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Chapter 15

Methodological advances in Neanderthal identification, phylogeny, chronology, mobility, climate and diet

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Abstract:

In the last decades, methodological advancements in the natural and exact sciences have increasingly been used to study the past. In this chapter, we review how such developments can be applied to address questions regarding Neanderthal identification, phylogeny, chronology, mobility, climate and diet. These examples illustrate how prehistoric studies are becoming inherently multi-disciplinary, as each research strategy brings forward a different

type of information. Piecing these various data together can enrich our understanding of Neanderthals, allowing us to gain a more comprehensive view of our past.

Key Words (5-10): Isotopes, Radiocarbon dating, Palaeoproteomics, Palaeogenetics, dental wear, diet, mobility, phylogeny, biomolecular palaeoanthropology

1. Introduction

What did Neanderthals eat, how did they move around the landscape, how are they related to other hominins, were they able to cope with changing climate and environment, until when did they exist? These are some of the questions that were almost impossible to answer a few decades ago. Fortunately, the use of new techniques during the past decades allows nowadays scientists to provide clearer answers to these essential questions to understand human evolution. This chapter will present some of these new approaches and the main results regarding Neanderthal identification, phylogeny, chronology, mobility, climate and diet (Figure 15.1).

< place Figure 15.1 around here >

2. Neanderthals and their contemporaries

Traditionally, morphological and increasingly geometric morphometric methods are used to determine the distribution of hominin populations in space and time. Once determined, subsequent debates often include aspects related to biological adaptation evident within the skeletal remains, or techno-typological and behavioural manifestations through association of hominin skeletal remains with the archaeological record. Recently, molecular methods have been adopted to address such and related aspects, first by the advent of ancient DNA analysis and more recently through the development of ancient protein analysis. Although both approaches have to work with fragmented, sometimes highly modified, ancient biomolecules, information retrieved from Neanderthal and other hominin fossils, as well as sediments, is starting to address biological and behavioral questions through a new, molecular lens.

2.1. Ancient DNA

The first Neanderthal DNA

In 1984, two studies reported the retrieval of DNA from ancient tissues (Higuchi et al., 1984; Pääbo, 1984), thereby pioneering the field of ancient DNA research. The first Neanderthal DNA fragments, sequenced in 1997, pertained to a 379-base pair region of the mitochondrial (mt) genome of the Neanderthal type specimen. The Neanderthal mtDNA fell outside the variation of present-day mtDNA genomes, showing that Neanderthals did not contribute mtDNA to modern humans (Krings et al., 1997; see also Rosas et al., 2021 [Chapter 5 in this book]). Since then, the mitochondrial genome sequences of 25 Neanderthals have been reconstructed, originating from individuals who lived from western Europe to the Altai Mountains between ~38 and ~120 thousand years ago (ka) (Green et al., 2008; Briggs et al., 2009; Gansauge and Meyer, 2014; Skoglund et al., 2014; Prüfer et al., 2014; Rougier et al., 2016; Posth et al., 2017; Hajdinjak et al., 2018; Douka et al., 2019; Peyrégne et al., 2019; Mafessoni et al., 2020; Romandini et al., 2020), as well as that of a ~430 ka hominin from Sima de los Huesos in Spain (Meyer et al., 2014).

Nuclear DNA from Neanderthals

Mitochondrial DNA is a useful target when screening ancient samples for DNA preservation, due to its presence in multiple copies per cell. However, to gain a comprehensive view of the population from which an individual comes, one needs to recover and analyze nuclear DNA. In 2010, the first Neanderthal genome sequence was reconstructed, composed of nuclear DNA from three Neanderthal bones from Vindija Cave in Croatia (Green et al., 2010). Comparisons between this draft Neanderthal genome and the genomes of present-day humans revealed that Neanderthals had a greater genetic affinity to non-Africans than to Africans. From this, it was hypothesized that Neanderthals and ancient modern humans admixed outside of Africa, leaving the traces of Neanderthal DNA still found in genomes today. So far, the genomes of three Neanderthals have been sequenced to high coverage¹ (27-fold or more) (Prüfer et al., 2014, 2017; Mafessoni et al., 2020), while low coverage (between 1- and 3-fold) genomes have been reconstructed from five other individuals (Prüfer et al., 2017; Hajdinjak et al., 2018), and sparse nuclear data have been generated from 12 more (Green et

¹ Coverage refers to the average number of sequenced reads that cover each base in the reference genome. Higher coverage allows to overcome errors that may affect the genetic data, such as sequencing errors, amplification errors, and in the case of ancient DNA, base modifications due to damage over time. Therefore, high-coverage genomes can be used for more in-depth analyses than low-coverage ones.

al., 2010; Castellano et al., 2014; Meyer et al., 2016; Bockelmann et al., 2019; Peyrégne et al., 2019). Among other things, analyses of these datasets revealed additional evidence for admixture between Neanderthals and modern humans (Kuhlwilm et al., 2016; Meyer et al., 2016; Petr et al., 2020). By sequencing DNA from individuals across a large timescale and from different parts of the known Neanderthal range, it was shown that there was long-term genetic continuity in Neanderthal populations in western Europe, whereas there were population changes in the Caucasus and eastern Europe (Hajdinjak et al., 2018; Slon et al., 2018; Peyrégne et al., 2019; Mafessoni et al., 2020). Lastly, the genetic evidence points to Neanderthals, particularly in the easternmost part of their range, living in smaller, more isolated populations than ancient modern humans (Sikora et al., 2017; Mafessoni et al., 2020).

The Denisovans and their relationship to Neanderthals

In 2010, the mtDNA genome of a phalanx from Denisova Cave was shown to fall basal to both Neanderthals and modern humans, suggesting that it belonged to a previously unknown group of archaic hominins (Krause et al., 2010b; see also Viola, 2021 [Chapter 4 in this book]). Its nuclear genome showed that this individual belonged to a sister-group to Neanderthals, henceforth dubbed the “Denisovans” (Reich et al., 2010; Meyer et al. 2012). The two groups split from each other ~400-440 ka and from modern humans ~520-630 ka (Prüfer et al. 2017). To date, three additional remains, all teeth from Denisova Cave, have been identified as pertaining to Denisovans based on their DNA (Reich et al., 2010; Sawyer et al., 2015; Slon et al., 2017b). Denisovans admixed with ancient modern humans, leaving genetic traces in some present-day populations (Reich et al., 2010). Denisovans also admixed with an unknown archaic hominin group that diverged more than one million years ago from the lineage leading to Denisovans, Neanderthals and modern humans (Prüfer et al., 2014). Lastly, traces of Neanderthal ancestry in the Denisovan genome sequenced to high coverage indicated that Neanderthals and Denisovans admixed as well (Prüfer et al., 2014). More direct evidence for admixture between the two groups was found when the genome of “*Denisova 11*”, a ~90 ka individual from Denisova Cave represented by a single bone fragment (Brown et al., 2016) was sequenced, as she was identified to be the daughter of a Neanderthal mother and a Denisovan father (Slon et al., 2018).

