VIJFENVEERTIGSTE KROON-VOORDRACHT

FRIDO WELKER

PROTEIN TALES OF THE HUMAN PAST

GERRIT HEINRICH KROON
(1868-1945)

PROTEIN TALES OF THE HUMAN PAST

VIJFENVEERTIGSTE KROON-VOORDRACHT

GEHOUDEN VOOR DE
STICHTING NEDERLANDS MUSEUM
VOOR ANTHROPOLOGIE EN PRAEHISTORIE

TE AMSTERDAM OP 27 OKTOBER 2023

DOOR

ASSOC. PROF. DR. FRIDO WELKER

Section for Molecular Ecology and Evolution,
University of Copenhagen
Denmark

INTRODUCTION

The majority of the skeletal archaeological and palaeontological record is rather unassuming. Instead of the glitter and glamour associated with hominin fossils, the fossil skeletal record largely consists of bone fragments of undetermined taxonomic origin (Figure 1). Archaeologists painstakingly excavate these remains from unique, precious, and increasingly protected archaeological sites. In some cases, these bone fragments will be measured and photographed in-situ, while in other cases they are discovered during wet or dry sieving. All these hundreds or thousands of skeletal specimens are normally cleaned, registered, and sometimes photographed. They will be deposited in a curatorial facility, for example a museum or a university collection, where they are stored for potential future analysis.

Figure 1. A typical skeletal assemblage from a Pleistocene archaeological site. *Normally, the majority of excavated skeletal material contains few, if any, morphological characteristics that allow for taxonomic identification. Musea and university collections are therefore to a large extent composed of boxes and shelves containing this type of material. Credit: F. Welker.*

In most cases, during this analytical process a Pleistocene bone fragment would be studied visually, often by a trained zooarchaeologist. In the process, the specimen might be assigned to a body size class based on gross morphological characteristics such as fragment size and cortical thickness. In some cases, it can be established what skeletal element a bone fragment represents, for example if it is a distal tibia, a rib fragment, or a mandible. A zooarchaeologist might also observe the presence of anthropogenic modifications, such as cut marks, or the presence of carnivore or rodent modifications, for example indicating hyaena denning activities. Similarly, the bone surface might reveal information about the depositional environment during site occupation, or insights into taphonomic processes that affected the archaeological site since its initial formation. In a few cases, a taxonomic identity might be assigned, specifying whether a specific bone fragment might stem from a hominin, a type of deer, a species of carnivore, etcetera.

In the majority of cases, however, the taxonomic identity of a Pleistocene bone fragment remains unknown. Such bone fragments typically make up more than half of a bone assemblage, sometimes over 90% (Table 1). Ecological and behavioural interpretations about the content and formation of bone assemblages is therefore frequently based on a small proportion of the skeletal assemblages recovered and curated from Palaeolithic archaeological sites. The fragmentary nature of the Palaeolithic skeletal record has also limited the discovery of hominin remains at dense spatial and temporal scales. This has implications on the strength of our evidence with which particular hominin populations (or species - both terms are used interchangeably here) are associated with the behavioural information contained within the sedimentary, lithic, and faunal datasets recovered from archaeological sites.

	% identified morphologically	% identified ZooMS
Fumane Cave	**3.1 (n=19955)**	**97.8 (n=684)**
Bacho Kiro	**20.7 (n=7013)**	**96.7 (n=1595)**
Les Cottés	**36.9 (n=5169)**	**96.9 (n=523)**
La Ferrassie	**17.6 (n=809)**	**98.3 (n=527)**
Abri du Maras	**5.9 (n=827)**	**80.7 (n=280)**
Portuguese Late Pleistocene sites	*NA*	**57.1 (n=21)**

Table 1. Reported success rates for visual and proteomic taxonomic identifications to subfamily level or more precisely, for a selected number of Late Pleistocene archaeological sites. *Unless indicated otherwise, counts are sums for multiple archaeological assemblages at each site. Data taken from Sinet-Mathiot et al., 2019 (Fumane Cave), Sinet-Mathiot et al., 2023 (Bacho Kiro, Les Cottés, La Ferrassie), Daujeard, 2008 and Ruebens et al., 2022 (Abri du Maras, Layer 1), and Rüther et al., 2022 (Portuguese Late Pleistocene sites). Note that the number of faunal specimens from Abri du Maras and Fumane Cave includes specimens recovered through sieving, which are generally small and therefore less identifiable based on morphological characteristics.*

Archaeologists and researchers in associated disciplines have for a long time recognised the relevance of both hominin fossils and the fauna with which they are associated contextually (Fuhlrott, 1857; Schaaffhausen, 1857). Over the past decades, the molecular contents of the organic component of skeletal remains, of both hominins and associated fauna, have become increasingly central to palaeoanthropological and archaeological investigations. This organic component includes a comparatively large amount of ancient proteins, which can be studied through a variety of protein mass spectrometry methods. In the following, an attempt will be made to show how the field of ancient protein analysis, palaeoproteomics, is contributing new data and insights through the taxonomic identification of new hominin fossils, by

unlocking the zooarchaeological potential of complete Pleistocene skeletal assemblages, and via the analysis of ever-larger skeletal proteomes. Although the focus will be on Palaeolithic archaeological sites, the observations made are equally applicable to palaeontological contexts and more recent archaeological sites. There, too, the majority of skeletal specimens recovered are unidentifiable through morphological observation, a significant number of human skeletons cannot be sexed securely, and many bone artefacts have unknown taxonomic origins.

PART 1: SOLVING HOMININ DISCOVERIES

toen de nacht ons omgaf
en meenam in de duistere vragen
wanneer en waar en wie

From "Toen de Nacht", in Toen ik dit zag
(R. Kopland, 2008)

Historically, the discovery of Pleistocene hominin remains has, to some extent, been based on chance. Places of hominin occupation tend to be locations of camp sites of some kind, and are therefore unlikely to contain remains of the hominins themselves. The comparatively large number of deciduous dental remains from hominins is likely explained by this phenomenon (Benazzi et al., 2015). Likewise, there are several instances where hominin remains have been recovered in cave sites or open air settings, but where they are without archaeological contexts, for example those from Oase and Zlatý kůň (Prüfer et al., 2021; Trinkaus et al., 2003). These limit the direct association of hominin remains with archaeological material.

Several approaches can be taken to infer the biological identity of the makers of specific technocomplexes of material culture. The most straightforward approach would be the recovery of hominin remains in direct stratigraphic association with an archaeological assemblage. Other arguments, that used to be rather common, are techno-typological and rely on the association of particular tool types with particular hominin populations. These arguments frequently include a chronological component, and sometimes a geographic one too. The argument that because site A contains hominin remains, for instance Neanderthal specimens, in direct and secure connection with a particular type of technocomplex, site B with the same technocomplex *but* without hominin re-

mains would also have been the result of Neanderthal occupation, is pervasive in archaeological debates, even though this approach has been questioned on various occasions. More recently, ancient DNA directly recovered from sediments has opened up the possibility to determine the past presence of a hominin group at a site in the absence of hominin skeletal remains and/or in the absence of lithic or even faunal remains (Slon et al., 2017). The assumption that the hominin evidence, whether skeletal or molecular, is associated with the other archaeological material should be demonstrated, however, and these associations are therefore inferred.

Another option to enrich the hominin fossil record is to return to the vast majority of bone fragments that are stored within archaeological collections. In life, the bones and teeth that compose the skeleton contain DNA, proteins, lipids, and other small biomolecules. These biomolecules are embedded within the inorganic mineral matrix that makes up most of the mammalian skeleton. The organic components end up in the skeletal matrix through a variety of mechanisms and with a range of biological functions. More importantly, they also survive, to different extents, the molecular degradation processes that occur over archaeological and geological time scales. As a result, for temperate environments like those found in Europe, it is now well established that DNA can survive in skeletal elements from the Late Pleistocene, and in some cases even from the Middle Pleistocene. Some skeletal proteins survive beyond DNA, and have been extracted even from Early Pleistocene contexts in temperate environments (Welker et al., 2020). Their survival is partly dependent on local temperatures and humidity, meaning that biomolecules survive longer in the permafrost, where the oldest DNA sequences published to date are almost 2 million years old, compared to tropical environments, where the oldest DNA sequences published are just a couple of thousand years old. The relative order of biomolecule sur-

vival, however, remains the same, with proteins, and some lipid breakdown products, surviving beyond the local preservation limits of DNA.

