Integrating palaeoproteomics into the zooarchaeological analysis of Palaeolithic bone assemblages

Sinet-Mathiot, V.

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Chapter One

Introduction

After *Homo sapiens* moved out of Africa they spread across the globe, reaching western Europe around 50,000 years ago. About 10,000 years later, traces from local populations of Neanderthals, who had been occupying these territories for more than 400,000 years, disappeared from most of western Europe (Higham et al., 2014; Hublin, 2015). Understanding the complex patterns of interactions between these populations are central to assessing the development of modern groups and the reasons why local hominin groups went extinct, while considering that interactions may have ranged from mutual exclusion to admixture, including several episodes of interbreeding (Fu et al., 2015). In Europe, the period of transition between the Middle Palaeolithic cultural complexes (MP), biologically assigned to Neanderthal groups, and the Upper Palaeolithic, starting with the Initial Upper Palaeolithic (IUP) technologies and biologically assigned to *Homo sapiens* is denominated as the Middle to Upper Palaeolithic transition (MUPT). The spread of Upper Palaeolithic *Homo sapiens* groups in western Europe alongside the progressive extinction of Neanderthals and the genetic interactions between these two populations has led researchers to address this transitional phase, seeking to explore potential behavioural and cognitive differences eventually leading to the predominance of one taxon over the other (Clark & Speth, 2013). The emergence of behavioural complexity and changes in the lithic technology have possible consequences on resources procurement. Investigating shifts and prey selection variability within the context of transitional periods during human evolution contributes to a better apprehension of past hominin behaviour and potential differences between human groups. However, Late Pleistocene bone assemblages are most often highly fragmented, as the result of taphonomic processes or the action of accumulation agents, limiting the amount of morphologically identifiable material for interpretation.

Obtaining a clear understanding of human subsistence practices requires the assessment of bone assemblages in their *entirety*. Fragmented and morphologically unidentifiable bone components have constituted, up to now, an extensive taxonomically uninformative proportion of Pleistocene bone assemblages. Nonetheless, this component potentially contains information about hominin behaviour not previously included in interpretations related to subsistence strategies, possibly restraining our understanding of behavioural patterns occurring at certain sites. The recent development of biomolecular methods, for example proteomic analysis through Zooarchaeology by Mass Spectrometry (ZooMS), provides the opportunity to assign taxonomic information to bone remains for which this has not been possible using traditional methods. Up to now, ZooMS screening studies have largely focused on the identification of additional hominin specimens, generally representing less than 1% of bone remains found on an archaeological site. The other 99% of specimens identified have been used most often to answer ecological questions, trying to improve the faunal spectrum or documenting the spread of domesticated species (Buckley et al., 2017; Coutu et al., 2021; Welker et al., 2016; Welker et al., 2015). However, investigating the relationship between faunal composition and bone fragmentation, in particular, remains to be explored and what such patterns might mean in terms of past human subsistence behaviour during transitional periods such as the MUPT in Europe.

This first chapter will briefly introduce background information on zooarchaeology and human subsistence during the MUPT, and will present basic concepts of Zooarchaeology by Mass Spectrometry (ZooMS) as well as outlining the aims of this dissertation.

1. Reconstructing human subsistence and diet

Since the eighteenth century, ancient animal remains have raised questions about human (pre)history (Grayson, 1983). The discovery of artifacts and human bones associated with extinct fauna led to new perspectives on the antiquity of the human species (de Perthes, 1847; Lubbock, 1865), and by the mid-nineteenth century, vertebrate remains started to play a major role in the reconstruction of past environments and human diet. The analysis of archaeofaunal remains aims to contribute to the archaeological investigation of human evolution (Steele, 2015) and to provide evidence of past population subsistence behaviour (Reitz & Wing, 2008)). Thus, subsistence has become crucial to understanding past human group's relationships with their environments, and the technologies they create to exploit it, as well as their relation to each other on a social and economic level. Humans respond to interactions between themselves and their environment in a variety of ways resulting in different subsistence strategies (Peres, 2010). The study of animal remains from archaeological sites aims to address changes in subsistence behaviours through time and space, and to explore the patterns that influenced them. More specifically, the faunal record offers an invaluable resource for examining human responses to climate changes and the possible impact of technological variation on hunting and processing strategies (Clark & Speth, 2013).

1.1. Vertebrate zooarchaeology: definitions and role

In 1865, Sir John Lubbock used for the first time the term “zoologico-archaeologists” to designate specialists studying animal remains (Lubbock, 1865). Defined in 1971 by Olsen (Olsen, 1971), the term ‘zooarchaeology’ refers to the analysis and interpretation of faunal remains from archaeological deposits. Vertebrate zooarchaeology consists of integrating the analysis of vertebrate remains, such as mammals, fish and birds, into a multi-methodological approach to answer questions about human-animal interactions (Reitz et al., 1999). The work presented in this thesis will focus on mammals as they represent the most extensively exploited class of animals by human groups and are, generally, the most prevalent remains on archaeological sites.

Zooarchaeological analysis of faunal remains provide a wealth of information related to site formation and chronology, relationship between humans and other species with their environment, and behavioural strategies related to food procurement and raw material exploitation (e.g., Behrensmeyer, 1983; Broughton, 2015; Gifford-Gonzalez, 2018). These inferences are based on primary data such as taxonomic identification, skeletal identification (bone element or body part), age at death, health and sex of the animal, as well as the presence and type of bone surface modifications. Through the interpretation of secondary data (e.g. relative species frequencies, patterns of butchery, proportions of bone accumulators), the analysis of bone assemblages provides substance for the understanding of how past human groups were selecting and acquiring resources from the surrounding environment, and how these assemblages have been modified during burial.