How to sequence a Neanderthal?

Since the early days of ancient DNA research, advances in sequencing technology have allowed the field to shift from strategies involving direct amplification of DNA fragments from

an extract by PCR² to library-based techniques. The latter have the advantage of allowing the sequencing all DNA fragments in an extract, even very short ones, while retaining the entirety of the fragments and allowing for their authentication based on damage patterns accumulating over time (Briggs et al., 2007; Krause et al., 2010a; Sawyer et al., 2012; Dabney et al., 2013; Skoglund et al., 2014). To generate sequencing data from a Neanderthal, one first removes a small amount of bone or tooth powder using a sterile drill. If needed, the powder can be treated to reduce the amount of contaminating bacterial and/or present-day human DNA affecting it (Korlević et al., 2015). DNA is then extracted, typically using a silica-based method adapted to recover short DNA fragments (Dabney et al., 2013; Glocke and Meyer, 2017), and converted into a DNA library, ideally using a single-stranded, rather than double-stranded, library preparation procedure to increase the efficiency of the conversion (Gansauge and Meyer, 2013; Gansauge et al., 2020). Optionally, DNA fragments containing uracils can be enriched for (Gansauge and Meyer, 2014; Slon et al., 2017b; Bokelmann et al., 2019), as these residues are the result of the deamination of cytosine bases and constitute a hallmark of ancient DNA (Briggs et al., 2007). Following double-indexing and amplification (Kircher et al., 2012), the libraries can be sequenced directly by shotgun sequencing or undergo targeted enrichment for regions of interest (*e.g.*, mtDNA or Y-chromosome, Maricic et al., 2010; Fu et al., 2013; Petr et al., 2020).

The potential of DNA from sediments

The generation of sequencing data from Neanderthals is often limited by the availability of skeletal remains. As an alternative, research has been developed to recover, isolate and identify ancient hominin mtDNA from sediment samples. The reconstruction of partial to full mtDNA genome sequences from sediments, as demonstrated using nine samples from four archaeological sites, showed the feasibility of recovering Neanderthal and Denisovan mtDNA from sediments even in the absence of skeletal remains (Slon et al., 2017a). This approach was recently applied to the site of Baishiya Karst Cave on the Tibetan plateau, where Denisovan mtDNA was recovered from four layers dated to between 100 and 45 ka, constituting the first paleogenetic evidence for the presence of Denisovans outside of Denisova Cave (Zhang et al., 2020). More recently, a strategy to recover nuclear DNA of Neanderthals and Denisovans was established, opening the possibility of conducting population genetic analyses on archaic hominins using sediments as a source material (Verrot et al., 2021). Although the complexity of sedimentary and stratigraphic contexts need to be

² PCR – Polymerase Chain Reaction

taken into account, the recovery of ancient DNA from sediments at additional sites may prove to be instrumental in retracing the dispersals of Neanderthals and Denisovans through time and space, at a higher resolution than is currently possible based on the fossil record.

2.2. Palaeoproteomics

In recent years, ancient protein analysis has emerged as an alternative or supplementary approach to identifying Neanderthals in the archaeological fossil record. Two approaches specifically have been proposed. The first, collagen peptide mass fingerprinting, primarily as a means to identify additional hominin fossils, the other, tandem mass spectrometry, to distinguish between hominin populations directly. Proteins are made up of a chain of amino acids linked together. The type and order of the amino acids that compose these protein sequences are, ultimately, determined by the DNA sequence of the relevant gene within the genome. As the nucleotide sequence might differ between individuals or species, the protein sequences might differ as well, providing an effective means to both assign taxonomic identity and conduct phylogenetic analysis. One can observe protein sequence differences directly (in the case of tandem mass spectrometry) or indirectly (in the case of peptide mass fingerprinting) using protein mass spectrometry methods, as most types of amino acids have a unique mass. The applicability of this approach has been demonstrated for proteins preserved in bone, dentine, and enamel, as well as for extinct mammals (Welker et al., 2015), extinct great apes (Welker et al., 2019), and archaic hominins (Chen et al. 2020; Welker et al., 2020).

Collagen peptide mass fingerprinting.

Collagen peptide mass fingerprinting, also known as Zooarchaeology by Mass Spectrometry (ZooMS), is a mass spectrometry-based tool to identify tissues rich in collagen type I, such as bone and dentine. Collagen type I is composed of three chains forming a triple helix, two of which derive from *COL1 α 1* and one of which derives from the *COL1 α 2* gene. The protein sequences translated from both genes differ from each other but also slightly from species to species (Buckley et al., 2009). Analysis of the peptide masses resulting after trypsin digestion produces a “barcode” that can be observed following MALDI-TOF MS analysis in which homologous peptides with a different sequence will have a different mass. Based on a selected set of peptide markers, which are selected as both their masses and peptide

sequences are known for a range of relevant mammalian species, one can then achieve a taxonomic assignment (Buckley et al., 2009; Buckley et al., 2016; Kirby et al., 2013). Such identifications are generally at the subfamily level, but can be genus- or even species-specific.

ZooMS has found widespread application in a range of historical, archaeological, and even paleontological settings. In part, this is because collagen is the dominant organic component in bone and because of the long-term preservation of collagen compared to other proteins (Wadsworth and Buckley, 2014; Buckley and Wadsworth, 2014), as well as its longer survival in comparison to other biomolecules, such as DNA (Welker et al., 2015b). As a result, ZooMS has been shown to provide an effective means for taxonomic identifications across the Pleistocene, and even into the Pliocene (Rybczynski et al., 2013). Additionally, collagen peptide mass fingerprinting has relatively small sample size requirements, generally in the 5-10 mg range, allowing sampling even of highly fragmentary bone specimens or unique objects.