Compared to DNA, proteins are the more abundant organic component. Of the proteins present in bone and dentine, type I collagen is the most abundant protein. It is composed of two strands of collagen type I, alpha-1 (hereafter COL1A1) and one strand of collagen type I, alpha-2 (hereafter COL1A2) that wind together to form a triple helix. The triple helix and its association with the hydroxyapatite crystals of the bone provides a structure resistant to degradation, implying that of the proteins present in bone and dentine, type I collagen (hereafter COL1) tends to preserve longer than other proteins. As with any other protein, the amino acid sequences of both COL1A1 and COL1A2 are determined by the nucleotide sequence at the genetic level. Over time, nucleotide mutations accumulating at the genetic level result in variations of the amino acid sequence at the protein level. For these protein sequences as a whole, their amino acid sequences therefore reflect the evolutionary relationships between species, genera, and families, in a manner consistent with the evolutionary relationships determined through genomics research (Figure 2). Protein amino acid sequences therefore provide some evolutionary information.

Analysing and determining protein amino acid sequences can be done through a variety of approaches established in the field of protein mass spectrometry. Over the past two decades, some of these mass spectrometry methods, such as MALDI-ToF MS and LC-MS/MS, have been adopted to study the ancient proteins preserved in archaeological and palaeontological materials, including skeletal materials (Hendy, 2021; Warinner et al., 2022). Prior to protein mass spectrometry, the proteins have to be liberated from the skeletal material and prepared for analysis. For skel-

Mammuthus primigenius (mammoth)	P	G	H	A	G	A	V	G	P	A	G	V	R
Felis catus (cat)	T	G	H	P	G	S	V	G	P	A	G	V	R
Canis lupus (dog)	T	G	Q	P	G	T	V	G	P	A	G	I	R
Equus caballus (horse)	S	G	Q	P	G	T	V	G	P	A	G	V	R
Ceratotherium simum (white rhino)	S	G	Q	P	G	T	V	G	P	A	G	V	R
Sus scrofa (pig)	T	G	Q	P	G	A	V	G	P	A	G	I	R
Rangifer tarandus (reindeer)	A	G	Q	P	G	A	V	G	P	A	G	I	R
Cervus elaphus (red deer)	T	G	Q	P	G	A	V	G	P	A	G	I	R
Ovis aries (sheep)	T	G	Q	P	G	A	V	G	P	A	G	I	R
Capra hircus (goat)	T	G	Q	P	G	A	V	G	P	A	G	I	R
Bos primigenius (aurochs)	I	G	Q	P	G	A	V	G	P	A	G	I	R
Macaca mulatta (rhesus macaque)	T	G	H	P	G	T	V	G	P	A	G	I	R
Homo sapiens (human)	T	G	H	P	G	T	V	G	P	A	G	I	R

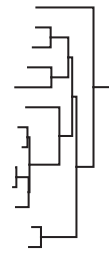


Figure 2. Partial sequence alignment and phylogenetic relationships of the COL1 sequences of selected mammalian species. *The aligned collagen peptide is the COL1α2 978–990 peptide marker used in ZooMS. Its sequence is slightly different between some of the species listed. The phylogenetic tree on the right is based on the complete COL1 sequences of the same species. The branching pattern, the topology, of the phylogenetic tree based on the protein sequences is similar to that observed for the same species based on genomic information.*

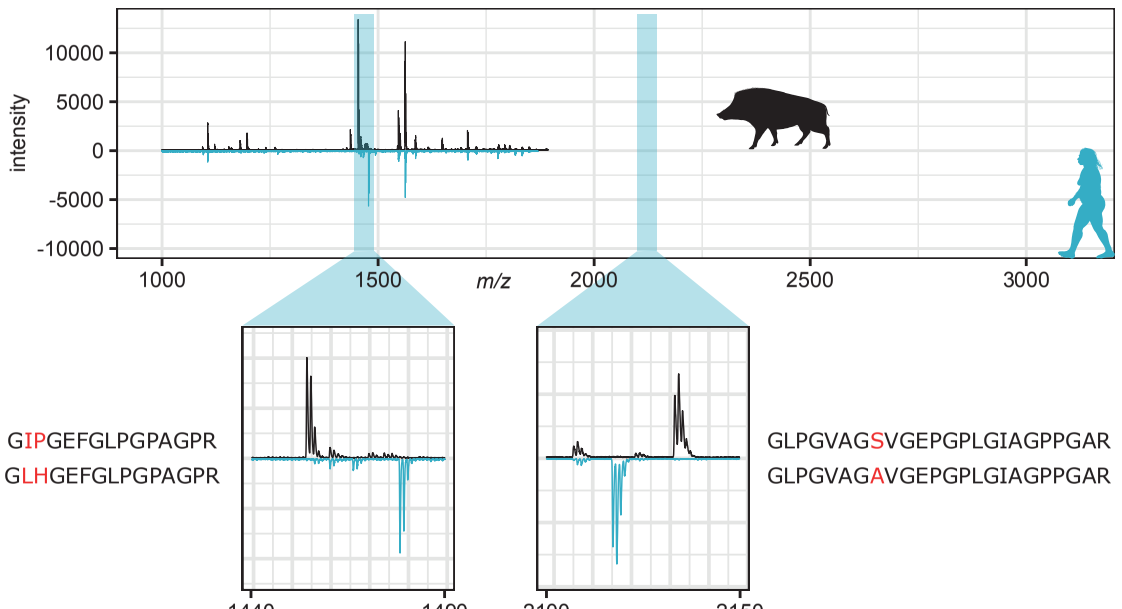
etal material, protein extraction methods generally involve the removal of the inorganic mineral matrix, often by using an acid, such as hydrochloric acid (HCl), or EDTA (ethylenediaminetetraacetic acid). Subsequently, the three-dimensional organisation of the proteins is disrupted (for example the triple helix of COL1), which can be done by mild heating or chemically. Once the three-dimensional structure is lost, the protein sequences are normally digested with a protease, commonly trypsin. Proteases break the peptide bonds of the amino acid sequence at particular locations, creating smaller protein fragments called peptides. In the case of trypsin, digestion of a protein using this protease results in cleavages of the peptide bond after lysines (abbreviated with the letter K) and arginines (R).

In probably the simplest and most widely adopted proteomic approach in palaeoproteomics, ZooMS (short for Zooarchaeology by Mass Spectrometry), the masses of the complete COL1

peptides generated after trypsin digestion are measured (Buckley et al., 2009). Of all these peptide masses measured, the masses of a selected subset of peptides, the peptide markers, are compared to the peptide masses present in a reference database containing COL1 peptide masses for a large range of animal species. Through this comparison a taxonomic identity is assigned, for example effectively distinguishing between wild boar and hominin skeletal fragments (Figure 3). This peptide mass fingerprinting (PMF) approach generally allows taxonomic identifications at the subfamily or genus level for mammalian taxa.

ZooMS, due to its simplicity in terms of protein extraction and protein mass spectrometry, allows for the analysis of a relatively large number of bone fragments in short periods of time, at reasonable costs. By screening hundreds or even thousands of bone fragments, this has allowed the identification of hominin bone specimens that were not recognizable by morphology alone (Figure 4). As the Palaeolithic skeletal record is so fragmentary, and hominin remains comparatively rare, these newly recovered hominin remains that have little to no morphological value become molecular treasure chests.

One context in which hominin fossils are in high demand concerns the time period when Neanderthals disappeared and modern humans started arriving in western Eurasia. Often described as a "transition", this biological change coincides with changes observed in lithic technologies and associated, archaeologically visible behaviours. What is more, so-called "transitional" industries exist at the chronological "interface" of the Middle Palaeolithic (MP) and the Upper Palaeolithic (UP), especially in Europe. These transitional industries, such as the Châtelperronian in France and the Uluzzian in Italy, display technological characteristics that are viewed as a combination of the MP and UP. Identifying the hominin population responsible for these transi-


intensity
10000
5000
0
-5000
-10000
1000
1500
m/z
2000
2500
3000
GIPGEFGLPGPAGPR
GLHGEFGLPGPAGPR
1440
1490
2100
2150
GLPGVAGSVGEPGPLGIAGPPGAR
GLPGVAGAVGEPGPLGIAGPPGAR

tional industries, as well as the preceding late Middle Palaeolithic industries or the subsequent Initial Upper Palaeolithic (IUP), has therefore been central to the chronological models surrounding Neanderthal extinction and modern human dispersal across the continent. Similarly, the genome sequencing of ancient DNA from hominin fossils dating to this time range is vital to understanding the full genetic legacy of the "transition" period. As with other Pleistocene contexts, the scarcity of hominin remains has made this a lively debate.

Proteomic screening through ZooMS, and more novel methods such as SPIN (Species by Proteome Investigation, see below), has proven remarkably successful in the identification of hominin remains associated with the MP/UP transition. Hominin remains are now being identified at many Late Pleistocene archaeological sites where proteomic screening is employed at reasonably large scales. For example, we managed to identify additional hominin remains at Grotte du Renne, a key Châtelperronian site where previously recovered Neanderthal remains are associated with bone artefacts and ornaments. The hominin remains identified through ZooMS were directly radiocarbon dated, demonstrating that they fit chronologically with the Châtelperronian occupation of the site. Ancient mitochondrial DNA demonstrated the hominin remains are, at least in the maternal line, Neanderthals, and not modern humans (Welker et al. 2016). These specimens are therefore among the youngest directly-dated Neanderthals in Europe, and belong to the few molecularly-studied hominin remains associated with a transitional assemblage.

