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Late Middle and Early Late Pleistocene Hominin Systematics

Paleoproteomic Contributions, and Current Limitations, to Understanding Middle and Late Pleistocene Human Evolution

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ABSTRACT

Over the past three decades, a new picture of our own ancestral past has emerged through the elucidation of the complex genetic relationships between Neanderthals, Denisovans, and modern humans by the direct analysis of ancient hominin genomes. These genetic insights have largely been derived from ancient genomes dating to the Late Pleistocene. How preceding and additional contemporary hominin populations fit into their story is partly unknown. It has become clear that Middle and Late Pleistocene hominin populations were highly diverse, however. These hominins were present across Africa and Eurasia, with large portions of the hominin fossil record (far) beyond the reach of ancient DNA research. Paleoproteomic analysis of skeletal proteomes has recently emerged as a potential additional biomolecular approach across the Pleistocene, providing molecular evidence on hominin evolutionary relationships, as well as insights into their behavior through the paleoproteomic analysis of associated zooarchaeological assemblages. Here, we summarize the state-of-the-art of Middle and Late Pleistocene paleoproteomics, and its relevance to refining both our evolutionary as well as our ecological and behavioral understanding of the human past within this chronological window.

INTRODUCTION

The idea that the study of proteins preserved in archaeological specimens holds relevance to understanding the past has a deep history, extending into the first half of the 20th century (Abelson 1954; Boyd and Boyd 1937). Since the turn of the millennium, advances in soft ionization mass spectrometry techniques have driven the adoption of paleoproteomics in archaeological sciences (reviewed fully in Warinner et al. 2022). Meanwhile, the study of modern biomolecules has shown tremendous promise in the context of human evolution. Based on immunological analysis of albumin, a common blood protein, Sarich and Wilson (1967) demonstrated that humans, chimpanzees, and gorillas share a common ancestor and that the evolutionary divergence between these taxa and orangutans was much more recent than estimated based on morphological differences. The subsequent advent of genetic sequencing technologies brought biomolecular research on human evolution into the genetic domain, providing confirmation of the phylogenetic position of humans among great apes (Sibley and Ahlquist 1984), as well as on the origins, divergence, and dispersal of modern humans across the globe (Cann et al. 1987; Vigilant et al. 1991).

In current contexts, the analysis of Pleistocene hominin biomolecules is dominated by that of ancient DNA. Mostly recovered from skeletal and, increasingly, sedimentary resources, ancient hominin DNA has provided unprecedented insights into the population genetic relationships between Neanderthals, Denisovans, and early modern humans (Meyer et al. 2012; Prüfer et al. 2014, 2017), allowed for insights into the social structures in the past (Skov et al. 2022), enhanced insights into hominin behavior (Lalueza-Fox et al. 2011), and by extension further clarified the ecological and environmental conditions in which these hominins operated. Such analyses have been, largely, restricted temporally to the Late Pleistocene, with only a handful of cases reported of exceptional Middle Pleistocene contexts with ancient hominin DNA preservation in skeletal remains or sediments (Brown et al. 2022; Meyer et al. 2016; Posth et al. 2017; Prüfer et al. 2014; Zavala et al. 2021). Ancient DNA has also been recovered from a range of Middle Pleistocene and even Early Pleistocene faunal remains as well as ancient sediments (Dalen et al. 2023). Due to DNA fragility, most Pleistocene ancient DNA has been recovered from temperate or northern latitude archaeological sites (Orlando et al. 2021). As a consequence, there is limited direct molecular evidence from (sub)tropical regions of the world, as well as from older chronological time periods, excluding some regions and/or chronological windows with relevance to understanding human evolution from direct genetic analysis.

Over the past two decades or so, in parallel with the advent of paleogenetics, and fueled by significant changes in protein mass spectrometry instrumentation, the study of ancient proteins has received increasing attention in the archaeological sciences. Some of this research concerns the analysis of the surviving proteins and peptides recovered from Pleistocene time periods. In this chronological period, paleoproteomics research is largely motivated based on the observation that (some) protein fragments survive longer compared to ancient DNA (Demarchi et al. 2022; Rybczynski et al. 2013; Welker et al. 2015), and also by the lower cost of some applications, such as ZooMS (Buckley et al. 2009). Amino acid sequences in proteins are determined by the coding regions of the genome. As such, homologous protein sequences may show amino acid variations reflecting single nucleotide polymorphisms, which, in turn, resulted in changes to the incorporated amino acids. This proteomic sequence information then becomes relevant in phylogenetic research. Furthermore, the presence and absence of peptides belonging to specific proteins can convey meaningful information, for example, in relation to dental enamel sexing, as can the study of differences in whole proteome composition. As outlined below, paleoproteomics applied to human evolutionary scenarios is not without limitations, however, and it currently faces some significant open questions across the range of applications and methodological steps.