In the context of Neanderthals, collagen peptide mass fingerprinting has been applied to a range of sites, primarily to discover new hominin remains by screening hundreds or even thousands of morphologically unidentifiable bone specimens. This has proven successful in a range of Eurasian sites, for example Grotte du Renne (Welker et al., 2016), Bacho Kiro (Hublin et al., 2020), Vindija (Devièse et al., 2017), and Denisova Cave (Brown et al., 2016; Douka et al., 2019). Based on the limits of the peptide markers selected, such ZooMS identifications are, strictly speaking, within the *Pan/Homo* clade. Additional information, either molecular, geographic, or chronological, could then allow determination of a hominin bone as a Neanderthal or some other hominin population.

The bone assemblages screened using ZooMS to identify hominin remains are themselves also informative on both ecological (Welker et al., 2015a) and behavioural (Sinet-Mathiot et al., 2019; Bouchard et al., 2020) aspects of hominin occupation. For example, they might enhance the number and kind of bone specimens displaying anthropogenic traces, thereby providing a more substantiated, and potentially nuanced, view on Neanderthal dietary ecology. Likewise, due to its small sample requirements, ZooMS can also be used to taxonomically identify bone tools. This recently provided evidence that certain groups of Neanderthals selected *Bos/Bison* rib fragments to produce *lissoirs*, a type of bone tools first identified in association with Neanderthal occupations (Martisius et al., 2020; Soressi et al., 2013).

Shotgun proteomics

As mentioned previously, ZooMS has limited taxonomic resolution and utilizes a single protein present in Pleistocene bone or dentine. In contrast, many hundreds of proteins are present in skeletal tissues during life, dozens of which can survive over prolonged timescales (Cappellini et al., 2012), even beyond the preservation of DNA (Welker et al., 2015b). By utilizing LC-MS/MS technology, also known as tandem mass spectrometry, one can analyze all such surviving proteins at once, providing a complete look at the preserved proteome (Cappellini et al., 2012, 2018).

Not only do these proteins survive, but their sequences remain phylogenetically informative even when comparing the protein sequences of modern humans, Neanderthals, and Denisovans (Figure 15.2). By reconstructing the ancient protein sequences preserved in an ancient hominin specimen, and comparing these sequences with the known protein sequences from modern humans and the predicted protein sequences for extinct hominins like Neanderthals and Denisovans, one can propose a phylogenetic hypothesis on the placement of the ancient hominin. Such phylogenetic trees will be based on a comparatively small number of informative positions, primarily due to the high level of sequence conservation between closely related hominin populations. Still, in the absence of ancient DNA preservation and the prolonged timescales over which ancient proteomes survive, this approach is increasingly used in geographic regions and time periods for which no hominin biomolecular sequence data was available previously (Welker et al., 2020).

For example, on the basis of proteomic sequence evidence and mtDNA sequences, it has been proposed that Neanderthals were the hominin population responsible for the Châtelperronian artefacts at Grotte du Renne (Welker et al., 2016). This has some implications for our interpretation of Neanderthal extinction in western Europe, as it indicates the adoption of new kinds of symbolic behaviour towards their demise (Hublin, 2015). In similar ways, the identification of a hominin mandible recovered from the Tibetan Plateau, in China, and its subsequent identification as a “Denisovan”, in the broadest sense, by palaeoproteomic methods challenges the notion that archaic hominins would not have been able to colonize the high-altitude Himalayas during glacial periods (Chen et al., 2019).

The recent recovery of even older Early and Middle Pleistocene hominin proteomes indicates that the limit for the preservation of hominin proteomes has not yet been reached (Welker et al., 2020). As a result, one can expect palaeoproteomics to provide phylogenetic information on (early) Neanderthal and other archaic hominin fossils, which will inform on the

processes and order by which Neanderthal and archaic hominin acquired their morphological traits. In combination with the proteomic analysis of associated fauna and cultural material, such a proteomic framework of Middle and Late Pleistocene hominins will provide new information on their evolutionary trajectories.

3. The radiocarbon dating and the last Neanderthal. Present status quo.

3.1 The Radiocarbon from two-hands clock to three-hand precision

From the out-of-date to the up-to-date radiocarbon calibration

After Libby won the Nobel Prize for Chemistry in 1960, radiocarbon has evolved into a universal dating 'clock' and now represents the backbone of chronological reconstructions for the time up to 55,000 years ago.

The radioactive isotope, ^{14}C , is produced by cosmic radiation interacting with atmospheric nitrogen (Masarik and Beer, 2009) and enters the carbon cycle as $^{14}\text{CO}_2$, which is well mixed within the atmosphere. Conventional ^{14}C ages originally assumed that the initial atmospheric ^{14}C concentration, ($\Delta^{14}\text{C}$), has remained constant through time. However, it has been shown that the $\Delta^{14}\text{C}$ levels have varied in the past (Stuiver and Braziunas, 1993; Suess, 1970), primarily due to changes in either the rate of ^{14}C production in the atmosphere (a function of the geomagnetic field intensity and solar variability) or the distribution of ^{12}C and ^{14}C between different reservoirs in the global carbon cycle (atmosphere, biosphere, ocean). Detailed records of past changes in atmospheric $\Delta^{14}\text{C}$ are therefore critical to the accuracy of ^{14}C dating and form the basis of the calibration process of ^{14}C ages. The IntCal working group has been responsible for the creation of an internationally agreed calibration data set, culminating in the most recent version, IntCal20 (Stuiver et al., 1998; Reimer et al., 2004, 2013, 2020).

The archive that best records the ^{14}C level of the atmosphere is tree-rings. In fact, trees incorporate atmospheric CO_2 directly into their tissues via photosynthesis, avoiding complications from unknown changes in the local reservoir age of archives coupled indirectly to the atmosphere, such as marine corals, sediments, and speleothems. In addition, tree-ring chronologies are constructed and replicated using numerous individual trees, providing true annual calendar ages. Finally, trees provide a large amount of woody tissue for ^{14}C dating, allowing precise, high-resolution chronologies to record even annual to decadal scale ^{14}C variability.