1.2. From the biosphere, through the lithosphere to present time

Due to their high inorganic mineral content, bones, teeth and antlers can survive over time and represent some of the most abundant finds from archaeological sites. The accumulation of faunal remains resulting from various agents (e.g. carnivores, humans, environment) form the faunal assemblages. However, due to the impact of these various agents faunal assemblages may not accurately reflect either the living faunal community around an archaeological site during human occupation or human subsistence behaviour itself. Indeed, the formation of these assemblages pass through different stages (*life, death, deposited, fossil* and *sampled assemblage;* **Figure 1**), increasing the sample bias over time (Behrensmeyer, 1984; Brain, 1981; Grayson, 1984; Katzenberg & Grauer, 2018; Kidwell & Flessa, 1995; Lawrence, 1968; Lyman, 1994b). Understanding these biases provide a clearer picture of the site formation process and the human involvement. The initial stage is the *life assemblage* represented by the taxa available in the immediate environment of the archaeological site. This assemblage is defined by the social structure of the herds (age and sex) and their distribution around the year, influenced by the climatic and vegetation conditions. The *death assemblage* is represented by the community of animals hunted or scavenged by human groups and carnivores. The composition of this assemblage relies directly on the hunting choices taken by humans and carnivores. The *deposited assemblage* corresponds to faunal remains processed and deposited at a site. The deposition can be selective and depend on a number of factors including the accumulation agents and the intensity of their action on faunal remains. The *fossil assemblage* represents the deposited component that was preserved over time until its discovery during the excavation. The survivorship and preservation of the remains are determined by the burial environment and a number of 'diagenetic parameters'. These involve both mechanical and chemical factors, such as porosity and crystallinity, potentially altering the nature of the specimen, as well as biomolecular information it may contain, e.g. the collagen content (Hedges, 2002; Hedges et al., 1995). Moreover, not all skeletal elements nor every taxa will preserve in the same way, which is also called 'differential preservation'. The processes leading to the formation of the fossil assemblage can generate a bias in the composition of a faunal assemblage that can be independent from the death and deposited assemblages. Assessing these processes provides the possibility to untangle hominin behavioural signatures. Finally, the *sampled assemblage* refers to the material excavated, recorded, washed, stored, manipulated and analysed by one or several zooarchaeologists/ palaeontologists. The degree of representativity of the initial fossil assemblage depends on the methods of excavation and recording of the finds (Brain, 1969; Lyman, 1994b; Lyman, 2004). In addition, the biomolecular preservation of the remains also depend on post-discovery practices, e.g. consolidation treatment processes or surface cleaning using chemicals or organic glues and varnishes possibly altering the collagen content of the specimen (Le Cabec & Toussaint, 2017).

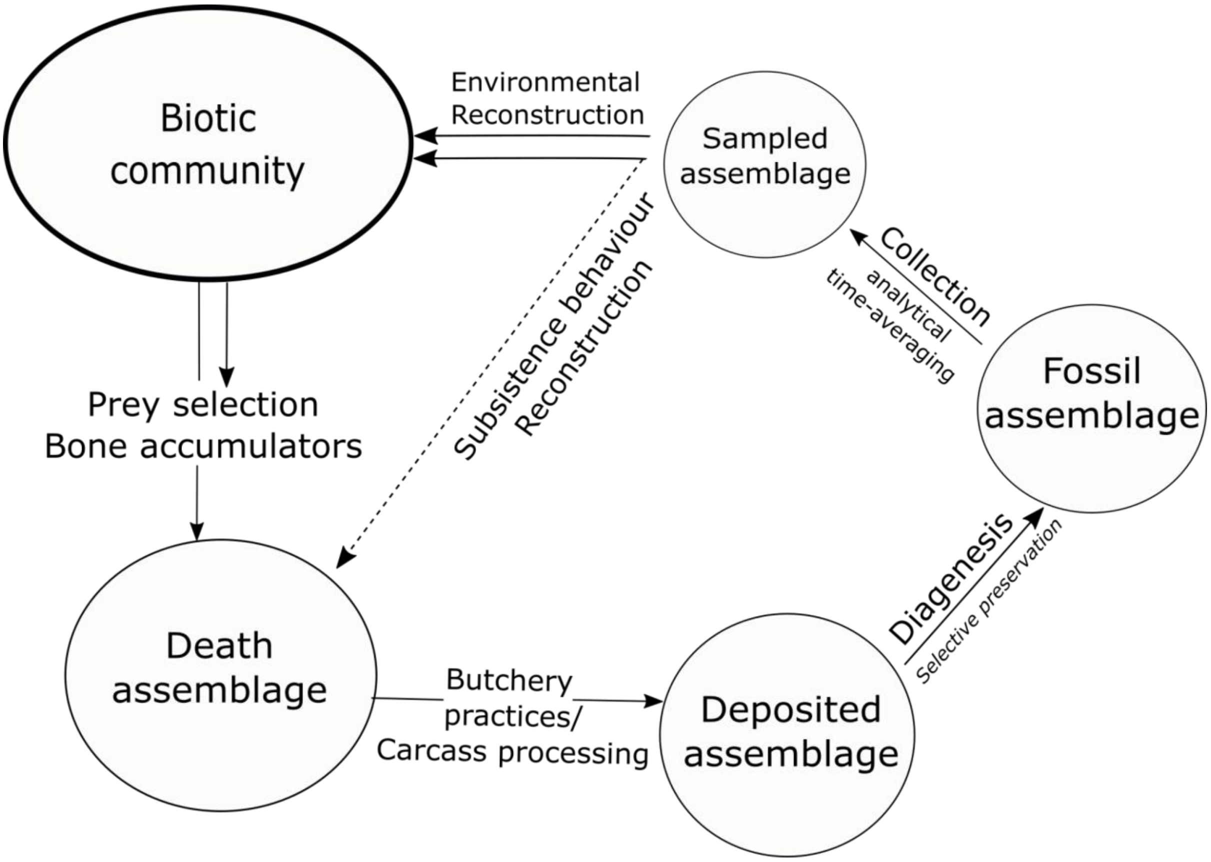

Figure 1: Simplified schematic representation of the taphonomic processes affecting the integrity of Palaeolithic faunal assemblages. Modified after Kidwell & Flessa, 1995, and Lyman, 1994b.

1.3. Bone modification agents

Identifying the agents of surface modification and bone accumulation is key to relate these bone specimens to past human activity and behaviour. Several organic and inorganic processes affect the preservation of faunal remains from the death of the animal until studied by zooarchaeologists (Brain, 1981; Rogers, 2000; Villa & Mahieu, 1991). Zooarchaeology relies heavily on taphonomic analysis. Initially defined as a subfield of palaeontology (Efremov, 1940), taphonomy is the discipline studying all events occurring between the death of an organism and the eventual discovery of the fossil remains or traces of this organism (Lyman, 1994b). It refers to the study of the action of agents affecting the remains of past living organisms constituting the prehistoric record (Fernandez-Jalvo & Andrews, 2016). These taphonomic agents generate modifications of the specimens (Lyman, 1994a) which can inform on the archaeological context and conditions during the decay and burial of the remains. Indeed, each agent generates diagnostic traces specific to their type of interaction with bone specimens. However, different agents can lead to equifinality in signatures, often resulting in debates among taphonomists (Behrensmeyer et al., 1986; Blumenschine et al., 1996; Domínguez-Rodrigo et al., 2017; Olsen & Shipman, 1988) Determining the taphonomic history of a bone assemblage provides a better understanding of the formation of the archaeological record and of the impact of these processes on the integrity of the faunal assemblage. The identification of the causal agents behind the bone modifications is key for the recognition of potential sources of biases for subsequent measures and interpretations (Binford, 1978; Gifford-Gonzalez, 1991; Lyman, 1987; Upex & Dobney, 2020).

