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Author: Geordie Laidlaw

Thesis Title: Mitochondrial and Metagenomic Analysis of Iberian Individuals from the Neolithic and Chalcolithic Periods

Abstract

From the end of the Mesolithic to the onset of the Early Bronze Age (~5700-2000 BC), Iberia experienced a multitude of cultural changes reflected in the archaeological record. Genetic and genomic analyses of ancient populations have been able to provide insight into how these changes related to population shifts, as well as uncovering others not related to cultural changes.

This study presents mitochondrial genomic results from 3 individuals from the Middle Neolithic site of Algar do Barrão in the Tagus River valley of Portugal and 7 individuals from the Bell Beaker context of Convento do Carmo in the Estremadura region of Portugal. Two more individuals from this site were also analysed, as well as individuals from the Late Neolithic sites of La Sagrera in Barcelona (N=10) and Casais da Mureta in the Tagus River valley (N=3), but reliable results could not be obtained.

Based on analysis from these individuals and previously published material, a migration event carrying HV, V, K, N, T2, and J mitochondrial lineages replacing the U5 hunter-gatherer associated lineages in the Early Neolithic period, followed by a 'resurgence' of hunter-gatherer associated genetic components that was especially visible in Portugal in the Middle and Late Neolithic periods, were observed. Subsequently, the Chalcolithic period saw the introduction of steppe-related ancestry in continental Europe, which this study shows was unrelated to the expansion of the Bell Beaker cultural complex.

Metagenomic screening revealed the first instance of *Spirometra erinaceieuropaei*, which also marked the oldest known tapeworm DNA discovered in an ancient sample, found in a tooth sample from Algar do Barrão dated to 3760-3650 cal BC.

Mitochondrial and Metagenomic Analysis of Iberian Individuals from the Neolithic and Chalcolithic Periods

Geordie Laidlaw

This thesis is submitted for the qualification of Master by Research
Department of Archaeology
Durham University

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Data Access Statement

Supplementary material mentioned in this work can be accessed at:

https://github.com/GeordieL9/MRes_Supplementary_GL

1. Introduction

This study aims to understand past populations in Iberia through the Mitochondrial and Metagenomic analysis of Neolithic and Chalcolithic individuals. This chapter will provide a brief overview of the characteristics and types of DNA (deoxyribonucleic acid) evidence utilised, the archaeological and genetic context, and the specific aims and objectives of this study.

1.1 Ancient DNA

1.1.1 Characteristics

Ancient DNA, or aDNA, is DNA evidence extracted from long deceased organisms (Hofreiter et al., 2001b). During life, DNA in the cells of organisms is constantly being repaired and maintained (Lindahl, 1993). After death, these processes stop, and DNA eventually breaks down over time due to physical and chemical processes resulting in a set of characteristic properties of ancient DNA such as short fragment length (typically between 10-150 base pairs long), high fragmentation and low relative quantity of endogenous DNA (Dabney et al., 2013b; Pääbo et al., 2004). Inter and intra molecular crosslinks between DNA molecules (Hansen et al., 2006; Sczepanski et al., 2010) and polymerase chain reaction (PCR) inhibitors (Kemp et al., 2014) are also common in ancient DNA samples, causing difficulty in the amplification process.

More recently, an excess of cytosine (C) to thymine (T) transitions mostly at the ends of individual reads (raw sequence of base pairs from a single fragment of DNA generated during sequencing) has been uncovered as an important characteristic used to identify endogenous ancient DNA (Briggs et al., 2007; Brotherton et al., 2007; Dabney et al., 2013b; Hofreiter et al., 2001a). These C to T mutations occur when a cytosine base, typically on the exposed overhanging end of a DNA strand that has broken, is transformed into a uracil due to hydrolytic deamination, as seen in Figure 1 (Gilbert et al., 2007b, 2007a). As Uracil (U) is an RNA base structurally very similar to Thymine (T) and complementary to the DNA base adenine (A), this will result in C to T or guanine (G) to A mutations during amplification based on the strand sequenced (Dabney et al., 2013b).

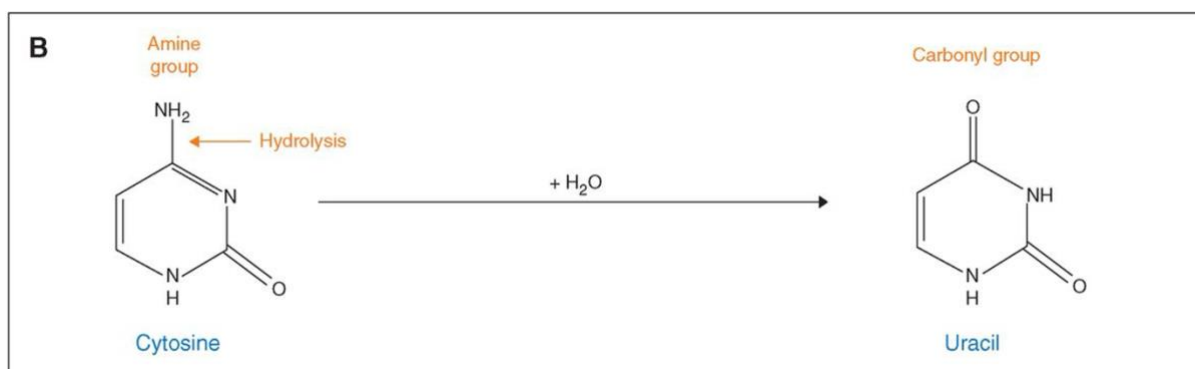


Figure 1: Image of hydrolytic deamination of a cytosine to uracil base. From (Dabney et al., 2013b)

1.1.2 Preservation

Preservation in ancient DNA varies based on the burial environment and age of the sample. High humidity, heat, frequent precipitation, high soil acidity, and fluctuating environmental conditions tend to result in poor DNA preservation due to the increased potential for microbial growth, hydrolysis, and other chemically damaging processes (Alaeddini et al., 2010; Bär et al., 1988; Kravanja et al., 2025; Latham and Miller, 2019). Preservation is best in cold, dry, and stable environments, as these restrict microbial growth and hydrolytic processes (Alaeddini et al., 2010; Bär et al., 1988; Kravanja et al., 2025; Latham and Miller, 2019). Cave environments are an excellent example, as they often provide a cool stable temperature year-round as well as protection from rain and other environmental conditions (Burger et al., 1999). In human subjects, DNA is best preserved within the interior portion of the *pars petrosa* of the temporal bone (Pinhasi et al., 2015), auditory ossicles (Sirak et al., 2020) and tooth cementum (Damgaard et al., 2015), as the higher density of these elements provides protection against environmental factors (Gilbert et al., 2003; Higgins et al., 2015; Höss et al., 1996; Lindahl, 1993; Poinar et al., 1996). A study by Parker et al (2020) determined that the *pars petrosa* was the best option for successful endogenous DNA recovery, with molars being the next best option if the *pars petrosa* is not available for sampling.

1.1.3 History of Ancient DNA

The study of aDNA is a relatively new scientific field that endured a rocky start which led to the extensive revision of previously published literature and the establishment of authentication criteria that allowed for aDNA analysis to be a useful tool for archaeological studies today. The first study of aDNA examined a quagga museum specimen by using bacterial cloning to amplify the quagga DNA (Higuchi et al., 1984). The first analysis of ancient human DNA was done using a similar method on an ancient Egyptian mummy (Pääbo, 1985). After the invention of the Polymerase Chain Reaction technique (PCR) (Saiki et al., 1985) for amplifying DNA and the automation of Sanger Sequencing in the late 1980s (Sanger et al., 1977), which allowed for relatively easy and cheap sequencing of DNA strands, aDNA studies exploded in frequency and mainstream popularity.

During this period, multiple papers were published in high impact journals that claimed they had sequenced DNA from 5-135 million year old specimens from amber and fossil samples (Cano et al., 1993; DeSalle et al., 1992; Golenberg et al., 1990; Poinar et al., 1993; Soltis et al., 1992; Woodward et al., 1994). These eye-catching publications drew attention from the molecular biology community, and sparked debate about the authenticity and usefulness of aDNA research (Allard et al., 1995; Hedges and Schweitzer, 1995; Lindahl, 1997; Zischler et al., 1995). These studies were later determined to be unreproducible, and DNA sequences were deemed to have originated from contaminant sources. To avoid similar instances in the future, strict criteria of authenticity were established and compiled (Cooper and Poinar, 2000; Pääbo et al., 2004). Original aDNA authentication criteria included:

1. The use of exclusive laboratory and equipment for ancient DNA analyses
2. Physically isolated pre and post PCR work areas
3. Analysis by a single researcher
4. Use of extraction and amplification blanks
5. Comparison of obtained sequences to archaeologists and researchers associated with samples
6. Replication in a second laboratory
7. Independent extractions and amplifications from the same extract
8. Independent assessment of biomolecular preservation
9. Bacterial cloning of amplified products and sequencing of multiple clones (Cooper and Poinar, 2000; Pääbo et al., 2004).

From this point onward, studies of aDNA abided by these criteria to ensure authentic results. Under these criteria, the oldest truly ancient DNA recovered is from a 2-million-year-old sedimentary sample recovered from a permafrost environment in Greenland (Kjær et al., 2022).

1.1.4 Next Generation Sequencing in Ancient DNA Studies

The development of Next Generation Sequencing (NGS)(Margulies et al., 2005) has drastically changed aDNA research (Rizzi et al., 2012). The most popular NGS technology in ancient DNA research is the Illumina sequencing by synthesis (SBS) chemistry. After extraction and purification, short strands of DNA ('adaptors') necessary for amplification and sequencing are attached to both ends of the DNA strands, creating DNA 'libraries' (Meyer and Kircher 2010). During the library building process, specific DNA 'barcode' sequences are also attached to both ends of the DNA, with a unique combination of sequences for each sample. This allows for pooled or 'multiplex' samples to be sequenced, as the barcode indexes can be used to differentiate which DNA reads came from which sample after sequencing, also known as 'demultiplexing'. Pooling of samples greatly reduces the cost of sequencing, as fewer lanes are necessary (Knapp et al. 2015).

As primers do not target specific DNA in this method, all DNA present in a sample is amplified and sequenced. Samples can be submitted for sequencing in this 'Shotgun' form, where all DNA amplified in the sample is sequenced, or can undergo an enrichment process in which specific types of DNA – such as mitochondrial reads or

reads with common whole genome SNP positions – are separated and amplified (Rohland et al. 2022).

During sequencing, adapters on DNA Illumina library strands attach to sequences connected to a flow cell. Strands are then copied by polymerase chain reaction onto those sequences. The opposite end then anneals to another point on the flow cell. Strands are copied before denaturing and beginning the process again. This process of copying and denaturing creates clusters of forward and reverse copies of a single strand, and reverse strands are washed away to leave one signal. Fluorescent nucleotides are attached to each nucleotide in the DNA strands and recorded by a camera one at a time, generating a sequence of nucleotides for each cluster (“NGS Workflow Steps | Illumina sequencing workflow,” 2025).

Sequences can be aligned to reference genomes using bioinformatic alignment tools that rely on statistical probabilities to position reads to specific positions in the genome. ‘Shotgun’ sequencing of all DNA present within a sample allows for sequencing of DNA not only from the endogenous organism, but also from any other DNA present in the sample. Using latest common ancestry based metagenomic analysis techniques, which competitively align reads to all available reference genomes, DNA from organisms such as pathogens that were sequenced in parallel to host DNA can be uncovered (Herbig et al., 2016; Pochon et al., 2023).

In comparison to the PCR approach, this sequencing method allows for faster output of comprehensive data from reads of any part of any genome, elevating analysis potential from the genetic to the genomic scale. As the PCR approach can only sequence one read at a time, constructing an entire genome could take years depending on the size of the genome (13 years to sequence ~85% the human nuclear genome, for example)(Lander et al., 2001). With NGS, this can be done in a matter of hours. The downside of NGS, however, is that it is less sensitive to minute amounts of DNA, as many reads are lost during the library building process. While this makes NGS studies less susceptible to minor contamination, it becomes an issue when dealing with ancient DNA samples, which usually contain very small amounts of endogenous DNA.

The ancient DNA criteria of authenticity have adapted to the NGS approach. Bacterial cloning, replication in a second laboratory, and preservation assessment of other biomolecules are no longer necessary. Instead, metrics obtained from NGS sequencing results, such as C to T deamination plots, average length of DNA strands, and endogenous DNA percent are used to ensure the DNA is ancient and belongs to the targeted organism (Davidson et al., 2025; Palencia-Madrid and de Pancorbo, 2015; Rizzi et al., 2012). NGS data also allows for the detection of heterogeneity in mtDNA and heterozygosity in the X chromosome (Knapp et al., 2015). As polymorphic positions are not expected in these sequences, detection of heterogeneity or heterozygosity can be a potential indicator of contamination of the sample (Knapp et al., 2015; Rasmussen et al., 2010).

1.2 Mitochondrial DNA

Mitochondrial DNA is a type of genetic material found within cell mitochondria and organised in a circular chromosome. Mitochondria are passed down through the

egg cells produced by females during sexual reproduction, resulting in mitochondrial DNA being inherited maternally. With the exception of new mutations arising in the germ lines or in specific tissues during the life of the individual, mitochondrial DNA will be exactly the same in the mother and the offspring (Dawid and Blackler, 1972; White et al., 2008). Mitochondrial DNA is typically 16,569 base pairs long, contains a light (L) and heavy (H) strand, and is highly packed into 37 genes within the coding region of the mitogenome responsible for mitochondrial function (Taylor and Turnbull, 2005). The non-coding control region, seen in Figure 2, contains three regions often targeted by genetic studies utilising a PCR approach. These specific regions are called Hypervariable Regions (HVR) due to the abundance of mutations usually observed within them (Árnadóttir et al., 2024). HVRI covers positions 16024-16569, HVRII positions 1-340, and HVRIII positions 438-574. HVRI is usually preferred over other hypervariable regions, as it had been established that the mutation rate in this region was higher (Meyer et al., 1999), but a recent study places HVRII of the control region as the region with the highest rate of mutation in the mitochondrial DNA, with approximately 4.0×10^{-5} mutations per base pair per generation (Árnadóttir et al., 2024).

Figure 2: Location of genes in the mitochondrial DNA, and detail of the control region and the positions of HVRI(HVSI) and HVRI(HVSI)I within. From: (Carolina Azevedo de Sá, 2021).

When traced, mutations in the mitochondrial DNA can be used to determine maternal biological relationships and wider population maternal affinity of individuals in ancient populations (Haak et al., 2008, 2015a). Studies utilising PCR and sanger sequencing technology often targeted specific areas within the mtDNA genome - primarily the HVRI and HVRII - due to limitation of only being able to sequence one DNA fragment at a time (Palencia-Madrid and de Pancorbo, 2015). Next generation sequencing, however, allows for analysis of the entire mtDNA genome due to the ability to sequence multiple reads at once (Palencia-Madrid and de Pancorbo, 2015).

The combination of mutations in the mitochondrial genome of an individual is referred to as a 'mitochondrial haplotype' (Zhang et al., 2003). These mutations are typically identified in genetic studies by comparing DNA sequences to a reference. The Cambridge Reference Sequence (CRS) was the first published mitochondrial DNA sequenced (Anderson et al., 1981), and its revised and reanalysed version (rCRS)(Andrews et al., 1999) is used as a worldwide reference for haplotype determination (Bandelt et al., 2014). The Reconstructed Sapiens Reference Sequence (RSRS)(Behar et al., 2012), which is based on the estimated sequence of the deepest root of all modern human ancestry, is also used as a reference sequence within human population genetics, although not as commonly.