Figure 3. A Homo sp. MALDI-ToF MS spectrum (bottom, in blue) in comparison to that of a pig (Sus scrofa, top). *Selected insets show differences in peptide marker m/z of the collagen peptide markers COL1α2 484–498 (left) and COL1α2 793–816 (right). The hominin spectrum is flipped to enable visual comparison with the pig spectrum.*

Figure 4. Three hominin bones recovered from the Kleine Feldhofer Grotte (Germany). *In this case, a small assemblage of mixed Pleistocene and Holocene bone fragments was analysed through ZooMS to identify additional hominin remains potentially belonging to the Neanderthal type specimen. Credit: J. Vogel, from Lanigan et al., 2020.*

ZooMS has allowed similar advances in our understanding of some of the first modern humans arriving in Europe. Proteomic screening performed at the site of Bacho Kiro (Bulgaria) allowed the identification of several hominin remains. Again, radiocarbon dating and isotopic analysis provided chronological and dietary contexts (Fewlass et al., 2020; Hublin et al., 2020), associating these hominin remains with an IUP industry. Subsequent ancient DNA analysis of these hominin remains assigned them to some of the first modern human populations to enter the European continent. Their genomes are more closely related to current populations in East Asian and the Americas, rather than western Eurasian populations. Furthermore, full genome analysis showed that the humans present at Bacho Kiro had Neanderthal ancestry

only a few generations back in their family tree, providing further data on the frequency and timing of Neanderthal-modern human introgression (Hajdinjak et al., 2021).

These are far from the only examples of hominin specimens recovered through proteomic screening. At Denisova Cave (Russia), a bone specimen was identified as a hominin through ZooMS (Brown et al., 2016). Ancient DNA analysis of this specimen revealed her genome derived from a Neanderthal mother while her father was a Denisovan – the only first generation hominin hybrid individual identified to date (Slon et al., 2018). By adopting ZooMS, or more novel approaches such as SPIN, palaeoanthropology now has a tool available to identify hominin remains at a large scale and at a high spatial and chronological density. As the discovered hominin fragments have little to no morphological value, their true relevance lies in the molecular (ancient DNA, proteins, lipids) and isotopic (radiocarbon dating, stable isotopes) information contained within them. This enables the generation of new models of Middle and Late Pleistocene human evolution, especially in genomic and chronological contexts, that would otherwise have been based on the chance discovery of hominin remains.

PART 2: ADDRESSING HOMININ BEHAVIOUR

Mijn gebeente vertelt de vertaling
van leven in dood
Daarmee ben ik niet verdwenen

From "Skelet", in Aas (C. Nooteboom, 1982)

The large-scale screening of Pleistocene bone fragments through proteomic methods does not only result in the identification of hominin remains. The vast majority of the proteomic identifications relate to other taxa, and the hominins make up only a small proportion of the total identifications obtained. These faunal identifications are insightful in their own ways. They provide complementary data to more traditional, morphology-based analysis of the faunal assemblages associated with hominin occupations.

In general, proteomic screening studies are finding that the taxa they identify correspond to the taxa identified through morphological analysis of the same assemblages, as long as sample sizes are large enough for both. In cases where a faunal assemblage is comparatively small, and morphological analysis provides taxonomic identifications for an even smaller number of specimens, ZooMS analysis might be a particularly powerful approach and provide a more comprehensive understanding of the faunal communities with which hominins interacted (Sinet-Mathiot et al., 2023). Given that ZooMS studies have higher identification rates compared to morphological studies, they identify rare species at a higher rate as well. This not only leads to the recovery of additional hominin remains, but also to the identification of species that may not be abundant, but that are ecologically meaningful. For example, the ZooMS-recovery of a wild boar (*Sus* sp.) specimen in the Châtelperronian layer at Les Cottés (France) indicates

that there were at least patches of woodland in an otherwise open steppe landscape (Welker et al., 2015).

Integration with traditional zooarchaeological practices can be made in other, potentially more informative ways too. Although only a small proportion of a faunal assemblage can be identified to some taxonomic level through morphological observations (Table 1), a larger number of specimens present anthropogenic traces, such as cut marks or percussion marks, traces of carnivore activity, or traces related to site formation processes, for example those resulting from extensive bone surface weathering or fluvial processes. By providing taxonomic identifications for a larger number of specimens, proteomic screening also allows for enhanced insights into the occurrence of each of these types of bone surface modifications. One example of this derives from the late Neanderthal occupations at Fumane Cave (Italy). Here, ZooMS screening resulted in a rather similar species composition when compared to morphological analysis of the same assemblage (Figure 5). Interestingly, though, the abundance of the major taxa (*Cervus elaphus*, *Capra* sp., and *Bos* sp./*Bison* sp.) is rather different between the ZooMS-identified and the morphologically-identified component of the same bone assemblage – there is a roughly 6-fold increase in the number of bone fragments identified as *Bos* sp./*Bison* sp. in the ZooMS-identified component. No explanation for this could be found in terms of bone fragmentation, bone surface modifications related to taphonomy or carnivore activity, or spatial distribution within the site. Instead, we observed that percussion marks, which are thought to be the result of breaking open a long bone with a stone tool to access the bone marrow stored within, are largely observed only on *Bos* sp./*Bison* sp. bone fragments identified through ZooMS (Figure 5). The implication could be that Neanderthals deliberately broke *Bos* sp./*Bison* sp. bones to extract their marrow, to such an extent that the bone remains of this taxon are less identifiable through

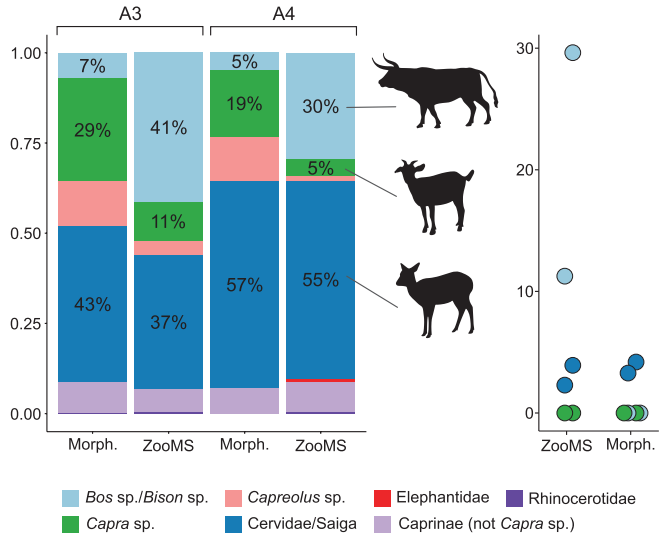


Figure 5. Taxonomic composition (left, as a fraction of the total number of identified specimens) and frequency of percussion marks (right, in percentages) of layers A3 and A4 at Fumane Cave (Italy). *Morph. = Morphologically-identified component of the skeletal assemblage. ZooMS = ZooMS-identified component of the same skeletal assemblage. Data taken from Sinet-Mathiot et al., 2019. Credit: V. Sinet-Mathiot and Z. Fagernäs.*

morphological means. The existence of this Neanderthal behaviour at Fumane Cave could therefore only be established through the molecular identification of the resulting bone fragments (Sinet-Mathiot et al., 2019).

Similarly, taxonomic identifications provided by proteomic methods present a unique opportunity to study bone artefacts. Through the shaping involved in their production, as well as through the abrasive action of their usage, bone artefacts can generally not be identified to genus or species level. One such case

concerns lissoirs, elongated bone tools normally made from ribs that were used in the preparation of animal hides. They are among the first formal bone tools identified and associated, initially, with Neanderthals. There are very few lissoirs known from the Middle Palaeolithic archaeological record (Soressi et al., 2013). Through proteomic means, we were able to identify these MP lissoirs as *Bos* sp./*Bison* sp. in all cases where COL1 preservation was sufficient for a taxonomic classification. Interestingly, *Bos* sp./*Bison* sp. are not abundant at any of the archaeological levels from which these lissoirs were recovered – instead, Neanderthals appear to have deliberately selected *Bos* sp./*Bison* sp. ribs to make lissoirs (Martisius et al., 2020). Such specific insights into raw material selection for bone tool production have been made for the IUP humans from Bacho Kiro as well (Martisius et al., 2022), and in numerous cases for more recent Upper Palaeolithic, Mesolithic and Neolithic contexts. In contrast to the large-scale screening of bone fragments, here the focus lies on the proteomic analysis of a small number of bone artefacts, where each taxonomic identification can provide a unique insight into hominin behaviour that would not have been possible through morphological methods alone.

