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The palaeoproteomic identification of pleistocene hominin skeletal remains

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Chapter 6:
Discussion and Conclusion

1. Biological implications for the MP-UP transition

This thesis aimed to provide a palaeoproteomic workflow based on ZooMS screening and LC-MS/MS analysis that would allow the identification of hitherto unrecognized hominin fossils in Middle to Upper Palaeolithic contexts and provide ancient protein evidence on the genetic ancestry of these hominin specimens. Several aspects of this workflow were specifically developed, such as a COL1 faunal database (Welker et al., 2016), and how to build this (Welker et al., 2015a). The thesis also provides an initial exploration of possible differences in species composition between morphologically identified bone assemblages and those identified through ZooMS (Welker et al., 2017; Welker et al., 2015b). Furthermore, the final chapter of this thesis demonstrates that ancient proteins enable the taxonomic and phylogenetic placement of ancient hominin specimens (Welker et al., 2016).

ZooMS screening is successful if the correct taxonomic identifications can be obtained in a cost-effective manner. We demonstrated that ZooMS provides molecular taxonomic identifications in agreement with morphological identifications obtained in a blind-test comparison (Welker et al., 2015b). In addition, ZooMS screening results demonstrate that ZooMS success rates are high (>90%) for all three Châtelperronian sites included in this thesis (Figure 6.1A). Such success rates are comparable to those obtained on smaller assemblages for younger time periods (Charlton et al., 2016; Evans et al., 2016).

This thesis proposes to routinely use ammonium-bicarbonate extraction prior to acid demineralization extraction for selected specimens as this allows reliable COL1 deamidation values to be calculated for specific glutamine and asparagine positions (Welker et al., 2016). Ammonium-bicarbonate extraction is also compatible with subsequent ancient DNA analysis or LC-MS/MS analysis, thereby minimizing the need for additional samples to be taken from the original bone specimen (von Holstein et al., 2014; Welker et al., 2016; 2017). In addition, ZooMS screening with ammonium-bicarbonate (Quinçay and the Grotte du Renne) is as successful as those studied with acid demineralisation (Les Cottés) and only requires a small number of bone specimens to be demineralised after initial ammonium-bicarbonate buffer extraction (≈5%; Figure 6.1A). Together with the completion of an adequate COL1 sequence and peptide marker database (Welker et al., 2016), it seems that all individual components for successful large-scale applications of ZooMS screening on the MP-UP transitional assemblages are in place.

Palaeoproteomic analysis is now of relevance to the study of hominin phylogeny, not merely by screening for additional hominin specimens through ZooMS (Welker et al., 2016). The phylogenetic relationships between ancient hominins, early modern humans and present-day modern humans is currently routinely explored by analyzing ancient DNA for ever increasing numbers of individuals (Fu et al., 2016; Green et al., 2010; Krings et al., 1997; Meyer et al., 2012). This ancient DNA “revolution” largely takes place in the Late Pleistocene, with only limited genetic data available for Middle Pleistocene hominin

specimens (Meyer et al., 2016, 2014). This is not unexpected given the expected degradation of DNA over time, and technical progress will likely increase our ability to retrieve, sequence and analyze ancient DNA from Middle Pleistocene time periods. Nevertheless, the ability of ancient proteins to provide insights into the phylogenetic relationships between extant and extinct hominid populations provides an alternative biomolecular approach to several current phylogenetic issues (Dembo et al., 2016; Stringer, 2016). The survival of proteins in contexts where ancient DNA no longer survives is especially promising in this regard (Welker et al., 2015a).

1.1 Ecological and taphonomic observations

One of the ecological observations made during ZooMS analysis is that the studied fragmented bone assemblages have a rich species composition. This is of interest as such bone specimens are good candidates for subsequent stable isotope analysis, thereby providing an ecological, dietary and environmental context for any newly identified hominins or the transitional assemblages they derive from (Welker et al., 2016). Future ZooMS screening of transitional assemblages across Europe therefore has the potential to provide insights into the ecological regimes present during the period, which is characterized by large climatic fluctuations. For example, some have linked the arrival of AMHs with specific climatic periods such as Greenland Interstadial 10-9 (Proto-Aurignacian) and Heinrich Stadial 4 (Early Aurignacian; Banks et al., 2013). Others have contested such claims, to a large extend based on chronological arguments (Higham et al., 2013; Nigst et al., 2014; Timmermann and Friedrich, 2016). Detailed ecological information for the transitional technocomplexes, including the Châtelperronian, is currently lacking, however.

We noted previously that the Châtelperronian ZooMS assemblage of Les Cottés has a richer species composition compared to the related morphologically identified bone assemblage (Figure 6.1B; Welker et al., 2015b). This is not the case for units Ej+Sj and Em+Sm from Quinçay (Figure 6.1C) and units IX+X for the Grotte du Renne (Figure 6.1D), where the species richness rarefaction curves are nearly identical. A clear explanation for this additional richness in some ZooMS faunal assemblages, but not others, is absent (Welker et al., 2015b). This observation is of interest as both types of analyzed bone assemblages are subsamples from the same bone population at a level/site. One could therefore expect that they result in similar species composition and frequency if enough specimens are identified taxonomically.