PALEOPROTEOMICS IN ZOOARCHAEOLOGY: IMPLICATIONS FOR UNDERSTANDING PLEISTOCENE HOMININ EVOLUTION

Paleoproteomics has become an invaluable tool in zooarchaeology, offering new methods for taxonomic identification and ecological reconstruction. These advances have opened the door to more detailed understandings of hominin interactions with their environments, their dietary practices, and broader behavioral patterns. Below, we discuss the primary paleoproteomic techniques employed in zooarchaeology, with an emphasis on their (potential) applications to human evolutionary studies.

ZOOMS AND SPIN

Being among the most commonly retrieved remains from archaeological sites, faunal skeletal elements represent a rich and complex source of information about past human populations. Not only informative about subsistence, zooarchaeological analyses can grant access to details about cultural practices, ecology, cooking techniques, symbolic behavior, resource acquisition and transport decisions, environmental processes present at the time of deposition, as well as a wealth of subsequent taphonomic processes, among others. However, accessing this information can often become a challenge when the morphological integrity of the remains is highly altered, as is common in Pleistocene contexts. During the past two decades, paleoproteomics has been used principally to overcome the challenges of morphological identification in zooarchaeological studies, primarily through the development of ZooMS and, more recently, SPIN (Smith et al. 2024).

Zooarchaeology by Mass Spectrometry (Buckley et al., 2009), or ZooMS, employs peptide mass fingerprinting (PMF), comparing archaeological samples with known species databases to taxonomically identify bone fragments. Although based on whole-proteome extracts, these PMFs primarily, if not exclusively, contain peptide-level information from collagen type I, the dominant protein in bone and dentine. Using matrix-assisted laser desorption ionization mass spectrometry (MALDI-ToF MS), it allows the identification of hundreds of samples in one analytical session. ZooMS reference libraries of PMFs and their informative peptide marker masses are available for over 300 mammalian species (Xia et al. 2024), as well as a smaller but growing number of fish, reptiles, amphibians, and birds. In general, and depending on the taxonomic context, ZooMS enables taxonomic identifications at the subfamily or genus level in most cases. The applications of ZooMS are widely distributed both in space, with studies representing all continents except for Antarctica, and time, with examples ranging from the Pliocene (Rybczynski et al. 2013) to the Medieval period in the Holocene (Brandt et al. 2018).

In Pleistocene contexts, where skeletal assemblages generally consist of many different taxa, ZooMS has become particularly useful in providing a more accurate taxon representation in faunal assemblages by enabling the identification of bone fragments belonging to taxa that are in low abundance (Sinet-Mathiot et al. 2019), including hominins. These low-abundance taxa might be particularly useful to provide ecological indicators (Welker et al. 2015b), with the specimens themselves generally being suitable for enhanced dietary ecology reconstructions through stable isotope analysis (Jaouen et al. 2019; McCormack et al. 2022; Smith et al. 2024). Beyond the recovery of faunal taxa at low abundance, the integration of ZooMS into Pleistocene zooarchaeological data has focused on exploring faunal exploitation and mobility patterns of Middle and Upper Paleolithic societies (Brown et al. 2021; Pothier Bouchard et al. 2020; Ruebens et al. 2023; Silvestrini et al. 2022; Sinet-Mathiot et al. 2019; 2023; Wang et al. 2023). The comparison of both ZooMS and zooarchaeological data has revealed, at some sites, taxonomic differences between the two components, which have been linked to differences in carcass processing (Sinet-Mathiot et al. 2019; 2023). The integration of ZooMS into faunal studies has also provided new data to evaluate zooarchaeological methodologies, showing that mammal size classifications, a well-established method in zooarchaeology, can be misleading and should be used with caution (Brown et al. 2021; Torres-Iglesias et al. 2024; Sinet-Mathiot et al. 2019, 2023). Taxonomic identification using ZooMS has also made it possible to analyse other aspects of Paleolithic human behavior, such as raw material procurement, revealing strategic selection of certain taxa to produce bone tools and ornaments (Martisius et al. 2020; Pétillon et al. 2019; Talamo et al. 2021).

Regarding paleoenvironmental studies, obtaining proper taxonomic identifications is crucial for ecological reconstructions when inferences are made based on animals' dietary patterns or habitat preferences, including hominins and fauna alike. Therefore, ZooMS has also been used in stable isotope studies to confirm animal taxon identity and thus, to properly interpret isotopic data (McCormack et al. 2022; Reade et al. 2021). Peptide mass fingerprinting has also shown its utility in Pleistocene paleoenvironmental reconstruction by enhancing the identification of micromammals at Klipdrift shelter in South Africa, taxa that are very constrained in terms of ecological niche (Nel et al. 2023).