Phylogenetic analysis has been able to organise the variation in mtDNA into haplogroups and sub-haplogroups that represent different branches in the mitochondrial evolutionary tree (Van Oven and Kayser, 2009). The simplified phylogenetic tree can be seen in Figure 3 below. All living lineages can be traced back to a most recent common ancestor (MRCA), sometimes referred to as 'mitochondrial Eve' (Behar et al., 2008; Cann et al., 1987). Time to the most recent common ancestor (TMRCA) estimations can be made within haplogroups, offering insight into when and how different lineages emerged (Walsh, 2001; Zhou and Teo, 2016). Due to genetic drift and population differentiation, haplogroups or a combination of haplogroup lineages can sometimes be associated with populations and used as a marker for migration and gene flow (Haak et al., 2015a).

The relative abundance of mitochondrial to nuclear DNA (several thousand copies per cell as opposed to two copies of nuclear DNA per cell) (Taylor and Turnbull, 2005) and the rate of mutation make mtDNA a useful tool for understanding past population maternal ancestry, movement and in certain instances, maternal close biological kinship. The smaller size of the genome also makes it ideal for studies with time and budget constraints. Despite these advantages, mitochondrial DNA also has many disadvantages. Its low resolution in comparison to nuclear DNA means that more nuanced population changes can be missed. The restriction of mitochondrial DNA analysis to maternal lineage history only is also disadvantageous, as population

changes occurring solely in males or disproportionately between males and females cannot be perceived (Rizzi et al., 2012).

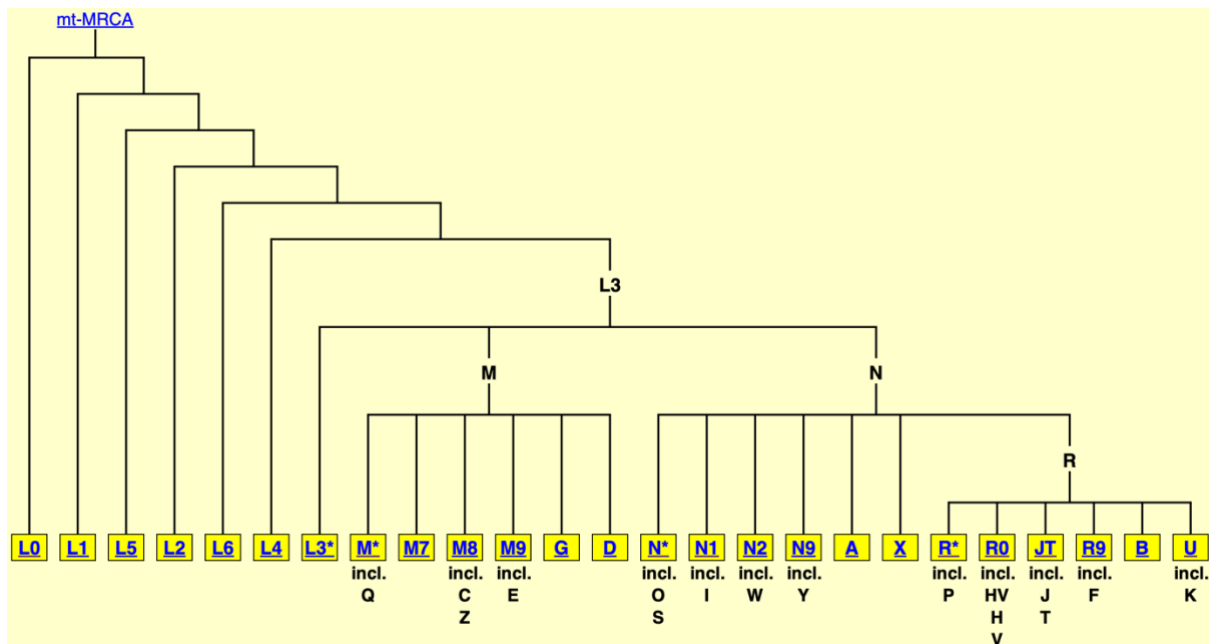


Figure 3: Simplified phylogenetic tree of mtDNA haplogroups. From PhyloTree.org - Phylotree mtDNA tree Build 17 (Van Oven and Kayser, 2009). MRCA = Most Recent Common Ancestor.

1.3 Archaeological Background of the Mesolithic to Bronze Age Periods in Iberia

The Mesolithic period in Iberia (~8,250-5,650 cal BC) is characterised by the emergence of microlithic tools (Cucart-Mora et al., 2022; Fuertes-Prieto et al., 2023). The Early Mesolithic (~8,250-6,650 cal BC) is associated with the use of Notche and Denticulated lithic technology, while the Late Mesolithic (~6,650-5,650 cal BC) is accompanied by the use of geometric (typically triangular and trapezoidal) microliths (Cucart-Mora et al., 2022; Fuertes-Prieto et al., 2023). Variable exploitation of marine resources resulting in 4-23% marine protein diets determined from carbon and nitrogen stable isotope studies is also observed throughout the Mesolithic (Cubas et al., 2019; Salazar-García et al., 2014). This is supported by the substantial amount of shell middens (>130 in the Asturias region alone) found in Iberia during this period (Fano, 2019). Funerary practices throughout the Mesolithic, while rarely found, are usually reported as single or multiple pit burials including shells and/or lithic artefacts (Gibaja et al., 2012). In Portugal, a large concentration of Mesolithic shell middens and other occupational sites have been observed in the Muge valley region near the Tagus river,

with a period of use in the Mesolithic between 5,950-5,450 cal BC and later reuse in the Neolithic after 5,350 cal BC (Bicho et al., 2013). Due to the concentration of settlements along the coasts in both the Mesolithic and subsequent Early Neolithic periods, it should be noted that a large number of sites from these periods have likely been lost to coastal erosion and rising sea levels in the time since their occupation (Aguirre-Uribesalgo et al., 2024; García-Artola et al., 2018).

The Iberian Neolithic saw the spread of agriculture into the region, alongside a collection of associated artefacts and cultural practices (Antolín, 2016; Defranould, 2021). Pottery, polished stone tools, a collection of domesticated flora and fauna, agricultural practices, sedentary lifestyle, and subsistence on a predominantly terrestrial diet are all part of the 'Neolithic package' of cultural and technological changes that originated in Anatolia and the Near East and defined the Neolithic transition in Europe (Çilingiroğlu, 2005).

The earliest evidence of the Neolithic arrival in Iberia comes from ~5,500 cal BC (Martins et al., 2015) along the Mediterranean coastline, and is characterised by the Cardial pottery tradition (Defranould, 2021). This style of pottery is similar to the slightly earlier (~6,000-5,600 cal BC) Impressa style found in Italy and the Adriatic Sea coastline (Defranould, 2021; Gomart et al., 2017), suggesting the Neolithic spread out along Mediterranean coastlines from Anatolia and the Near East, eventually reaching Iberia. The leading theory for the process of Neolithic spread in Iberia is the movement and migration of pioneering farming groups along the Iberian coasts (Drake et al., 2017; Zilhão, 2001a), arriving in Portugal and North Atlantic Iberia by ~5,400 cal BC (Antolín, 2016; Drake et al., 2017). The Cardial pottery tradition was followed by the similar Epicardial pottery tradition, which originated in the central inland areas of Iberia around ~5,350 cal BC and spread outward to coastal regions by ~5,100 cal BC (Cámara Manzaneda et al., 2022).

While most burials in Neolithic Iberia have been observed in cave or hypogeum contexts, dolmens and other megalithic tombs emerge as a significant portion of burial contexts in Atlantic and West Iberia by the Middle Neolithic, around ~4,300 cal BC (Alt et al., 2016; Rojo and Garrido-Pena, 2012; Scarre, 2019; Tavares da Silva and Soares, 2000; Vijande-Vila et al., 2022). Archaeologists and geneticists still debate whether

1 - these sites constitute a cohesive cultural or genetic group

2 – when such a culture emerged, and

3 – if they are directly linked to WHG groups

(Carvalho et al., 2023; Scarre, 2019; Tavares da Silva and Soares, 2000).

Alongside the use of Megaliths in the West, the Middle and Late Neolithic period in Iberia (~4,500-3,200 BC) also sees the emergence of pit burials around ~4,770 cal BC

and stone box burials around ~4,230 cal BC, both in the Northeast region (Albizuri et al., 2019; Morell Rovira et al., 2018). Although the pit burial style emerged earlier, these burial styles coexisted until around ~3,490-3,180 cal BC (Morell Rovira et al., 2018). Agricultural changes have also been observed in this period (Antolín, 2016). An increase focus was placed on stockbreeding and the production of wheat (Antolín, 2016). This led to a shift in human settlements to open countryside sites more conducive to these agricultural practices (Albizuri et al., 2019).

The Chalcolithic period in Iberia (~3,200-2,200 BC) is separated from the Late Neolithic by the first use of copper metallurgy. Early Chalcolithic grave goods were similar to those of the Neolithic, with an abundance of stone tools, pottery, and bone adornments, with the addition of occasional copper items such as awls (Alday et al., 2015; Gibaja Bao et al., 2012; Linares-Catela and Vera-Rodríguez, 2023; Valério et al., 2023). During this period, one of the most prominent archaeological signatures in Iberia is the emergence of Bell Beaker shaped pottery near the Tagus river estuary in Portugal around ~2,750 cal BC (Cardoso, 2014). This style of pottery spread across the rest of Iberia by ~2,500 cal BC, and is found in similar contexts throughout Western and Central Europe and North Africa between ~2,500-1,800 cal BC (Olalde et al., 2018), leading to the classification of the ‘Beaker Folk’ archaeological cultural group (Harrison, 1980).

Bell Beaker burials in Iberia specifically were often single inhumation burials in the left lateral decubitus position, although collective and double inhumation burials have also been observed (Soriano et al., 2021). Alongside the eponymous Bell Beaker shaped pottery, these burials typically included flint arrowheads, stone wrist guards, as well as V-perforated buttons, copper awls, and other copper, gold, stone, or ivory jewellery typically (but not exclusively) found alongside osteological females (Soriano et al., 2021). Occasionally, Bell Beaker burials contained more elaborate grave goods such as gold, copper daggers typically (but not exclusively) found with osteological males (Herrero-Corral et al., 2025), and ivory or ostrich eggshells suggesting trade networks between Iberia, North Africa, and the Near East (Gibaja Bao et al., 2012; Schuhmacher et al., 2009). These items possibly indicate the existence of a social elite (Soriano et al., 2021). Non-Bell Beaker burials in the latter half of the Chalcolithic period, in contrast, rarely contained any grave goods at all (Herrero-Corral et al., 2025).

In Atlantic Iberia, Dolmens and other megalithic tombs were often reused as burial sites, both for Bell Beaker (Soriano et al., 2021) and non-Bell Beaker burials (Sousa and Gonçalves, 2022). Cave burials continue to be the most commonly occurring sites across Iberia, with hypogeum also making up a substantial proportion (Linares-Catela and Vera-Rodríguez, 2023; Sousa and Gonçalves, 2022). These types of burials are less common for Bell Beaker contexts (Soriano et al., 2021).

The Early Bronze Age in Iberia (~2,200-1,500 BC), distinguished by the emergence of bronze metallurgy, did not see a widespread cultural change but rather increased regional cultural diversity within Iberia. In the Southeast of Iberia, the Argaric period (beginning ~2,200 BC) saw an increased economic divide between social elites and the rest of the population, based on the appearance of 'elite' graves including goods such as copper halberds and, while most Argaric graves only included simple undecorated pottery (Micó, 2011). Settlements of this archaeological culture were also heavily concentrated in hilltop settlements, also suggesting a hierarchical social structure (Lull et al., 2021). Interestingly, this archaeological group sees no evidence of continuing to use Bell Beaker style pottery, in contrast to the rest of Iberia (Micó, 2011).

The Cogotas I group, named for the earliest phase at the site of Las Cogotas, existed between ~1,700-1,000 BC and originated in the north-central Meseta Duero valley region. This group is identified by the use of black pottery with geometric designs (Fernández-Posse et al., 2012). Funerary contexts associated with this group rarely included any grave goods, and most often secondary pit burials (Esparza et al., 2017).

In Portugal, Atlantic groups lived primarily in lowland areas and continued funerary traditions from the Chalcolithic albeit with more individual than collective burials (Blanco-González et al., 2018). Groups in the inland eastern regions of Portugal made settlements on hilltops and have been found with Bell Beaker and copper halberd grave goods (Blanco-González et al., 2018).

1.4 The Genetic Landscape of Mesolithic to Bronze Age Iberia

Although the sites analysed in this study are dated to the Neolithic and Chalcolithic periods of Iberia, an analysis of populations from the Mesolithic to the Bronze Age was considered. This allowed for a more complete contextualisation of the Neolithic and Chalcolithic populations by allowing for comparisons to previous and later population groups.

During the Last Glacial maximum (~25,000-19,000 years ago) (ya), harsh conditions forced human populations into two areas of refuge in Europe: the Iberian Peninsula and Southern Europe (Italy, the Balkans) (Stewart and Stringer, 2012). Mitochondrial DNA studies have revealed a homogeneous European Mesolithic mitochondrial gene pool made of entirely U haplogroup lineages, with a majority being further defined as U5b, resulting from this bottleneck event (Posth et al., 2016).

Genomically, these refugia developed two genetically distinct groups: one associated with the Iberian refugium and Magdalenian culture, and another associated with the Southeastern refugium and the Epigravettian culture (Posth et al., 2023; Villalba-Mouco et al., 2019). These genomic signatures are often identified in the

genetic literature by the associated sites of the Goyet Caves in Belgium for the Magdalenian, resulting in GoyetQ-2 ancestry, and Ripari Villabruna in Northern Italy for the Epigravettian, resulting in Villabruna ancestry (Villalba-Mouco et al., 2019).

Following the last glacial maximum, a warming event led to the recolonisation of North and Central Europe by approximately 14,000 years ago (Allentoft et al., 2024; Posth et al., 2023). During this recolonisation, Villabruna ancestry replaced GoyetQ-2 ancestry in almost all of Western Europe (Allentoft et al., 2024; Posth et al., 2023). This genetic ancestry is therefore referred to as Western Hunter-Gatherer (WHG) ancestry in the genetic literature, although it is important to note that these genetic classifications do not directly relate to archaeological cultures (Eisenmann et al., 2018).

Figure 4 displays that Iberian hunter-gatherers, however, are genetically intermediate between the GoyetQ-2 and Villabruna groups (Villalba-Mouco et al., 2019). A ~19,000 year old individual at the Magdalenian site of El Mirón in Cantabria shows the first evidence of this dual ancestry, and all other Iberian hunter-gatherers of later dates that have been genomically analysed also carry it (Villalba-Mouco et al., 2019). This Iberian hunter-gatherer dual ancestral signature is different from the WHG signature seen throughout the rest of Western Europe and will be referred to as ‘HG’ throughout this work. No distinction can be made between WHG and Iberian HG mtDNA (Villalba-Mouco et al., 2019).