When considering Palaeolithic faunal assemblages, various taphonomic agents can cause bone modifications. These agents include natural processes such as weathering, geological or other mechanical forces, soil pH and water content, insects, plants and fungi, temperature changes, and biological agents. In addition, human butchering and processing practices can generate modification, resulting notably in the presence of cut marks, scrape marks, percussion traces, burning, and non-human activity from carnivores or rodents (Backwell et al., 2022; Binford, 1981; Blumenschine et al., 1996; Fisher, 1995; Lyman, 1994b; Olsen & Shipman, 1988; Soulier & Costamagno, 2017). These modifications can consist of alteration to the surface or the shape of the specimen, but also penetrate the bone tissue and cause erosion (Fernandez-Jalvo & Andrews, 2016). Bone modifications provide evidence for the reconstruction of human activities on a site through the identification of traces associated with human action. As an example, body part representation, the occurrence and location of cut marks, and percussion traces can inform about specific transport decisions and if certain resources were particularly targeted (Morin & Ready, 2013). The involvement of human groups in the formation of the bone assemblage and interpretations around subsistence behaviour and diet is made possible through the recognition of hominin behavioural signatures and the attempt to extricate the potential relative contribution of humans, carnivores and other bone accumulating agents (Domínguez-Rodrigo et al., 2014; Grayson & Delpech, 2003; Lyman, 2004; Marean et al., 2000; Patou-Mathis, 2000).

The effect of these taphonomic factors on bone preservation can ultimately lead to bone fragmentation. Addressing the fragmented portion of a bone assemblage requires the implementation of bone surface modification analysis on every fraction of a bone assemblage in order to have a better understanding of the origin of all components of the archaeological record.

1.4. Bone fragmentation in Palaeolithic faunal assemblages

Several pre- and/or post-depositional taphonomic processes, which are not mutually exclusive, can lead to high bone fragmentation particularly in the context of Palaeolithic bone assemblages. The characterisation of the fragmentation patterns through zooarchaeological analysis aims to recognize the taphonomic processes involved and to identify the bone accumulators and potential human activities.

Such patterns of fragmentation may be caused by natural actions among which high stages of weathering, effects related to the environmental conditions prior to burial of the remains (Behrensmeyer, 1978; Madgwick & Mulville, 2012), gelifraction or the impact of frost (Pokines et al., 2016), sediment compression (Müller et al., 2018), and can provide details about site formation and depositional processes (Bartram et al., 1999; Bonnichsen, 1979; Lam & Pearson, 2005; Madgwick & Mulville, 2015; Nielsen-Marsh et al., 2007; Stiner, 1994). Bone breakage can be induced by non-human biological agents (e.g. carnivores/other animals) through trampling and gnawing (Marean, 1991), but also resulting from excavation, transport and storage of the remains (Behrensmeyer et al., 2000; Brugal, 2017; Durocher et al., 2022). Moreover, fragmented assemblages can infer a wide range of anthropogenic activities and human behaviours notably related to butchery practices: the production of worked bones (Gummesson et al., 2019; Smith & Poggenpoel, 1988), the percussion of bones for marrow extraction (Vettese et al., 2020), and the use of bone as fuel or skeletal fat rendering (Bovy et al., 2019; Morin, 2010, 2020; Morin & Soulier, 2017; Outram, 2001; Théry-Parisot et al., 2005; Todd & Rapson, 1988). Due to their high calorific value, bone fats play an important role in subsistence economies. The exploitation of bone fat usually requires the breakage of the medullary cavity for the extraction of bone marrow from the long bone shafts, and separation of the epiphyses for the extraction of grease from the spongious bone, while the bone is relatively fresh. Thus, the intensity of the exploitation can be reflected by the degree of fragmentation related to fat exploitation activities (Outram, 2002). Certain taxa such as reindeer store large quantities of bone marrow fat relative to their body size (West, 1996). As a result, these fragmented and morphologically unidentifiable fragments may carry information about bone marrow and grease exploitation, and can be of high interest for the interpretation of past subsistence strategies related to bone fat exploitation (Outram, 2001)

However, such highly fragmented bones are less likely to contain morphologically diagnostic features, affecting their anatomical and taxonomic identification (Lyman, 2002; Morin et al., 2017a). Three attributes can be identified on faunal specimens: the skeletal element from which the fragment originates, the taxonomic group, and possible surface modifications retracing its taphonomic history (Driver et al., 2011). The attribution of the bone fragment to an anatomical part will be necessary to assign the identified element to a taxonomic group. However, fragmentation of long bones or ribs, notably, tends to generate diaphysis fragments which do not contain many characteristic features, thus leading to undiagnostic specimen morphology. Without the possibility to identify a bone specimen, they are categorised as unidentifiable or assigned to body size classes (Lyman, 2002). In addition, differences of identifiability between anatomical parts and taxa can ultimately affect measures of quantification, representing another source of bias for the interpretation of Palaeolithic faunal assemblages (Morin et al., 2017a).

Late Pleistocene bone assemblages are most often highly fragmented (Villa et al., 2004) resulting in a small proportion of remains identifiable using traditional methods through visual comparison of the bone morphology, for example at Pech de l'Azé IV (France) (Niven, 2013), at Abri Peyrony (Martisius et al., 2015), or Les Pradelles (Costamagno et al., 2006) (other examples include Gaudzinski, 1996; Niven, 2007; Terlato et al., 2019). Previous studies of past human behaviour relies on morphologically identifiable fauna which, in many cases, represent a small portion of the complete sampled bone assemblages uncovered in a site, leaving a potentially incomplete picture of human subsistence (Dirrigl & Frank, 2002; Marean, 1991; Marean & Kim, 1998; Morin, 2004; Morin et al., 2017a, 2017b; Pickering et al., 2006). Thus, bone fragmentation leads to a loss of taxonomic identification but also of hominin behavioural information and with the interpretation based on a relatively small proportion of identifiable remains.

Figure 2: Example of morphologically unidentifiable bone remains. Bone material from Layer 1 Collection Gilles, Abri du Maras, France. Credit: Virginie Sinet-Mathiot.

2. Hominin subsistence during the Middle to Upper Palaeolithic Transition (MUPT)

The Middle to Upper Palaeolithic Transition, starting around 47,000 years BP (Fewlass et al., 2020; Hublin et al., 2020), is marked by the replacement of local populations of Neanderthals by modern *Homo sapiens* groups across western Eurasia. This crucial period during recent human evolution in Europe has been perceived as a period of changes marked by important biological and behavioural changes and the development of innovations, both cultural and technological (Mellars, 1989). The abrupt aspect has been smoothed out in favour of a progressive emergence of modern behaviours (d'Errico, 2003; d'Errico et al., 2009; Teyssandier et al., 2010; Zilhão et al., 2010). While it is now widely accepted that modern humans are not derived from local archaic forms, interactions between the two populations have been attested by the identification of a gene flow from Neanderthals into modern *Homo sapiens* (Fu et al., 2015; Prüfer et al., 2014). Some scholars considered the MUPT as a period of "human revolution" (Bar-Yosef, 1998; Binford, 1985; Klein, 1989, 1995, 2008; Mellars, 1996b; Mellars, 1989; Noble & Davidson, 1991; Tattersall, 1995; Trinkaus, 1989), while others have proposed alternative models emphasising the emergence of so-called "behavioural modernity" in the late Middle Stone

Age of Africa and possibly in the late MP of Europe. Central to these debates lies a critical difference between the two continents: Europe witnessed a major population replacement (Neanderthals by modern *Homo sapiens*), while in Africa there is essentially a biological continuity of the human peopling.