Despite its widespread applications in a range of contexts, ZooMS has some relevant limitations, especially in certain taxonomic and preservation contexts. Firstly, because it is solely based on the observation of whole peptide masses (MS1-level information), ZooMS does not observe amino acid sequences of those peptides directly. In some cases, peptide marker masses are widely shared among the same family or sub-family. They may contain single amino acid substitutions (SAPs) of isobaric amino acids, or inversions of the same amino acids at two different positions in the same peptide (A…V and V…A for instance). Although the peptide amino acid sequence might therefore differ, the mass of the resulting peptide does not, making it impossible to distinguish between the two. In addition, the use of a selected number of peptide markers, to the exclusion of most peptide information within the PMF, limits the taxonomic resolution obtained through ZooMS. Secondly, a range of studies demonstrate that there are preservation conditions

and timescales within which ZooMS is not routinely successful in providing taxonomic identifications (Jensen et al. 2023; Nel et al. 2023; Peters et al. 2023; Rüther et al. 2022; Wang et al. 2021; Welker et al. 2015a). This makes the large-scale application of ZooMS in such contexts unattractive, although it could still resolve the taxonomic identity of selected specimens of special interest. Thirdly, computational approaches to ZooMS data analysis and sharing are only beginning to be developed (Hickinbotham et al. 2020; Végh and Douka 2024). As a result, ZooMS data analysis takes a disproportionate amount of time.

To circumvent some of the challenges existing with ZooMS, a number of studies have utilized whole-proteome analysis through shotgun proteomics to determine taxonomic identities of (Pleistocene) bone fragments (Engels et al. 2024; Gilbert et al. 2024; Jensen et al. 2020; Le Meillour et al. 2020). Utilising a variety of standardized computational approaches to assign taxonomic identities, they promise enhanced taxonomic specificity compared to ZooMS analysis. In the case of Species by Proteome INvestigation (SPIN), this concerns a novel combination of sample preparation, medium-throughput data acquisition, and computational scripts for taxonomic analysis (Rüther et al. 2022). It presents an LC-MS/MS-based taxonomic identification algorithm coupled to a novel protein aggregation capture (PAC)-based extraction method that combines sequencing-based reliability from conventional LC-MS/MS with the speed and cost-effectiveness of ZooMS. Subsequent data analysis is entirely automated in a set of R scripts. This approach allows taxonomic assignments at a higher taxonomic resolution than ZooMS when reference data is available. However, the proposed SPIN-PAC extraction method, which can be partly automated and therefore achieve higher throughput than manual extraction sessions, has limited potential for highly degraded samples (Mylopotamitaki et al. 2023; Rüther et al. 2022). Hence, optimizing methods of skeletal proteome extraction with poor preservation becomes relevant. In addition, updating reference databases is necessary for any shotgun proteomic approach to skeletal proteome analysis for taxonomic identification, since publicly available databases often contain a subset of the taxa for which ZooMS markers have been studied.

ENAMEL SEXING OF FAUNAL REMAINS

Identifying the biological sex of faunal remains offers valuable insights not only into the ecological dynamics of Pleistocene environments but also into the behaviors and resource-use strategies of hominin populations. By analyzing faunal sex ratios and associated patterns, we can gain a better understanding of hominin hunting practices, seasonal movements, and potentially even social organization. Although initially explored on primates (both human and non-human), the genetic sexing of archaeological and paleontological faunal remains is gaining increased interest in the paleoproteomics community. In mammals, the AMELX and AMELY genes, generally located on the non-recombining parts of the X and Y chromosomes, respectively, encode for slightly different amino acid sequences of the two amelogenin proteins. The protein isoforms of these genes are expressed during amelogenesis, the process by which enamel is formed (Lau et al. 1989). This chromosomal distinction enables researchers to determine genetic sex by identifying the presence of both AMELX and AMELY, indicating a male individual, or only AMELX, suggesting a female individual.

Sex assignment of archaeological remains with ancient DNA methods is already established and makes use of the ratio between the X chromosome and either the Y chromosome, or other chromosomes of comparable length (Bro-Jørgensen et al. 2021; Rey-Iglesia et al. 2024). Genetic sexing has been applied to a range of taxa, from cetaceans to ruminants (Elsner et al. 2016; Macé and Crouau-Roy 2008; Nistelberger et al. 2019), which demonstrates the potential applications of obtaining biological sex information of archaeological materials.

The genetic sexing through paleoproteomic methods is particularly useful in cases where DNA is not well-preserved, but enamel protein fragments remain present, or when large numbers of faunal remains are studied. So far, applications of dental enamel sexing to the paleontological and archaeological faunal records have been scarce, with only a few non-primate species studied, including rhinoceros (Cappellini et al. 2019), extinct mastodon *Notiomastodon* and Pleistocene rodents (Nogueira et al. 2021), equids (Zazueta et al. 2024), mammoths (Rey-Iglesia et al. 2024), and bovines (Berezina et al. 2024; Kotli et al. 2024). Considering it is crucial to provide an accurate reconstruction of the past interactions between humans and their environment, there is no doubt that these first exploratory paleoproteomic amelogenin sexing studies will be followed by more enamel protein sexing of archaeological fauna.