In the examples of ZooMS taxonomic identifications mentioned before, it has become clear that many taxonomic assignments are at the family or subfamily level, and rarely at the genus or species level. There are several reasons for this. One aspect concerns the choice of peptide markers utilised in ZooMS - some informative peptides are not taken into account or rarely observed in MALDI-ToF MS spectra. Another aspect concerns the sensitivity of MALDI-ToF MS to detect peptide masses - many less-abundant peptides are not observed in a MALDI-ToF MS spectrum, even when COL1 is relatively well preserved. This means that the ZooMS analysis of Palaeolithic bone assemblages has been less successful in those regions where COL1 preservation is not optimal,

for example when applied to Mediterranean contexts (Table 1). Finally, taxonomic restrictions in ZooMS are also due to the measurement of complete peptide masses, without observing the amino acid sequence of the peptides directly. Recently, a new approach termed SPIN, Species by Proteome INvestigation, has been developed to directly address some of the taxonomic restrictions imposed by the ZooMS methodology (Rüther et al., 2022). In SPIN, high-throughput liquid-chromatography tandem mass spectrometry (LC-MS/MS) methods are used that, through extensive bioinformatics analysis, allow for the determination of the amino acid sequences that compose a peptide. In this manner, low-abundance peptides and proteins become observable.

ZooMS and SPIN were both applied to 21 bone specimens from several Late Pleistocene archaeological sites from Portugal, all roughly related to the Middle to Upper Paleolithic transition (Rüther et al., 2022). With ZooMS, 57% of the specimens could be assigned a taxonomic identity at the subfamily level or more precisely. This is worse than what we commonly observe in archaeological deposits of the same chronological age in temperate preservation conditions (Table 1). Of the studied samples, a third failed to provide any taxonomic identities. In contrast, SPIN was able to assign taxonomic identities to subfamily level, or more precisely, in 95% of the extracts, with only one extract remaining unidentifiable (Figure 6). Where a comparison is possible, SPIN taxonomic identifications are consistent with those obtained by ZooMS. Excitingly, SPIN identifications are in several cases more precise than what is possible with ZooMS. ZooMS cannot distinguish between members of the genus *Equus*, for example, while SPIN analysis allowed one extract to be assigned to asinine members of the genus (wild asses and zebras, in this case likely a European wild ass) and three extracts to be assigned to caballine members of the genus (horses, in this case likely a wild horse). SPIN analysis is therefore suited for the taxonomic identification of

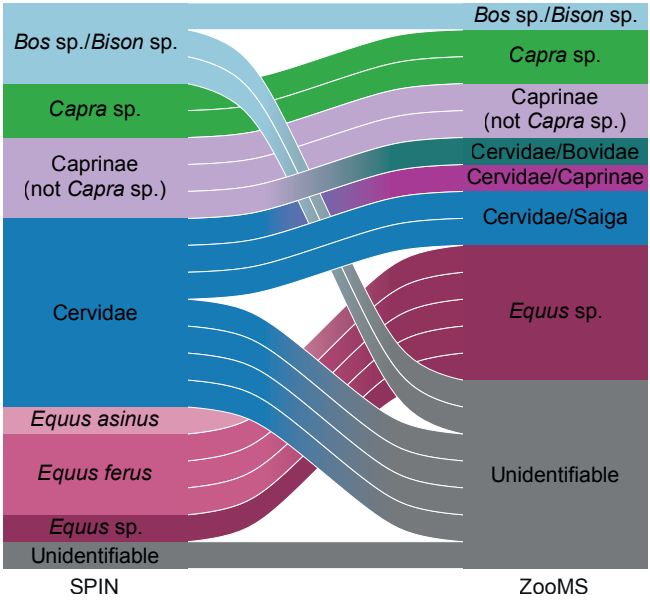


Figure 6. Comparison of taxonomic identifications made through ZooMS (right) and SPIN (left) for 21 Late Pleistocene bone specimens from Portugal. *Each ribbon is one specimen analysed through both SPIN and ZooMS. Data taken from Rüther et al., 2022.*

highly-degraded skeletal assemblages, or can be employed in contexts where taxonomic identifications are required beyond the specificity offered by ZooMS.

In this manner, ZooMS, SPIN and other proteomic methods provide an exciting toolkit for zooarchaeologists to further explore the ecological, behavioural, and taphonomic data contained within the skeletal assemblages they study. These proteomic approaches are not replacing traditional morphological research, but have the potential to become an integral part of zooarchaeological research practices.

PART 3: BUILDING HOMININ PHYLOGENIES

maar de mens is er niet om te vergeten
al zou hij willen
het lukt hem niet

From "Voorlopig gedicht voor Jan Wolkers",
in Ode aan mijn jas (R. Campert, 1997)

ZooMS makes use of the abundance of COL1 in bone and dentine, which partly explains its success. COL1 is not the only protein present in skeletal materials, however. Research using sophisticated shotgun proteomics (LC-MS/MS) methods has demonstrated that Pleistocene and Holocene bone and dentine can preserve dozens, if not hundreds, of different proteins. Enamel, the hardest tissue in the mammalian skeleton, only contains around 10 proteins in life, but several of these survive for extremely long periods of time, at least 1.9 million years into the past (Cappellini et al., 2019; Welker et al., 2020). Ancient proteomes therefore preserve beyond the survival limits of ancient DNA in the same environmental conditions.

Studying entire skeletal proteomes through shotgun proteomic methods has some benefits compared to the MALDI-ToF MS technology used for ZooMS. Firstly, tandem mass spectrometry data analysis allows determination of the amino acid sequences of the peptides present in a protein extract (Figure 7). This enables the reconstruction of protein amino acid sequences. Secondly, shotgun proteomic methods are generally more sensitive compared to MALDI-ToF MS. This enables detection of peptides that are less abundant, allowing the study of specimens with more degraded proteomes, for example hominin fossils without ancient DNA preservation. Thirdly, using shotgun proteomic methods allows determination of the presence of proteins

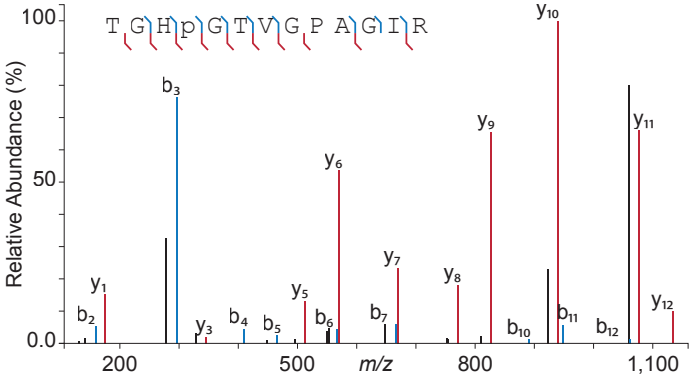


Figure 7. An example of a tandem mass spectrum. *In this case, the peptide TGHPGTVGPAGIR was fragmented, resulting in b (in blue) and y (in red) fragment ions of varying intensity. Fragmentation sites of assigned fragment ions are indicated across the peptide sequence inset. This peptide is known as peptide marker COL1α2 978–990 in ZooMS studies, appearing at 1,235.6 m/z in MALDI-ToF MS spectra of COL1. The peptide contains a number of phylogenetically informative positions across Mammalia (Figure 2). The peptide is placed at amino acid positions 1068 to 1080 in COL1α2 of the human reference protein sequence, Uniprot: P08123. Figure generated with assistance of the Interactive Peptide Spectral Annotator (Brademan et al., 2019).*

---

beside COL1. Through shotgun proteomic methods we therefore gain access to the entire skeletal proteome preserved in a sample.