The archaeological record from this period has played a key role in discussions related to the detection of signs of behavioural modernity (d'Errico, 2003; Henshilwood & Marean, 2003; Klein et al., 1999; Mcbrearty & Brooks, 2000; Wadley, 2001). Such evidence is illustrated by the multiplication of symbolic behaviours and archaeological manifestations of complexity, such as the manufacture of bones and teeth for personal ornaments and tools (Martisius et al., 2022; Vanhaeren & d'Errico, 2006), the proliferation of a symbolic culture with figurative art (both parietal and portable), geometric signs or musical instruments (Conard et al., 2009; White et al., 2012), or the change in lithic technological complexity and appearance of stereotypical lithic artefacts and long distance projectiles used for hunting (O'Driscoll & Thompson, 2018). The implications of these findings have been widely discussed regarding their inference with various aspects of behavioural complexity, such as the use of symbols and abstract thinking (d'Errico & Henshilwood, 2011; Mcbrearty & Brooks, 2000; Nowell, 2010; Wadley, 2006, 2010) or the appearance of language (d'Errico et al., 2009; d'Errico & Henshilwood, 2011). Some scholars attribute cultural changes associated with Neanderthals to a parallel development towards "cultural modernity", independently of any cultural changes observed among modern *Homo sapiens* throughout their expansion across Europe (d'Errico, 2003). However, cultural interactions between local populations of Neanderthals and modern *Homo sapiens*, introducing new behaviours and techniques across Europe through their progressive migration from the Levant/Africa, might have influenced the development of certain cultural changes (Hublin, 2012, 2015; Hublin et al., 1996; Roussel, 2013; Soressi & Roussel, 2014).

Behavioural variability has historically been linked to distinct lithic production systems, and understanding what influences these changes is essential to assessing cultural variation and population dynamics among Pleistocene groups. Regional-specific transitional technocomplexes are defined on the base of their chronostratigraphic intermediate position between Middle Palaeolithic Mousterian and Upper Palaeolithic Aurignacian assemblages, but chronologically overlapping with the Initial Upper Palaeolithic and the beginning of the Aurignacian complex. The Initial Upper Palaeolithic relates to the first known dispersal of modern *Homo sapiens* out of Africa and takes its origin in southwest Asia. The transitional notion of these lithic industries rely on the identification of shared features from both of these assemblages, although to varying degrees (Ruebens et al., 2015). These techno-complexes are distributed in limited territories across Europe: the Châtelperronian (present across central-east of France until northwestern Spain), the Uluzzian (present in Italy and on the west coast of Greece), the Szeletian (present in Czech Republic and Hungary), the Lincombian-Ranisian-Jermanowician (present across northern Europe including the south of the United Kingdom), the Bohunician and the Bachokirian (both present in eastern Europe, notably in the Balkans). The direct association of these industries with a particular biological maker is rare, and has only been described for the Châtelperronian with the identification of Neanderthals remains at Grotte du Renne (Bailey & Hublin, 2006). The Châtelperronian assemblage is characterised by Châtelperron points and the presence of personal ornaments and worked bones (d'Errico et al., 2003; Granger & Lévêque, 1997). The recent dating between 46,790 and 42,810 cal BP of *Homo sapiens* remains associated with an Initial Upper Palaeolithic industry at Bacho Kiro Cave extend the period of contact between Neanderthals and modern *Homo sapiens* in eastern Europe (Hublin et al., 2020).

The emergence of behavioural complexity and changes in the lithic technology occurs in a period of fluctuating climatic and environmental conditions. The Late Pleistocene corresponds to the last 100 ka-climatic cycles, comprising several Marine Isotope Stages (MIS 5-2) and is marked by an alternation of abrupt warming and cooling events (Sánchez Goñi, 2022). During the Middle to Upper Palaeolithic transition (MIS 3), a climatic deterioration occurs during the Upper Palaeolithic (Banks et al., 2013; Sánchez Goñi et al., 2013), as arctic species slowly replace temperate woodland and cold steppic species in the archaeological record (Discamps et al., 2011; Rendu et al., 2019). Considering that primary consumers, such as herbivores, depend on the availability of vegetal resources, climatic shifts can cause significant ecological changes (Rivals et al., 2022). Understanding how these climatic fluctuations might have impacted human ecology can enable us to determine whether the groups changed their species procurement or made deliberate choices related to subsistence strategies. Although the contemporaneity between climatic and archaeological events does not necessarily imply that one was the cause of the other, understanding subsistence behaviour during a period of environmental change is key to address changes of hunting strategies and the emergence of new cultures.

Faunal exploitation is related to a wide range of behaviours and cognitive capacities such as mobility, social organisation and technological development. Thus, investigating shifts and prey selection variability lead to the assessment of past hominin behaviour. Ungulates, such as wild horse, reindeer, large bovine, red deer and fallow deer, or occasionally caprines, wild ass, saiga and woolly mammoth, were the basis of the economy of these groups. These taxa were not consistently hunted in the same proportions, and the regional variability in human diet seen during this time period seems to be more related to changes in the abundance of large herbivores rather than shifts in the spectrum of hunted prey

(Morin et al., 2016; Yravedra-Sainz de los Terreros et al., 2016). Such consideration has led some authors (Klein 1998) to describe a change in some of the ungulate abundance as an indicator of hunting skills.