At present, the tree-ring based part of the calibration curve that is dendrochronologically linked reaches back to 13,900 cal BP (Calibrated ages Before Present). A brand-new method that connects floating tree-ring chronologies to the ice-core age scale through comparison with ^{10}Be data measured in ice cores is added to the new IntCal20. This connection is possible due to the fact that ^{10}Be and ^{14}C are both produced in the atmosphere by the cascade of nuclear reactions induced by high-energy galactic cosmic rays. Therefore, ^{10}Be and ^{14}C are both influenced by changes in solar activity and geomagnetic field intensity that modulate the incoming galactic cosmic ray intensity (Adolphi et al., 2017; Muscheler et al., 2020;).

The new IntCal20 incorporates the floating tree-ring sequences in three different sections (14,700 cal BP, 30,000 cal BP and 42,000 cal BP). The precision of the ^{14}C measurements and the resolution of sampling is best visible in the 14,700 cal BP section, published in Adolphi et al. (2017). This section is on a decadal scale and demonstrates a substantial advance in the new IntCal20. Most importantly, the improvement of the precision of ^{14}C measurements on tree-ring samples of 10 years resolution, would allow sequences on the time scale of a generation to be resolved (Talamo et al., 2017).

Before the tree-ring dataset interval (i.e., during glacial times) back to 55,000 years, the IntCal20 curve is composed of ^{14}C data from marine and terrestrial archives (i.e., marine sediments from the Cariaco Basin and the Iberian and Pakistan margins; marine corals; varved sediment cores from Lake Suigetsu, Japan; and stalagmites from the Bahamas and from Hulu cave, China), which do not have the same high resolution as the tree-ring data and the speleothem and marine data do not directly reflect the atmospheric ^{14}C concentration. However, between 48 to 40 ka cal BP the new IntCal20 shows more structure, which allows a better resolution and increased calendar age precision (Bard et al., 2020). This situation will favour the chronological reconstruction of the events that characterized our evolutionary past (Fig. 15.2).

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From conventional to AMS measurements

Since the 1950s gas proportional counters and liquid scintillation were used to detect and measure beta decay of ^{14}C in archaeological samples (Kromer and Münnich, 1992). Obtaining a precise radiocarbon date with this method could take more than a week and required up to 20 g of carbon. From c. 1980 the radiocarbon field improved with the creation of the Accelerator Mass Spectrometers (AMS) which directly counted the ^{12}C , ^{13}C , and ^{14}C ions in a

sample. This innovation has led to a real revolution in terms of sample size and measurement time. On the one hand, the dates obtained via AMS are made on very small samples (around 500 mg), which require a more rigorous samples pretreatment due to the fact that reducing the sample size will result in an increase in exogenous carbon contamination (Ramsey, 2008; Taylor and Bar-Yosef, 2014). On the other hand, reducing the sample size reduces the destruction of precious archaeological samples, strengthening the archaeologists to further use the ^{14}C method.

Normally, all AMS laboratories convert pretreated samples into CO_2 , through combustion, and then into graphite. This requires between 500 - 1000 μg of carbon, yet direct measurement of the CO_2 gas is possible and has the advantage of using only 5 - 100 μg carbon (Middleton, 1984; Ramsey and Hedges, 1997). However, even if, with the CO_2 gas, it was possible to further reduce the sample size the precision of the measurement would decrease (Ramsey et al., 2004). Modifications to the gas ion source were possible thanks to the new MIni CARbon DAtIng System (MICADAS) that, in 2007, was developed at ETH Zurich at the Laboratory of Ion Beam Physics (Synal et al., 2007). The MICADAS is a compact AMS which is furnished with an ion source capable of accepting both solid and gas samples (Ruff et al., 2007, Synal et al., 2007), and which greatly increases the level of precision possible for CO_2 samples. The system was successfully applied in Fewlass et al. (2018, 2019) with the conclusion that samples older than 35,000 years are still challenging for obtaining high precision.

The importance of constraints

To securely evaluate the relationship between dated samples and their archaeological context, the Bayesian statistics approach should be taken. In fact, Bayesian techniques combine chronometric information (^{14}C ages) with archaeological knowledge or 'prior' information (e.g., stratigraphy). This allows much finer chronologies to be built for the site in question. The most frequently used software is OxCal (Ramsey, 2009; Bronk Ramsey and Lee, 2013). This type of software uses IntCal20 as the reference curve and allows individual radiocarbon dates to be modelled statistically. However, the accuracy of these models strongly depends on the kind of data the users introduced: sample selection, sample pretreatment, 'prior' information, and the error range of the ^{14}C dates (Bayliss et al., 2007).

3.2. Why bones?

Obtaining the age of bones with radiocarbon is crucial for many archaeologists since it can directly target the event being dated (i.e., dating a human itself) or closely associated to the

event of interest (i.e., animal remains from a site, bone artefacts). Bone is characterized by a complex structure, ca. 65% of bioapatite (mineral, crystalline carbonate–hydroxylapatite, the inorganic phase), between 20-30% of proteins (collagen, the organic fraction), and c. 10% of water.

Collagen is the protein that permits strength and elasticity for the bone and is the most desired for radiocarbon dating (Arnold and Libby, 1951) and stable isotopic analysis (Collins et al., 2002). However, archaeological bones often have problems with contamination and degradation (DeNiro, 1985; van Klinken, 1999). Degradation involves structural alteration and the gradual breakup of the protein chains. Background contamination can then come from the inclusion of exogenous carbonaceous contaminants, either *in situ*, during the excavation, or in the laboratory. As the bone deteriorates, the collagen amount decreases, and with less collagen of the bones retrieved, higher is the danger for the collagen to be contaminated. Moreover,, as mentioned above, the low-collagen bone samples may result in less precise dates or no date at all (Talamo et al. 2021).

For these reasons, the preservation of collagen is crucial when we are dealing with ^{14}C dating on bone. In the following paragraph, key issues are considered in order to obtain a good quality and quantity of collagen for radiocarbon dating.

Sample selection and collagen extraction

The sampling strategy for bones in an archaeological site is a crucial step in obtaining a reliable radiocarbon date; hence it must be planned in detail. The first step is, of course, to select bone samples that display an obvious connection with human activities, like, for example, the presence of cut-marks or any type of fracture imposed by humans. As we all know, a radiocarbon date is not just a number, it is an expensive number. For this reason, we must choose with parsimony the samples in order to not exceed the budget dedicated to attesting the chronology of a single site. However, by increasing the number of samples for radiocarbon dating, we increase the precision and the details of the chronological sequence at the site in question (Hublin et al., 2012; Fewlass et al., 2020;). Once these fundamental aspects are contemplated, the collagen preservation can be considered.