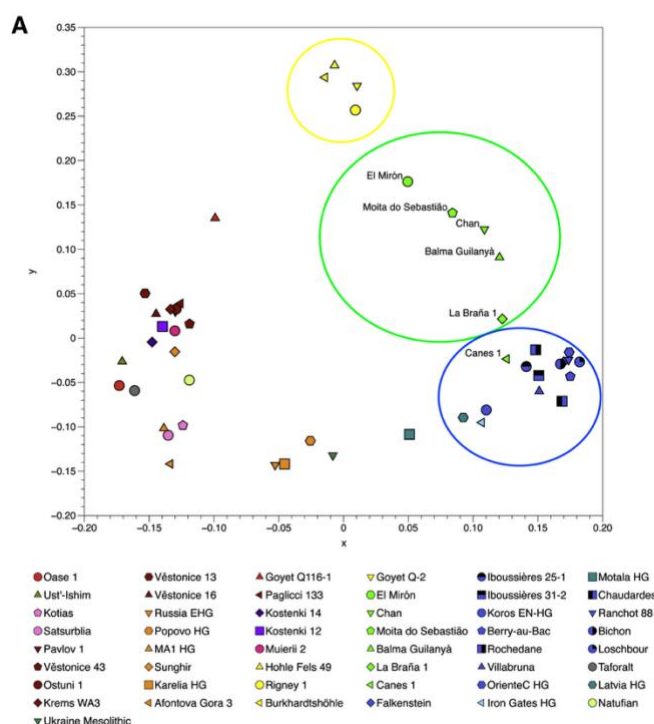


Figure 4: MDS plot of genetic distances between hunter-gatherer individuals in Eurasia. Yellow circle indicates GoyetQ-2 cluster, blue circle indicates Villabruna cluster, and

green circle indicates Iberian hunter-gatherer cluster. From: (Villalba-Mouco et al., 2019). Cluster circles added by author according to (Villalba-Mouco et al., 2019).

Different models of Neolithic spread have been proposed prior to the application of genetic studies. The cultural diffusion model saw Neolithic practices and artefacts spread through trade and social networks (Dennell, 1983), while the demic diffusion model saw the direct migration and movement of people as the primary means of the Neolithic spread (Ammerman and Cavalli-Sforza, 1984). Intermediate models have been suggested more recently, such as the migration of a small group of elites (Renfrew, 1989), infiltration of small groups (Neustupný, 1982), frontier mobility along established social networks (Zvelebil, 2001, 1996), and the spread of maritime pioneering farmers coexisting with local groups along the coastlines (Gamba et al., 2012; Zilhão, 1998, 2001a). Genetic implications of these different models of Neolithic diffusion were modelled by Fernández-Domínguez and Reynold (2017), which can be seen in Figure 5 below.

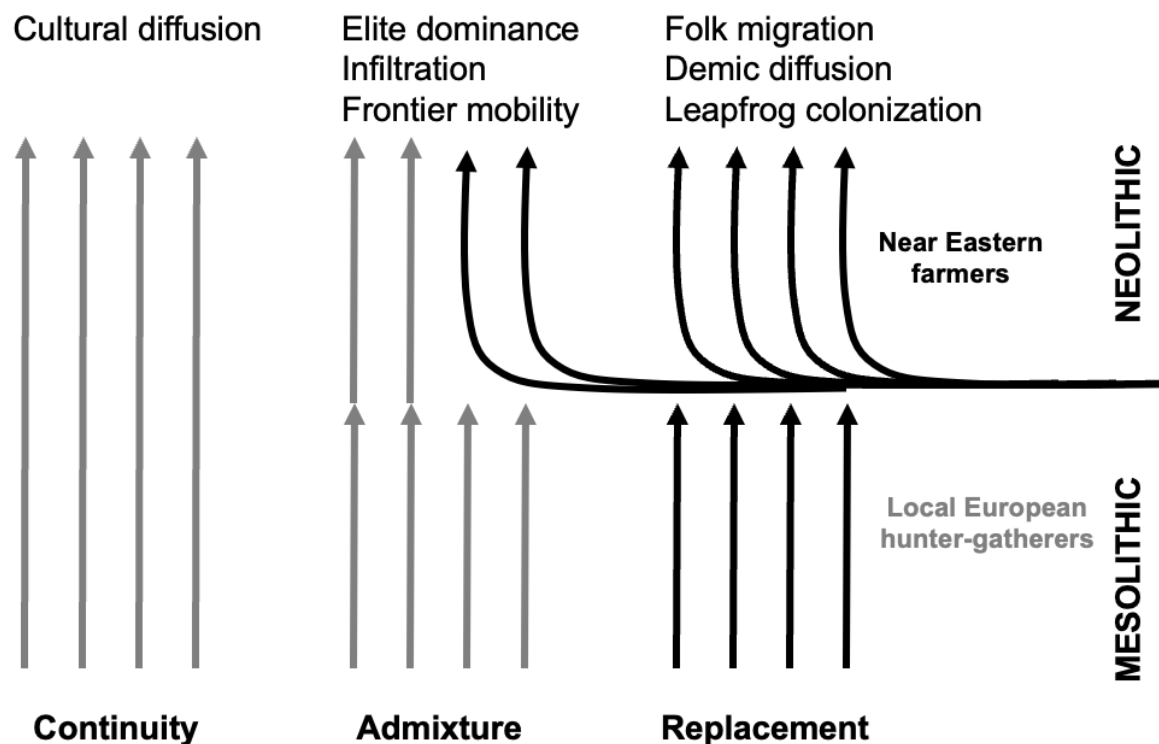


Figure 5: Genetic implications of different models of Neolithic diffusion. From (Fernández-Domínguez and Reynolds, 2017: Fig 12.1).

Mitochondrial haplogroups H, K, T2, X2, and N are observed in Iberia at the onset of the Neolithic, with no evidence of lineage U5b in the Early Neolithic to date (Brandt et al., 2013; Olalde et al., 2019; Szécsényi-Nagy et al., 2017), although an undefined U5 haplogroup has been found in an individual at Can Sadurní (5475-5305 cal BC) (Gamba et al., 2012). Brandt et al (2013) proposed the idea of a ‘mitochondrial Neolithic package’ based on the appearance of N1a, T2, K, J, HV, V, W, and X haplogroups in Early Neolithic Linear Bandkeramik culture populations of the Mittelelbe-Saale region of Germany that arrived alongside Neolithic practices. Based on the appearance of these haplogroups in earlier periods in Anatolia, it has been postulated as the origin of this ancestry (Brandt et al., 2013; Fernández-Domínguez et al., 2017; Haak et al., 2010; Szécsényi-Nagy et al., 2017). It is debated whether this ancestry reached Iberia either through migrations across central Europe (Brandt et al., 2013; Szécsényi-Nagy et al., 2017) or along Mediterranean coastlines (Fernández-Domínguez et al., 2017; Gamba et al., 2012; Sampietro et al., 2007). Genomic studies have confirmed the introduction of Anatolian ancestry into Iberia alongside the Neolithic (Fregel et al., 2018; Haak et al., 2015b; Olalde et al., 2019; Valdiosera et al., 2018), although they provide a more nuanced picture than mtDNA analyses. Rather than indicating a complete genetic replacement, Early Neolithic populations in Iberia contain between 10-20% HG ancestry, with the rest of the ancestry being of Early European Farmer (EEF) origin (Olalde et al., 2019). In Portugal, HG components account for 27-43% of ancestry (Allentoft et al., 2024). Interestingly, this area also sees a high concentration of hunter-gatherer groups as previously discussed, although Early Neolithic genomic evidence only comes from 2 individuals at the site of Gruta do Caldeirão (Allentoft et al., 2024). Genomic evidence suggests that the truth of Neolithic diffusion lies somewhere between the two extreme theories of demic and cultural diffusion, with a migration of populations of Anatolian or Near East origin admixing with local hunter-gatherer populations *in situ* and/or on their way to Europe.

Local HG ancestry sees a ‘resurgence’ in the Middle to Late Neolithic (Brandt et al., 2013; Olalde et al., 2019; Szécsényi-Nagy et al., 2017). Genomically, HG ancestry rises to ~18-35% in Middle and Late Neolithic Iberian populations (Lipson et al., 2017; Olalde et al., 2019). This is reinforced by the reappearance of the HG associated U5b mitochondrial haplogroups during this period (Brandt et al., 2013; Szécsényi-Nagy et al., 2017). In Portugal, this resurgence is even more pronounced (Carvalho et al., 2023), with HG components contributing between ~25-40% of whole genome ancestry (Roca-Rada et al., 2024). The resurgence of HG ancestry is similarly observed to varying degrees throughout the rest of Europe in this period (Haak et al., 2015b).

The genetic landscape of Chalcolithic Iberia remains mostly similar in the early phase to that of the Neolithic (Olalde et al., 2019; Roca-Rada et al., 2024). In the latter half of the Chalcolithic from around ~2,400 cal BC, steppe-related ancestry that had spread across the rest of Europe from the Pontic-Caspian steppes, consisting of a mix

of Eastern Caucasus and Iranian hunter-gatherer ancestry as well as local Neolithic ancestry picked up along the way, makes an appearance in the Cantabrian (north), Levantine (east) and 'Meseta' (central) parts of Iberia (Haak et al., 2015a; Olalde et al., 2018; Richards and Fernández-Domínguez, 2022). The steppe ancestry Y-chromosome signature primarily involves R1b-M269 lineages (Olalde et al., 2019). These lineages were found in ~93%(N=90) of Bell Beaker associated individuals outside of Iberia, but only ~23.5%(N=17) of Bell Beaker associated individuals in Iberia (Olalde et al., 2018). The mitochondrial signature of steppe ancestry is not yet thoroughly understood, but the unpublished work of Richards and Fernández-Domínguez (2022) suggest it could include U4, U5a, T1, and T2 haplogroup lineages, but further research is necessary to confirm this. Steppe-related ancestry accounts for anywhere between 0% and 50% of whole genome ancestry in Bell Beaker associated groups, with almost no steppe-related ancestry found in non-Bell Beaker associated Chalcolithic individuals (Olalde et al., 2019, 2018; Villalba-Mouco et al., 2021). Evidence of only very limited steppe-related ancestry reaching the Southeast or the West during this period has been found (Olalde et al., 2018; Roca-Rada et al., 2024). Although not significantly contributing to the Iberian gene pool until the Roman period, North African ancestry has been found in two individuals during this timeframe (Olalde et al., 2019). This supports the idea of networks linking Iberia and North Africa in this period previously suggested based on the archaeological material. Apart from the two individuals with North African ancestry, whose mtDNA belonged to haplogroups M1 and L1, haplogroup compositions are relatively consistent with Neolithic Iberian populations (Szécsényi-Nagy et al., 2017).

Central and Northern Europe experienced a large genetic influx of Steppe-related ancestry in the Chalcolithic and Early Bronze Age, including a 79% turnover of whole genome ancestry (Haak et al., 2015b) and an almost complete replacement of local Y-haplogroups mostly by R1b-M269 lineages (Olalde et al., 2019). In Iberia, this replacement encompassed ~40% of whole genome ancestry and almost 100% of Y-chromosome DNA by ~2,000 BC (Olalde et al., 2019). Steppe ancestry increased in West and Southeast Iberia in the Early Bronze Age (Martiniano et al., 2017; Roca-Rada et al., 2024; Villalba-Mouco et al., 2021). Although Y-haplogroup turnover was still almost complete in these regions, Steppe ancestry played less of a role in the whole genome composition of Portuguese and Andalusian groups than in Cantabrian, 'Meseta', and Levantine groups (Roca-Rada et al., 2024; Villalba-Mouco et al., 2021). Contrary to whole genome and Y-chromosome evidence, mitochondrial haplogroup composition show relative continuity to earlier Chalcolithic and Neolithic populations (Olalde et al., 2019; Roca-Rada et al., 2024). Consequently, a sex-biased migration of predominantly males was suggested for the integration of Steppe ancestry into Iberian populations (Goldberg et al., 2017; Olalde et al., 2019), but this has been disputed (Lazaridis and Reich, 2017).

1.5 Aims and Objectives

Research questions

This study sets out to answer the questions:

- How did the mitochondrial composition of different regions of Iberia change between the Mesolithic and Bronze Age periods?
- Did genetic shifts correlate with archaeological cultural shifts?
- Was there any pattern of close biological kinship in burial placements of individuals in mass graves?
- Were there pathogens present in the individuals that could have impacted their health?

To answer these questions, this study aimed to perform shotgun sequencing as well as mitochondrial capture sequencing using next generation sequencing techniques on DNA extracted from the teeth of 25 individuals from four sites in the Spanish Levant and Portugal from the Neolithic and Chalcolithic periods in order to increase the available genetic data from underrepresented regions and periods in Iberia. The genetic data was used to:

- I. Determine the presence or absence of material biological kinship between individuals within sites.
- II. Uncover patterns between burial placement within sites and genetic affinities or kinship.
- III. Combine the genetic data from this study to a comprehensive ancient database to explore genetic shifts in Iberian populations throughout the Mesolithic, Neolithic, Chalcolithic, and Bronze Age.
- IV. Contextualise the genetic data within the wider archaeological context

2. Description of Archaeological Study Sites and Previous Work

Four sites were chosen for study based on sample availability, archaeological period, and preservation. These sites range from the Middle Neolithic to the Chalcolithic (approximately 3760-2290 cal BC) and are all located on the Iberian Peninsula, seen in Figure 6. Twenty-five samples were analysed, 10 from La Sagrera (2883-2461 cal BC, Late Neolithic, Barcelona, Spain), 9 from Convento do Carmo (2550-2290 cal BC, Chalcolithic, Torres Novas, Portugal), 3 from Algar do Barrão (3760-3100 cal BC, Middle Neolithic, Monsanto (Alcanena), Portugal), and 3 from Casais da Mureta (3260-3020 cal BC, Late Neolithic, Monsanto (Alcanena), Portugal).



Figure 6: Approximate location of archaeological sites analysed in this study. Created using ArcGIS Pro v3.0.6.

2.1 La Sagrera

During the building of the La Sagrera high speed (AVE) rail station in 2010, a Late Neolithic mass grave was discovered. The site was then excavated between 2011 and 2012 (Balaguer et al., 2013; “LAV estructura de l’estació de la Sagrera,” 2012). In total, the remains of 207 individuals were uncovered. Within the site, 3 separate phases of use were identified.

The first phase consisted of approximately 100 individuals. Archaeological and anthropological analysis revealed that individuals in this phase were initially buried in the centre of the site, and then moved to secondary positions at the sides of the chamber at a later point to make space for newly deceased individuals (Balaguer et al., 2013). This pattern likely occurred over a long period of use. Radiocarbon dating of two individuals from this phase placed it between 2916-2590 cal BC. Two ceramic vessels, a white stone necklace, and a flint arrowhead were found alongside the individuals in this phase. These artefacts are typical of Late Neolithic burial sites in Catalonia, supporting the radiocarbon date (Balaguer et al., 2013).

The first phase burials were covered directly by the second phase. This was a mass inhumation consisting of at least 100 individuals, seen in Figure 7 (Balaguer et al., 2013). A small number of artefacts were also discovered deposited with the individuals, but it was concluded that their origin and relationship to the burials could not be determined. Radiocarbon dating of four individuals from this phase, seen in Table 1, gave a range of 2883-2461 cal BC. This places the second phase also in the Late Neolithic period.

The final stage only contained one individual. Radiocarbon dating of this individual gave a range of 2871-2640 cal BC. Although this phase was stratigraphically later than the previous phase, it was suggested based on the similar radiocarbon date and position of burial that this individual could have been from the same social group as the individuals in phase 2 (Balaguer et al., 2013).

Site	Individual	Skeletal Element	Years BP	Cal BC	Archaeological Period
La Sagrera	7144-IND1	Unspecified	4184±45	2883-2680	Late Neolithic
	7144-IND27	Unspecified	3998±60	2620-2461	Late Neolithic
	7144-IND79	Unspecified	4125±45	2862-2590	Late Neolithic
	7144-IND106	Unspecified	4074±40	2836-2498	Late Neolithic

Table 1: Radiocarbon dating results from 4 individuals of the second period of use at La Sagrera (Balaguer et al., 2013).



Figure 7: Overhead view of the second phase with human remains in situ at the site of La Sagrera. Obtained from (Balaguer et al., 2013).