In principle extendable to a whole range of mammals (Warinner et al. 2022), there is, however, not a general understanding for what taxa amelogenin sexing would be feasible in paleoproteomic contexts. Furthermore, so far, confident female biological sex assignments are proving difficult. The primary reason for this is the lower abundance of AMELY compared to AMELX during amelogenesis, levels of AMELY transcripts being roughly 10% of those of AMELX (Santos and Line 2006). In Pleistocene contexts, and assuming that AMELX and AMELY degradation rates are similar, this creates the possibility that AMELY has already degraded to the point where it is no longer observable, while AMELX is still present. This would risk falsely identifying male specimens as female. In addition, for non-primate taxa it is unknown whether all taxa express AMELY during amelogenesis, while for other taxa the amino acid sequences of AMELX and AMELY are identical. This issue requires exploring across taxa before widespread applications are proposed.

The biological sex identification of faunal remains can provide information that would go beyond the strict identification of the biological sex of a single specimen only. It holds potential to unveil hunting practices during the Pleistocene by the molecular identification of males over females, thus disclosing targeted hunting practices (Wein-

stock 2000; Sanz et al. 2019). For many herbivores, herd composition often follows a seasonal pattern including sex-related aspects, often resulting in adult male individuals being disassociated from females during part of the year (Carranza 2007; Fisher 2018; Geist 1998; Grange et al. 2018). Establishing the sex composition of a taxon within a faunal assemblage might therefore inform on whether individuals or whether herds were targeted. In turn, together with additional zooarchaeological information, this could then provide further insights into seasonality as well. Additionally, in the case of personal ornaments made out of dental tissues, uncovering the animal's sex from which personal ornaments were made could provide us with a deeper understanding of the gender, social status, or ethnic affiliation of the holder (D'Errico and Vanhaeren 2002).

HOMININ PROTEOMES

Proteomic screening conducted via ZooMS and SPIN has on numerous occasions resulted in the discovery of hominin specimens, either through large-scale, untargeted analysis or when applied to selected specimens spatially connected to known hominin remains (Balzeau et al. 2020; Brown et al. 2016; 2022; Devièse et al. 2017; Hublin et al. 2020; Lanigan et al. 2020; Mylopotamitaki et al. 2024; Rüther et al. 2022; Slimak et al. 2024; Welker et al. 2016; Xia et al. 2024). Hominins can be considered one of the low abundance taxa present in many Pleistocene skeletal assemblages. These newly discovered hominin specimens are then available for stable isotope dietary analysis, radiocarbon dating, as well as genetic and proteomic analysis, providing a rich and integrated record of biomolecular information related to hominin occupation histories, and have done so particularly across Eurasia in relation to late Neanderthal and early modern human dispersals across the continent. It should be noted, however, that ZooMS and SPIN generally provide proteomic evidence for an attribution to the *Pan* sp./*Homo* sp. clade, or the hominin lineage, without providing further taxonomic and/or population genetic detail.

To move beyond the taxonomic limits imposed by shallow proteomic techniques such as ZooMS and SPIN, full proteome analysis conducted via tandem mass spectrometry (LC-MS/MS) techniques has been explored. Hitherto, such an approach, which aims to identify the largest number of peptides, and by extension proteins, preserved in a skeletal sample, has been employed on a small number of specimens. In this context, ancient hominid proteins have been recovered from a (sub)tropical specimen of *Gigantopithecus blackii*, the largest ape to have ever lived, effectively demonstrating through the partial protein sequences recovered that *Gigantopithecus* is most closely related to the genus *Pongo* (Welker et al. 2019). The availability of a large number of reference genomes, and therefore by extension reference proteomes of great ape individuals is especially relevant, since it allows assessing the power to determine phylogenetic relationships for ancient proteomes confidently (Zazueta et al. 2023).

Among hominins, studies have determined the taxonomic identity and phylogenetic placement of hominin specimens for taxa with reference proteomes available, such as Denisovans (Chen et al. 2019; Xia et al. 2024), as well as for specimens of taxa for which no prior genetic information exist, such as *Paranthropus* (Madupe et al. 2023) and *Homo antecessor* (Welker et al. 2020). This type of analysis relies on the availability of reference proteomes for Neanderthals, Denisovans, and modern humans (Froment et al. 2021; Patramanis et al. 2023), a range of primates including all the great apes (Zazueta et al. 2024), and advances in proteomics bioinformatics that allows the confident retrieval of protein amino acid sequences outside of previously known sequence variation (Welker 2018). The shotgun proteomic analysis of Middle and Late Pleistocene proteomes is suitable for any skeletal tissue, is not restricted to the analysis of collagen type I, and has also been used to clarify the taxonomic status of ambiguous hominin specimens, for example, those that could potentially represent *Pongo* (Bacon et al. 2021).