The proteins and peptide sequences identified are not like the modern proteins and peptide sequences that one would observe in a modern skeletal sample (Figure 8). The individual amino acids making up the protein sequences will have undergone a variety of diagenetic modifications through the addition or removal of chemical compounds. Additionally, the amino acid sequences that constituted the protein will have undergone partial hydroly-

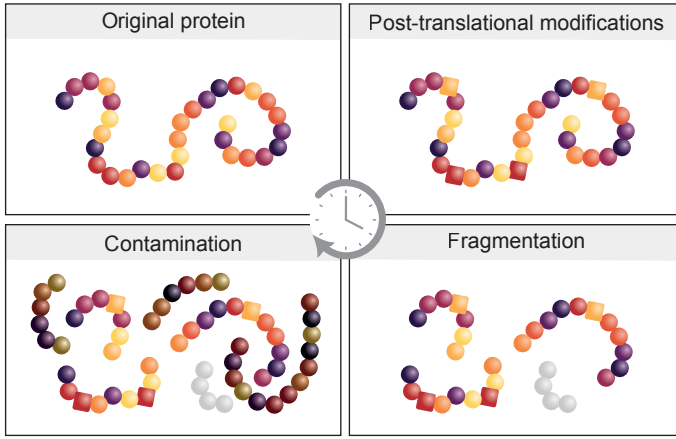


Figure 8. Proteome degradation and contamination. *Over time, amino acids acquire diagenetic modifications, peptide bonds break, and contamination is occurring, resulting in a complex mixture of diagenetically altered ancient proteins and modern contamination. Credit: Z. Fagernäs.*

sis at some peptide bond locations, fragmenting the protein into short peptides (also called diagenetiforms). Through this process, parts of some proteins will almost never survive into the Pleistocene, while for other proteins peptide bond hydrolysis will be so extensive that these proteins do not survive at all. As a result, the skeletal proteomes that survive over extended chronological periods are small, and composed of fragmented and modified amino acid sequences. Both extraction methods and computational approaches have to take this into account.

Finally, as with any ancient biomolecular study, the endogenous skeletal proteomes have become contaminated with proteins from the bacteria, fungi, and other organisms that have lived in and on the skeletal material since its deposition in the archaeological record, as well as the proteins deposited onto them

during excavation, storing, and human handling during research activities. The extraction of ancient skeletal proteomes therefore has to be conducted in clean laboratory environments where additional contamination is minimised. In addition, there are several approaches available to detect the presence of protein contamination. First, many proteins are specific to particular cell types or tissues, meaning that their spatial distribution within an organism, or across its lifetime, might be rather restricted. One example of such a protein group are keratins, many of which are specific to skin or hair, and which are present in most skeletal protein extracts. Due to their specific expression in hair and skin, however, they are not endogenous to the skeletal proteomes but are present as the result of human handling of a skeletal element prior to protein extraction. Second, we can quantify the extensive damage that characterises ancient proteins, and compare this to the level of protein damage observed in positive and negative controls. This could show, for example, that very ancient proteins have shorter peptide lengths compared to peptides recovered from younger, less degraded samples (Welker et al. 2020). Such measures to prevent contamination from happening within the laboratory environment, and subsequent data analysis strategies to authenticate proteome composition and its damage serve to validate the interpretations made based palaeoproteomic datasets.

The composition of these skeletal proteomes is generally thought of as being highly similar between skeletal elements of the same skeleton, but can provide some surprising insights. For example, some of the hominin bones discovered through ZooMS at the site of Grotte du Renne were also analysed through shotgun proteomic techniques. This allowed for the retrieval of amino acid sequences unique to the genus *Homo*, thereby confirming the assignment of these specimens to our genus. The skeletal proteome also included peptide sequences unique to the collagen type X, alpha-1 protein (COL10A1). This protein is secreted by a par-

ticular cell type, hypertrophic chondrocytes, during the final stages of initial endochondral bone ossification. The analysed bone specimen therefore likely derives from a foetal or juvenile bone specimen, as COL10A1 would not be expressed at later stages of skeletal development (Welker et al., 2016). This observation was subsequently confirmed through stable isotope analysis (Jaouen et al., 2019).

Another example concerns the protein amelogenin. When analysing enamel proteomes, the protein dominating the enamel proteome is amelogenin, which is located on both the X and Y chromosomes in primates. The region of these chromosomes where the amelogenin genes are located does not undergo recombination, and as a result the amelogenin-X (AMELX) and amelogenin-Y (AMELY) proteins have accumulated slightly different amino acid sequences over evolutionary timescales. Observing peptide sequences unique to AMELY therefore determines that a particular specimen was a biological male. AMELX and AMELY are not secreted at equal amounts, instead, AMELX is far more abundant than AMELY. Only observing AMELX-specific peptides could therefore indicate that a specimen was a biological female. Alternatively, this observation could be due to extensive proteome degradation, where AMELY is no longer preserved. Finally, some male primates do not carry any AMELY gene on their Y chromosome, and they would appear as a biological female through dental enamel proteomics (Skov et al., 2022). It has therefore become feasible to determine the biological sex of hominins, particularly of biological males, through protein mass spectrometry analysis of dental enamel samples, even for specimens almost two million years old (Welker et al., 2020).

Of course, one primary reason to analyse entire skeletal proteomes concerns the phylogenetic information contained within the amino acid sequences. It should be realised that this informa-

tion is rather limited in comparison to the wealth of information obtained from ancient genomes. The proteomes expressed in skeletal tissues derive from only a small fraction of the genomic nucleotide sequences, and only a part of those survive the challenges of long-term preservation. The protein sequences themselves will be under significant selection pressure, ensuring that only some amino acid positions can be substituted without negatively impacting protein function, further limiting the amino acid sequence variation that is possible. Nevertheless, comparison of genomes of modern humans, Neanderthals, Denisovans, and other great apes shows that protein sequence variation exists and that some of this is located in the part of the proteomes we can retrieve from Pleistocene skeletal material.

Denisovans are an enigmatic hominin population initially described based on the ancient genome recovered from a fossil derived from Denisova Cave, Russia (Meyer et al., 2012). They were more closely related to Neanderthals than to modern humans. Analysis of a high-coverage Denisovan genome has demonstrated that Denisovans and modern humans produced fertile offspring in the past, probably repeatedly. Levels of Denisovan introgression in modern human populations are highest in southeast Asian human populations, suggesting that Denisovans were widespread across eastern Eurasia. In Himalayan populations, Denisovan introgression introduced an allele essential for the high-altitude adaptation necessary to survive in the hypoxic conditions that characterise this environment (Huerta-Sánchez et al., 2014). Subsequent analysis of other hominin fossils recovered from Denisova Cave has provided additional Middle and Late Pleistocene Denisovan remains, but all of these are rather fragmentary. As a result, although the East Asian hominin fossil record is large, there is little morphological comparison possible with the Denisovan material recovered from Denisova Cave.

Many hominin fossils turn out to contain little to no ancient DNA, and the so-called Xiahe mandible is one of them (Figure 9). Uranium-thorium dating of an adhering carbonate crust demonstrated that the specimen is late Middle Pleistocene in age. Based on its curatorial history, the mandible derives from Baishiya Karst Cave, a sacred site associated with the local Tibetan Buddhist monastery and located on the lower reaches of the Tibetan Plateau in China. As with many other Middle Pleistocene hominin fossils, its population attribution through morphological methods is complicated, making it impossible to assign to Neanderthals or modern humans with any certainty. Although current archaeogenetic methodology failed to retrieve ancient DNA from the specimen, the palaeoproteomic analysis of a dentine sample from this mandible allowed the recovery of a small, highly degraded proteome (Chen et al., 2019). Based on the phylogenetic analysis of the reconstructed protein sequences from the mandible, the specimen is placed closest to the high-coverage Denisovan genome from Denisova Cave (Figure 9). The Xiahe mandible is therefore stemming from a Denisovan or Denisovan-related hominin.

Since the Xiahe specimen preserves an almost complete hemimandible as well as two molars, it allows for morphological comparisons to be made with other eastern Eurasian hominin mandibles and molars. Several Middle and Late Pleistocene hominins, such as the Penghu mandible from the Taiwan Strait, could therefore also represent Denisovan or Denisovan-related hominin populations. Comparison of morphological characteristics of the Xiahe molars demonstrated that a molar from Tam Ngu Hao 2 (also known as Cobra Cave, Laos) is rather identical. If it is indeed considered a Denisovan, then it brings the physical evidence of the spatiotemporal distribution of Denisovans into the late Middle Pleistocene of southeast Asia, a time period when several hominin populations are known to have been present in the re-

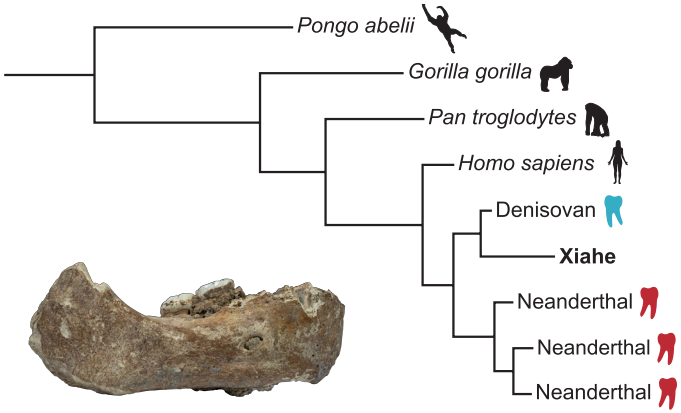


Figure 9. The Xiahe mandible, from Baishiya Karst Cave (China). *Based on morphology, the taxonomic identity of this mandible could not be determined with certainty, while a phylogenetic analysis of ancient and modern protein sequences demonstrated that the Xiahe mandible is, in this comparative context, a Denisovan or Denisovan-related hominin. Data taken from Chen et al., 2019. Credit: Z. Fagernäs.*

---

gion (Demeter et al., 2022). Future morphological and molecular research on these and other hominin fossils, including palaeoproteomic studies, is therefore likely, and necessary to further improve our understanding of Denisovans.