This study will focus only on the second burial phase. Within this phase, burials seemed to be clustered into three groups of simultaneous deposit based on the analysis of joint placements (Balaguer, 2016; Balaguer et al., 2013). There were no signs that bones were displaced when individuals were deposited on top of each other in these clusters. This suggests that connective tissue around the joints was still present, keeping the bones in place. Balaguer et al suggest that the only two possibilities that could have led to the large simultaneous death event observed in this phase are war or epidemic (Balaguer et al., 2013). During the excavation, little to no perimortem trauma was observed in the remains. Upon further anthropological study of the remains, only a few signs of trauma were identified. All of these had either healed or were in the process of healing, and therefore not likely related to the death of the individuals (Balaguer, 2016). Therefore, it can be concluded that war was likely not the cause of the mass death event. A complete lack of material evidence associated with conflict, such as arrowheads and daggers, also supports this conclusion (Balaguer et al., 2013). Very few signs of infection were observed in the osteological remains, and most of these cases were attributed to anaemia (Balaguer, 2016). Many infections do not result in pathologies visible in bone, such as the plague (Bramanti et al., 2018). Therefore, of the two hypotheses an epidemic remains the most likely cause of this mass death event. Metagenomic analysis done as part of this study may be able to shed light on this by providing genetic evidence of the presence of pathogens in the individuals studied.

The burial clusters were designated A, B, and C. Cluster A was located in the northeast corner of the hypogeum, Cluster B in the south-central area and Cluster C located in the southwest corner. Individuals from each cluster were selected by the archaeologists for anthropological analysis (Balaguer, 2016) to determine if there were any patterns in the distributions of individuals across the clusters. As seen in Table 2, each cluster contains a variety of ages at death and osteological sex. No patterns based on this analysis could be discerned. This led to the suggestion that the clusters could be arranged based on kinship or population groups.

To attempt to confirm or deny this suggestion, isotopic and genetic analyses of 13 individuals were undertaken as part of two master's projects at Durham University (DiRenno, 2016; Robinson, 2018). From Cluster A, individuals SA4 and SA8 were selected for study. Individuals SA18, SA56, and SA68 were selected from Cluster B. Finally, individuals SA25, SA26, SA39, SA43, SA44, SA45, SA46, and SA53 were selected from Cluster C. Osteological information and burial groups can be seen in Table 2 below. In situ positions of individuals within the clusters can be seen in Figure 8.

Site	Individual	Age at Death	Osteological Sex	Burial Groups
La Sagrera	SA4	8--10	Undetermined	Cluster A
	SA8	>25	Female	Cluster A
	SA18	45	Male	Cluster B
	SA25	Infant, ~18 months	Undetermined	Cluster C
	SA26	5--6	Undetermined	Cluster C
	SA39	~8	Undetermined	Cluster C
	SA43	17	Female	Cluster C
	SA44	18 to 22	Male	Cluster C
	SA45	18--24	Male	Cluster C
	SA46	25--34	Male	Cluster C
	SA53	?	Female	Cluster C
	SA56	?	Male	Cluster B
	SA68	18--23	Female	Cluster B

Table 2: Results of osteological analysis of the individuals focussed on in this study (Balaguer, 2016).

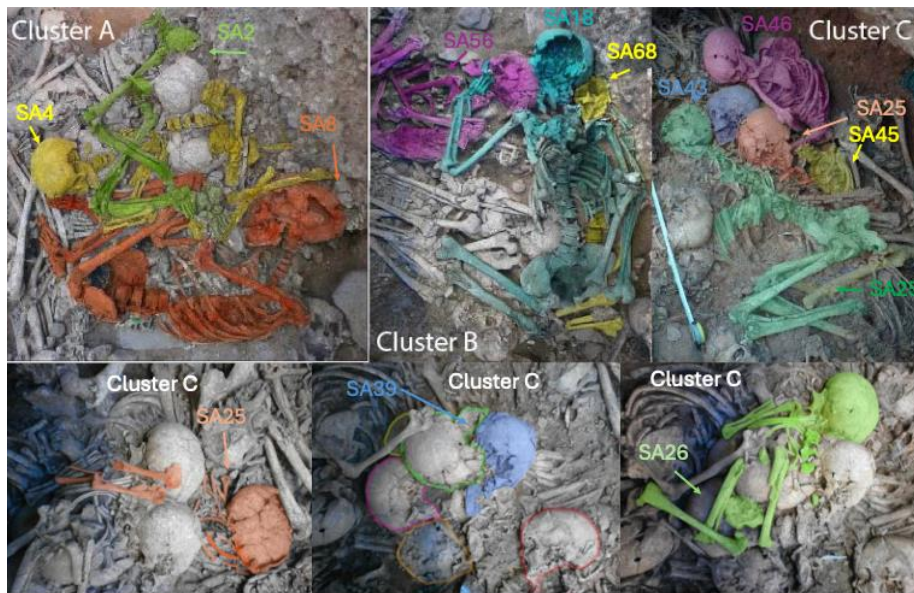


Figure 8: Positions of individuals from clusters A, B, and C of La Sagrera in situ. From (Balaguer, 2016). Labels added by author.

Results from the isotopic studies revealed that 7 individuals (53.8%) plotted outside the expected local range for both strontium and oxygen isotope ratios, while all of the successfully analysed individuals fell outside the expected local oxygen range ($\delta^{18}\text{O}$ between -6‰ and -5‰) (DiRenno, 2016). This can be seen in Table 3 and Figure 9. The local bioavailable strontium isotope ratio ($^{87}\text{Sr}/^{86}\text{Sr}$) range (0.7092-0.7098) in this study was calculated based on the values obtained from dentine of three of the individuals as well as the geological data. This is because dentine absorbs strontium from the local environment after deposition (Montgomery et al., 2007). These values only relate to the exact location of burial, and it was suggested that the true local values for the nearby surrounding area likely had a much larger range (DiRenno, 2016). Along with this, the geology of the surrounding region is very diverse, including areas of higher elevation. Consuming drinking water from these areas of higher elevation would account for the lower oxygen values. One possibility is that individuals lived in these areas earlier in life before moving to the area of La Sagrera.

Site	Individual	Skeletal Element	Expected Local Range	$^{87}\text{Sr}/^{86}\text{Sr}$ ratio	Within Local Range?
La Sagrera	SA4	Molar	0.7092-0.7098	0.709276	Yes
	SA8	Molar	0.7092-0.7098	0.710446	No
	SA18	Molar	0.7092-0.7098	0.711248	No
	SA25	Undetermined tooth	0.7092-0.7098	0.709198	Yes
	SA26	Undetermined tooth	0.7092-0.7098	0.709302	Yes
	SA39	Molar	0.7092-0.7098	0.709187	Yes
	SA43	Molar	0.7092-0.7098	0.709826	No
	SA44	Molar	0.7092-0.7098	0.708308	No
	SA45	Molar	0.7092-0.7098	0.709794/0.709865	No
	SA46	Molar	0.7092-0.7098	0.70929	Yes
	SA53	Molar	0.7092-0.7098	0.709649	Yes
	SA56	Molar	0.7092-0.7098	0.710256	No
	SA68	Molar	0.7092-0.7098	0.709062	No

Table 3: Strontium isotope results from La Sagrera (DiRenno, 2016).

As strontium and oxygen enter the body through food and drinking water, it is also possible that the individuals outside the local ranges were consuming food or drinking water from these surrounding areas, resulting in their different isotopic signatures. Although there was some variety in strontium and oxygen ratios across the entire population, there was no distinct difference between the burial clusters (Figure 10). These results suggest that there was no organisation of clusters based on provenance of individuals in the population.

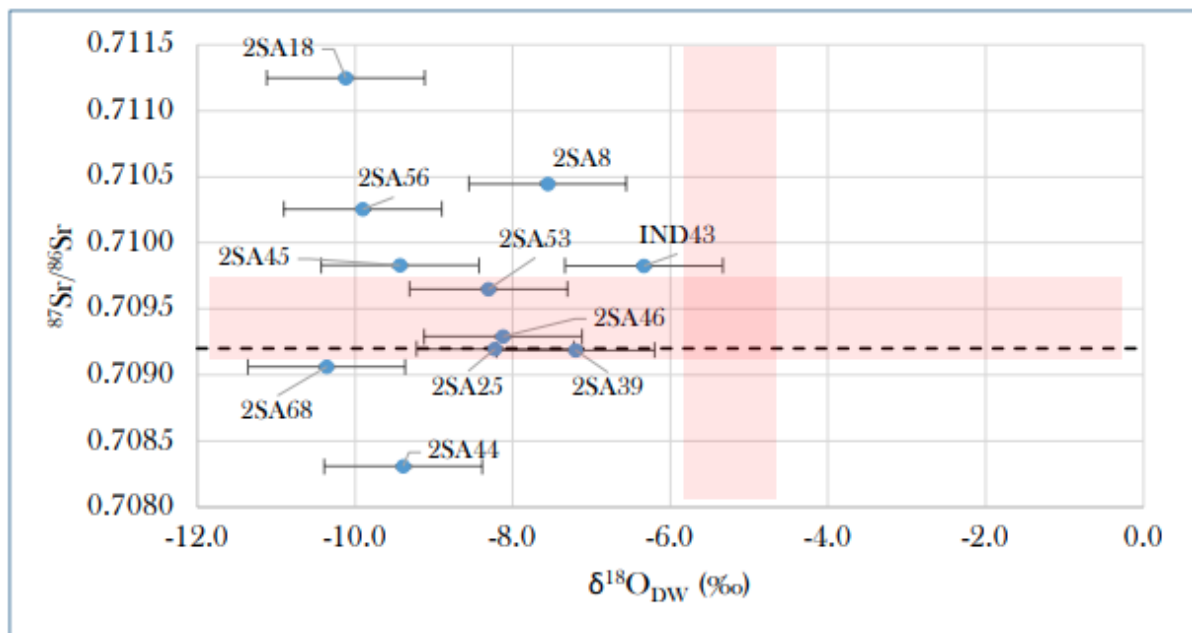


Figure 9: Plot of $^{87}\text{Sr}/^{86}\text{Sr}$ ratios and $\delta^{18}\text{O}_{\text{DW}}$ values for individuals at the site of La Sagrera. Red areas represent the local ranges calculated through dentine for strontium and modern precipitation for oxygen. Individuals SA4 and SA26 are not present due to unsuccessful oxygen isotope analysis, but both plot within the local strontium range. Obtained from (DiRenno, 2016).

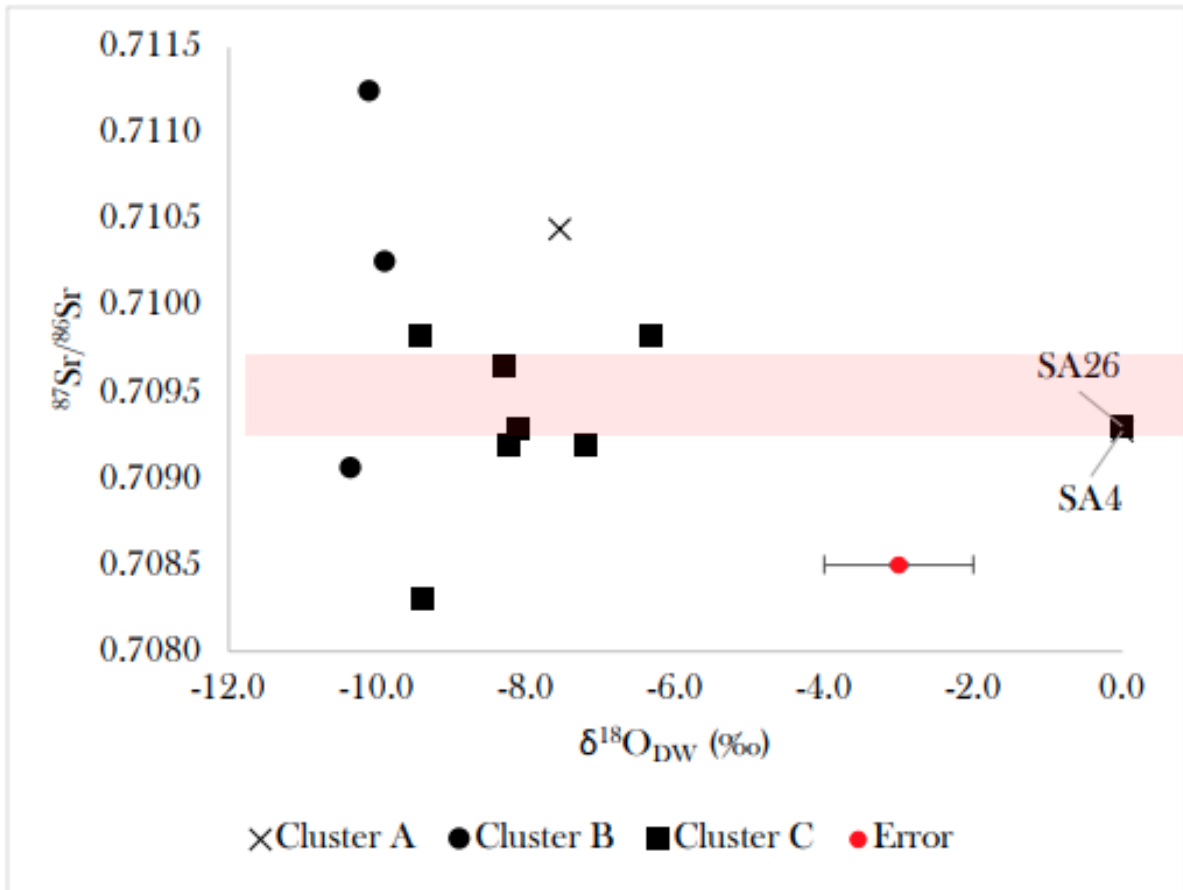


Figure 10: Plot of strontium and oxygen values of individuals identified by cluster. The red area is the expected local strontium ratio values based on dentine. Oxygen isotope analysis of SA4 and SA26 was unsuccessful, so they were placed at 0.0. Obtained from (DiRenno, 2016).

Carbon and nitrogen isotopic incremental dentine studies revealed a potential shift to marine diet in the juvenile individuals studied at La Sagrera near their time of death as evidenced by an increase in $\delta^{15}\text{N}$ values in later increments (DiRenno et al., 2016). Following this, a third hypothesis was suggested for the reason behind the mass burial. Agricultural disaster could have led the population to rely on a marine diet and eventually led to starvation (DiRenno et al., 2016). This would explain the rise in $\delta^{15}\text{N}$ values observed in the developing increments near the time of death and the lack of evidence in bulk analysis of adult individuals due to their teeth being fully developed at the time of the event. This pattern has been observed previously in Neolithic populations in the Shetland Isles (Montgomery et al., 2013).

Partial consensus mitochondrial haplotypes based on PCR amplification and Sanger sequencing of the Hypervariable Region 1 (HVR1) and targeted SNPs from the coding region of the mitochondrial DNA (mtDNA) were obtained for 10 of the individuals, as seen in Table 3 (DiRenno, 2016; Robinson, 2018). Individuals SA4 (Cluster A) and SA25 (Cluster C) were assigned the same haplotype (positions 16126-16389). This haplotype was also not found in modern populations, so the Likelihood Ratio of matrilineal relation between these two individuals was reported to be 99.98% (Robinson, 2018). Individuals SA43 and SA45 also shared the same haplotype for HVR1, but both sequences were partial, only covering positions 16105-16280. Also, further

SNP analysis revealed that individual SA43 had a C at position 10873, whereas individual SA45 had a T. It is therefore unlikely that these two individuals were related.