For hominin taxa where no ancient DNA information is available, placement in phylogenetic trees have thus far been reliant on morphological studies. For some taxa, such as *Homo antecessor*, the placement based on morphology has, however, been highly debated. A study of protein sequences preserved in enamel of a *H. antecessor* individual has been able to shed light on this debate, placing the specimen, in comparison to the available reference sequences, basal to modern humans, Denisovans, and Neanderthals (Welker et al. 2021). Further, paleoproteomic studies of unidentified hominin skeletal material have been able to show both an extended geographic distribution and lifeways of Denisovans (Chen et al. 2019, Xia et al. 2024, Demeter et al. 2022), in accordance with genetic models of Denisovan introgression into present-day human genomes (Ongaro and Huerta-Sanchez 2024). Paleoproteomic analyses can therefore extend our molecular understanding of hominin evolution and behavior further back into the past than previously possible, and complement other methods within archaeology and paleoanthropology.

So far, these analyses have been restricted to the recovery of a few phylogenetically informative positions in the context of the handful of hominin reference genomes and proteomes available, limiting the general applicability and population proteomic insights gained. In some cases, this has implied that assignments could go no further than confirmation that a specimen represents a hominin (Demeter et al. 2022). Future work should therefore explore whether this is due to limited protein sequence preservation in Middle and Late Pleistocene proteomes, due to the computational approaches to data analysis employed, or whether enhanced extraction approaches can increase sequence recovery significantly.

Of the skeletal proteomes, and similar to developments within faunal paleoproteomics, the genetic sex identification through the recovery of AMELX-specific and AMELY-specific peptides is receiving increasing attention. Applicable across the Pleistocene (Madupe et al. 2023), a number of Middle and Late Pleistocene hominin dental enamel proteomes have been studied in this regard (Demeter et al.

2022; Shaw et al. 2024; Welker et al. 2020). Here, the absence of the AMELY gene in some Neanderthals (Skov et al. 2022) is particularly noteworthy, as this might artificially inflate the number of archaic hominin females identified in dental enamel sexing studies based on amelogenin peptides.

ANALYTICAL CHALLENGES

The paleoproteomic workflow faces challenges when applied to Pleistocene materials. A variety of approaches to extraction, proteomic data generation, and proteomic data analysis have been explored, often dependent on the tissue type available for study, as well as (relative) protein preservation and the research question at hand (Taurozzi et al. 2024). Below, we will highlight three areas of active research: specimen and sample selection criteria, developments in extraction and protein digestion methods, and computational approaches to proteomic data analysis, which we believe show promising results.

SAMPLING ETHICS

Despite the excitement associated with addressing existing analytical challenges in the paleoproteomics field, and given the rapid growth of the number of ancient protein studies coupled to the implications of destructive sampling on the remaining and modified archaeological record, it is important to consider the ethical aspects of working with archaeological and paleoanthropological remains. They constitute a universal heritage, representing the past of our own species and many other hominin populations. Working with past extinct hominins therefore raises unique ethical considerations about scientific responsibility and long-term preservation of unique archaeological objects. The use of destructive sampling methods, even minimally destructive, should be thoroughly evaluated to avoid unnecessary sampling, and damage, to preserve the remains. Additionally, establishing international collaborations as well as transparency throughout the collaborations is important as these remains are shared heritage resources.

Ethical implications of the zooarchaeological discipline have received much less attention than those related to the study of human remains (Pálsdóttir et al. 2019). However, Pleistocene faunal remains are not an unlimited resource, and this must be taken into account when considering paleoproteomic studies, which imply the destruction of this archaeological heritage, even if in a minimally invasive way. This is especially relevant in the case of the large-scale application of ZooMS-based studies in which hundreds, if not thousands, of small bone samples are taken from archaeofaunal collections.

SPECIMEN SELECTION AND SAMPLING

The archaeological skeletal record is a limited resource and great care must thus be taken when selecting specimens and during sampling to both limit destructive sampling and to protect the specimen's morphology. Sampling strategies within the field are currently mostly based around sample availability. Gaining empirical data on variation in protein preservation and composition between skeletal elements would be a valuable addition to the current strategies.