There are several ecological, taphonomic and behavioural scenarios that could result in these observations. Ecological factors include differences in initial taxonomic abundance, with species comprising a small proportion of the total NISP adversely identified morphologically. Taphonomic differences between the fragmentation rate depending on bone density, bone thickness and/or bone length might have an influence too, although such variation is mostly present between different skeletal elements rather than between species. Behavioural factors that could contribute are different bone fragmentation strategies, for example to obtain bone marrow, transport decisions when specific anatomical regions are preferentially transported towards camp sites, or bone breakage as a raw material source for fire (Castel et al., 2016; Hodgkins et al., 2016; Munro and Bar-Oz, 2005; Rendu, 2010). These would influence the skeletal element composition, which has a strong impact on the “identifiability” of an individual bone specimen. Decisions made during the excavation process, for example when using a minimum size cutoff for the retention of bone specimens,

might also cause an under representation of smaller species. In addition, the Les Cottés dataset presented in this thesis is the smallest of all four Châtelperronian assemblages studied. So, sample size might be having a confounding effect on estimated species richness rarefaction curves for both the ZooMS and the morphologically identified assemblages at Les Cottés. Given the future increase in ZooMS assemblages studied, upcoming comparative studies might be able to provide more conclusive answers, including possible implications for hominin behaviour or site formation. At the moment it seems untimely to make behavioural or taphonomic interpretations, as it is unclear whether the increase in species richness is a consistent feature of ZooMS bone assemblages.

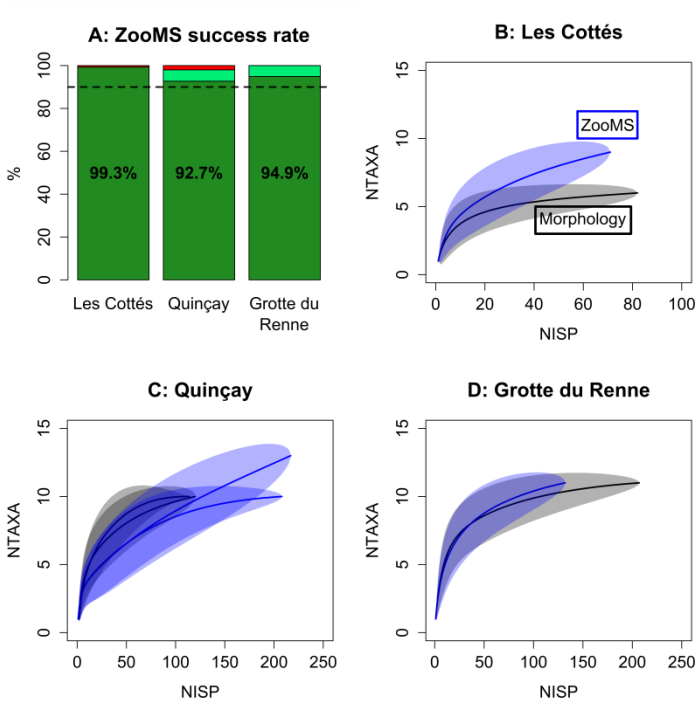

Figure 6.1. Success rates and rarefaction curves for Châtelperronian faunal assemblages. A) ZooMS success rates observed for Les Cottés, Quinçay and the Grotte du Renne, Arcy-sur-Cure. Dashed line indicates 90% successful identifications upon COL1 extraction. Light green indicates bone specimens requiring acid demineralisation after initial ammonium-bicarbonate buffer extraction for Les Cottés and Quinçay. **B-D)** Rarefaction curves for fauna assemblages identified through ZooMS (blue) or morphology (gray) for Les Cottés US-06 (B; data from Welker et al., 2015b), Quinçay Ej+Sj and Em+Sm (C; data from Lavaud-Girard, 1980 and Welker et al., 2017) and the Grotte du Renne IX+X (D; data from David et al., 2001 and Welker et al., 2016). Human and micromammal specimens are excluded in B-D.

1.2 Châtelperronian hominins

The research presented in three of the four preceding chapters focuses on the Châtelperronian, with ZooMS analysis of bone assemblage deriving from Les Cottés, Quinçay and the Grotte du Renne. This decision is justified as the Châtelperronian is the classic transitional industry with, arguably, the most intense research history. Given this research history there is a comparatively large sample of Châtelperronian sites available, including some sites with excellent bone preservation. Ruebens et al. (2015) list eight Châtelperronian sites with bone preservation and good contextual data, three of which have been included in the preceding chapters. These three sites include two sites (Quinçay and the Grotte du Renne) directly involved in theoretical debates on the Châtelperronian and the MP-UP transition due to the presence of bone ornaments in the Châtelperronian (d'Errico et al., 2003; Granger and Lévêque, 1997), while the third site (Les Cottés) is currently under

excavation (Soressi et al., 2010; Talamo et al., 2012). Furthermore, compared to the other transitional technocomplexes, the Châtelperronian has a well-constrained chronology with excellent AMS radiocarbon datasets available for a number of archaeological sites (Higham et al., 2014; Hublin et al., 2012; Talamo et al., 2012) including Les Cottés and the Grotte du Renne. The presence of this chronological data has allowed previous research to suggest that the Neanderthal skeleton from Saint-Césaire originates from Châtelperronian time periods based on direct AMS radiocarbon dating, despite the specimen being derived from a possible mixed context comprised of Late Mousterian and Châtelperronian material (Hublin et al., 2012; Soressi, 2011).