Haplogroup assignments for the individuals can also be seen in Table 4. Five individuals were assigned to haplogroup N, including SA4 from Cluster A, SA18 from Cluster B, and three individuals from Cluster C (see Table 4). Four individuals were assigned to major haplogroups HV or H, all from Cluster C. Finally, L3 was found in one individual from Cluster C. Based on this analysis, it was suggested that the population at La Sagrera is similar to other Neolithic populations in Iberia in haplogroup diversity and the appearance of new haplogroups such as N and HV, and that the appearance of these lineages supports the theory of a Neolithic migration into Iberia in the Early Neolithic (Robinson, 2018). While insightful, the haplogroup assignments of the La Sagrera individuals are based on haplotypes of HVRI and target just a small number of targeted positions in the mitochondrial genome. Analyses in this study will aim to enhance the resolution by studying positions throughout the entire mitochondrial genome.

Samples from 10 individuals will be analysed in this study. This includes all previously analysed individuals with the exception of individuals SA8, SA25, and SA39 due to lack of available sample material. Results from previous genetic analysis and osteological analysis can be seen in Table 4 and Table 2 respectively.

Site	Individual	Haplotype relative to rCRS	Haplogroup Assignment	Burial Groups
La Sagrera	SA4	16129A 16223T 16261T	Within N	Cluster A
	SA8	No Results		Cluster A
	SA18	12705T 16179T 16223T 16311C 16354T	N	Cluster B
	SA56	No Results		Cluster B
	SA68	No Results		Cluster B
	SA25	16129A 16223T 16261T	Within N	Cluster C
	SA26	7028T 10550A 16311C	HV1/R1	Cluster C
	SA39	7028T 16320T	HV	Cluster C
	SA43	10873C 16223T	L3	Cluster C
	SA44	CRS	H	Cluster C
	SA45	16223T	N	Cluster C
	SA46	16221T	H or HV	Cluster C
	SA53	16223T 16362C	N	Cluster C

Table 4: Results of previous genetic studies of individuals at La Sagrera (Robinson, 2018).

2.2 Convento Do Carmo

The historic site of Convento do Carmo, located in Torres Novas, Portugal, was excavated between 2012 and 2015 during the construction of a new town hall. During the excavation, a Chalcolithic hypogeum was discovered. Excavation of this context took place between 2014 and 2015. The building of later historical structures is thought to have led to the destruction of an unknown portion of the hypogeum (Carvalho, 2019). Archaeological artefacts from this context included a minimum of 24 ceramic vases,

including at least six bell-beaker ones (Carvalho, 2019). Buttons, stone beads, gold beads, a gold ring, a copper pin, two copper tongue daggers, and chipped stone fragments were also found within the hypogeum. These artefacts identified the site as belonging to the ‘bell-beaker culture’ archaeological group (Carvalho, 2019).

Four shells of the *Trivia* genus were found in the hypogeum, and were likely used as decorative adornments in the funerary context (Valente, 2019). As species of this genus are found along the rocky North Atlantic coasts, this is also evidence of mobility or trade networks extending to the coast. Except for these shells, faunal remains at the site showed no signs of human modification and were not associated with the Chalcolithic hypogeum.

The hypogeum contained 861 human remains. Of these remains, there were 740 classified bones, 33 unclassified bones, and 88 teeth (Granja, 2019). There was determined to be a minimum number of 15 individuals present in the site. There were a few instances of periosteal reactions and osteophytes in some bones, but the fragmentation at the site made it impossible to assign these to specific individuals (Granja, 2019).

The remains were arranged into four areas of the site, called ‘ossuaries’. The level of disarticulation in the remains suggests that ossuaries 3 and 4 may have contained primary depositions, while ossuary 1 was likely a secondary burial site (Granja and Carvalho, 2019). The human remains were likely deposited on the surface. Animal scavenging could have led to the high levels of scattering of archaeological materials and human remains. This is supported by evidence of extensive burrowing found throughout the site (Granja and Carvalho, 2019).

Ten mandibles were recovered from the hypogeum. Locations of the mandibles in the site as well as the ossuaries can be seen in Figure 11. Although caries were present in at least five of the mandibles, it was determined that the individuals likely had a low cariogenic diet based on the amount and severity of the lesions (Granja, 2019). Eight mandibles were selected for radiocarbon, isotopic, and genetic studies. These were assigned the individual codes of CVC1-CVC8. A ninth mandible, CVC9, was also made available in this study for genetic analysis. Osteological sex and age at death estimations for these individuals can be seen in Table 5, although it should be noted that the fragmentation of the remains made this analysis difficult (Granja, 2019).

Site	Individual	Age at Death	Osteological Sex	Burial Groups
Convento do Carmo	CVC1	Mature	Possibly Female	Ossuary 1
	CVC2	Mature	Undetermined	Ossuary 1
	CVC3	Mature	Possibly Female	Ossuary 1
	CVC4	Mature	Undetermined	Ossuary 1
	CVC5	Mature	Male	Ossuary 3
	CVC6	Mature	Male	Ossuary 3
	CVC7	Mature	Male	Ossuary 1
	CVC8	Immature	Undetermined	Ossuary 2
	CVC9	Mature	Undetermined	Ossuary 1

Table 5: Osteological analysis of individuals at Convento do Carmo (Granja, 2019).

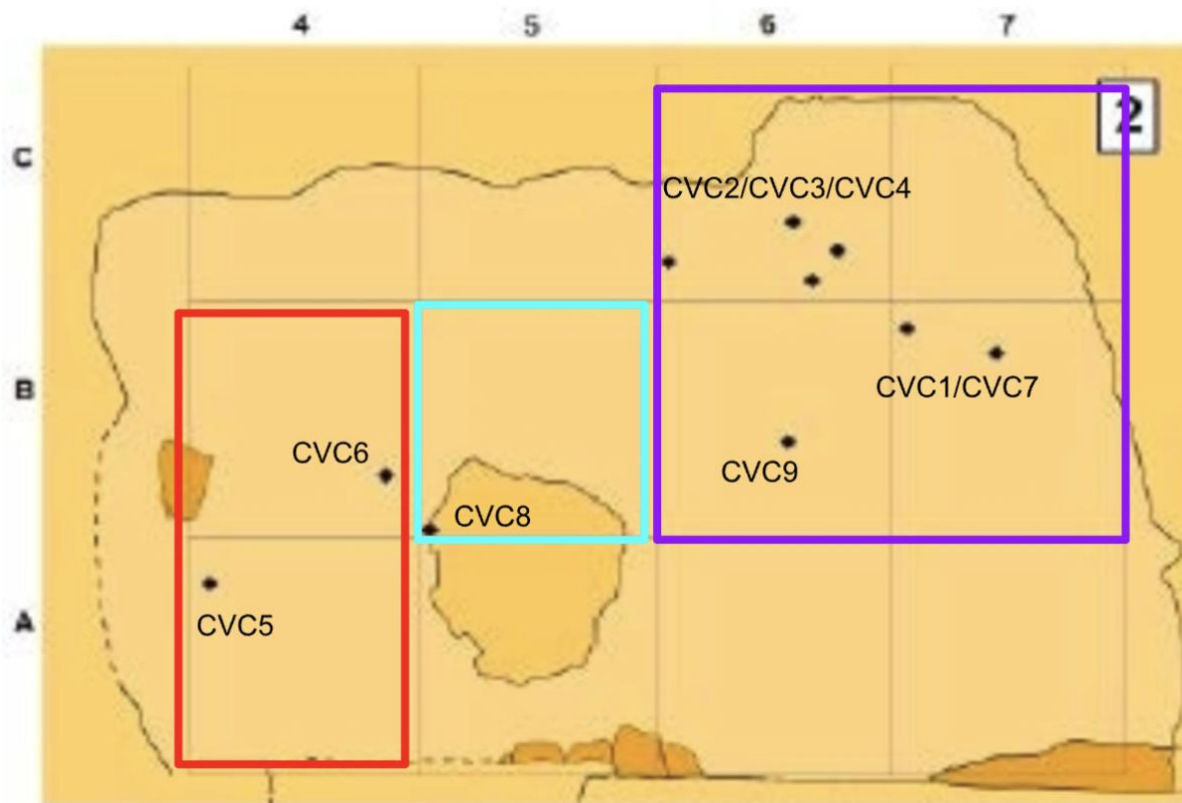


Figure 11: Distribution of mandibles between the different ossuaries of the hypogeum. Grid squares represent 1 square metre. Ossuary 1: purple. Ossuary 2: blue. Ossuary 3: Red. Ossuary 4: along the edge to the left of ossuary 3, not highlighted. Positions of mandibles for individuals CVC2, CVC3, CVC4, CVC1, and CVC7 are not distinguished within their respective grid spaces. A tenth mandible is also present in square C6. Obtained from (Granja and Carvalho, 2019). Ossuary outlines and labelling of individual mandibles added by author based on information in (Granja and Carvalho, 2019).

Radiocarbon dating was performed on mandibles 1 to 8 (Petchey, 2019). The results, seen in Table 6, confirm the archaeological dating of the site to the Chalcolithic period. The radiocarbon analysis also suggests that the site was in use for a long period of time. This period of use was estimated to be around two and a half centuries (Petchey, 2019). Although radiocarbon dating was only successful for two of the individuals, the context of the site places all other individuals likely within this period of use.

Site	Individual	Skeletal Element	Years BP	Cal BC	Archaeological Period
Convento do Carmo	CVC1	Mandible	No results	No results	
	CVC2	Mandible	3965±18	2550-2460	Chalcolithic
	CVC3	Mandible	3875±19	2460-2290	Chalcolithic
	CVC4	Mandible	No results	No results	
	CVC5	Mandible	No results	No results	
	CVC6	Mandible	No results	No results	
	CVC7	Mandible	No results	No results	
	CVC8	Mandible	No results	No results	

Table 6: Radiocarbon Dating results from Convento do Carmo. Calibrations at 68.2% (Petchey, 2019).

Strontium isotope analysis was also completed on teeth of the eight individuals (Fernández-Domínguez et al., 2019; Neff, 2016). These results can be seen in Table 7. A local baseline $^{87}\text{Sr}/^{86}\text{Sr}$ ratio was established based on analysis of dentine from individual CVC5. Individuals CVC1, CVC3, CVC5, CVC6, CVC7, and CVC8 were all determined to be within the local range based on their proximity to the baseline (Fernández-Domínguez et al., 2019). Individuals CVC2 and CVC4 were outliers, with much higher strontium isotope ratio values. These higher values are consistent with the granite geology of the nearby Beira region to the North, and it was suggested that this could possibly be the place of origin of these two individuals (Fernández-Domínguez et al., 2019). No oxygen isotope analysis has been done on these individuals.

Site	Individual	Skeletal Element	Expected Local Range	$^{87}\text{Sr}/^{86}\text{Sr}$ ratio	Within Local Range?
Convento do Carmo	CVC1	Canine	~0.711124	0.711199	Yes
	CVC2	Premolar	~0.711124	0.717848	No
	CVC3	Molar	~0.711124	0.710982	Yes
	CVC4	Molar	~0.711124	0.720209	No
	CVC5	Incisor	~0.711124	0.711226	Yes
	CVC6	Molar	~0.711124	0.712485	Yes
	CVC7	Molar	~0.711124	0.711912	Yes
	CVC8	Molar	~0.711124	0.712153	Yes

Table 7: Strontium Isotope results from Convento do Carmo (DiRenno et al., 2016).

Carbon and nitrogen isotope analysis was completed on these individuals (Petchey, 2019). As seen in Table 8, most of the individuals consumed a mostly terrestrial diet. Three individuals, however, had significant levels of over 20% aquatic protein. This is more than most Neolithic sites in the region, and it was suggested that there was a shift to a more aquatic diet between the Neolithic and Chalcolithic periods in Portugal (Carvalho et al., 2019; Carvalho and Petchey, 2013; Petchey, 2019).

Site	Individual	Skeletal Element	% Nitrogen	% Carbon	Carbon:Nitrogen	$\delta^{15}\text{N}(\text{‰})$	$\delta^{13}\text{C}(\text{‰})$	% Marine protein	% Aquatic protein
Convento do Carmo	CVC1	Mandible	15.06	42.79	3.32	8.52	-19.83	2	6
	CVC2	Mandible	15.20	43.50	3.34	11.01	-19.81	2	33
	CVC3	Mandible	15.10	42.80	3.31	9.30	-19.59	5	14
	CVC4	Mandible	15.19	43.09	3.31	10.49	-19.38	8	28
	CVC5	Mandible	14.11	41.54	3.44	10.68	-19.34	8	30
	CVC6	Mandible	15.74	44.79	3.32	8.13	-20.25	No results	14
	CVC7	Mandible	14.77	42.03	3.32	9.19	-19.93	No results	13
	CVC8	Mandible	15.69	43.89	3.26	8.99	-20.05	No results	11

Table 8: Dietary Isotope results from Convento do Carmo (Fernández-Domínguez et al., 2019).

Genetic analyses were also performed on teeth from the 8 mandibles using PCR and Sanger sequencing (Fernández-Domínguez et al., 2019; Neff, 2016). Haplotypes and haplogroup assignment can be seen in Table 9. Only partial haplotypes of the HVR1 were able to be confirmed for individuals CVC1, CVC2, CVC3, and CVC8 (See Table 9). Therefore, no haplogroup assignments were made for these individuals. CVC4, CVC6, and CVC7, however, were all able to be assigned to the major haplogroup K. CVC4 and CVC7 contained an additional SNP and were able to be further defined to haplogroup K1. It was suggested that individuals that did not have haplogroups assigned could also have belonged to haplogroup K, and that the population at the site was genetically homogeneous (Fernández-Domínguez et al., 2019). It was noted, however, that the size of the fragments of DNA analysed were limited, and some of the mutations found were also present in one of the researchers (MN). Analysis of the entire mitogenome may reveal more diversity between the individuals. As well as samples of the previously genetically analysed individuals, a tooth sample from individual CVC9 was also made available for study in this project.

Site	Individual	Haplotype relative to rCRS	Haplogroup Assignment	Burial Groups
Convento do Carmo	CVC1	16311C 16362C	?	Ossuary 1
	CVC2	16311C 16362C	?	Ossuary 1
	CVC3	CRS	?	Ossuary 1
	CVC4	16224C 16311C 16362C	K1	Ossuary 1
	CVC5	No Results		Ossuary 3
	CVC6	16224C 16311C	K	Ossuary 3
	CVC7	16224C 16311C 16362C	K1	Ossuary 1
	CVC8	16311C 16362C	?	Ossuary 2

Table 9: Results of previous genetic studies of individuals at Convento do Carmo (Fernández-Domínguez et al., 2019).

2.3 Algar do Barrão

The Middle Neolithic cave site of Algar do Barrão is located within the Tagus River valley on the eastern edge of the Limestone Massif in Estremadura, Portugal. Three areas of the cave were excavated. These were designated Sondage 1, 2, and 3, and their locations can be seen in Figure 12. The site contained a minimum number of 20 individuals, with at least 16 adults and 4 subadults. Of the adults, the

osteological sex of five were determined to be male and three female. The sex in the remaining individuals could not be determined.

Sondage 1 contained the remains of at least 2 adults, including one probable male. Of the 25 teeth found in this context, caries were not observed in any, and erosion of the occlusal surface ranged from moderate to moderate-high (Carvalho et al., 2003). Sondage 2 contained at least 9 adults and 3 subadults. The osteological study of the adults established that 3 were males and two were females. Osteological sex of the remaining individuals could not be determined. Slight bone remodelling was noticed in some of the bones, indicating healing from an infectious disease (Carvalho et al., 2003). At least two adults, including one probable male, were found in Sondage 3 (Carvalho et al., 2019). Remains of at least three adults, including a possible female, and one subadult were found on the surface layer of the cave, not part of any Sondage. Possible signs of infection were observed in three of the bones from this layer (Carvalho et al., 2003). Although some signs of infection were observed in bones from Sondage 2 and the surface layer, the high level of post-mortem fragmentation of the remains makes it possible that more cases exist but were not observed (Carvalho et al., 2003).