Recently a non-destructive method to assess the collagen preservation of bone, prior to sampling, was developed (Sponheimer et al., 2019). Using Near Infra-Red (NIR) spectroscopy, we can screen the collagen preservation in archaeological bones directly at the site. This innovative method allows us to not only sample bones where the chances of successful

collagen extraction are high, but also to not destroy more samples or even precious archaeological samples.

Once we recognize the samples suitable for the radiocarbon dating method, a rigorous pretreatment in the laboratory must be undertaken. It is worth remembering that for samples older than 30,000 years the remaining ^{14}C activity is below 2%, hence these samples are especially vulnerable to contamination. Fortunately, in the last two decades, great improvements in the extraction of genuine carbon have been made (Talamo and Richards, 2011; Brown et al., 1988; Brock et al., 2013; Fewlass et al., 2019; Talamo et al. 2021). In most cases, this involves the use of the ultrafiltration step, and its specific way of cleaning, as well as the kind of ultrafilter used and the time that the bones spend in acid to be demineralized. Once the collagen is extracted, the carbon and nitrogen content should be considered in order to understand the quality of the extracts. The ranges are nowadays agreed upon and follow the rules imposed in van Klinken (1999) and DeNiro (1985).

3.3. The 'statistic' surrounding Neanderthals and *Homo sapiens* chrono-interaction

Around 40 ka, Europe was at the heart of the most fascinating events in Human Evolution. This time period saw the unfolding of the dramatic events between our closest cousins, the Neanderthals, and us, *Homo sapiens*.

When *Homo sapiens* groups crossed into Europe and walked into forested lands, they encountered the Neanderthal species. Neanderthals had been living for several hundred thousand years in variable climatic conditions (Sanchez Goñi 2021 in this book), from cold episodes with open steppe and tundra, to warm interstadial periods when florid forests were present in Mediterranean regions, showing that they possessed the behavioural flexibility to cope with different distributions and availabilities of biotic resources. However, after 37,000 years BP Neanderthals were gone, leaving *Homo sapiens* the sole species inhabiting Eurasia and finally the entire world (Zilhão et al., 2017; Higham et al., 2014). Pinpointing exactly when *Homo sapiens* arrived in Europe will improve our understanding of the interactions between Neanderthals and our species, and what, if any, responsibility we took in their demise. Accurate ^{14}C dating is of paramount importance to solving this puzzle of human history.

The most recent discoveries show that *Homo sapiens* were already in Europe around 46,000 years ago, in the Balkan Peninsula at Bacho Kiro, a site in Bulgaria (Fewlass et al., 2020; Hublin et al., 2020), and that they propagated east-west along the Mediterranean rim within a fairly short time, reaching the westernmost part of Europe between 41,000 and 38,000 years ago (Haws et al., 2020, Picin et al. 2021 in this book). All in all, these findings suggest *Homo sapiens*

and Neanderthals coexisted in Europe for roughly 8,000 years; this overlap would have been ~1,600 years in France (Talamo et al., 2020) and longer in southern Iberia.

However, despite progress being made, the resolution of the most used 'clock' still needs improvement in order to resolve archaeological disputes in a convincing way. In particular, more work needs to be done in order to substantially increase the resolution of the radiocarbon calibration curve in the Middle-Upper Palaeolithic period, as well as the tight error ranges that characterizes the ^{14}C age (Reimer et al., 2020; Fewlass et al., 2020).

Reducing the amount of bone taken is an ongoing concern in dating exceptional archaeological artefacts or human bones and obtaining precise date using the gas ion source installed in the new MICADAS (Fewlass et al., 2018, 2019).

4. Reconstructing Neanderthal's way of life (Diet, habitat, weaning, mobility)

4.1 Neanderthal isotopes

Diet and habitat reconstruction

Neanderthals are the first extinct hominins for which an isotopic approach, based on carbon and nitrogen isotopic abundances ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) in bone collagen, was used to determine their diet. This approach has been applied to individuals from Western France (Marillac, Charentes), Belgium (Scladina) and Slovenia (Vindija) (Bocherens et al., 1991, 1999, 2001; Richards et al., 2000). In these early works, Neanderthals appeared to have high nitrogen isotopic ratios compared to the coeval fauna, including predators, suggesting a very carnivorous diet. However, this conclusion was based on an oversimplification of the meaning of nitrogen isotopic ratios in the diet of omnivorous mammals. Indeed, even if $\delta^{15}\text{N}$ values increase with trophic position (from prey to predators), it is necessary to remember that collagen is synthesized from the protein fraction of the diet, i.e., the meat fraction for an omnivorous hominin, and that different plants exhibit various nitrogen isotopic ratios in a given ecosystem, leading to various $\delta^{15}\text{N}$ values in different herbivore species depending on their foraging preferences (Bocherens, 2003, 2009). With this in mind, it appears that Neanderthals consumed, in higher proportion than other predators such as hyena, prey with higher $\delta^{15}\text{N}$ values, which were, in the mammoth steppe biome, the woolly mammoth and to some extent the woolly rhinoceros (Bocherens, 2003; Bocherens et al., 2005; Wißing et al., 2016). Moreover, this result, concerning the meat fraction of the Neanderthal diet, does not

preclude the consumption of plants in addition to meat. This plant contribution, however, would not be visible in the isotopic composition of bulk collagen, since meat would provide almost all the consumed proteins (Bocherens, 2009). When more Neanderthal specimens were analyzed, this pattern of higher $\delta^{15}\text{N}$ values in Neanderthals than in predators prevailed (e.g., Beauval et al., 2006; Richards et al., 2008b; Bocherens et al., 2013; Wißing et al., 2016), suggesting that the high amount of mammoth meat in their diet was rather general, at least in the northern part of the Neanderthal range for the late representatives of the species. For Neanderthals from the beginning of the Late Pleistocene, isotopic results indicated either the consumption of meat from open landscape herbivores, as in Scladina (Bocherens et al., 1999) or from more closed forest environment, as in Hohlenstein-Stadel (Posth et al., 2017).