Prior to considering sampling methods themselves, recent research has explored the value of non-destructive or minimally invasive pre-screening methods to determine the likelihood of sufficient amounts of protein surviving for paleoproteomic analysis. Specifically, spectroscopic approaches such as infrared spectroscopy (IR) or Raman spectroscopy are used to determine the elemental composition of a sample, ultimately allowing estimation of the collagen preservation of archaeological remains. Fourier transform infrared spectroscopy (FTIR) has proven to require only small sample sizes and is efficient in assessing the organic preservation of bone prior to protein mass spectrometry analysis (Kontopoulos et al. 2020; Le Meillour et al. 2018; Pal Chowdhury et al. 2021; Pothier Bouchard et al. 2019), although contested regarding the presence of secondary mineralization, altering the IR spectral quality and reliability (Presslee et al. 2021). Nitrogen content (%N) has been proposed as a useful screening approach prior to conducting ZooMS studies (Wang et al. 2021), and a protein weight percentage in bone and dentine of 3% or higher, generally expressed as col%, to correspond with good proteome retrieval (Le Meillour et al. 2018; Presslee et al. 2021). Borrowed from the heritage sciences, Raman spectroscopy has also proven to be an alternative approach for assessing collagen preservation in the context of collagen isotopic studies (Halcrow et al. 2014; Pestle et al. 2015). More recent studies have explored the use of non-invasive near-infrared (NIR) spectroscopy in the context of sampling bone for the radiocarbon dating of collagen (Legan et al. 2020; Malegori et al. 2023; Sponheimer et al. 2019). NIR pre-screening in paleoproteomics would be advantageous since proteomic assessment can happen remotely and entirely non-destructively. Interestingly, based on the small sample sizes required for MALDI-ToF MS analysis, some authors have also explored the use of ZooMS as a pre-screening method in itself (Harvey et al. 2016).

Sampling methods, such as the most common ones, drilling and cutting, are destructive and will alter a specimen's morphology. Minimally invasive sampling methods are in constant development and include the eraser approach (Evans et al. 2023; Hansen et al. 2024; Sinet-Mathiot et al. 2021) as well as the use of a variety of polishing films (Evans et al. 2023; Gilbert et al. 2024; Hansen et al. 2024; Kirby et al. 2020). Truly non-invasive approaches have been developed as well, targeting the sampling of membrane box surfaces and plastic storage bags in which bone objects have been stored for prolonged periods of time (Hansen et al. 2024; Martisius et al. 2020; McGrath et al. 2019). These minimally- and non-invasive methods are, however, outperformed by the conventional, more destructive, methods, especially in cases of poor protein preservation (Hansen et al. 2024). Furthermore, it should be noted that although sampling methods may be non-invasive, they are always destructive, since biomolecules originally from an archaeological or paleoanthropological object, be it a hominin specimen or an undeterminate faunal bone fragment, are

irreversibly removed. In addition, non-invasive sampling methods may introduce exogenous compounds onto specimen surfaces, including chemical reagents, which could potentially hinder future biomolecular studies.

The hominin skeleton can be divided into three distinctive proteomes that are commonly used in paleoproteomic research. The smallest proteome belongs to dental enamel and consists of roughly ten proteins unique to the enamel (Lacruz et al. 2017). The other two, the dentine and bone proteomes, are more similar to each other in terms of size and composition. Both are large, consisting of around a thousand different proteins (Alves et al. 2011; Widbiller et al. 2019) and, while they share the majority of the proteins, each contains unique proteins not found elsewhere in the skeleton.

Bone makes up a large portion of the archaeological record, whether fragmented or intact. The bone proteome is generally considered to be uniform across the skeleton within the paleoproteomics field. This is, however, unlikely to be the case as there are two different ossification processes, endochondral and intramembranous, that form the skeleton and are reliant upon different cell types in the forming, living bone. With endochondral ossification the mineralization takes place in a cartilage template of the forming bone, while intramembranous ossification takes place directly within the soft tissues (Hallett et al. 2021). This suggests that bones formed through endochondral ossification contain cartilage-related proteins, while bones formed through intramembranous ossification do not, at least initially and prior to the extensive remodelling that takes place during life. Some evidence suggests that these differences in proteome composition are indeed observable in skeletal material, including in Pleistocene contexts (Welker et al. 2016). Further, most bones are made up of two types of bone, cortical and trabecular bone, which differ in terms of structure and maintenance. A recent study (Ásmundsdóttir et al. 2024) showed that the two bone types are different when it comes to protein preservation, with the archaeological cortical bone proteome being larger and showing a lower rate of degradation compared to trabecular bone. Further research should therefore explore how proteome composition and preservation is influenced by mineral density and water content, as well as bone ossification.

Dentine is one of the major parts of the tooth, alongside dental enamel, and regularly used in paleoproteomic research. During dentine formation, dentinogenesis, odontoblasts form pre-dentine, which becomes dentine when mineralized. The most abundant proteins secreted by odontoblasts are collagens, mainly collagen type I (Jágr et al. 2012; Widbiller et al. 2019). The dentine proteome also contains non-collagenous proteins and proteins specific to the dentine such as dentine sialophosphoprotein (DSPP) and its three byproducts—dentine sialoprotein, dentine glycoprotein, and dentine phosphoprotein (Jágr et al. 2012). Given its higher density compared to bone, as well as the absence of large-scale remodelling during life, protein preservation might be enhanced in dentine compared to bone. There are few direct comparisons available that empirically demonstrate this, however, and future work could therefore explore whether this is generally true (Chen et al. 2019; Welker et al. 2020).