Grave goods such as polished stone tools, bone tools, flint blades, plain pottery, and shell adornments were also found within the site (Carvalho et al., 2019, 2003). The grave goods at this site are typical of those found in other Middle Neolithic cave sites in Portugal (Carvalho and Cardoso, 2014; Carvalho and Zilhão, 1996; Carvalho, 2019; Carvalho et al., 2003). Radiocarbon dating of samples from 7 individuals supports this, with a date range of 3760-3100 cal BC (Carvalho et al., 2019). Radiocarbon dating results from the three individuals analysed in this study can be seen in Table 10. Whitish sediments intrusive to the cave environment were found with the human remains, which suggests a primary burial elsewhere before the defleshed bones were moved to the cave site. This was supported by evidence of post-mortem fractures of long bones consistent with burial and the deliberate arrangement of the bones (Carvalho et al., 2003).

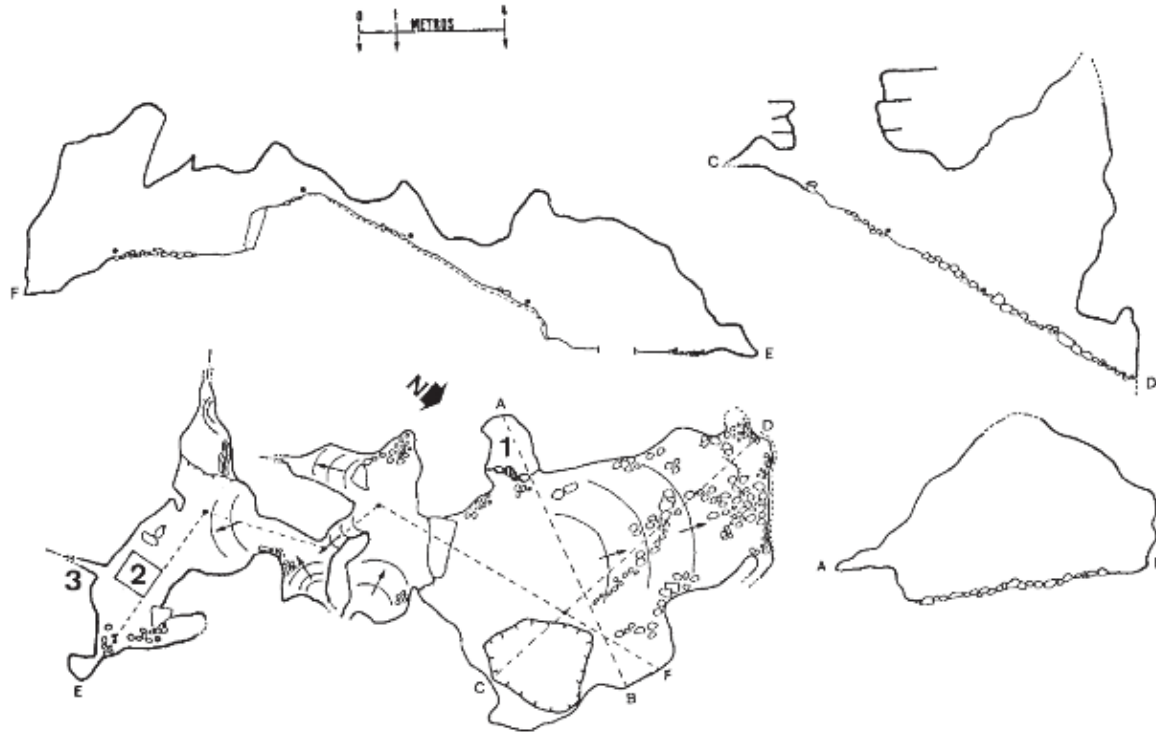


Figure 12: Site plan of Algar do Barrão with location of sondages. Drawn by João Maurício and Pedro Souto of the Sociedade Torrejana de Espeleologia e Arqueologia. Image obtained from (Carvalho et al., 2003).

Site	Individual	Skeletal Element	Years BP	Cal BC	Archaeological Period
Algar do Barrão	BAR3	Premolar	4929±39	3760-3650	Middle Neolithic
	BAR4	Molar	4551±36	3370-3120	Middle Neolithic
	BAR5	Mandible	4485±22	3330-3100	Middle Neolithic

Table 10: Radiocarbon Dating results from Algar do Barrão (Carvalho et al., 2019). Calibrations at 68.2% (Carvalho et al., 2019).

Faunal remains were found scattered across the surface of the cave. These faunal remains were determined to be from a different period and not associated with Neolithic use (Carvalho et al., 2019, 2003). No evidence of anthropogenic processes was found on any of the faunal remains. The animal remains also appear to be in a better state of preservation than the human remains, suggesting they were deposited more recently. As it can be seen in Figure 12, the entrance to the cave is a steep drop. It has been suggested that this served as a natural trap for passing animals, which fell in and died during a later period (Carvalho et al., 2003).

A previous Master's project analysed samples from three individuals at Algar do Barrão, chosen for their macroscopic preservation (Robinson, 2018). These were individuals #03(BAR3), #04(BAR4), and #05(BAR5). Samples from these same three individuals will be analysed in this study. In Robinson's study, DNA results were obtained from all three. Haplotypes based on analysis of mtDNA HVR1 and 10 coding region SNPs can be seen in Table 11. Based on these results, individuals BAR3 and BAR5 were assigned to haplogroup U5, and individual BAR4 was assigned to

haplogroup K. Haplogroup assignment for each individual achieved at least an 88% quality score (Robinson, 2018).

Site	Individual	Haplotype relative to rCRS	Haplogroup Assignment	Burial Groups
Algar do Barrão	BAR3	7028T 16270T	U5	Sondage 2
	BAR4	16129A 16224C 16311C	K	Sondage 2
	BAR5	10550A 16270T 16311C	U5	Sondage 2

Table 11: Results of previous genetic studies of individuals at Algar do Barrão (Robinson, 2018).

Due to the lack of available contemporary faunal remains and the proximity of this site to the site of Casais da Mureta, the expected local $^{87}\text{Sr}/^{86}\text{Sr}$ isotope range for this site was based on the faunal remains at Casais da Mureta (Carvalho et al., 2019). As seen in Table 12, each of the three individuals from Barrão that were analysed in this study showed $^{87}\text{Sr}/^{86}\text{Sr}$ ratios within the local range.

Site	Individual	Skeletal Element	Expected Local Range	$^{87}\text{Sr}/^{86}\text{Sr}$ ratio	Within Local Range?
Algar do Barrão	BAR3	Undetermined Tooth	0.7083-0.7115	0.711131	Yes
	BAR4	Premolar	0.7083-0.7115	0.708943	Yes
	BAR5	Premolar	0.7083-0.7115	0.710602	Yes

Table 12: Strontium Isotope results from Algar do Barrão (Carvalho et al., 2019).

2.4 Casais da Mureta

Not much is known about the small Middle Neolithic cave site of Casais da Mureta. The site is located about 1.5 km northeast of Algar do Barrão, also within the Tagus River valley region. The cave was discovered during construction work in the late 1990s (Carvalho et al., 2019). During this construction work, a single deposit of commingled remains was removed and studied. From the commingled remains, there was determined to be a minimum of three adult and two subadult human individuals. The deposit also included plain potsherds as well as 989 faunal specimens. This deposit was an unknown fraction of the entire site, which was destroyed during the construction work.

The faunal assemblage was unusual in the context of Middle Neolithic cave sites. The large and diverse assemblage, along with the fact that it was of the same context as the human remains, make this site unique (Carvalho et al., 2019). Three hundred and twelve of the 989 specimens were able to be classified at species or genus level. The taxa present in the assemblage were red deer (*Cervus elaphus*), swine (*Sus* sp.), dog (*Canis familiaris*), rabbit (*Oryctolagus cuniculus*), and caprines. The age at death estimations for dog and swine specimens was mature (Valente et al., 2017). Rabbits varied in age at death and could have potentially been deposited naturally and not by humans. Only one red deer bone was identified. This red deer scapula was also the only bone to show evidence of cut marks. 90% of the classified faunal remains were identified as caprines, which included domestic goat (*Capra hircus*) and sheep (*Ovis*

aries), as well as a few remains that were potentially from Iberian ibex (*Capra cf. pyrenaica*) (Valente et al., 2017). The age at death estimations of these caprine specimens varied from neonatal to elderly. Goats and sheep were not separated for this analysis. This could account for some of the age at death variance, as sheep are often kept alive longer for wool and milk production (Marom and Bar-Oz, 2009). The large presence of caprines at the site could indicate that the cave was used as a shelter for shepherds during the period of deposition. Without the direct radiocarbon dating of all specimens, it is difficult to be certain that they come from the same context. In most cases, however, the sediments attached to the faunal specimens match up with each other and with those attached to the human remains (Carvalho et al., 2019).

Of the three human adults, the osteological sex of two were determined to be probable males, and the third was a probable female. The sex of the subadults was undetermined. Three mandibles were selected from these individuals by the archaeologists for radiocarbon, isotopic, and genetic studies (Carvalho et al., 2019). These were individuals #01(CMR1), #02(CMR2), and #03(CMR3).

Radiocarbon dating was performed on the mandibles of each of these three individuals. Individual CMR2 did not produce a result due to the lack of preserved collagen, but it was deemed to be of the same archaeological context as the other two individuals (Carvalho et al., 2019). As seen in Table 13, individual CMR1 was dated to between 3100-3020 cal BC, and individual CMR3 was dated to between 3260-3030 cal BC. These dates place the site near the end of the Middle Neolithic period in Portugal.

Site	Individual	Skeletal Element	Years BP	Cal BC	Archaeological Period
Casais da Mureta	CMR1	Mandible	4431±20	3100-3020	Late Neolithic
	CMR2	Mandible	No results	No results	
	CMR3	Mandible	4436±20	3260-3030	Late Neolithic

Table 13: Radiocarbon dating results from Casais da Mureta (Carvalho et al., 2019).

Strontium $^{87}\text{Sr}/^{86}\text{Sr}$ isotope ratios were used to estimate locality of the individuals (Carvalho et al., 2019). Eight samples from fauna recovered from the site were used to create a baseline for local $^{87}\text{Sr}/^{86}\text{Sr}$ strontium isotope ratios (0.7083-0.7115). Individual CMR1 was not analysed, but teeth from individuals CMR2 and CMR3 showed values within the expected local range, as seen in Table 14. Carbon and nitrogen isotopes were also analysed in these individuals (Carvalho et al., 2019). This analysis, the results of which can be seen in Table 15, indicate that individuals CMR1 and CMR3 consumed a predominantly terrestrial diet, consistent with other inland Neolithic sites. No results were obtained from CMR2. Oxygen isotopes were not analysed at this site.

Site	Individual	Skeletal Element	Expected Local Range	$^{87}\text{Sr}/^{86}\text{Sr}$ ratio	Within Local Range?
Casais da Mureta	CMR1	Not Analysed			
	CMR2	Canine	0.7083-0.7115	0.711117	Yes
	CMR3	Undetermined tooth	0.7083-0.7115	0.71147	Yes

Table 14: Strontium Isotope results from Casais da Mureta (Carvalho et al., 2019).

Site	Individual	Skeletal Element	% Nitrogen	% Carbon	Carbon:Nitrogen	$\delta^{15}\text{N}(\text{‰})$	$\delta^{13}\text{C}(\text{‰})$	% Marine protein	% Aquatic protein
Casais da Mureta	CMR1	Mandible	15.57	44.32	3.3	9.42	-19.56	6	16
	CMR2	Mandible	No Results	No Results					
	CMR3	Mandible	15.82	43.95	3.3	7.18	-19.69	4	NA

Table 15: Dietary Isotope results from Casais da Mureta (Carvalho et al., 2019).

Previous genetic work on these individuals was also carried out as part of a Master's project (Robinson, 2018). Robinson's project was unable to get reliable results from the individuals at Casais da Mureta due to the poor state of preservation, and no genetic information was reported. Recent improvements in techniques for ancient DNA analysis that will be utilised in this study may be able to extract meaningful genetic data from the three individuals at Casais da Mureta.

3. Methods

3.1 Sampling

Skeletal samples were chosen by the archaeological and/or anthropological teams of each site for genetic study (Carvalho et al., 2019; Granja, 2019; Robinson, 2018). Due to the high level of fragmentation and disarticulation in most of the sites, teeth were deemed the best option for preservation (Hansen et al., 2017) and to avoid sampling of the same individual multiple times for different biomolecular analyses. Archaeological sample names and corresponding codes in this study can be found in [Supplementary Table S1](#).

Sampling of all La Sagrera, Algar do Barrão, and Casais da Mureta teeth was carried out by a previous student as part of a Masters project in the DNA facilities at Liverpool John Moores University (Robinson, 2018). The surfaces of the teeth were cleaned with a Base 3 pneumatic sandblaster using 90um aluminium oxide particles, followed by UV radiation for 15 minutes on each side. The teeth were then pulverised into powder using a liquid nitrogen cooled SPEX 6775 Freezer/Mill cryogenic grinder.

From the ten mandibles identified in the Bell Beaker context of Convento do Carmo, 8 were originally selected for DNA and isotopic studies. From each of these 8 mandibles, one tooth was sent for isotopic analysis, and another was sent for DNA analysis. The 8 teeth sent for DNA study were sampled at the Archaeo-DNA Lab at Durham University in a previous study (Fernández-Domínguez et al., 2019; Neff, 2016). After cleaning the surface, most teeth were sampled by drilling into the neck and collecting the powder from dentine. In some cases, samples were too small for this method, and the crown of the tooth was instead separated and pulverised with a Freezer Mill refrigerated with liquid nitrogen (Neff, 2016).

For this study, one more tooth from a ninth mandible, B6.1 (1CVC9), was selected and made available by the director of the excavation Prof. António Faustino Carvalho. As well as this tooth, the teeth 1CVC2 and 2CVC3 were sampled again, as there was not enough powder for extraction remaining from the previous study. Drilling of the three teeth was completed in a separate and specialised sampling room within the Archaeo-DNA Lab in the Department of Archaeology at Durham University. This is a state-of-the-art ancient DNA laboratory equipped with HEPA filters, a positive pressure system, and built in UV irradiating lights in each room. Access to the laboratory is restricted and monitored, and anyone using the lab is required to wear full sterile PPE including a Tyvek suit, gloves (two pairs, put on before entering the lab and again after entry, with the second pair being replaced periodically or between samples), an N95 mask, goggles, a hairnet, and a beard net if applicable, to prevent any possible

contamination. The laboratory was also designed with a lobby to change to clothes and prepare to enter the working rooms, which included separate working rooms for sampling, extraction, and library preparation.

Tinfoil was laid down to cover the inside of a LEV hooded cabinet and an additional piece of UVed tinfoil was laid down to capture the powder from the drilled tooth. The tooth 1CVC9 was drilled using the method described in Adler et al (2011) with a Marathon 7 dental drill. Drill bits were subjected to UV radiation for at least 30 minutes prior to use and were cleaned with 70% ethanol solution and deionised water before being UVed again after every sample. The outside of the tooth was cleaned of potential contaminants by removing an external thin layer with a rounded drill bit. Next, the tooth was sliced along the transversal axis with a circular saw drill bit, separating the crown from the root. Powder was then drilled using a rounded drill bit from dentine around the inside of the crown, stopping every couple of seconds to prevent the sample from overheating and damaging the DNA. The other two teeth were already sliced and only needed to be drilled inside the crown. Around 50mg of powder captured on the tinfoil for each sample was transferred to a 15ml sterile and UV irradiated tube for extraction, and the rest was transferred to a 15ml sterile and UV irradiated tube to be kept for future research. Tinfoil and gloves were changed between samples to prevent contamination. This tinfoil method was also used when transferring the 50mg of powder necessary for extraction from the stored powder of the other samples to new 15ml sterile and UV irradiated tubes. This was done to prevent contamination as well as be able to capture any spilled powder and return it to the tubes to minimize sample loss. The remaining tooth from 1CVC9 was returned to the excavation director to be sent for future isotopic analysis. All other teeth and powder were stored inside a -20°C freezer.