Subsequent archaeological research conducted at Baishiya Karst Cave has resulted in the recovery of archaeological material spanning approximately 150,000 years, including stone tools and a large, highly fragmented faunal assemblage (Zhang et al., 2020). The recovery of ancient sedimentary DNA demonstrated the presence of Denisovan mitochondrial DNA, both confirming the palaeoproteomic observations of the Xiahe mandible as well as

extending the time range of Denisovan presence at the site. The material being recovered from the site will enable environmental reconstructions of this part of the Tibetan Plateau, contextualising hominin occupation in a highly challenging hypoxic environment. The stone tool and faunal assemblage will allow for assessment of Denisovan behaviour, for example in terms of faunal procurement and butchery practices. As only two to three archaeological sites are known to contain Denisovan archaeological material, observations made at Baishiya Karst Cave will be very informative on Denisovan archaeology generally. Finally, the recovery of a Denisovan-related hominin from the Tibetan Plateau has implications by itself, as previous research suggested that only "modern human" populations would be able to adapt their survival strategies to such a challenging environment. With the recovery of the Xiahe dentine proteome and the excavation of Baishiya Karst Cave, we can now say with certainty that archaic hominins, such as Denisovans, were equally able to exploit these environments.

CONCLUSION

Palaeoproteomics provides tremendous opportunities to enhance our understanding of human evolution. Protein mass spectrometry methods can contribute significantly to the number of hominin fossils available for molecular and isotopic analysis, which will alleviate the growing pressure on the finite hominin fossils record. Additional hominin remains enable improvements of our understanding of the past spatiotemporal distributions of hominin populations, for example when modelling the dispersal of modern humans across Europe in conjunction with the disappearance of Neanderthals. Proteomic screening, as well as shotgun proteomic methods, allow the contextualization of these hominin remains in relation to the faunal communities with which they interacted, including patterns of prey preference, butchery practices, and the acquisition of raw materials for bone artefact production. Finally, the palaeoproteomic analysis of hominin remains, whether discovered morphologically or through proteomic screening, allows for the recovery of molecular phylogenetic information beyond the preservation limits of ancient DNA.

Through these various applications, palaeoproteomics will contribute new perspectives on human evolution, just like ancient DNA has changed our understanding of the field over the past decades. To accomplish this, palaeoproteomics will have to further develop extraction and analytical methods, for example in terms of sample selection procedures, measures to decontaminate skeletal specimens, optimization of data acquisition strategies during mass spectrometry, and spectral identification strategies after protein mass spectrometry. This will allow the recovery of ever-older skeletal proteomes, but also the retrieval of larger, phylogenetically more informative proteomes, for an increasing number of animal and hominin fossils. With these methodological developments in mind and the ubiquity of proteins in archaeological and palaeo-

anthropological contexts, it is clear that we are only at the beginning of an exciting period for palaeoproteomic applications in human evolutionary contexts and beyond.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the Board of the Stichting Nederlands Museum voor Anthropologie en Praehistorie for their invitation to provide the 45th Kroonvoordracht. It was an unexpected honour to be invited to present this lecture. Ragnheiður Diljá Ásmundsdóttir, Dorothea Mylopotamitaki, Gaudry Troché, Geoff M. Smith, Huan Xia, Jakob Hansen, Jorsua Herrera Bethencourt, Karen Ruebens, Louise Le Meillour, Virginie Sinet-Mathiot and Zandra Fagernäs should be thanked for discussing and reading drafts of this text. I want to express my gratitude to collaborators allowing us to work on their precious archaeological and palaeoanthropological material, whether hominin fossils or small(er) faunal bone fragments, and institutions and funders that have made our work possible over the past years. I would also like to thank members of the Biomolecular Palaeoanthropology research group, as well as close collaborators and friends scattered far and wide across the globe, for being a source of daily inspiration. Wherever we are heading scientifically, it is exciting and an honour to be on this journey with you. This lecture is therefore dedicated to you (although any mistakes in it are strictly mine!).

REFERENCES

Benazzi, S., Slon, V., Talamo, S., Negrino, F., Peresani, M., Bailey, S. E., Sawyer, S., Panetta, D., Vicino, G., Starnini, E., Mannino, M. A., Salvadori, P. A., Meyer, M., Pääbo, S., & Hublin, J.-J. (2015). The makers of the Protoaurignacian and implications for Neandertal extinction. *Science*, *348*(6236), 793–796.

Brademan, D. R., Riley, N. M., Kwiecien, N. W., & Coon, J. J. (2019). Interactive Peptide Spectral Annotator: a versatile web-based tool for proteomic applications. *Molecular & Cellular Proteomics: MCP*, *18*(8 suppl 1), S193–S201.

Brown, S., Higham, T., Slon, V., Pääbo, S., Meyer, M., Douka, K., Brock, F., Comeskey, D., Procopio, N., Shunkov, M., Derevianko, A., & Buckley, M. (2016). Identification of a new hominin bone from Denisova Cave, Siberia, using collagen fingerprinting and mitochondrial DNA analysis. *Scientific Reports*, *6*, 23559.

Buckley, M., Collins, M., Thomas-Oates, J., & Wilson, J. C. (2009). Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry: RCM*, *23*(23), 3843–3854.

Cappellini, E., Welker, F., Pandolfi, L., Ramos-Madrigal, J., Samodova, D., Rüther, P. L., Fotakis, A. K., Lyon, D., Moreno-Mayar, J. V., Bukhsianidze, M., Rakownikow Jersie-Christensen, R., Mackie, M., Ginolhac, A., Ferring, R., Tappen, M., Palkopoulou, E., Dickinson, M. R., Stafford, T. W., Jr, Chan, Y. L., … Willerslev, E. (2019). Early Pleistocene enamel proteome from Dmanisi resolves *Stephanorhinus* phylogeny. *Nature*, *574*(7776), 103–107.

Chen, F., Welker, F., Shen, C.-C., Bailey, S. E., Bergmann, I., Davis, S., Xia, H., Wang, H., Fischer, R., Freidline, S. E., Yu, T.-L., Skinner, M. M., Stelzer, S., Dong, G., Fu, Q., Dong, G., Wang, J., Zhang, D., & Hublin, J.-J. (2019). A late Middle Pleistocene Denisovan mandible from the Tibetan Plateau. *Nature*, *569*(7756), 409–412.

Daujeard, C. (2008). *Exploitation du milieu animal par les Néanderthaliens dans le Sud-Est de la France* [Lyon 2]. http://www.theses.fr/2008LYO20003

Demeter, F., Zanolli, C., Westaway, K. E., Joannes-Boyau, R., Duringer, P., Morley, M. W., Welker, F., Rüther, P. L., Skinner, M. M., McColl, H., Gaunitz, C., Vinner, L., Dunn, T. E., Olsen, J. V., Sikora, M., Ponche, J.-L., Suzzoni, E., Frangeul, S., Boesch, Q., … Shackelford, L. (2022). A Middle Pleistocene Denisovan molar from the Annamite Chain of northern Laos. *Nature Communications*, *13*(1), 2557.

Fewlass, H., Talamo, S., Wacker, L., Kromer, B., Tuna, T., Fagault, Y., Bard, E., McPherron, S. P., Aldeias, V., Maria, R., Martisius, N. L., Paskulin, L., Rezek, Z., Sinet-Mathiot, V., Sirakova, S., Smith, G. M., Spasov, R., Welker, F., Sirakov, N., … Hublin, J.-J. (2020). A 14C chronology for the Middle to Upper Palaeolithic transition at Bacho Kiro Cave, Bulgaria. *Nature Ecology & Evolution*, *4*(6), 794–801.

Fuhlrott, J. C. (1857). Theile des menschlichen Skelettes im Neanderthal bei Hochdahl. *Verhandlungen Des Naturhistorischen Vereines Der Preussischen Rheinlande Und Westphalens*, *14*, 50.

Hajdinjak, M., Mafessoni, F., Skov, L., Vernot, B., Hübner, A., Fu, Q., Essel, E., Nagel, S., Nickel, B., Richter, J., Moldovan, O. T., Constantin, S., Endarova, E., Zahariev, N., Spasov, R., Welker, F., Smith, G. M., Sinet-Mathiot, V., Paskulin, L., … Pääbo, S. (2021). Initial Upper Palaeolithic humans in Europe had recent Neanderthal ancestry. *Nature*, *592*(7853), 253–257.