To solve the issue of plant consumption by Neanderthals, a more advanced technique was used: the single compound amino acid nitrogen isotopic composition, which compares the $\delta^{15}\text{N}$ values of source amino acids with those of trophic amino acids within bone collagen (Naito et al., 2016; Jaouen et al., 2019). In the Belgian site of Spy, this approach showed that Neanderthals were not fully carnivorous, but consumed about 20% plants (Naito et al., 2016). This study also showed that fish was virtually absent from the diet of Neanderthals in this context. However, further isotopic studies of animal predators and fish in a cave in the Caucasus, Kudaro 3, suggest that salmon may have been collected by Neanderthals rather than by predators such as bears or lions (Bocherens et al., 2014).

In addition to carbon and nitrogen isotopic abundances, bone collagen can provide an additional isotopic tracer based on sulphur stable isotopes. In terrestrial contexts, these isotopic ratios depend on geological bedrock and soil conditions and are therefore useful for investigating mobility. One study recently applied this approach to late Neanderthals from Goyet in Belgium, where it appeared that Neanderthal bones had been extensively butchered and were of non-local origin when compared to the local fauna (Wißing et al., 2019).

Most isotopic studies on collagen in Neanderthal bones have been performed for late specimens, as collagen preservation usually becomes poorer with greater age and is exceptional for ages higher than 100,000 years (Kuitens et al., 2015). In cases where collagen cannot be retrieved from bones, an alternative support for isotopic tracers is the mineral fraction of tooth enamel, where a small fraction of carbonate can record the isotopic composition of carbon and oxygen in diet and drinking water, indicative of subsistence and habitat, at the time of tooth formation; this was applied to Neanderthal teeth from the site of Payre in southeastern France (Bocherens et al., 2016). This study could tell differences in the

habitats of Neanderthal individuals from two levels with no climatic differences, showing that Neanderthal changing land use was not necessarily linked to environmental change.

Neanderthal mobility and isotope studies

The cost of Neanderthal mobility may be different from what modern humans experience due to their shorter limbs, higher body mass, and possibly more elevated basal metabolic rate (Verpoorte, 2006). It has been suggested that, within a year, they were probably changing sites more frequently than modern humans in order to reduce the daily number of kilometers required for foraging. This hypothesis can be tested through different methods, such as 1) the study of raw materials found within a site (often local even though exceptions occurred (e.g., Delpiano et al., 2018; Fernandes et al., 2008; Spinapolice, 2012; Turq et al., 2017; de Soler et al., 2020) and 2) investigating the investment in hearth making at a site (which seem to suggest short site occupation periods (Verpoorte, 2006; Leierer et al., 2019), but also, 3) sulphur (S, mentioned above) and strontium (Sr) isotope studies.

Most of the work focusing on Neanderthal mobility through isotopes has focused on Sr isotopes. The ratio of interest is $^{87}\text{Sr}/^{86}\text{Sr}$, where the ^{87}Sr derives from the radioactive decay of ^{87}Rb and ^{86}Sr is a stable isotope. This ratio is dependent of the local geology: the type of rock and its age are the main parameters influencing it and the measured values in animal teeth – or other tissues– directly reflects the bioavailable Sr in soils where their food comes from (Bentley, 2006). When studying human mobility, local plants and/or animals are first collected and analyzed to identify the isotope values of the bioavailable Sr in the region of interest. One can then see if an individual of interest spent its childhood at the found place, and if not, what is the closest location where it could have come from. Recent major advancements in isoscapes (geographical map of an isotope distribution), using small and large scale measurements of Sr isotope variability and machine learning models, have helped develop a precise idea of expected Sr isotope ratios in Western Europe and Israel (Willmes et al., 2014, 2018; Bataille et al., 2018; Snoeck et al., 2020; Kootker et al., 2016; Britton et al., 2020). Teeth are the material of choice because dental enamel is resistant to diagenesis. Therefore, Sr isotope studies reveal the foraging mobility of children, since enamel is not renewed through life, or that of their food providers.

The first application of Sr isotopes for reconstructing Neanderthal mobility involved a 40 000 year old individual from the site of Lakonis, in Greece (Richards et al., 2008a). The

enamel analyzed was formed when the Neanderthal was between 7 and 9 years old. The tooth values were more elevated than that of the local soil, and the closest location with such a value was about 20 km away. Following studies have reported similar observations : Benson et al. (2013) showed that a Neanderthal from Moula-Guercy, France, dating from the end of MIS 5 to the beginning of MIS6 (Willmes et al., 2016), had a more elevated Sr isotope value than the local one, which would suggest that they had moved from at least 50 km away since childhood. In a communication, Pike et al. (2016) also describe the mobility of Neanderthals (~35.000 and ~70.000 BP) between 6 areas located in a range of 30 km around the site, but without seasonal occupation patterns, in the region of Gruta da Oliveira, Portugal. Finally, a recent study documented the absence of mobility for three Neanderthals of the Nadale 1, Riparo Broion and Fumane sites (Italy). The teeth analyzed were deciduous, which would suggest the absence of mobility during the pregnancy of their mothers (Nava et al. 2020). Another study performed on the same site and one of the teeth analyzed by Nava et al confirmed this conclusion and reinforced it by adding baseline data of the regions obtained on plants and common vole teeth (M. P. Richards et al. accepted).

These analyses were performed using laser ablation³, a technique which is not always considered reliable for Sr isotopes (Nowell and Horstwood, 2009). However, different ways have been found to correct those potential analytical biases (Lugli et al., 2017b; Nowell and Horstwood, 2009; Horstwood et al., 2008; Willmes et al., 2016). Using one of the correction methods, Lugli et al., (2017a) studied the deciduous incisor of a Middle Pleistocene individual from Isernia La Pineta. Given the fact that this tooth is formed in utero, the results suggests that the woman who carried this child did not move for at least several months during her pregnancy. Using another correction method, Moncel et al. (2019) studied three Neanderthals from Payre, France. Two Neanderthals from the same layer (G) show Sr isotope ratios compatible with that of sediments from the Rhone Valley, where the site is located. The ratios of the third Neanderthal (layer F), however, indicate that it could have originating from the higher limestone plateaus. Those conclusions are in agreement with C and O isotope analyses, which allowed the identification of different ranges for the herbivores hunted in those two regions (Ecker et al., 2013), as well as dental micro wear data which suggested shorter

³ Laser ablation is a technique consisting in removing material from a solid (here teeth) through irradiation using a laser beam. The removed material is then transported and analyzed in a mass spectrometer in order to obtain isotope ratios. The technique is considered “quasi nondestructive” as it only destroys small areas of the teeth (<1mm).

occupation times for Neanderthals from layer F compared to those of layer G (Rivals et al., 2009).