3.2 DNA extraction

DNA extraction took place in a separate and specialised ancient DNA extraction room within the Archaeo-DNA Lab at Durham University. Work was done within a HEPA filtered PCR cabinet fitted with UV light. All materials and equipment were subjected to UV radiation for at least 30 minutes prior to use inside the PCR cabinet or in a UVP AnalytikJena CL 1000 cross-linker. After each extraction batch (consisting of 7, 8, and 11 samples plus a blank) was completed, all workspaces and equipment were cleaned with a 50% bleach solution or, in the case of metals, a 70% ethanol solution, and then with deionised water. The UV light in the cabinet was then left on for 30 minutes and the laboratory built-in UV lights for 3 hours after each day of work.

Rohland and Hofreiter (2007a, 2007b) demonstrated that binding the DNA to silica was the most efficient method for extracting ancient DNA. The protocol used in Dabney et al. (2013) made the process more efficient for short fragments of 30-50 bp,

common in ancient DNA, by modifying the binding buffer to contain GuHCl, isopropanol, and sodium acetate, and increasing the amount of binding buffer in relation to the extraction buffer. It also utilised commercially available silica spin columns as opposed to doing silica suspension. This study uses a slightly modified version of the protocol from Dabney et al. (2013).

1ml of extraction buffer composed of 0.45M EDTA, 0.25mg/ml proteinase K, and nuclease-free water was added to each of the 15ml tubes with 50mg of sample powder. The tubes were sealed with parafilm and placed in a rotating incubation chamber at 37°C. Tubes were left for approximately 12-14 hours overnight. The rotation in the large 15ml tubes overnight allowed for the buffer to thoroughly mix with the powder, and the incubation at 37°C allowed for the buffer to efficiently demineralise the powder (Rohland et al., 2009). After this incubation period, the tubes were centrifuged for 5 minutes at 4000 rpm in an Eppendorf 5702 centrifuge to concentrate the powder in a pellet at the bottom. The supernatant, containing the DNA, was then transferred to a new 15ml tube and mixed by inversion with 400µl of sodium acetate and 10ml of a binding buffer solution of 5M GuHCl and 40% isopropanol. These mixtures were then loaded into Roche High Pure extender assemblies with silica columns and centrifuged at 1500 rpm in the same centrifuge for 3-5 minutes, or until all liquid had passed through the column. The columns were then removed from the assembly and placed into 2ml collection tubes and dry spun at 14000 rpm in an Eppendorf Minispin centrifuge for 2 minutes to ensure all liquid had passed through the silica filter of the column. Liquid that passed through was discarded, and 450µl of wash buffer reconstituted with absolute ethanol from the Roche High Pure kit was added to each of the columns. The columns were then spun at 6000 rpm in the same centrifuge for one minute, and the flow-through was again discarded along with the collection tube. This wash step was repeated with a new collection tube, and flow-through was discarded. The columns were then dry spun in the collection tubes at 14000 rpm for 30 seconds, rotated vertically 180° and spun again to ensure all wash buffer had passed through. The collection tubes were discarded, and the columns were transferred to 1.5ml Eppendorf LoBind tubes. To elute the DNA, 50µl of TET (10mM Tris, 1mM EDTA, 0.05% Tween 20), was added to the centre of each column and incubated at room temperature for 3 minutes. The columns were then spun for 1 minute at 14000 rpm. This elution step was then repeated, resulting in a total eluate of 100µl.

Extraction was completed in three batches. Each batch included one extraction blank to identify and monitor contamination. 1µl of each extract were pipetted into new 0.2ml tubes and taken to the Modern DNA lab for quantification with an Invitrogen Qubit fluorometer and Quant-iT High Sensitivity (HS) assay kit. 1µl of sample and 199µl of qubit master mix (99.5% dsDNA HS Buffer and 0.5% Quant-iT reagent) were combined in Invitrogen Quant-iT assay tubes. Standards were prepared for each batch of

quantification by combining 10µl of Invitrogen Quant-iT High Sensitivity Standard 1 with 190µl of qubit master mix in an Invitrogen Quant-iT assay tube and 10µl of Invitrogen Quant-iT High Sensitivity Standard 2 with 190µl of qubit master mix in another Invitrogen Quant-iT assay tube. Standard and sample tubes were vortexed for approximately 3 seconds and left to incubate at room temperature for 2 minutes. Standards were then placed in the Invitrogen Qubit Fluorometer until processed to calibrate the machine. Samples were then placed in the machine one at a time and calibrated for the input of 1µl of sample. Results of these quantifications can be seen in [Supplementary Table S1](#). Quantification ensured all extractions were successful before proceeding to the next step. Extracts were stored in a -20°C freezer in the Archaeo-DNA Lab.

3.3 Library Preparation

Extracted DNA was transformed into Illumina libraries following Meyer and Kircher, 2010. As with DNA extraction, all materials and laboratory equipment for this step were subjected to UV irradiation for at least 30 minutes prior to use. All work was carried out in a specialised room for library preparation within the Archaeo-DNA Lab at Durham University. After a batch, consisting of 10 or 18 samples and 1 or 2 library blanks, was completed, all workspaces and equipment were cleaned with a 50% bleach solution or 70% ethanol solution, and then with deionised water. The UV light in each cabinet was then left on for 3 hours after each day of work.

Two PCR cabinets equipped with HEPA filters and UV lights were used in the library building process. The first cabinet was designated as 'DNA free', and a master mix of 1.2 X NEBuffer 2, 1.2 mM ATP, 0.2mg/ml BSA, 0.072 U USER enzyme from New England Biolabs (NEB), and nuclease-free water was prepared in this cabinet. This was done to prevent any contamination of materials and reagents from previously prepared libraries. After all the DNA extracts including extraction blanks had been completely thawed and vortexed, 25µl of extract and 25µl of master mix were combined in 0.2ml tubes in the second, or 'DNA' hood. This resulted in a mixture with a final concentration of 1.2x NEBuffer 2, 1.2mM ATP, 0.2mg/ml BSA, and 0.072U USER enzyme. The tubes were then mixed by flicking and spun down in a table-top spinner before being incubated for 30 minutes at 37°C followed by 1 minute at 12°C in a DNA Engine PTC-200 Thermal cycler. This process, called UDG or uracil-DNA-glycosylase treatment, removed uracil bases common in ancient DNA that cause C to T deamination as explained in section [1.1.1](#) (Rohland et al., 2015). This study utilised partial UDG treatment, which removes some Uracil bases, but the process is stopped before removing all. This makes the libraries useful for analysis by removing most of the damage while still leaving enough uracil bases on the ends of the DNA molecules to be able to authenticate the samples by observing C to T deamination (Rohland et al.,

2015). To stop the reaction, the tubes were placed in a frozen rack, and 3.6µl of UGI (2 U stock, 0.1343 U final concentration, New England Biolabs), or UDG inhibitor, was added to each tube. Tubes were again mixed by flicking and spun down before being incubated for 30 minutes at 37°C followed by 1 minute at 12°C.

Next, the samples went through blunt-end repair. This process filled in or removed overhanging ends of the double stranded molecules with a T4 DNA polymerase (Meyer and Kircher, 2010). 3µl of 10U T4 PNK and 1.65µl of 3U T4 polymerase were added to each sample while in a frozen rack to prevent the reaction from starting prematurely. The samples were then mixed by flicking and spun down before being incubated at 25°C for 20 minutes followed by 10 minutes at 12°C in a DNA Engine PTC-200 Thermal cycler.

Following this, the samples were purified using the MinElute kit from Qiagen to remove any remaining reagents. The samples were transferred to new 1.5ml LoBind tubes and mixed with 650µl of PB buffer. After vortexing, the solution was loaded into MinElute columns with collection tubes and incubated at room temperature for 2 minutes. The columns were centrifuged at 13000 rpm for 1 minute in an Eppendorf Minispin centrifuge and flow-through was discarded. 700 µl of PE buffer was added to the columns, and they were again centrifuged at 13000 rpm for 1 minute, discarding the flow-through. Columns were dry spun at 13000 for 1 minute, flipped, and spun again to ensure all liquid had passed through. Columns were then placed into new 1.5ml LoBind tubes. 20µl of TET heated to 50°C was added to the centre of the columns to elute the DNA. After incubation for 1 minute at room temperature, the columns were centrifuged for 1 minute at 13000 rpm, resulting in around 18µl of eluate for each sample in the 1.5ml LoBind tubes.

To prepare for the indexing step, adapters were ligated to the DNA molecules. This was done by adding 20µl of 2 X quick ligase buffer (NEB) and 1µl of 10µM adapter mix to each of the libraries for a final concentration of 1 X quick ligase buffer and 0.25µM adapter mix. This adapter mix contains DNA oligonucleotides that attach to DNA strands in the sample and create a site for primers to bind to. The libraries were mixed by pipetting, then 1µl of 5U quick ligase was added to the libraries before being mixed by pipetting again. The libraries were then incubated at room temperature for 60 minutes before being purified with the MinElute kit in the same process previously described with the exception that elution was done with 22µl of TET in this case. Adapter fill-in was completed by adding 4µl of 10x isothermal buffer, 0.5µl of 10mM dNTPs, 2µl of 8U bst polymerase, and 13.5µl of nuclease-free water to each of the libraries. The libraries were then incubated for 30 minutes at 55°C followed by 5 minutes at 80°C in a DNA Engine PTC-200 Thermal cycler. Libraries were not purified after this step.

The first library batch consisted of ten samples and one extraction blank. One library blank was also included with the samples in this batch. The second batch was larger, consisting of 16 samples and 2 extraction blanks, and therefore 2 library blanks were included with this batch. After library preparation, all libraries were stored in a freezer at -20°C. 1µl of each library was quantified by qubit following the method described in section 3.2. Quantification values can be found in [Supplementary Table S1](#).

3.4 Indexing

Samples would eventually be pooled into one 1.5ml LoBind tube and sequenced on one lane. For the sequencing machine to be able to identify which reads came from which samples during the demultiplexing process, indexes first had to be added to each of the samples after the library preparation stage. These indexes are short sequences of known base pairs within the library adapters. Two different indexes are added to each read, one p7 index and one p5 index. This combination of indexes is unique for every sample. This dual indexed approach increases accuracy and decreases the chance of mistakes caused by cross-contamination during the demultiplexing process (Kircher et al., 2012).

The first steps of indexing took place in the library preparation room within the Archaeo-DNA Lab at Durham University following Kircher et al. (2012). All materials and laboratory equipment were subjected to UV radiation for at least 30 minutes prior to use, and all work was done inside PCR cabinets equipped with HEPA filters and UV lights. Two 0.2ml tubes were prepared for each sample. Two indexing reactions were conducted per library using 10µl per reaction to make the reactions more efficient. In the 'DNA free' hood, a master mix of nuclease-free water, Pfu turbo buffer, dNTPs, BSA, and Pfu turbo Cx hotstart DNA polymerase was created. 80µl of master mix was added to each of the 0.2ml tubes. Both p5 and p7 primers were added to each of the tubes. The same unique combination was added to both tubes from the same sample. The primers and index sequences can be seen in [Supplementary Table S1](#). Tubes were then moved to the 'DNA' hood, where 10µl of library were added to each corresponding tube. The final concentrations of reagents in each tube were 1X Pfu turbo buffer, 0.3mM dNTPs, 0.3mg/mL BSA, 0.05U/µl Pfu turbo Cx hotstart DNA polymerase, 0.25µM p5 index, and 0.25µM p7 index. All workspaces and equipment were cleaned with a 50% bleach solution or 70% ethanol solution, and then with deionised water. The UV light in each cabinet was then left on for 30 minutes and the laboratory built-in UV lights for 3 hours after each day of work. Tubes were kept in a frozen rack while cleaning took place in order to prevent the reaction from beginning prematurely.

Tubes were then taken to the Modern DNA lab in the Department of Archaeology at Durham University for PCR amplification. In a TECHNE 3Prime thermal cycler, the samples were initially denatured at 95°C for 2 minutes. They then ran for 20 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute. They then went through a final extension at 72°C for 10 minutes. Sample splits were then pipetted into the same tube and purified using the MinElute kit (Qiagen) as described in the previous section and eluted in 25µl of the kit elution buffer.

Samples were first analysed by Qubit to determine DNA concentration (see [Supplementary Table S1](#)) and then with the Agilent 2200 TapeStation system using the High Sensitivity D1000 kit. Based on the qubit results, 2µl of each sample were diluted to 1:100 with nuclease-free water to fit the range of 10-1000pg/µl required by the tape station. 2µl of diluted sample and 2µl of D1000 buffer (Agilent) were combined in a 0.2ml tube and vortexed in an IKA vortexer at 2000 rpm for 1 minute. Samples were then spun down in a tabletop spinner and loaded into the tape station along with an Agilent High Sensitivity D1000 ScreenTape. The tape station automatically ran the samples through the tape by electrophoresis and analysed the results. The outputs of this system allow for visualisation of the lengths of the different DNA fragments in the sample as seen in the example in Figure 13. This was useful for ensuring that samples exhibited the expected normal distribution peaking at the length of approximately 200bp for ancient DNA and that they have amplified correctly, as seen in Figure 13. After the first two batches of indexing, tape station results showed that the samples had not amplified successfully. It was suspected that amplification failed due to the presence of PCR inhibitors in the samples. To account for this, the indexing protocol was modified slightly. The final concentration of the p5 and p7 indices and the Pfu Turbo Cx enzyme were increased to 0.5µM and 0.08U/µl in the reactions to overcome possible non-specific interactions between primers and inhibiting agents and increase amplification efficiency. The final concentration of BSA was raised to 0.5mg/mL, as BSA binds to PCR inhibitors to prevent them from interacting with DNA template (Kreader, 1996). This modified protocol was used to index the last two batches as well as to redo the indexing for the samples in the first two batches. Amplification of samples with the modified protocol was successful when analysed with the tape station, showing expected peaks at approximately 200bp lengths. All indexed libraries were stored in a freezer at -20°C.

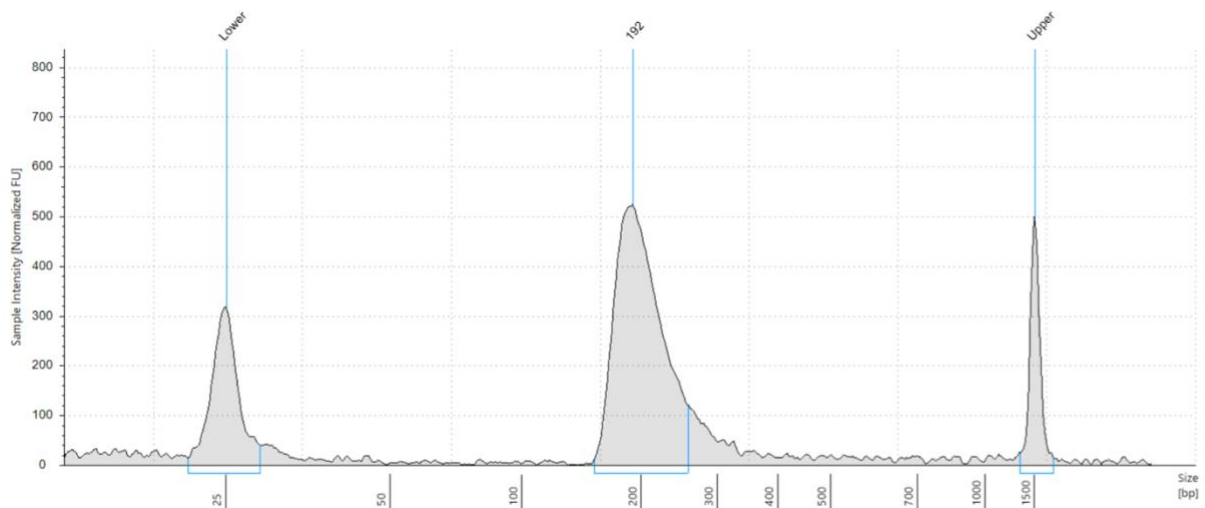


Figure 13: Example of tape station results from a successfully amplified sample showing a peak of at 192bp length.