Even though methodological frameworks have improved, the question of how Late Pleistocene hominins lived and subsisted continues to be debated, notably in relation to the identification and the timing of the emergence of 'behavioural modernity' (Bar-Yosef, 2004; Burke, 2000; Burke, 2004; d'Errico, 2003; Discamps et al., 2011; Gaudzinski, 2006; Grayson et al., 2001; Grayson & Delpech, 2002, 2006; Marean & Assefa, 1999; Mcbrearty & Brooks, 2000; Mellars, 1996b, 2004; Münzel & Conard, 2004; Speth, 2004; Stiner, 2001; Straus, 2013). In terms on subsistence, several signatures have been proposed to indicate a behavioural modernity (d'Errico, 2003; Henshilwood & Marean, 2003; Klein, 1989; Mcbrearty & Brooks, 2000; Mellars, 1996a; Mellars, 1989) such as the emergence of specialised hunting (Mellars, 1973, 1989; Mellars, 2004), the use of throwing weapons (Shea, 2009), a more diverse diet with the exploitation of small game and marine resources (Álvarez-Fernández, 2011; Marean et al., 2007; Richards et al., 2001; Stiner et al., 2000), or the optimised exploitation of animal resources through seasonal hunts and a more exhaustive exploitation of carcasses (Binford, 1984; Klein, 1995). Many scholars have discussed the occurrence of these criteria among Middle Palaeolithic and Upper Palaeolithic faunal assemblages (Costamagno et al., 2006; Gaudzinski, 2000, 2006; Gaudzinski & Roebroeks, 2000; Gaudzinski-Windheuser et al., 2014; Grayson & Delpech, 2002, 2006; Klein, 1995, 2003, 2008; P. Mellars, 1973, 1989, 1996b; Mellars, 2004; Morin, 2004, 2008, 2012; Rendu, 2007, 2010; Rendu et al., 2012, 2019; Smith, 2015; Smith et al., 2021).

Thus, taxonomic abundance plays a major role for the investigation of diet variability, subsistence behaviour, prey selection and environmental adaptation. Faunal assemblages showing a single dominant taxon, based on the morphological identification of bone specimens, and denominated as monospecific, have been argued to result from "specialised hunting". In Upper Palaeolithic contexts, this concept refers to the communal hunting of large numbers of animals and the processing of their meat for storage (Costamagno et al., 2006; David & Enloe, 1993), and potentially imply a drastic change in hunting strategies and group organisation. Some scholars have argued that this specific hunting strategy has been brought by modern *Homo sapiens* through their expansion across Europe (Costamagno et al., 2006; Mellars, 1996b; White, 1989), hence the progressive multiplication of reindeer-dominated faunal assemblages throughout the MUPT. Specialised hunting is often used as a criterion to differentiate the MP generalised hunting from the UP specialised hunting. In numerous Mousterian sites, however, a single taxon dominates the faunal assemblage, as it does at La Borde [Citation error], Mauran (Farizy et al., 1994), Coudoulous I (Jaubert et al., 2005), Jonzac (Airvaux, 2004) or Salzgitter (Gaudzinski & Roebroeks, 2000). Nonetheless, such specialised hunting strategies associated with Neanderthal occupations are still widely debated (Binford, 1982; Mellars, 1996a; Morin, 2004). The causal association between deliberate selection of a particular taxon and behavioural modernity has been questioned (Stanford, 1995). The difficulty to identify the stratigraphic signature of a mass kill event compared to the repetitive individual hunting episodes of a specific taxon (Speth, 2004) is problematic as they do not require the same set of skills and group organisation. Nonetheless, the formulation of such a hypothesis rests upon the ability to securely identify bone fragments to species using comparative bone morphology. Obtaining a secure interpretation of a faunal assemblage requires understanding the context of these bone assemblages and more specifically their formation and exhaustive taxonomic composition.

3. Palaeoproteomics in archaeology

Palaeoproteomics refers to the field dedicated to the identification and study of ancient proteins retrieved from archaeological, historical, palaeoanthropological, palaeontological remains and environmental samples (Hendy, 2021; Hendy et al., 2018; Richter et al., 2022; Warinner et al., 2022; Welker, 2018). Multiple approaches exist to identify ancient proteins such as amino acid analysis (Abelson, 1954), immunoassays (Child & Pollard, 1992), peptide mass fingerprinting (Ostrom et al., 2000), and liquid chromatography–tandem mass spectrometry (LC-MS/MS). The application of palaeoproteomics in archaeology and evolutionary anthropology ranges from the phylogenetic reconstruction of extinct species to the investigation of past human diets and lifeways, or can provide insights into prehistoric and historic material culture. Although ancient DNA has been and will remain a highly informative source of biological information, recent years have witnessed the development of alternative biomolecular methods for species identification which analyses proteins, more stable than DNA, that survive in organic remains (Cappellini et al., 2014; Collins et al., 2010).

3.1. Collagen: structure and interests

Taxonomic identification methods require biomolecules that differ between taxa, and their resolution will be determined by the degree of taxonomic variation. As proteins are coded by DNA, amino acid sequences of a protein are directly specified by the gene sequence, through the translation of a mRNA (copy of a portion of DNA corresponding to one or more genes of a biological organism) into amino acids in the ribosomes. Mutations of the gene sequences over evolutionary time causing variation in the protein sequence, are of interest for taxonomic identification and phylogenetic analyses. Collagen type I represents the major protein in connective tissues (Henriksen & Karsdal, 2016) and consists of a triple helix made from three polypeptide α-chains (COL1α): two identical ones, COL1α1, and one slightly different in its chemical composition, COL1α2 (Cowan et al., 1955; Rich & Crick, 1955; Vuorio & de Crombrugghe, 1990). Both COL1α1 and COL1α2 result as protein sequences from two different genes. These triple helices are assembled into microfibrils, bundled together to form the fibrils which compose the collagen fiber (**Figure 3**). The high resistance of the structure of the protein is enhanced by the repeated amino acid motif in the sequence (Glycine-Proline-X or Glycine-X-Hydroxyproline, X being any various other amino acid), and through hydrogen bonding provided by hydroxyprolines (Némethy & Scheraga, 1986; Szpak, 2011). Collagen varies between organisms as its sequence possesses enough amino acid variation allowing for taxonomic discrimination. This long-term preservation biomolecule persists in archaeological samples and is routinely extracted for radiocarbon and stable isotope analyses (Buckley, 2018). Thus, the protein collagen type I is phylogenetically informative (Welker et al., 2015), easily accessible, and survives beyond the temporal preservation range of ancient DNA (Demarchi et al., 2016; Rybczynski et al., 2013), which strengthens its advantage for an application on Palaeolithic material.

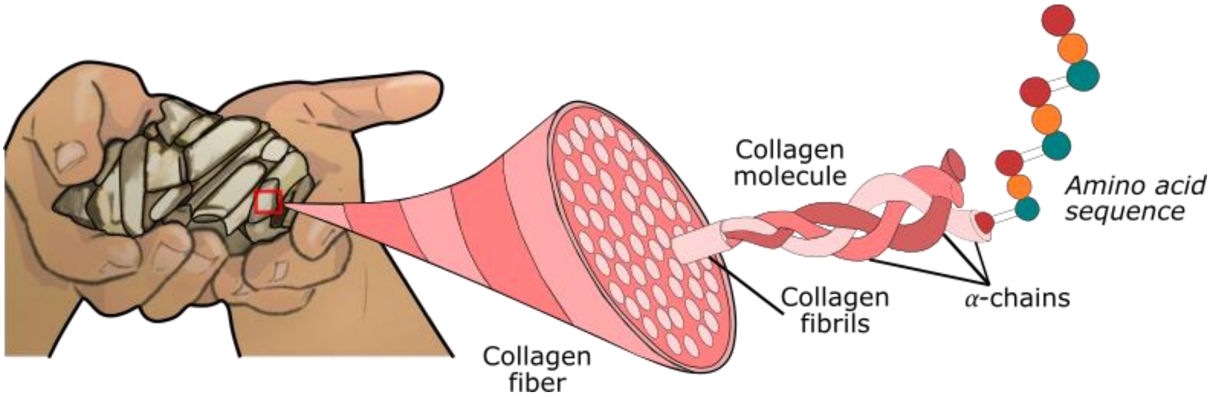

Figure 3: Structure of collagen type I. Drawing “bones in hands” credit: Anna Goldfield