In addition to direct measurements on hominin teeth, Sr isotope studies have sometimes been conducted on the fauna that they were hunting (e.g., Kelly et al., 2007). For instance, Britton et al. (2011) observed, using sequential measurement in teeth at Jonzac, France, that reindeer were seasonally migrating, that they were from the same herd due to the resemblance of the observed patterns between individuals, and that they were all killed over a short period of time. Those observations suggested a seasonal occupation by Neanderthals at the site where they were found. Hartman et al. (2015) also studied the evolution of Sr isotope ratios in gazelle teeth in different layers of Amud Cave, Israel. They linked the isotopic shift observed in animals to a change in the hunting territories of Neanderthals over time as they responded to climate changes.

Whatever technique is used, all these results seem to indicate that Neanderthals were, in general, mobile over the course of their lives, on a distance of several dozens of kilometers at least. Interestingly, the two sites where mobility was not indicated through S (Spy) or Sr isotopes (Payre, layer G) correspond to sites without any trace of cannibalism, whereas the sites showing clear mobility (Goyet and Moula-Guercy) are sites with evident proofs of this practice. No mobility was also detected in Fumane (Nava et al. 2020, Richards et al. in press) but the analysed teeth were formed In utero or during breastfeeding period, and therefore during or shortly after the pregnancy of the Neanderthals ' mothers. More results are needed to confirm those trends, but many Sr isotope data are currently unpublished due to the increased concern surrounding laser ablation data precision.

4.2. Dental microwear and microwear, dietary traits, and tooth-use in Neanderthals

Molar macrowear is a long term proxy that forms during the lifespan of an individual and thus reflects diet over long periods of time. It is analysed using occlusal fingerprint analysis derived from optical 3D topometry (Fiorenza et al., 2011). The analysis of dental microwear patterns is used as a high-resolution proxy to infer Neanderthal dietary traits. Tooth microwear has a high turnover rate, i.e., the wear pattern changes quickly when new food items are consumed, and it reflects a snapshot of diet over a short period before death (Grine, 1986). Dental microwear reveals diet but specific patterns of anterior tooth wear observed in Neanderthals

can be the result of paramasticatory activities i.e., the use of teeth as tools (e.g., Spencer and Demes, 1993).

Dental microwear and dietary variability in Neanderthals

The analysis of dental microwear in Neanderthal populations from Europe indicates significant eco-geographic variation in the dietary traits. Populations from Mediterranean evergreen habitats show a high variability in dietary traits in comparisons to the narrow dietary breadth observed in population that lived in steppe/coniferous forest environments in Northern Europe (Fiorenza et al., 2011).

Dental microwear and dietary traits in Neanderthals

The first studies of microwear and dietary traits in Neanderthals were focusing on the analysis of the buccal (non-occlusal) microwear patterns on microphotographs obtained through a Scanning Electron Microscope (Lalueza-Fox et al., 1996; Pérez-Pérez et al., 2003). A new approach to dental microwear analysis has been introduced by Ungar et al. (2003). This technique uses confocal microscopy and scale-sensitive fractal analyses to quantitatively study the microwear features. Dental microwear texture analysis has been successfully applied to Neanderthal dental remains (El Zaatari, 2007; Karriger et al., 2016; Estalrich et al., 2017; Krueger et al., 2017; Williams et al., 2018, 2019; Droke et al., 2020).

Since the end of the 2000s, there has been a growing body of data suggesting that Neanderthals did not have a diet exclusively based on meat. Recent studies based on microwear texture analysis show that Neanderthals had complex and diverse diets, which varied across space and through time (El Zaatari, 2007; Droke et al., 2020). The Neanderthals from the southern latitudes, i.e., in the Mediterranean area, had a diet that included meat but also significant amounts of plants yet relatively few hard items such as seeds and nuts (El Zaatari, 2007; El Zaatari et al., 2011; Karriger et al., 2016; Droke et al., 2020). In contrast, the diet of Neanderthals in northern Europe was made of softer items, including meat and plant foods. Dental microwear texture analysis also permitted the identification of differences between sexes (Estalrich et al., 2017) and among age groups (Pinilla Pérez et al., 2011) in Neanderthal populations.

Dental microwear and non-dietary anterior tooth-use behaviour and activities

Evidence of non-dietary scratches on the labial surfaces of the anterior teeth in Neanderthals has been reported since the beginning of the 20th century. These scratches were first observed on an incisor from La Quina (Henri-Martin, 1923), and later they were identified on other Neanderthal individuals (Koby, 1956; Patte, 1960; de Lumley, 1973; Trinkaus, 1983; de Castro et al., 1988; Lalueza-Fox, 1992). These studies provided evidence for the use of teeth as tools related to two types of activities: 1) some scratches, on the labial and occlusal side of the teeth, are related to the action of grasping materials between teeth; 2) other scratches, located in the centre of the labial surface, correspond to cutmarks produced when stone tools, involved in processing materials held between the anterior teeth, came into contact with the labial enamel face. The orientation of these scratches permitted the recognition of handedness and indicated that right-handedness is the dominant pattern in 90% of Neanderthals (Lalueza-Fox and Frayer, 1997). Activities are also related to the habitats where Neanderthals lived (see also Chap. 3). Recent advances in microscopy techniques, such as the use of confocal profilers, to analyse tooth microwear patterns are permitting new interpretations to be reached. Neanderthals from closed-habitats had highly diverse anterior tooth-use behaviours and exploited a wider variety of plant and animal resources than did those from open habitats (Krueger et al., 2017). As no differences in microwear were found between Neanderthals and early modern humans, it is hypothesized that they performed the same activities using their anterior teeth (Krueger et al., 2019).