Dental enamel is the hardest and the most mineralized tissue found within the mammalian skeleton, composed of 95% mineral, 1–2% organic material and 2–4% water (Lacruz et al. 2017). The enamel matrix proteins are secreted by ameloblasts during amelogenesis. What makes the dental enamel unique compared to other skeletal tissues is that during mineralization the proteins undergo *in vivo* digestion where two enzymes, MMP-20 and KLK-4, break down the proteins. Some of the resulting fragments remain entrapped within the enamel mineralized matrix. The dental enamel does not undergo remodelling during life (Lacruz et al. 2017). Due to this stable and extremely mineralized environment, the enamel is a great source of proteins bound to the mineral matrix. The most common enamel proteins are amelogenin X (AMELX), ameloblastin (AMBN), and enamelin (ENAM) (Lacruz et al. 2017). Amelogenin (X and Y) as well as enamelin have been identified in hominin material as old as 2.2 million years old (Madupe et al. 2023) and are, as discussed above, the focal point of dental enamel sexing studies based on amelogenin.

In addition to skeletal material, other sample sources may also preserve Pleistocene biomolecules. Archaeogenetic studies have shown preservation of DNA into the Pleistocene in hominin dental calculus (Weyrich et al. 2017; Fellows Yates et al. 2021), and hominin DNA in sediments has shown occupation histories across and within archaeological stratigraphies (Massilani et al. 2022; Rampelli et al. 2021; Slon et al. 2017; Zavala et al. 2021; Zhang et al. 2020). Additionally, studies of lipids preserved in Pleistocene coprolites have provided insights into Neanderthal diets (Sistiaga et al. 2014). Paleoproteomic studies of dental calculus, sediments from archaeological sites, or paleofaeces, may therefore provide additional insights into Pleistocene hominin lifeways and behavior.

EXTRACTION METHODS

After the initial quick growth of the field of paleoproteomics, efforts are currently increasingly being put into optimizing protein extraction protocols, to aim for obtaining high resolution data from every destructive sampling of archaeological material. These extraction methods are generally aimed either at enamel or at bone and dentine, although some approaches are applicable to all three tissue types.

For archaeological enamel, a protocol consisting solely of removal of the mineral fraction, followed by peptide cleanup, is often employed (Taurozzi et al. 2024). As enamel proteins are digested *in vivo*, no protein digestion is required. The added benefit of this approach is that it also removes the need for buffer exchange between a demineralizing agent and a buffer suitable for proteases. The enamel proteome is often studied to assign genetic sex to past human individuals, and significant progress has been made in standardizing this process through targeting sex-

specific peptides and streamlining the analysis, allowing for quantification-based sex assignment of a large number of samples per day (Koenig et al. 2024).

For archaeological bone and dentine, more complex protocols may be needed if the proteome is well-preserved enough to require enzymatic digestion prior to mass spectrometry. Recent studies (Mylopotamitaki et al. 2023) have shown that EDTA-based proteomic extraction methodologies are suitable for well-preserved archaeological specimens. However, highly degraded skeletal remains may require acid-based proteomic extraction protocols to generate high resolution mass spectra for taxonomic identifications (Mylopotamitaki et al. 2023; Wang et al. 2021).

Archaeological specimens have often been handled and stored for a long time prior to being analyzed through paleoproteomics. This, in addition to the burial environment, leads to modern contaminating proteins being present on the specimens, and may end up being analyzed together with the ancient endogenous proteins. This may lead to both an altered proteome being reconstructed, as well as low-abundance peptides being masked by the contaminants (Fagernäs et al. 2024a). Removal of surface-contaminating proteins has proven successful for a Pleistocene hominin tooth and is recommended before protein extraction from dentine and bone tissues (Fagernäs et al. 2024a).

A focus of recent studies has been the potential of using alternative proteases instead of the standard approach in the field, which is the use of trypsin. It has been found that although trypsin is indeed ideal for studying collagen, and does often result in the largest proteomes, using alternative enzymes such as Glu-C or chymotrypsin may allow for accessing additional proteins, increasing the fraction of low-abundance proteins, or low-abundance protein regions (Fagernäs et al. 2024b; Lanigan et al. 2020; Samodova et al. 2020). Additionally, consecutive digestion with two proteases may increase the fraction of desired non-collagenous proteins (Wilkin et al. 2024) or access low-abundance proteins and peptides (Fagernäs et al. 2024b). For studies looking to recover as many proteins as possible, with a high sequence coverage across the protein, such as phylogenetic studies, combining proteases in parallel or consecutively may therefore be beneficial.