The palaeoproteomic analysis presented in chapter 5 (Welker et al., 2016) allowed us to revisit the taxonomic status and stratigraphic position of the hominin specimens previously described from the Grotte du Renne, Arcy-sur-Cure (Bailey and Hublin, 2006; Hublin et al., 1996). In the past two decades, the debate on the stratigraphic position of these hominin specimens in relation to the Châtelperronian lithic technology and bone ornaments found in the same layers at the site has dominated the theoretical and factual debate on the MP-UP transition in Europe (Caron et al., 2011; d'Errico, 2003; Higham et al., 2010; Hublin et al., 2012). By identifying additional hominin specimens suitable for a variety of multidisciplinary analysis, we managed to demonstrate that these hominins date to the Châtelperronian at the Grotte du Renne. They have Neanderthal mitochondrial and archaic nuclear ancestry, and there is isotopic, morphological and palaeoproteomic evidence to suggest that these specimens belong to the same individual(s) as some of the previously described specimens (Welker et al., 2016). Chapter 5 thereby provides evidence for the first transitional hominin from Europe that is directly dated, contextualized in a well-dated stratigraphy, and for which molecular data is available on its genetic ancestry (ancient DNA and ancient proteins; Figure 6.2). Additional nuclear data on these newly described hominin specimens from the Grotte du Renne has the potential to provide further insights into potential introgression between incoming AMHs and the Châtelperronian "Neanderthals", while similar analysis on other Châtelperronian sites has the opportunity to provide further support for a Neanderthal-Châtelperronian association (including behavioral and chronological observations directly on hominin bone specimens).

The combined work presented in the preceding four chapters provides a methodological framework within which assemblages belonging to the LRJ, Uluzzian and Szeletian could be conducted. The resulting hominin specimens for these technocomplexes and the directly preceding MP and following UP should allow a detailed biological understanding of the transitional period in Europe to be formulated. As outlined in the introduction, there are no hominin specimens unambiguously associated with the Lincombian-Ranisian-Jerzmanowician, the Szeletian or the Uluzzian (Figure 6.2). In fact, for both the Uluzzian and the LRJ scholars have proposed opposite biological associations based on hominin fossils (Benazzi et al., 2011; Higham et al., 2011; Semal et al., 2009). Although the possibility of a dual authorship of these technocomplexes should not be excluded, the stratigraphic association of the hominin fossils from Kent's Cavern, Spy (directly dated) and Cavallo to the LRJ and the Uluzzian, respectively, is doubtful (White and Pettitt, 2012; Zilhão et al., 2015). The Châtelperronian is therefore the only classic transitional technocomplex for which biological data (ancient DNA, ancient proteins) is currently available. It needs to be realized that recent insight into Neanderthal>AMH introgression has opened up the possibility of more complex biological origins of these technocomplexes (d'Errico and Banks, 2015; Ruebens et al., 2015). Testing of such scenarios requires that hominin specimens are available from multiple sites for each

technocomplex, and future ZooMS screening of additional Châtelperronian bone assemblages is therefore of importance.

The biological data available for the IUP, Proto-Aurignacian and Early Aurignacian are slightly better. The IUP in the Levant is associated with hominin teeth at Üçağızlı Cave in Turkey. They are identified as belonging to modern human populations, although detailed examination of these teeth is not available yet and the authors note that at least one tooth has features sometimes associated with Neanderthals (Kuhn et al., 2009). The Bohunician and Bachokirian technocomplexes in Europe are technologically related to the IUP in the Levant by some authors. No direct hominin fossils are currently available for these technocomplexes as a hominin mandible from Bacho Kiro level 11 was lost after its initial description (Kozłowski, 1982). Hominin fossils morphologically identified as AMH are available for the Proto-Aurignacian and Early Aurignacian (Bailey et al., 2009; Bailey and Hublin, 2005; Benazzi et al., 2015; Verna et al., 2012). Although they are not directly dated, one tooth from the Grotte di Fumane has been studied using ancient DNA, confirming its assignment to AMH populations through its mtDNA (Benazzi et al., 2015). This is the only Aurignacian or IUP hominin for which genetic data is currently available. Additional and rather complete nuclear genomes from the Oase 1 and Ust'Ishim specimens indicate that these modern humans are not ancestral to any modern human populations in Eurasia (Fu et al., 2015, 2014). Direct dating of both specimens indicate that they fall in a time period compatible with the presence of IUP and early Aurignacian industries. Whether this is true generally requires the study of additional hominin specimens from IUP and early Aurignacian contexts.