3.2. Zooarchaeology by Mass Spectrometry (ZooMS)

Peptide mass fingerprinting (PMF) is a technique of protein identification using soft-ionization mass spectrometry such as Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry (MALDI-TOF MS), developed in the 1990’s (Aebersold & Goodlett, 2001; Ostrom et al., 2000; Pappin et al., 1993), and primarily used to identify organic glues and milk or egg-based paint blinders in art work (Hynek et al., 2004; Kuckova et al., 2007). This method was then developed as a taxonomic screening tool to identify fragmentary and morphologically unidentifiable remains, or so-called Zooarchaeology by Mass Spectrometry (ZooMS) (Buckley et al., 2009). ZooMS relies on the abundance of collagen type I in the vertebrate kingdom as well as the archaeological record (Shoulders & Raines, 2009), and its variation between organisms.

ZooMS was first developed slightly more than a decade ago (Buckley et al., 2009) and the use of this analytical technique is increasingly growing in the field of archaeology, ecology and cultural heritage (Brown et al., 2021; Richter et al., 2022). This method consists of the analysis of collagen type I through MALDI-TOF MS technology, with the aim to assign some level of taxonomic identity, normally via comparison to a reference list of peptide marker masses of possible species (PMF). It is a minimally destructive method, as it requires only a small amount of collagenous material (<20 mg), and can be performed with a low analytical cost per sample, allowing for rapid large-scale taxonomic investigations and providing robust taxonomic identities (Richter et al., 2022).

Different ZooMS extraction techniques can be used to obtain suitable peptide mass fingerprints. After a minimally destructive bone/dentin fragment is taken from each specimen (van Doorn et al., 2011; Welker et al., 2016; Welker et al., 2017), collagen molecules can be extracted from the mineralised tissues using hydrochloric acid (Buckley et al., 2009). This approach, called acid demineralisation (Buckley et al., 2009), is usually performed on poorly preserved samples. An alternative and less destructive method consists of extracting the soluble collagen from the surface of the osseous fragments by unfolding the molecule in an ammonium bicarbonate buffer using heat. The advantage of this semi-destructive ammonium-bicarbonate buffer extraction (AmBic; van Doorn et al., 2011; Welker, Soressi, et al., 2015) is to cause minimal damage to the bone sample allowing for subsequent analysis or duplication of the extraction (von Holstein et al., 2014; Welker, Soressi, et al., 2017). This step will prepare the primary amino acid structure of the protein for enzymatic digestion (**Figure 4**). Digestion with trypsin is used to cleave the sequence into peptides of different length and mass depending on the taxa. Peptides are then acidified to neutralise the enzyme and purified through C18 filters. Samples are then spotted onto a specialised plate, normally in triplicate, with a matrix which will co-crystallize with the peptides. Once placed inside the MALDI-TOF mass spectrometer, a laser will excite the matrix which will vaporise the peptides and ionise them with a charge of +1. Based on their time-of-flight to the spectrometer´s detector, the peptide mass(es) can be determined and converted into mass-to-charge ratio (m/z). A single mass spectrum is produced for each collagen sample, and peptide markers will be identified by associating an intensity peak with a characteristic m/z. The combination of the identification of nine selected peptide markers will allow the taxonomic identification of the sample specimen through comparison to a database of peptide marker series for all European Pleistocene medium- to large-sized mammals (Welker et al., 2016). The obtained taxonomic identifications are generally in the range of subfamily or genus.

Details of the protocols and the methodology used in this thesis are described in chapters two, three and four, but also published elsewhere (Buckley et al., 2009; Naihui et al., 2021; van Doorn et al., 2011; Welker, Soressi, et al., 2015).