Hendy, J. (2021). Ancient protein analysis in archaeology. *Science Advances*, *7*(3). https://doi.org/10.1126/sciadv.abb9314

Hublin, J.-J., Sirakov, N., Aldeias, V., Bailey, S., Bard, E., Delvigne, V., Endarova, E., Fagault, Y., Fewlass, H., Hajdinjak, M., Kromer, B., Krumov, I., Marreiros, J., Martisius, N. L., Paskulin, L., Sinet-Mathiot, V., Meyer, M., Pääbo, S., Popov, V., … Tsanova, T. (2020). Initial Upper Palaeolithic *Homo sapiens* from Bacho Kiro Cave, Bulgaria. *Nature*, *581*(7808), 299–302.

Huerta-Sánchez, E., Jin, X., Asan, Bianba, Z., Peter, B. M., Vinckenbosch, N., Liang, Y., Yi, X., He, M., Somel, M., Ni, P., Wang, B., Ou, X., Huasang, Luosang, J., Cuo, Z. X. P., Li, K., Gao, G., Yin, Y., … Nielsen, R. (2014). Altitude adaptation in Tibetans caused by introgression of Denisovan-like DNA. *Nature*, *512*(7513), 194–197.

Jaouen, K., Richards, M. P., Le Cabec, A., Welker, F., Rendu, W., Hublin, J.-J., Soressi, M., & Talamo, S. (2019). Exceptionally high $\delta^{15}N$ values in collagen single amino acids confirm Neandertals as high-trophic level carnivores. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(11), 4928–4933.

Lanigan, L. T., Mackie, M., Feine, S., Hublin, J.-J., Schmitz, R. W., Wilcke, A., Collins, M. J., Cappellini, E., Olsen, J. V., Taurozzi, A. J., & Welker, F. (2020). Multi-protease analysis of Pleistocene bone proteomes. *Journal of Proteomics*, *228*, 103889.

Martisius, N. L., Spasov, R., Smith, G. M., Endarova, E., Sinet-Mathiot, V., Welker, F., Aldeias, V., Horta, P., Marreiros, J., Rezek, Z., McPherron, S. P., Sirakov, N., Sirakova, S., Tsanova, T., & Hublin, J.-J. (2022). Initial Upper Paleolithic bone technology and personal ornaments at Bacho Kiro Cave (Bulgaria). *Journal of Human Evolution*, *167*, 103198.

Martisius, N. L., Welker, F., Dogandžić, T., Grote, M. N., Rendu, W., Sinet-Mathiot, V., Wilcke, A., McPherron, S. J. P., Soressi, M., & Steele, T. E. (2020). Non-destructive ZooMS identification reveals strategic bone tool raw material selection by Neandertals. *Scientific Reports*, *10*(1), 7746.

Meyer, M., Kircher, M., Gansauge, M.-T., Li, H., Racimo, F., Mallick, S., Schraiber, J. G., Jay, F., Prüfer, K., de Filippo, C., Sudmant, P. H., Alkan, C., Fu, Q., Do, R., Rohland, N., Tandon, A., Siebauer, M., Green, R. E., Bryc, K., … Pääbo, S. (2012). A high-coverage genome sequence from an archaic Denisovan individual. *Science*, *338*(6104), 222–226.

Prüfer, K., Posth, C., Yu, H., Stoessel, A., Spyrou, M. A., Deviese, T., Mattonai, M., Ribechini, E., Higham, T., Velemínský, P., Brůžek, J., & Krause, J. (2021). A genome sequence from a modern human skull over 45,000 years old from Zlatý kůň in Czechia. *Nature Ecology & Evolution*, *5*(6), 820–825.

Ruebens, K., Sinet-Mathiot, V., Talamo, S., Smith, G. M., Welker, F., Hublin, J.-J., & McPherron, S. P. (2022). The Late Middle Palaeolithic Occupation of Abri du Maras (Layer 1, Neronian, Southeast France): integrating lithic analyses, ZooMS and radiocarbon dating to reconstruct Neanderthal hunting behaviour. *Journal of Paleolithic Archaeology*, *5*(1), 4.

Rüther, P. L., Husic, I. M., Bangsgaard, P., Gregersen, K. M., Pantmann, P., Carvalho, M., Godinho, R. M., Friedl, L., Cascalheira, J., Taurozzi, A. J., Jørkov, M. L. S., Benedetti, M. M., Haws, J., Bicho, N., Welker, F., Cappellini, E., & Olsen, J. V. (2022). SPIN enables high throughput species identification of archaeological bone by proteomics. *Nature Communications*, *13*(1), 2458.

Schaaffhausen, H. (1857). Vortrag vom 2. Juni zu den Menschenknochen aus dem Neandertal. *Verhandlungen Des Naturhistorischen Vereines Der Preussischen Rheinlande Und Westphalens*, *14*, 50.

Sinet-Mathiot, V., Rendu, W., Steele, T. E., Spasov, R., Madelaine, S., Renou, S., Soulier, M.-C., Martisius, N. L., Aldeias, V., Endarova, E., Goldberg, P., McPherron, S. J. P., Rezek, Z., Sandgathe, D., Sirakov, N., Sirakova, S., Soressi, M., Tsanova, T., Turq, A., … Smith, G. M. (2023). Identifying the unidentified fauna enhances insights into hominin subsistence strategies during the Middle to Upper Palaeolithic transition. *Archaeological and Anthropological Sciences*, *15*(9), 139.

Sinet-Mathiot, V., Smith, G. M., Romandini, M., Wilcke, A., Peresani, M., Hublin, J.-J., & Welker, F. (2019). Combining ZooMS and zooarchaeology to study Late Pleistocene hominin behaviour at Fumane (Italy). *Scientific Reports*, *9*(1), 12350.

Skov, L., Peyrégne, S., Popli, D., Iasi, L. N. M., Devièse, T., Slon, V., Zavala, E. I., Hajdinjak, M., Sümer, A. P., Grote, S., Bossoms Mesa, A., López Herráez, D., Nickel, B., Nagel, S., Richter, J., Essel, E., Gansauge, M., Schmidt, A., Korlević, P., … Peter, B. M. (2022). Genetic insights into the social organization of Neanderthals. *Nature*, *610*(7932), 519–525.

Slon, V., Hopfe, C., Weiß, C. L., Mafessoni, F., de la Rasilla, M., Lalueza-Fox, C., Rosas, A., Soressi, M., Knul, M. V., Miller, R., Stewart, J. R., Derevianko, A. P., Jacobs, Z., Li, B., Roberts, R. G., Shunkov, M. V., de Lumley, H., Perrenoud, C., Gušić, I., … Meyer, M. (2017). Neandertal and Denisovan DNA from Pleistocene sediments. *Science*, *356*(6338), 605–608.

Slon, V., Mafessoni, F., Vernot, B., de Filippo, C., Grote, S., Viola, B., Hajdinjak, M., Peyrégne, S., Nagel, S., Brown, S., Douka, K., Higham, T., Kozlikin, M. B., Shunkov, M. V., Derevianko, A. P., Kelso, J., Meyer, M., Prüfer, K., & Pääbo, S. (2018). The genome of the offspring of a Neanderthal mother and a Denisovan father. *Nature*, *561*(7721), 113–116.

Soressi, M., McPherron, S. P., Lenoir, M., Dogandžić, T., Goldberg, P., Jacobs, Z., Maigrot, Y., Martisius, N. L., Miller, C. E., Rendu, W., Richards, M., Skinner, M. M., Steele, T. E., Talamo, S., & Texier, J.-P. (2013). Neandertals made the first specialized bone tools in Europe. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(35), 14186–14190.

Trinkaus, E., Moldovan, O., Milota, S., Bîlgăr, A., Sarcina, L., Athreya, S., Bailey, S. E., Rodrigo, R., Mircea, G., Higham, T., Ramsey, C. B., & van der Plicht, J. (2003). An early modern human from the Peştera cu Oase, Romania. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(20), 11231–11236.

Warinner, C., Korzow Richter, K., & Collins, M. J. (2022). Paleoproteomics. *Chemical Reviews*, *122*(16), 13401–13446.

Welker, F., Hajdinjak, M., Talamo, S., Jaouen, K., Dannemann, M., David, F., Julien, M., Meyer, M., Kelso, J., Barnes, I., Brace, S., Kamminga, P., Fischer, R., Kessler, B. M., Stewart, J. R., Pääbo, S., Collins, M. J., & Hublin, J.-J. (2016). Palaeoproteomic evidence identifies archaic hominins associated with the Châtelperronian at the Grotte du Renne. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(40), 11162–11167.