In the absence of hominin fossils for some technocomplexes and a scarcity in others, it seems premature to draw definitive biological conclusions based on the interpretation of behavioural observations when the exchange of behavioural concepts is likely or possible (Kroeber, 1940; Nigst, 2012; Roussel et al., 2016; Soressi et al., 2013, Tostevin, 2007). The palaeoproteomic approach presented in the foregoing chapters presents a radically different way of exploring this biological problem through the study of existing and new collections from transitional bone assemblages.

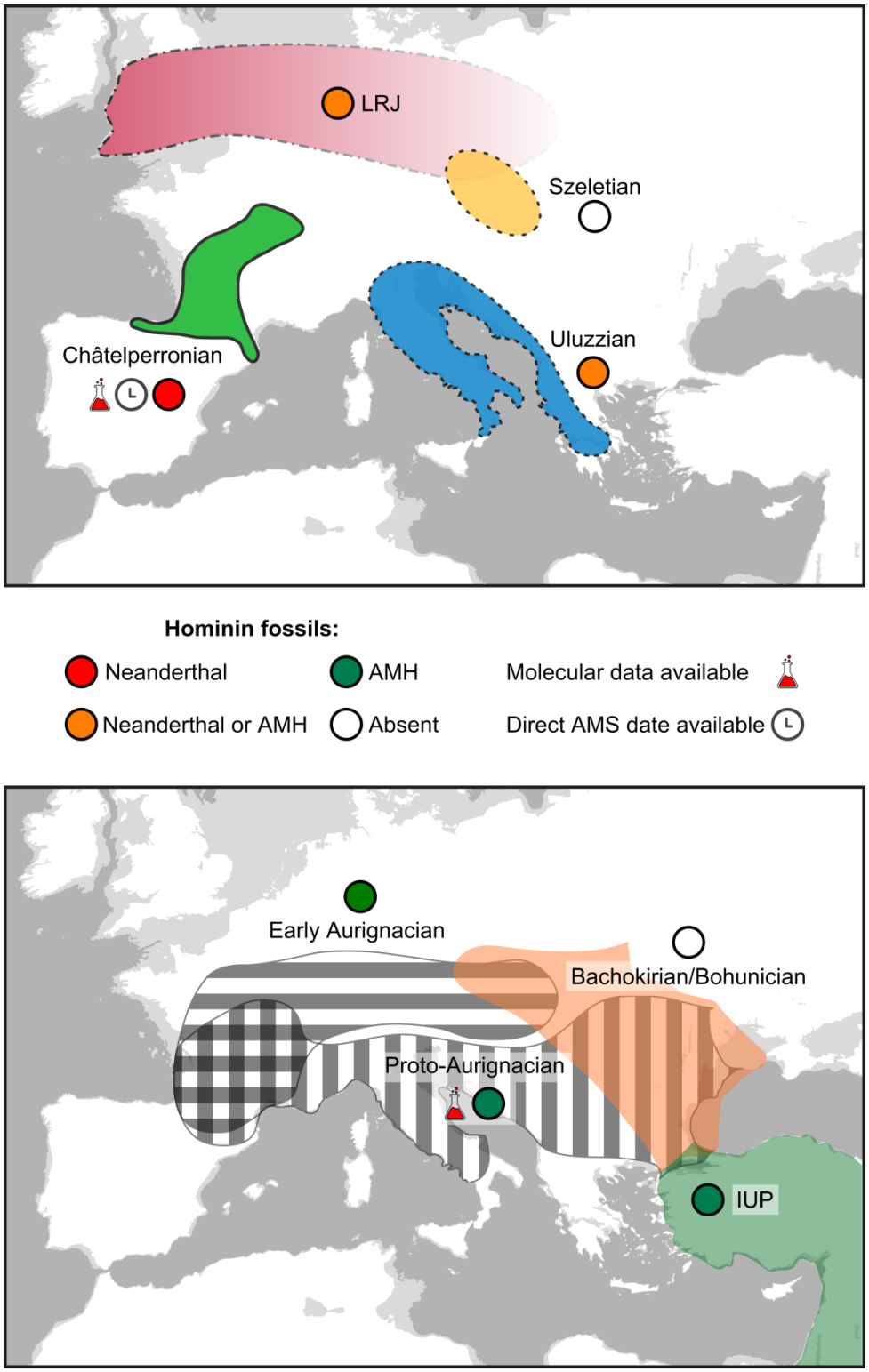

Figure 6.2. Distribution and proposed biological association of transitional (upper) and IUP/Early UP (lower) technocomplexes in Europe. Coloured circles indicate biological association of fossils, with orange for technocomplexes for which conflicting scenarios based on fossils have been proposed in the literature. Transitional and IUP/Early UP industries are preceded by regional Mousterian assemblages. Modified after Hublin, 2015.