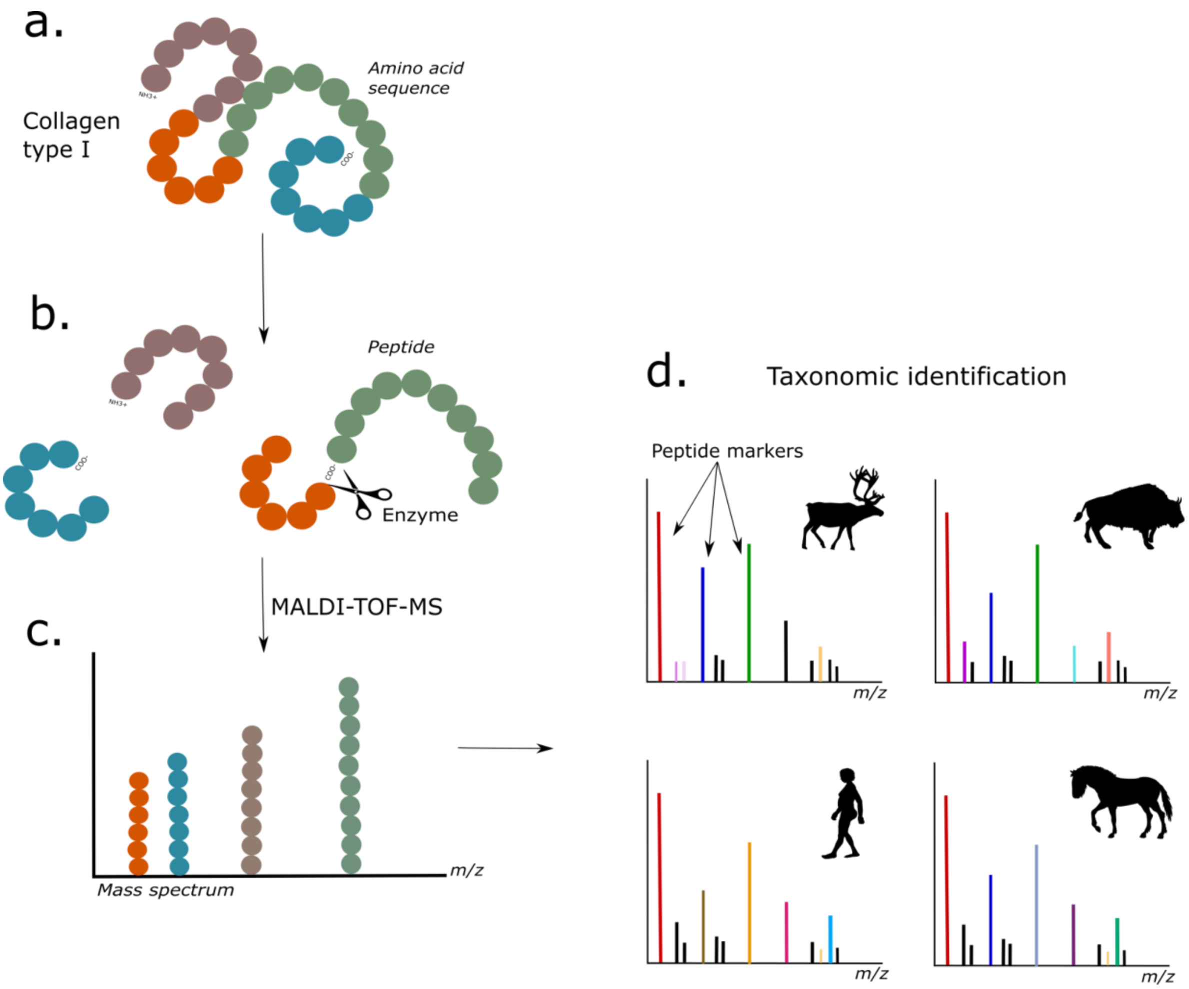

Figure 4: Schematic overview of ZooMS analysis of bone fragments. a. Denaturation of the amino acid sequence of collagen type I. b. Digestion with an enzyme to cleave the amino acid sequence into peptides. c. MALDI-TOF MS analysis generating a mass spectrum. d. Taxonomic determination of the sample through the identification of a combination of specific peptide markers. Animal silhouettes are from phylopic.org.

The AmBic approach has been preferentially used for the analysis of the large datasets allowing for a more rapid peptide extraction, and providing glutamine deamidation ratios (van Doorn et al., 2012; Welker et al., 2017; Wilson et al., 2012). The procedure of glutamine deamidation involves the transformation of glutamine into glutamic acid, resulting in an addition of 1 Da to the peptide. Glutamine (Gln) deamidation ratios are measured for the peptide COL1α1 508–519 following existing protocols (Wilson et al., 2012), to assess protein degradation and collagen preservation for the detection of outliers, thus permitting the identification of possibly intrusive material or differential bone preservation (van Doorn et al., 2012; Welker et al., 2017), although with varying success (Brown et al., 2021). The deamidation ratio ranges from %Gln = 1.0, indicating no deamidation from glutamine to glutamic acid, to %Gln = 0.0 indicating complete deamidation of glutamine to glutamic acid.

Because of the low sample input requirement, the recent development of non-invasive collagen extraction techniques for ZooMS analysis opens up the possibility for the analysis of rare and fragile specimens. Some of these non-invasive strategies are called electrostatic ZooMS (eZooMS), and rely on static electricity generated either by friction or by contact with a plastic polymer, in order to capture proteins from the surface of an organic tissue. The friction generates a triboelectric charge between the organic surface and the plastic polymer, releasing proteins from the specimen surface that binds to the plastic polymer (Richter et al., 2022; Welker et al., 2017). First developed in biocodicology (Fiddyment et al., 2015, 2021; Teasdale et al., 2017) by rubbing a soft polymer eraser on the surface of a parchment, alternative non-destructive sampling protocols have employed this technique to extract proteins from sample plastic bags or membrane boxes which had contained archaeological specimens (Martisius et al., 2020; McGrath et al., 2019). While these methods are increasingly raising interest and applied to various archaeological materials including worked bones (Brandt et al., 2018; Coutu et al., 2021; McGrath et al., 2019), the impact on bone surfaces has not been addressed. The application of the eraser sampling technique generates a friction on the bone surfaces, with the potential to remove, modify or even generate ancient features comparable to use-wear traces, thus bringing caution on its potential abrasiveness. Recently, new methods have been developed using polishing films with grit (Kirby et al., 2020), ethylene vinyl acetate films studded with strong cation and anion exchanges and C8/C18 resins (Zilberstein et al., 2020), enzyme functionalized films (Cicatiello et al., 2018) or hydrogels (Calvano et al., 2020). The rapid methodological development of minimally destructive extraction techniques opens the possibilities of non-invasive analyses while preserving the integrity of rare and culturally significant specimens, e.g. worked bones (Dekker et al., 2021; Martisius et al., 2020; McGrath et al., 2019).

3.3. ZooMS applications: an overview

Since 2018, the field has experienced a rapid growth of the application of ZooMS (Brown et al., 2021; Richter et al., 2022). It has provided taxonomic identification of collagenous material such as bone/ivory/antler including worked bone, bone points, arrowheads, daggers, rings and combs (Ashby et al., 2015; Bradfield et al., 2019; Dekker et al., 2021; Desmond et al., 2018; Martisius et al., 2020; McGrath et al., 2019; Pétillon et al., 2019;

Tomasso et al., 2018; von Holstein et al., 2014), but also parchment and soft tissues (Fiddyment et al., 2015; Kirby et al., 2013; Ruffini-Ronzani et al., 2021; Teasdale et al., 2017; Vnouček et al., 2020), leather (Ebsen et al., 2019), and bone fragments contained within dog palaeofaeces (Runge et al., 2021). The technique has been applied to a wide range of fragmentary and/or morphologically similar taxa from various archaeological and palaeontological assemblages.

In these cases, ZooMS is commonly performed in a targeted manner on individual bone specimens, for example to identify bone tools (as discussed previously), for radiocarbon or isotopic studies (Fewlass et al., 2020; McCormack et al., 2022), or to verify a taxonomic identity. However, ZooMS has been developed as a screening tool to distinguish closely related species that are difficult to identify morphologically (Buckley et al., 2011; Buckley & Kansa, 2011; Evans et al., 2016) and to identify species of interest among unidentifiable remains such as hominins or extinct megafauna (Brown et al., 2016; Buckley et al., 2017; Charlton et al., 2016; Devièse et al., 2017; Evans et al., 2016; Welker et al., 2016; Welker et al., 2017). When applied to faunal bone assemblages, ZooMS studies have often focused on ecological purposes to improve the faunal spectrum of a bone assemblage or to address domestic herd management, choices relating to secondary product use, exploitation of wild species, and appearance of commensal species (Eda et al., 2020; von Holstein et al., 2014). However, the assessment of the fragmented and morphologically unidentifiable component of bone assemblages through ZooMS may provide a clearer picture of assemblage composition and inform about specific hominin strategies in relation to faunal carcass processing and selection, and remain to be investigated. Indeed, the correlation of patterns of bone fragmentation revealed by bone surface modification analysis with taxonomic identification of morphologically unidentifiable faunal specimens may provide access to previously unavailable information on past hominin behaviour.