Several different extraction methods and approaches to increase protein recovery have therefore by now been developed for proteins of a range of preservation states. A well-preserved sample with a large number of relatively intact proteins needs a different extraction protocol to a poorly preserved sample with only a few analyzable peptides remaining. A major issue is, however, that it is not possible to know which method would be ideal, until an initial extraction and preliminary data analysis has been conducted, thereby potentially causing destruction of a larger portion of the sample than required for one extraction. This issue can be circumvented by optimizing the extraction protocol using a different specimen, e.g., using faunal skeletal remains prior to protein extraction from a hominin specimen, or by conducting a pilot extraction of several randomly selected morphologically unidentified specimens. In cases where protocol optimization on a different specimen is not possible, researchers are currently forced to base their protocol choice on an educated guess, based on the site age, environment, and comparison to similar, previously analyzed specimens.

COMPUTATIONAL NEEDS OF PALEOPROTEOMICS

The last couple of years have seen a real growth in our understanding of the performance of bioinformatics approaches to proteomic data analysis, as well as the standardization of data analysis after proteomic data generation. Several papers have, for example, explored the performance of common proteomic data analysis tools (see for an example Palomo et al. [2023]). They highlight the need for paleoproteomic-specific data analysis strategies to overcome the limits of most available workflows in relation to the diagenetic complexity of ancient (hominin) skeletal proteomes. Others have demonstrated that the vast majority of the generated paleoproteomic MS2 fragment ion spectra remain unidentified (Chiang et al. 2024), more so than commonly encountered in modern proteomic studies. This potentially means that there is a wealth of data being generated that is computationally inaccessible so far, for example, highly modified peptide sequences or peptide termini with undescribed terminal PTMs. Furthermore, further work dedicated to the validation and scoring of reconstructed amino acid sequences on a position-by-position basis, taking into account MS2 fragmentation efficiency of peptide bonds and uncertainty about amino acid assignments would provide welcome indicators of sequence quality. Resolving these issues would potentially unlock richer ancient proteome datasets.

For both ZooMS and SPIN, the two main and standardized approaches to obtaining taxonomic identities in Pleistocene paleoproteomics, computational workflows are now available. For ZooMS, both SpecieScan (Végh and Douka 2024) and bacollite (Hickinbotham et al. 2020) provide frameworks in which MALDI-ToF MS data can be analyzed and ZooMS taxon identities assigned. This greatly enhances the reproducibility of taxonomic assignments made by ZooMS. Nevertheless, the importance of a well-curated peptide marker database for ZooMS, and the validation of novel peptide marker masses by MALDI-ToF/ToF or LC-MS/MS, cannot be overstated, and efforts should therefore be made to continuously improve on this front. A range of tools now also exists to obtain deamidation ratios for selected peptides within MALDI-ToF MS data (Nair et al. 2023; Wilson et al. 2012), and these will enable researchers to consistently compare collagen degradation across and within archaeological sites. Similarly, central to SPIN is an R script that, from MaxQuant DDA output or Spectronaut DIA output, will assign a taxonomic identity in relation to a pre-constructed reference database (Rüther et al. 2022). Again, the importance of validity and content of a curated reference database for SPIN cannot be overstated, and efforts should be made to expand its taxonomic breadth in the future. Furthermore, ideally, for both ZooMS and SPIN

such resources would be easily accessible to all, amenable to change based on future datasets and corrections, as well as simple, so that individual researchers can make use of these resources in a variety of creative ways.

The growth in the field necessitates the creation of pipelines for the reconstruction of ancient protein sequences from the output of proteomic data analysis tools, as well as the subsequent phylogenetic placement of these partial, ancient sequences. The pipeline PaleoProPhyler has emerged as a promising tool for such phyloproteomic analyses (Patramanis et al. 2023). PaleoProPhyler joins the possibility of creating relevant reference datasets with reconstructing partial ancient protein sequences from proteomic data to conducting phylogenetic analysis. In addition, of particular relevance is the reference dataset of 10,058 protein sequences of great apes released with the package. Building on this, future work can now start to explore the extent of protein sequence variation between and within hominin taxa within a phyloproteomic framework.

CONCLUSION

Paleoproteomic analysis of Middle and Late Pleistocene skeletal proteomes is starting to contribute to the study of human evolution. Nevertheless, there are significant obstacles to this approach, and this seems true for large-scale taxonomic screening studies such as ZooMS or SPIN, for dental enamel sexing of hominin and faunal specimens, as well as for in-depth analysis of hominin proteomes for phyloproteomic purposes. Research should therefore continue to explore avenues to minimize sample destruction, use the most suitable skeletal elements, enhance the recovery of ancient peptides from such material, and optimize the sequence information retrieved computationally for taxonomic and/or phylogenetic purposes. Although improvements made in individual steps of the paleoproteomic workflow might be minimal, cumulatively they will enable the ethical analysis of our joint Pleistocene heritage.

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DATA AVAILABILITY STATEMENT

This paper is a review and has no primary data associated with it.

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