2. Collagen type I phylogenetics

One of the goals of thesis was to develop and explicitly test the application of *de novo*/error-tolerant proteomic search engines to Pleistocene bone COL1. We achieved this goal by searching COL1 spectral data from species for which COL1 sequences were available against a database not containing these COL1 sequences (Welker et al., 2016; Welker et al., 2015a). Although the protein-generated COL1 sequences contain gaps due to trypsin digestion, the retrieved COL1 sequences were the same as those available through translated genomic sources. Hence, we concluded that *de novo*/error-tolerant proteomic search engines result in correct amino acid sequences for this protein, and that it is an adequate approach to obtain phylogenetic information for specimens where ancient DNA is unlikely to survive. Our phylogenetic conclusions were subsequently confirmed by an independent project (Buckley, 2015), further lending support to both these papers, and have been applied to various other mammalian groups since (Buckley et al., 2015; Cleland et al., 2016).

One potential conflict that needs resolving in COL1 phylogenetics concerns tree-building. Currently, published COL1 phylogenetic trees resolve the deeper nodes within Laurasiatheria in different ways or with strikingly different support values (Buckley, 2015; Welker et al., 2015a). In part, this might be due to a general lack of adequate data for Xenarthrans; there are complete *COL1α1* and *COL1α2* sequences for the nine-banded armadillo (*Dasypus novemcinctus*), only a *COL1α2* sequence for the two-toe sloth (*Choloepus didactylus*), and partial COL1 sequences for several other extant and extinct sloths (Buckley et al., 2015; Welker et al., 2015a). The absence of a *COL1α1* sequence for *Choloepus* determined through genomic research is a serious problem that, preferably, needs solving through genetic analysis. The availability of such a *COL1α1* sequence would facilitate better error-tolerant COL1 protein sequencing within Xenarthrans, and will probably result in better topological support of the placement of Xenarthrans in relation to Afrotheria and Laurasiatheria. The problematic placement of Xenarthra in relation to Afrotheria and Laurasiatheria is not unique to COL1, as there is only a small group of potential Xenarthran species available for which molecular data could be obtained (Gibb et al., 2016). Different topologies are derived from molecular, morphological or combined approaches up to the present (Foley et al., 2016). Given the survival of COL1 for prolonged periods of time, the study of COL1 has the potential to provide important insights into the early radiations of mammalian clades by exploring a time-depth that is not reachable through conventional genetic or ancient DNA approaches.

More pertinent is how to adequately deal with the isoleucine/leucine problem. The isoleucine (I) / leucine (L) problem is unique to proteomics, as both amino acids are coded for by different genetic codons but the translated amino acids have an identical mass. Hence, proteomics cannot separate these two amino acids using common methods while protein translation from genomic sources can. Not accounting for this problem might cause incorrectly placed branches in phylogenetic trees. Scholars have dealt with this issue in slightly different ways. Welker et al. (2015a) have followed a conservative approach by converting all I into L. This leads to a loss of phylogenetic information as positions obtained through genomic information are converted too, but it is conservative as it does not involve any *a priori* decisions on phylogenetic information. It is unknown, however, whether converting to L or to I makes a difference in nodal support or topology. This could be the case as substitution matrices used in phylogenetic tree building score substitutions involving

any other amino acid to or from either L or I differently. Buckley (2015) and Cleland et al. (2016) used a different approach, where each isoleucine/leucine position was assigned either of the two amino acid states based on the state taken by the phylogenetic context using a majority-based system. Such an approach does not reduce phylogenetic information prior to tree-building and should lead to higher nodal support for deeper nodes, but does require that the phylogenetic position of the studied taxon is known to some extend. This might be justifiable in some cases (Cleland et al., 2016), but could be complicated in more diverged or older species.

3. Hominin palaeoproteomics

LC-MS/MS data on ancient hominins or archaeological human individuals was not available only a few years ago. Up to 2013/2014, palaeoproteomic publications on archaeological human or hominin material was largely restricted to MALDI-TOF/TOF-MS analysis of targeted proteins (Boros-Major et al., 2011; Nielsen-Marsh et al., 2009, 2005). This greatly reduced the possible number of identified proteins and physiological or phylogenetic interpretations that could follow. This has started to change, with publications on ancient non-human bone proteomes (Brown et al., 2016; Welker et al., 2016), human bone proteomes (Hendy et al., 2016; Kendall et al., 2016; Mikšík et al., 2016) and human non-bone proteomes (Hendy et al., 2016; Kendall et al., 2016; Maixner et al., 2013; Warinner et al., 2014a, 2014b). Together, these studies demonstrate that ancient protein analysis of hominin tissues has the potential to provide crucial and unique lines of evidence on ancient hominin phylogeny (Welker et al., 2016), diet (Warinner et al., 2014a), disease (Hendy et al., 2016; Kendall et al., 2016; Warinner et al., 2014b) and ontogeny (Welker et al., 2016).

These studies have demonstrated that, contrary to DNA, some proteins are differentially expressed in different tissues or cell types and that such differentially expressed proteins are preserved from Pleistocene time periods up to the present. Such proteins provide insights that cannot be gleaned from ancient DNA directly, as the genomic content of each cell is identical within an individual (with the exception of gametes or epigenetic modifications) while the proteome of cells and tissues can be very different (Kim et al., 2014).