5. Final remarks

The methodological advances of the past three decades have been decisive in deciphering the phylogeny, diet, mobility, age of Neanderthal fossils and to place these hominins more precisely in their chronological, environmental and evolutionary context. This trend does not seem to come to an end, as witnessed by pioneering works on the application of new isotopic tracers, such as those of calcium (Dodat et al., 2021), and zinc, if issues with diagenetic alteration can be sorted out for specimens as old as Neanderthals (Jaouen, 2018). Aspects of the life history of Neanderthals could be addressed using intra-tooth high resolution analysis of trace elements such as barium to track suckling and weaning (e.g. Austin et al., 2013). Single-compound isotopic analyses of organic molecules such as collagen have started to help

answering questions about plant consumption (e.g. Naito et al., 2016) and will certainly develop further in the near future.

Among the most recent breakthroughs in biomolecular approaches applied to Neanderthals, the possibility to detect and make genomic analyses of hominin ancient DNA in cave site sediments. This approach opens a wide range of possibilities for identify which hominins occupied sites where no skeletal fossils have been found and even reconstruct population history in the site (e.g. Vernot et al., 2021). Additionally, palaeoproteomics is emerging as an additional source of phylogenetic sequence information in cases where ancient DNA does not survive (Welker et al., 2020).

Another promising way of deciphering Neanderthal diet beyond their protein fraction is the palaeogenetic analysis of their oral microbiome. A recently published study by Fellows Yates et al. (2021) has shown that Neanderthals harbored in their mouths bacteria that help to digest starches, similarly to modern humans, suggesting that feeding on energy-rich sugars from plants was already performed by Neanderthals.

Technical developments in the various fields covered in this chapter have already been instrumental in shedding light on the life and history of Neanderthals. These methodologies, along with others that will certainly be brought forward in the next years, are likely to keep contributing valuable information, as we continue our quest to elucidate the mysteries of the past.

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Figure captions

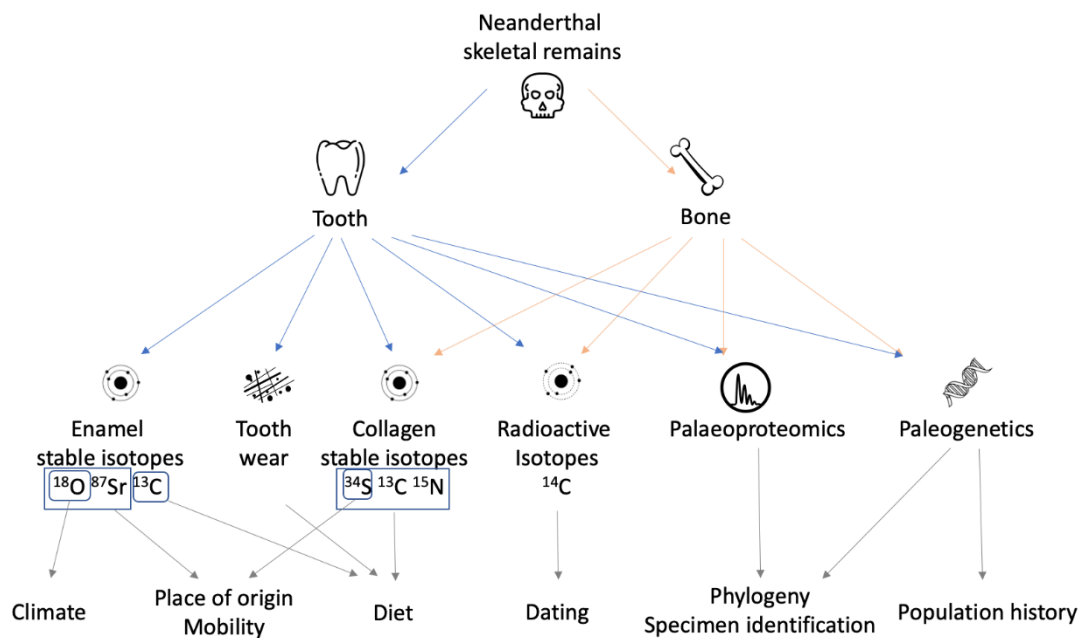


Figure 15.1: Schematic overview of the approaches described in this chapter and their implications.

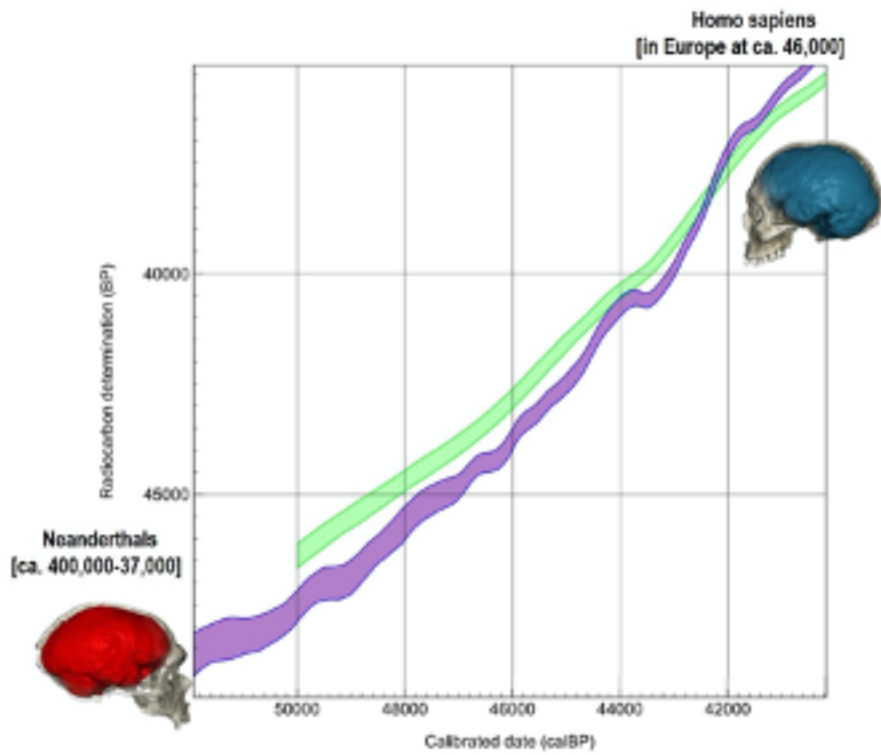


Figure 15.2: The differences between IntCal13 in green and IntCal20 in purple. The spread of *Homo sapiens* (blue rows) and Neanderthals (red zone) on a geographical map.