Welker, F., Ramos-Madrigal, J., Gutenbrunner, P., Mackie, M., Tiwary, S., Rakownikow Jersie-Christensen, R., Chiva, C., Dickinson, M. R., Kuhlwilm, M., de Manuel, M., Gelabert, P., Martinón-Torres, M., Margvelashvili, A., Arsuaga, J. L., Carbonell, E., Marques-Bonet, T., Penkman, K., Sabidó, E., Cox, J., … Cappellini, E. (2020). The dental proteome of *Homo antecessor*. *Nature*, *580*(7802), 235–238.

Welker, F., Soressi, M., Rendu, W., Hublin, J.-J., & Collins, M. (2015). Using ZooMS to identify fragmentary bone from the Late Middle/Early Upper Palaeolithic sequence of Les Cottés, France. *Journal of Archaeological Science*, *54*, 279–286.

Zhang, D., Xia, H., Chen, F., Li, B., Slon, V., Cheng, T., Yang, R., Jacobs, Z., Dai, Q., Massilani, D., Shen, X., Wang, J., Feng, X., Cao, P., Yang, M. A., Yao, J., Yang, J., Madsen, D. B., Han, Y., … Fu, Q. (2020). Denisovan DNA in Late Pleistocene sediments from Baishiya Karst Cave on the Tibetan Plateau. *Science*, *370*(6516), 584–587.

EERSTE KROON-VOORDRACHT (1971)
G. Bosinski, *Die Tierdarstellungen des Magdalénien Fundplatzes Gönnersdorf bei Neuwied*

TWEEDE KROON-VOORDRACHT (1975)
F. Anders, *Kunst und Kunstgewerbe Altmexikos*

DERDE KROON-VOORDRACHT (1977)
C.B.M. McBurney, *Archaeology and the Homo Sapiens Sapiens Problem in Northern Africa*

VIERDE KROON-VOORDRACHT (1978)
Raymond H. Thompson, *Mesoamerica and the Goals of Archaeology*

VIJFDE KROON-VOORDRACHT (1981)
J.K.S. St Joseph, *Air Photography and Archaeology: Achievements and Prospects*

ZESDE KROON-VOORDRACHT (1983)
Glynn Isaac, *Early Stages in the Evolution of Human Behavior: the Adaptive Significance of Stone Tools*

ZEVENDE KROON-VOORDRACHT (1984)
H.T. Waterbolk, *Archeologie en Landschap*

ACHTSTE KROON-VOORDRACHT (1985)
Tim D. White, *Acheulian Man in Ethiopia's Middle Awash Valley: The Implications of Cutmarks on the Bodo Cranium*

NEGENDE KROON-VOORDRACHT (1986)
M. Biddle, *The Thames and the Rhine: urban origins in the Channel-region (paper presented for the Ninth Kroon-Voordracht (1986); manuscript for publication never received)*

TIENDE KROON-VOORDRACHT (1987)
J.D. van der Waals, *De kolonisatie van het terpengebied – een ethno-archeologische benadering*

ELFDE KROON-VOORDRACHT (1988)
Jane E. Buikstra, *The mound-builders of eastern North America – a regional perspective*

TWAALFDE KROON-VOORDRACHT (1990)
David R. Harris, Settling down and breaking ground: *Rethinking the Neolithic Revolution*

DERTIENDE KROON-VOORDRACHT (1991)
W.A. van Es, *Van Maastricht naar Rhenen, een wandeling in de Laat-Romeinse tijd*

VEERTIENDE KROON-VOORDRACHT (1992)
J.D. Lewis-Williams, *Vision, Power and Dance: The Genesis of a Southern African Rock Art Panel*

VIJFTIENDE KROON-VOORDRACHT (1993)
B.W. Cunliffe, *Fertility, Propitiation and the Gods in the British Iron Age*

ZESTIENDE KROON-VOORDRACHT (1994)
J.T. Urtāns, *Latvian Hillforts: the Originality of the Archaeological Reality*

ZEVENTIENDE KROON-VOORDRACHT (1995)
Ph.V. Tobias, *The communication of the dead: earliest vestiges of the origin of articulate language*

ACHTTIENDE KROON-VOORDRACHT (1996)
O. Crumlin-Pedersen, *Archaeology and the sea*

NEGENTIENDE KROON-VOORDRACHT (1997)
George Eogan, *The discovery programme: Initiation, consolidation and development*

TWINTIGSTE KROON-VOORDRACHT (1998)
Leendert P. Louwe Kooijmans, *Between Geleen and Banpo, the Agricultural Transformation of Prehistoric Society, 9000 – 4000 BC*

EENENTWINTIGSTE KROON-VOORDRACHT (1999)
Colin Renfrew, *Loot, Legitimacy and ownership: The ethical crisis in archaeology*

TWEEENTWINTIGSTE KROON-VOORDRACHT (2000)
Erika Hagelberg, *Genetics in the study of human history: problems and opportunities*

DRIEENTWINTIGSTE KROON-VOORDRACHT (2001)
João Zilhão, *Anatomically archaic, behaviorally modern: The last Neanderthals and their destiny*

VIERENTWINTIGSTE KROON-VOORDRACHT (2002)
S. von Schnurbein, *Augustus in Germanien, neue archäologische Forschungen*

VIJFENTWINTIGSTE KROON-VOORDRACHT (2003)
S.W. Hillson, D.R. Brothwell and G.J.R. Maat, *Wealth, health and human remains in archaeology*

ZESENTWINTIGSTE KROON-VOORDRACHT (2004)
Miranda Aldhouse-Green, *Beyond the gods: biographies, values and cosmologies in 'celtic' iconography*

ZEVENENTWINTIGSTE KROON-VOORDRACHT (2005)
Maarten J. Raven, *Architectural principles in theory and practice: The New Kingdom Necropolis at Saqqara (Egypt)*

ACHTENTWINTIGSTE KROON-VOORDRACHT (2006)
Paul Halstead, *What's ours is mine? Village and household in early farming society in Greece*

NEGENENTWINTIGSTE KROON-VOORDRACHT (2007)
Peter de Knijff, *Archaeology by means of DNA: hype or here to stay?*

DERTIGSTE KROON-VOORDRACHT (2008)
Hermann Parzinger e.a., *Der Skythenzeitliche Krieger aus dem Eis: neue Entdeckungen im Mongolischen Altaj*

EENENDERTIGSTE KROON-VOORDRACHT (2009)
Bernard Wood, *The Human Fossil Record: Challenges and Opportunities*

TWEEENDERTIGSTE KROON-VOORDRACHT (2010)
Wil Roebroeks, *The Neandertal Experiment*

DRIEENDERTIGSTE KROON-VOORDRACHT (2011)
Johannes Müller, *Megaliths and Funnel Beakers: societies in change 4100-2700 BC*

VIERENDERTIGSTE KROON-VOORDRACHT (2012)
Albert Zink, *From Tutankhamun to Ötzi: the use of modern scientific methods in mummy research*

VIJFENDERTIGSTE KROON-VOORDRACHT (2013)
J. Francis Thackeray, *Human evolution from an African perspective: with reference to Charles Darwin palaeoclimate and the problem of defining a species*

ZESENDERTIGSTE KROON-VOORDRACHT (2014)
André F.L. van Holk, Jerzy H.G. Gawronsky, Jonathan R. Adams and Henk Dessens, *Maritime Archaeology*

ZEVENENDERTIGSTE KROON-VOORDRACHT (2015)
Lee R. Berger, *Five years after Australopithecus Sediba. New discoveries changing our understanding of human evolution*

ACHTENDERTIGSTE KROON-VOORDRACHT (2016)
Fred Spoor, *Evolving primates: a palaeontologist's view of his tribe*

NEGENENDERTIGSTE KROON-VOORDRACHT (2017)
Graeme Barker, *A tale of three caves: why was our species so successful at colonising new environments*

VEERTIGSTE KROON-VOORDRACHT (2018)
Michael Parker Pearson, *Science and Stonehenge: recent investigations of the world's most famous stone circle*

EENENVEERTIGSTE KROON-VOORDRACHT (2019)
Jean-Jacques Hublin, *The rise and spread of homo sapiens*

TWEEENVEERTIGSTE KROON-VOORDRACHT (2020)
Corrie C. Bakels, *Man: manipulator of his physical environment*

DRIEENVEERTIGSTE KROON-VOORDRACHT (2021)
David R. Fontijn, *'Give Peace a Chance' on Violence and Warfare in Prehistory and Why it Matters*

VIERENVEERTIGSTE KROON-VOORDRACHT (2022)
John D. Speth, *Archaeology, ethnohistory, early hominins, and some cherished scientific myths*

KROON-VOORDRACHTEN

REDACTIE: WILLY H. METZ

Printed by

DRUKKERIJ J. BOUT & ZN., HUIZEN

ISBN/EAN 978-90-829-481-5-8