One commonality apparent throughout these palaeoproteomic studies is a need to establish a coherent set of approaches towards identifying contaminating proteins or spectra, as in most cases protein amino acid sequence variation will be absent. Similar attempts have been successful in ancient DNA research (Sawyer et al., 2012), although sequence variation is far more abundant in DNA and can therefore be used to separate modern human contamination from endogenous Neanderthal DNA sequences (Meyer et al., 2016). We have explored possible ways towards estimating the endogenous or exogenous origin of identified proteins (Welker et al., 2016), but much remains to be explored in this regard (see Table 1.1). It is therefore promising that there is a growing interest in identifying contaminating sources in proteomics applied to modern tissues as well (Griss et al., 2016).

The combination of increasing sensitivity and precision of tandem mass spectrometry instruments, bioinformatic solutions, and development of more sophisticated protein extraction protocols for ancient tissues should lead to an increase in the number of proteins identified. Simultaneously, the aggregation of larger palaeoproteomic datasets will lead to a better understanding of the survival of *in vivo* post-translational protein modifications (Hill et

al., 2015) and the accumulation of diagenetic post-translational protein modifications (Cleland et al., 2015). Below, two lines of research are outlined that currently deserve (palaeoproteomic) attention and which have a direct impact on the approaches used in the analysis and interpretation of ancient bone proteomes.

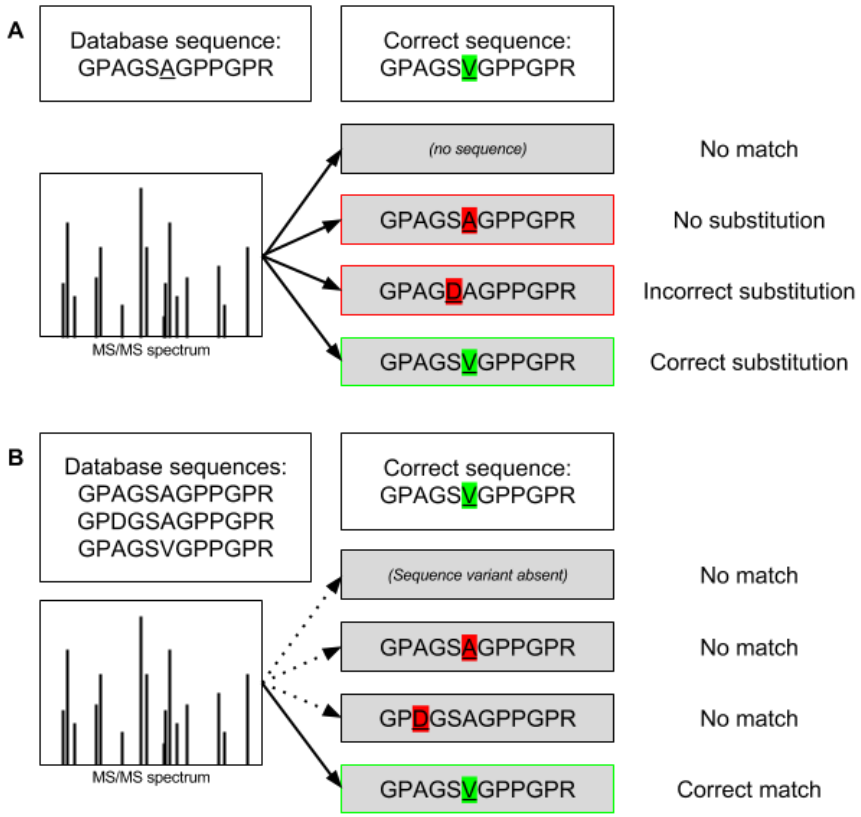

Figure 6.3. *De novo*/error-tolerant search approaches in (palaeo)proteomics. Generalized search possibilities when searching against a sequence database containing no sequence variation using error-tolerance enabled (A) or databases containing sequence variation without using error-tolerance enabled (B) for a hypothetical collagen peptide sequence. **A)** The result of a *de novo*/error-tolerant search has four possible outcomes for each considered MS/MS spectrum. There is either no PSM, there is a match identical to the database sequence, there is match to the database sequence with an incorrect substitution (or an incorrect placement of the substitution), or there is a match with a correctly placed substitution. *De novo*/error-tolerant search engines ideally optimize the latter and minimize the three former outcomes. The first outcome, an absence of PSMs, will not influence subsequent phylogenetic inferences but will influence overall experiment performance by lowering the number of identified peptides and proteins. **B)** Assuming that MS/MS spectrum interpretation is ideal *and* that the correct sequence is present in the protein database, database searches not employing *de novo*/error-tolerant searches should identify the correct peptide sequence.

