential effects of deamidation, as a signature of protein decay that results in the loss of side chain nitrogen, the overall relative abundance of these amino acids were evaluated and found to be more abundant in skin than bone (Fig. 5).

Conclusions

Although none of the thousands of Palaeolithic remains screened turned out to derive from human remains, some the ZooMS methodology was more productive in sedentary archaeological sites such as as Poggio Civitate. As the analysts continue to explore the human behaviours and depositional processes that resulted in “stray” bones of human babies being found among the animal debris, ZooMS has been helpful in distinguishing the more heavily fragmented and eroded specimens. This distinction is particularly important in aiding interpretation of the different functional areas of the site, one an “elite” residential building (which has produced few neonatal human bones) and one a workshop (which has produced the majority of the neonatal bones).

The use of proteomics techniques could also be used to aid our understanding of the differences in isotopic results between tissue types, particularly skin and bone, which appears to correlate to the types of proteins that are present in each tissue (e.g., more keratins), and potentially related to the overall levels of additional nitrogen in the side chains of asparagines and glutamines, which are more abundant in the skin proteome than the bone proteomes.

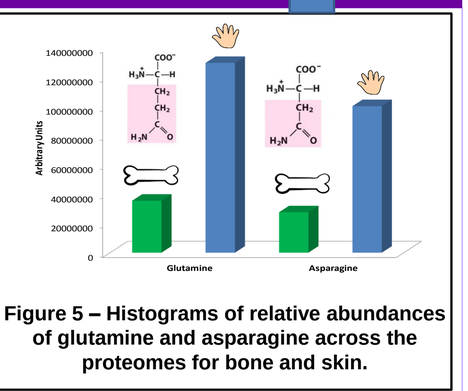


Figure 5 – Histograms of relative abundances of glutamine and asparagine across the proteomes for bone and skin.

References
Basha et al. submitted. Diet Reconstruction Through Stable Isotope Analysis of Ancient Mummified Soft Tissues From Kulubnarti (Sudanese Nubia). *JAS*.
Buckley et al. 2009. Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *RCM*, 23(23), 3843-3854.
Finucane, B.C., 2007. Mummies, maize, and manure: multi-tissue stable isotope analysis of late prehistoric human remains from the Ayacucho Valley, Peru. *JAS* 34: 2115-2124