4. Doctoral project aims and objectives

The overall objective of the research presented in this thesis is to integrate two complementary fields: palaeoproteomics, specifically ZooMS, and traditional zooarchaeology, by combining the analyses of bone surface modifications with biomolecular analysis, and to show the potential of the inclusion of ancient protein analysis within the current framework of zooarchaeological analysis at Palaeolithic sites. This project provides an integrative zooarchaeological and ZooMS workflow in order to assess the morphologically unidentifiable portion of Late Pleistocene bone assemblages and to yield complementary and comprehensive data on taxonomic abundance, site formation, human subsistence and site use. Because of high bone fragmentation and an often low proportion of identifiable bone remains based solely on morphology, this work focuses on the transition from the Middle to Upper Palaeolithic seeking to contribute to the understanding of human behavioural response to changing conditions (environmental, biological and technological changes). The ZooMS-identified and morphologically-identified bone specimens constitute two artificial components defined based on identifiability or size-cut-off, but in reality represent subsamples of the same death assemblage. The initial hypothesis previously presented expects that both components show similar taxonomic composition and abundances for the dominant taxa (Welker, 2017). Several ZooMS screening studies have shown that this hypothesis could be verified (Berto et al., 2021; Buckley et al., 2017; Welker et al., 2016; Welker et al., 2015). However, the increased taxonomic richness noted by Welker et al., 2015 within the ZooMS component of the faunal assemblage from Les Cottés but not among the assemblages of Quinçay (Welker et al., 2017) and Grotte du Renne (Welker et al., 2016) raises questions about the representativity of the morphologically identified component over the complete faunal assemblage.

Several objectives will be addressed throughout three projects:

- Demonstration that the morphologically unidentifiable bone components do not necessarily reflect the taxonomic abundance of the morphologically identified component of Palaeolithic faunal assemblages (Chapters 2 and 3)
- Further, through ZooMS identification, these previously unidentified bone components can provide new and complementary data about specific human subsistence behaviours and bone assemblage formation (Chapters 2 and 3)
- Demonstration that ZooMS analysis of the morphologically unidentified bone component allows a better understanding of potential biases, such as differential identification rates affecting the bone assemblage (Chapter 3)
- Development of a controlled sampling experiment in order to explore the effects of eraser sampling for eZooMS analysis on Palaeolithic bone surface microtopography (Chapter 4)

Chapters 2 and 3 focus on the integration of complementary datasets from zooarchaeological and ZooMS-based analysis and its contribution to the understanding of subsistence behaviour and hunting strategies during the MUPT. Chapter 4 emphasises the importance of testing the effect on bone surfaces and bone surface modifications of so-called “non-destructive” collagen extraction techniques such as the eraser sampling for ZooMS analysis.

4.1. Chapter Two - Project 1: Combining ZooMS and zooarchaeology at Fumane Cave (Italy)

This project presents, for the first time, the integration of complementary data sets from zooarchaeological and ZooMS-based analyses at Fumane Cave (Italy). This study aims to provide a more accurate picture of species proportions and explores the synthesis and analysis of comparable data for both the identifiable and morphologically unidentifiable portions of faunal assemblages from Final Mousterian and Uluzzian layers. In order to address these objectives, this work will:

- Provide taxonomic identifications of almost 700 bone morphologically unidentifiable specimens through ZooMS analysis, within a highly fragmented bone assemblage
- Explore the causes of a compositional difference between the ZooMS and morphology component, highlighted by frequency differences in the presence of a particular taxon within the same archaeological unit
- Investigate the involvement of human groups in the formation of the morphologically unidentifiable bone component from the Late Pleistocene bone assemblage of Fumane
- Examine the categorisation of taxonomically unidentifiable bone specimens into body size classes

This work has been published in Scientific Reports: **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: 12350. https://doi.org/10.1038/s41598-019-48706-z

4.2. Chapter Three - Project 2: Contribution of ZooMS to the understanding of subsistence strategies during MUPT

Following the results obtained for Project 1, this second project aims to explore the implications of incorporating the analysis of the morphologically unidentifiable bone components into the interpretation of several faunal assemblages covering the MUPT, both in terms of overall bone accumulation and, more specifically, human subsistence strategies. This project seeks to address methodological limits commonly faced during the morphological assessment of faunal assemblages and to demonstrate how the addition of biomolecular methods such as untargeted ZooMS screening can complement our understanding of subsistence behaviour by providing a clearer picture of prey selection and site occupation. In order to assess these aims, this project will:

- Integrate taxonomic identifications for more than 2,600 morphologically unidentifiable bone specimens from three key Late Pleistocene sites covering the MUPT (Bacho Kiro Cave (Bulgaria), Les Cottés and La Ferrassie (France)) into the zooarchaeological assessment of the faunal assemblages
- Investigate differences in taxonomic abundance of the dominant taxa between the ZooMS and morphology components
- Contribute to our understanding of prey selection, bone assemblage formation and subsistence strategies during the MUPT
- Address methodological limits commonly faced during the zooarchaeological analysis of faunal assemblages with the addition of ZooMS

This research is under review at Archaeological and Anthropological Sciences: **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., Hublin, J.-J., Welker, F., Smith, G.M. Identifying the unidentified fauna enhances insights into hominin subsistence strategies during the Middle to Upper Palaeolithic transition. *Archaeological and Anthropological Sciences.*

4.3. Chapter Four – Project 3: Testing the effect of a non-destructive collagen extraction method on Palaeolithic bone surfaces

With the expansion of ZooMS applications and the development of non-destructive collagen extraction techniques, this third project aims to address the impact of the eraser extraction method on ancient bone surfaces, and alert the community on the potential invasiveness of this sampling method when applied on Palaeolithic bone surfaces. In order to approach these objectives, this project will:

- Design a controlled sampling experiment measuring the force applied and the rate of the eraser movements
- Assess the macro- and micro-structure of the bone surfaces at multiple scales prior to and after eraser sampling using both qualitative and quantitative methods
- Discuss the implication of potential bone modifications for future analysis of the bone artefact

This project was published in Scientific Reports: **Sinet-Mathiot, V.,** Martisius, N.L., Schulz-Kornas, E., Van Casteren A., Tsanova T., Sirakov N., Spasov R., Welker F., Smith G. M., Hublin J.-J. (2021). The effect of eraser sampling for proteomic analysis on Palaeolithic bone surface microtopography. *Scientific Reports* 11: 23611. https://doi.org/10.1038/s41598-021-02823-w

4.4. Chapter Five: Discussion and Conclusion

The final section of this thesis will synthesise Chapters two to four by providing a brief conclusion of the outcomes of these projects. Next, it will give an overview for outstanding challenges and opportunities in the field of zooarchaeological research through palaeoproteomic methods, particularly in the context of Late Pleistocene hunter-gatherer subsistence strategies.