3.1 The cross-species proteomics problem

Chapter 6 of this thesis demonstrates that ancient protein sequence analysis has the potential to elucidate the phylogenetic relationship between hominin individuals (Welker et al., 2016). In order to achieve this, either i) error-tolerant search engines are employed to obtain amino acid substitutions compared to the searched reference proteome(s), or ii) non error-tolerant searches are performed against a protein database containing known and relevant amino acid sequence variation. The first route is favourable when one wants to minimize database size. It is currently unclear how big the loss of protein and peptide information is when a database search is performed against a single proteome, however, either in error-tolerant or non-error-tolerant approaches. This is known as the cross-species proteomics problem (Bayram et al., 2016; Wright et al., 2010). In fact, increases in protein sequence coverage and proteome complexity mean that amino acid sequence variation *within* populations is obtainable through proteomic methods when error-tolerant searches are employed. The second route is favourable when protein sequence variation is known beforehand and database size is not a constraining factor, and is the most commonly used approach in clinical studies. This second route is not of interest when sequence information for relevant taxonomic groups is limited or absent. This latter problem is the case with ancient hominins, despite the availability of some protein sequence variation for Neanderthals and Denisovans. These two routes can be combined, as was the case in the Welker et al. (2016) study.

In the case of ancient hominin specimens, relevant sequence variation is known for present-day modern humans (through the 1000 Genomes project; 1000 Genomes Project Consortium et al., 2015), three Neanderthal exomes (Castellano et al., 2014), one Denisovan high-quality genome (Meyer et al., 2012), and all major sub-groups of Hominidae (Prado-Martinez et al., 2013). Undoubtedly, the availability of amino acid sequence variation is going to increase in the coming years for ancient and modern individuals by 1 to 2 orders of a magnitude. The release of exome data (protein-coding genetic regions) of over 60,000 individuals by the Exome Aggretation Consortium illustrates such developments (Lek et al., 2016). This presents a challenge from a proteomic bioinformatics perspective.

Therefore, it would be beneficial to perform error-tolerant searches against a database containing a single reference proteome while knowing the biases this could introduce in protein and peptide identification. For example, are specific types of amino acid substitutions missed? Are peptides with multiple sequence substitutions compared to the reference proteome under-identified? Does peptide sequence length have an influence? When protein composition is of interest but not sequence variation, are some proteins more vulnerable to remain unidentified if error-tolerant searches are not employed?

In an ideal case, error-tolerant searches against a single reference proteome maximize correct amino acid substitutions while minimizing false amino acid substitutions and minimizing unidentified spectral matches (Figure 6.3). An explicit test of this issue is currently not available in the literature, but could easily be performed and would give an answer to the questions outlined above. The results of such a bioinformatic experiment could then guide future protein digestion strategies and bioinformatic workflows in palaeoproteomics as well as in proteomic applications in personalized medicine.

3.2 Hominin bone and dental (palaeo)proteomes

Mineralized tissues forming the skeleton perform distinct functions to support muscle attachment and body locomotion, to protect enclosed organs and tissues (within the thoracic cavity, the neurocranium, and around bone marrow and tooth pulp), metabolic regulation of fat and mineral storage, and, for teeth, mechanical breakdown of food items. Bone and dental tissues are thereby in intimate contact with a variety of additional tissues composed of extracellular matrices (ECM) or cells distinct from those composing the bone and dental tissues. These include cartilage, nerves, fat storage cells, nerves, saliva, and blood plasma, cells and vessels. In addition, bone is formed by osteoblasts in two highly different bone formation processes (endochondral and intramembraneous ossification) and remodelled by the combined efforts of osteoblasts and osteoclasts. Furthermore, osteocytes reside within the bone during life (Currey, 2002). Dentine and cementum are formed by odontoblasts, while enamel is formed by ameloblasts. They experience minimal (dentine, cementum) and no remodelling during life (enamel). Together, mineralized tissues represent a dynamic source of proteomic data from different physiological origins.

Clinical studies utilizing LC-MS/MS approaches have started to explore the human proteome of trabecular bone (Alves et al., 2011; Salmon et al., 2013), cementum (Salmon et al., 2013), enamel (Stewart et al., 2016) and dentine (Jágr et al., 2012; Park et al., 2009). These studies are of importance, as they provide reference information on the kinds of proteins that can be expected in palaeoproteomic studies. Several additional studies on non-human species are available as well (Jiang et al., 2007; Schreiweis et al., 2007; Silva et al., 2011), as are studies focusing on distinct cellular stages (Alves et al., 2010; Govey et al., 2014; Guo et al., 2010; Morhayim et al., 2015; Ruiz-Romero et al., 2008, 2005; Zhang et al., 2010). The palaeoproteomic implications of these latter studies are unclear, as a large proportion of the identified proteins in these cellular studies seem to be absent from mineralized tissues. Most of these proteomic studies do not explain detailed procedures to minimize contamination during protein extraction and analysis, and the implemented procedures to thoroughly remove soft tissue adhering to the mineralized tissues are often not explained in detail. As some of these studies explicitly acknowledge, there might be an unknown component of proteins included that are not truly part of the bone, dental or enamel proteomes. In this regard, current developments concerning the prevention and identification of protein contamination in palaeoproteomics appear ahead of those used in the clinical literature.

Tandem mass spectrometry studies mentioned above generally utilize different extraction techniques. Despite these methodological differences, they do converge on a rather similar and homogenous group of proteins comprising the bone and dentine proteomes. Fewer detailed studies are available for enamel, possibly because this tissue does not remodel during life and has a lower protein content than either bone or dentine.

It is of interest to note that explorative papers on the bone proteome in archaeological/palaeontological conditions are largely performed on cortical bone (Cappellini et al., 2012; Orlando et al., 2013; Wadsworth and Buckley, 2014), while no clinical study specifically focuses on this tissue. It can be expected that cortical bone and trabecular bones contain different sets of proteins, especially as trabecular bone is in more intimate contact with bone marrow and hematopoietic activities. In addition, trabecular bone gets remodelled at a higher rate compared to cortical bone, which also provides an opportunity for increased proteome complexity in trabecular bone compared to cortical bone (Cowin, 2001). There is no clinical exploration of ECM differences between endochondral and intramembraneous

bone formation and/or remodelling, as the only comparative study in this direction explores differences in proteome composition between dental cementum and alveolar bone present in associated jaws.

Bone and dental proteomes, either modern or ancient, are dominated by collagen type I (COL1) and a range of other typical bone proteins (for example *COL3α1*, *BGN* or *OMD*). There is consistent evidence for the incorporation of blood proteins (for example albumin) and vascular proteins into the "bone" proteome (Cappellini et al., 2012; Salmon et al., 2013; Wadsworth and Buckley, 2014; Welker et al., 2016). Only recently, however, has there been some evidence surfacing for the presence of cartilage and neuron-specific proteins within the mineralized bone matrix of archaeological specimens (Welker et al., 2016). Osteocyte-specific proteins are generally not identified, as are large proteoglycans (for example aggrecan and perlecan). Small proteoglycans are identified, however, such as biglycan and lumican (Buckley and Wadsworth, 2014). There is a general concern that low-abundance non-collagenous proteins (NCPs) might not be detected due to the dominance of COL1. Efforts to pre-digest COL1 during the extraction step have been unsuccessful as COL1 remained dominant and a decrease in the number of NCPs was observed (Wadsworth and Buckley, 2014). Alternative ways to explore the presence of low-abundance NCPs would be useful when studying the mineralized proteome of bone, dentine and enamel from both physiological and phylogenetic perspectives. These analysis increasingly rely on maximizing the number of distinct proteins identified, the sequence coverage obtained for these identified proteins, and the relative quantitative contribution (including their post-translational modifications) of these proteins. Efforts should therefore be made in improving the extraction of NCPs.

Despite some exploration of mineralized tissue composition, there is a lack of direct comparative data between different mineralized tissues, quantitative differences between bone proteomes, and the extent to which proteins from non-bone or non-dentine ECMs, cells or tissues are incorporated into the bone, dentine or enamel proteome. There is also no exploration of quantitative differences in PTM abundance and localization between skeletal locations or during life using LC-MS/MS, despite targeted studies indicating that quantitative differences in collagen PTM abundance might be present (Bank et al., 1998; Tang et al., 2007). As a result, current studies on bone and dental proteomes provide a static, homogenous view of bone and dental proteome composition. In contrast, bone and dental tissue formation, remodelling, and attachment to other tissues and cells suggest a dynamic, heterogenous composition. We have demonstrated that this dynamic nature of the bone proteome is obtainable through palaeoproteomic analysis (Welker et al., 2016). Conclusions and expectations drawn from such work will remain limited in the absence of a reference framework that explores bone proteome heterogeneity in detail, and so this requires additional (palaeo)proteomic research.

Conclusion

The research presented in the previous chapters has allowed the taxonomic analysis of morphologically unidentified bone assemblages, attributed to the Châtelperronian, through ZooMS in order to identify hitherto unrecognized hominin bone specimens. The analysis of these assemblages provides critical insights into faunal community composition and heterogeneity in bone protein preservation. For one of these assemblages, at the Grotte du Renne, 28 additional hominin specimens were identified. Ancient protein analysis of three of these hominin bone specimens indicated that they represented archaic hominins, likely Neanderthals. Both the ZooMS/COL1 screening stages and the palaeoproteomic analysis of ancient hominin bone specimens required establishing an analytical framework that allowed *de novo*/error-tolerant searches to be performed on Pleistocene bone specimens. This was successfully tested. Proteomic results were further contextualized in a multi-disciplinary framework (ancient DNA, stable isotope analysis and direct AMS radiocarbon dating) which allowed establishing that the Grotte du Renne hominins are indeed associated with the Châtelperronian at the site and that they contain mitochondrial DNA that falls within the genetic clade formed by other Neanderthals. Thereby, the ZooMS workflow established here and the resulting palaeoproteomic results provide the first directly dated, well-contextualized hominin for which biomolecular evidence (ancient proteins, ancient DNA) is available from a transitional technocomplex in Europe. Now, it is timely and technically feasible to perform a similar proteomic protocol on additional transitional sites in the region and elsewhere in Eurasia. Most importantly, the work demonstrates that palaeoproteomics is of interest when exploring ancient hominin phylogeny and physiology.

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