3.5 Capture of Mitochondrial DNA

Human mitochondrial DNA was used in this study for population and maternal kinship analysis due to budget and time constrictions. To enrich the fraction of human mitochondrial DNA in the indexed libraries, a commercial *in solution* capture approach was used. This study utilised the Arbor Bioscience myBaits mtDNA capture kit and the myBaits High Sensitivity protocol, which is well suited for ancient DNA. As samples had already been indexed, they could be pooled into groups of similar concentration to make more efficient use of the reactions, as each can capture up to a total of 12µg of sample. Up to four reactions can be pooled according to the protocol. Samples were pooled in groups with similar concentrations to avoid samples with much higher concentrations amplifying more and overpowering samples with much smaller concentrations. Concentrations, volumes and groupings for the different pools can be found in [Supplementary Table S2](#). Samples were pooled into 1.5ml LoBind tubes.

Pooled samples were first concentrated to make the capture reaction more efficient as described by the myBaits Hybridization Capture for Targeted NGS High Sensitivity protocol for ancient DNA. This concentration was done using the Nucleomag protocol for PCR clean up and the NGS clean-up and size select kit, which uses a magnetic bead solution that binds the targeted mtDNA to magnetic beads, allowing for non-target DNA to be washed away. Target DNA that remains can then be reamplified, resulting in a higher concentration of target DNA. A simplified diagram from the myBaits Hybridization Capture for Targeted NGS protocol can be seen in Figure 14. Around 50µl of pooled samples and 90µl of bead suspension were mixed by pipetting in 1.5ml tubes and incubated at room temperature for 5 minutes. During this step, DNA fragments bind

to the beads, which are covered in a magnetic coating. The tubes are then put onto a magnetic rack, which attracts the beads with DNA molecules. The supernatant was then carefully pipetted out and discarded. While still in the rack, 200µl of 80% ethanol solution was added to the tubes, completely submerging the beads. This was done to wash away impurities left over from the bead buffer solution. After 30 seconds, the ethanol solution was carefully pipetted away and discarded, making sure not to disturb the beads. This wash step was then repeated to ensure all impurities were removed. Beads were incubated at room temperature for 10 minutes to allow all remaining ethanol to evaporate. The beads were then resuspended in 10µl of the capture kit elution buffer and mixed by pipetting. This releases the DNA from the beads by rehydrating the DNA and dissolving the bonds between the baits and magnetic beads created by the high salt levels in the bead buffer. After incubating at room temperature for 5 minutes, the beads were separated from the eluate on the magnetic rack. This eluate of concentrated DNA was carefully transferred by pipette to new 1.5ml tubes.

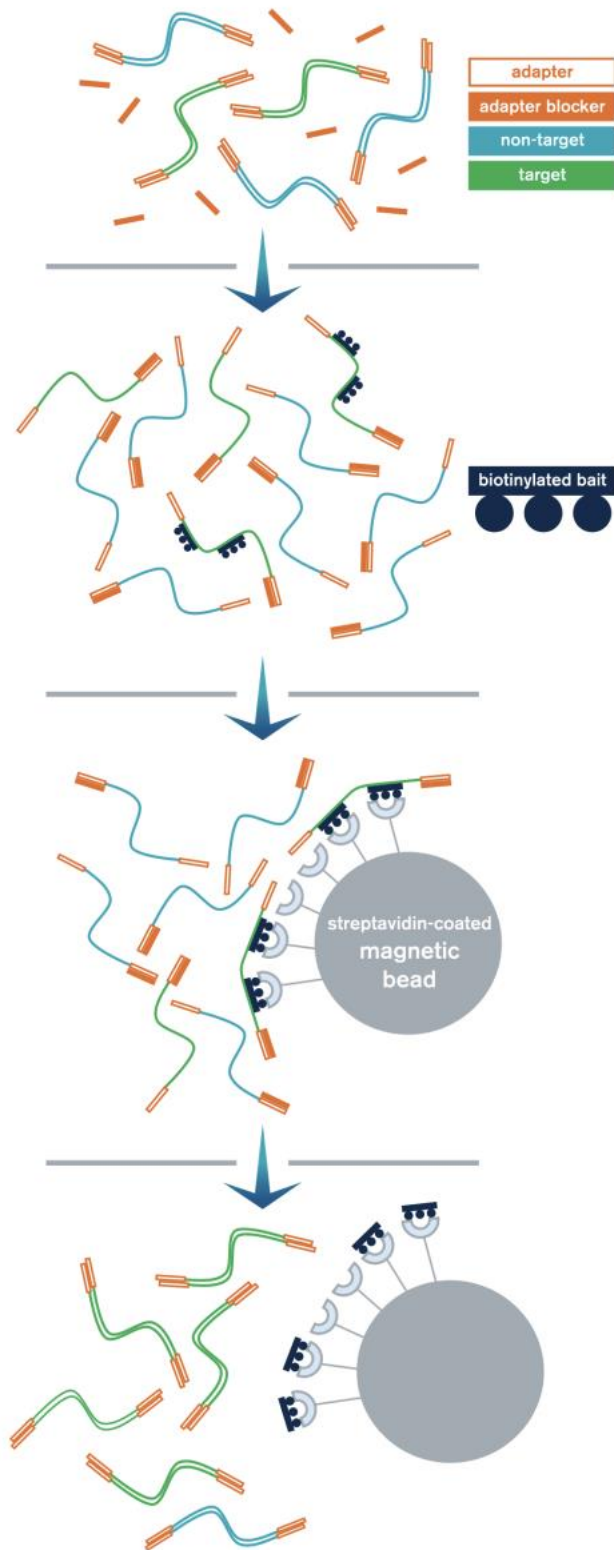


Figure 14: Model of the magnetic bead capture process. From: myBaits Hybridization Capture for Targeted NGS protocol

To begin the mtDNA capture, a hybridization master mix was prepared. This mix consisted of 9.25µl of Hyb N, 3.5µl of Hyb D, 0.5µl of Hyb S, 1.25µl of Hyb R, 1.1µl of nuclease-free water, and 4.4µl of baits per reaction for the first round of enrichment. The mix was incubated for 10 minutes at 60°C, and then 5 minutes at room temperature and 18.5µl were aliquoted to a 0.2ml tube for each pool. In another set of 0.2ml tubes, 7µl of concentrated pools were mixed with 2.5µl of Block O, 2.5µl of Block C, and 0.5µl of Block X. These blockers stop unwanted DNA from binding to magnetic beads during the process. These tubes were then put into a TECHNE 3Prime thermal cycler and heated at 95°C for 5 minutes. After this, the temperature was dropped to 60°C, and the tubes with the hybridization master mix were also put into the thermocycler. After 5 minutes, 18µl of the hybridization mixes were added into the pool tubes and mixed by pipetting. The tubes were then left in the thermocycler at 60°C overnight for around 18 hours.

Before the next step, 10ml of wash buffer consisting of 400µl Hyb S and 39.6ml nuclease-free water was prepared and heated to 60°C for at least 30 minutes prior to use. Beads were also prepared for the next step by aliquoting 30µl of bead solution into different 1.5ml Eppendorf tubes, one per reaction. The beads were pelleted using the magnetic rack, and the supernatant was discarded. 200µl of binding buffer was then added, and the tubes were vortexed to resuspend the beads. They were then placed back into the magnet rack for pelleting, and the supernatant was discarded. This binding step was repeated two more times. Beads were then resuspended in 70µl of binding buffer and the tubes were removed from the magnetic rack.

The bead solution was heated to 60°C for 2 minutes. After the overnight incubation, the pools were pipetted into the separate heated bead solution tubes and mixed. They were then incubated for 5 minutes at 60°C and mixed by flicking halfway through. Tubes were then placed on the magnetic rack to pellet the beads, and the supernatant was discarded. 375µl of heated wash buffer was added to each tube, and the tubes were placed in a heat block at 60°C for 15 seconds and vortexed to resuspend the beads. The tubes were heated at 60°C for 5 minutes, flicking to mix halfway through. The tubes were then placed back into the magnetic rack to pellet the beads, and the supernatant was discarded. This washing process was repeated two more times.

30µl of buffer E was added to the tubes, and the beads were resuspended by pipetting. 15µl of this solution was then put into two different 0.2ml tubes for each sample for a more efficient PCR reaction. 5µl of nuclease-free water, 25µl of KAPA HiFi HotStart ready mix, 2.5µl of 10µM p5 primer, and 2.5µl of 10µM p7 primer were added to each split enriched library pool for a final concentration of 1X KAPA HiFi HotStart ready mix, 500nM p5 primer, and 500nM p7 primer. The tubes were then put into a TECHNE 3Prime thermal cycler, starting with an initial denaturation for 2 minutes at 98°C. This was followed by 14 cycles of 20 seconds at 98°C, 30 seconds at 60°C, and 45 seconds

at 72°C. A final annealing step at 72°C was done for 5 minutes. PCR purification was then completed using the Nucleomag protocol for PCR clean up described earlier in this section. The sample was eluted in 20µl of elution buffer.

The entire process was repeated from the beginning of capture, using the eluate from the last step to mix with the blockers for a second round of enrichment instead of the concentrated pools. For this second round of enrichment, another hybridization master mix was created in the same way as the first, with the exception that the volumes of baits and water were switched from 1.1µl of nuclease free water and 4.4µl of Baits to 4.4µl of nuclease free water and 1.1µl of Baits. Also, the last PCR stage only went through 8 cycles for the second round. During the second round of PCR, the thermocycler malfunctioned, and the samples did not amplify correctly. To fix this, the failed samples were combined with 13µl of nuclease-free water and split into two tubes with 15µl each for PCR reamplification. This was done using the same method as before but using 14 cycles to account for loss of DNA. After the second round of PCR cleanup, tape station results showed that the pooled and captured libraries had expected peaks at ~200bp and were ready for sequencing.

3.6 Sequencing

Sequencing was carried out by Novogene LTD (Oxford) using the Illumina NovaSeq X Plus PE150 sequencing machine. To meet the requirements of ≥ 2 ng/µL in at least 70µL of nuclease free water stipulated by Novogene for single lane sequencing on an 8 lane 10 billion read flow cell, all the samples were pooled into one 1.5ml tube in amounts based on their concentration and the desired share of reads for each sample. Previously pooled and mtDNA captured samples were included as well as uncaptured indexed libraries for metagenomic (shotgun) analysis. Blank indexed libraries were also included. The final concentration of captured pools and blanks was lower than that of the uncaptured indexed libraries to give them a smaller share of reads, as the amount of DNA in the blanks and the size of the complete mitochondrial genome were much smaller in comparison to the nuclear human genome. The concentration calculations for this pooling step can be seen in [Supplementary Table S3](#). The pooled sample was packaged with dry ice and shipped to the Novogene sequencing centre in Cambridge, UK, where sequencing and demultiplexing took place.

3.7 Analysis of the Author's own mtDNA

The author's mitochondrial DNA was also analysed in this study to identify any potential contamination in the samples. DNA was extracted from cheek cells using a sterile buccal swab and the E.Z.N.A. extraction kit, following the protocol for DNA

extraction from tissues with some modifications for elution from swabs. The swab was rotated on the inside cheek of the mouth on both sides for 15 seconds each. After drying for 10 minutes, the tip of the swab was cut off into a 2ml tube. 180µl of QIAGEN buffer ATL and 20µl of QIAGEN proteinase K (>600 mAU/mL solution) was added to submerge the swab tip before incubation with shaking at 56°C for 1 hour. The swab tip was then removed, and the elution was purified using the E.Z.N.A kit following manufacturer's instructions. Due to time and financial constraints, only the hypervariable region one, or HVRI, of the mtDNA was amplified and sequenced. This was achieved by amplifying through Polymerase Chain Reaction (PCR) in a 50µl reaction, 3µl of the extracted DNA using 1µl each of primers L15996 and H16380 (Bramanti et al., 2009; Fernández et al., 2014) at a concentration of 10mM along with a PCR master mix of 82.5 mM Tris-HCl pH 8.5, 22 mM (NH₄)₂SO₄, 1.65 mM magnesium chloride, 0.22mM dNTPs, and 0.11 units/µl Ampliqon Taq DNA polymerase (VWR) (Mullis et al., 1986). The sample was denatured for 2 minutes at 95°C and then put through 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds in a TECHNE 3Prime thermal cycler. This was followed by a final extension for 5 minutes at 72°C. The sample was then purified using the Thermo Scientific GeneJET PCR purification kit and protocol following manufacturer's instructions. The DNA extract was quantified by qubit using the method described in section 3.2 which found it to be at a concentration of 30.4µg/ml. 20µl of purificate at a concentration of ~10ng/ml and 20µl of each primer at a concentration of 10µM were placed in separate 1.5ml tubes. These tubes were then packaged and sent to Genewiz UK for Sanger sequencing.

Sequences were aligned to the rCRS human reference sequence using the Mutation Surveyor software version 5.1.2 and mutations were reported according to the DNA Commission of the International Society for Forensic Genetics (Carracedo et al., 2000). Haplogroup assignment was completed using Haplogrep3 (Schönherr et al., 2023).

3.8 Sample Processing and Alignment to the Human Reference Genome

The nf-core Eager version 2.5.1 pipeline was used to process the raw sequencing output from Novogene's Illumina NovaSeq X Plus PE150 sequencing machine after demultiplexing (Peltzer et al., 2016). Data from samples were put through the pipeline twice. Once to analyse and map the mtDNA reads, and once to analyse and align the reads to the entire human genome. The analysis and mapping of the mitochondrial reads was done to generate data and files that could be used for population and kinship analysis in this study, while the alignment to the entire genome was done to gain insight on sample quality with the generated MultiQC report.

The pipeline began by clipping the adaptor sequences from each read using the AdapterRemoval tool, which also merged the paired end reads (Schubert et al., 2016). For the whole genome run, reads were aligned to the whole genome human reference hs37d5. To do this, the BWA aln tool was used with the parameter 0.01 (Li and Durbin, 2010). This older version of the BWA tool was used as it is better suited for short reads than newer tools (Dolenz et al., 2024; Li and Durbin, 2010). For the mitochondrial run, samples were aligned to the revised Cambridge Reference Sequence, or rCRS (Andrews et al. 1999), by using the circularmapper tool (Peltzer et al., 2016). For both runs, reads shorter than 30 base pairs and reads with a mapping quality score of less than 25 were discarded to avoid misaligned and low-quality reads. Unmapped reads were also discarded. The tool mapDamage generated plots for C to T deamination patterns for authentication of ancient DNA (Ginolhac et al., 2011). Two base pairs were then clipped from each end of every read using BamUtil to remove the deamination from downstream analysis. The DeDup tool was then used to remove duplicate reads (Peltzer et al., 2016). Variants from the rCRS reference were called by Eager using GATK UnifiedGenotyper for the mitochondrial run (McKenna et al., 2010). MultiQC reports were generated as the last step pipeline. These reports include overall statistics about sample quality and processes throughout the run.

3.9 Mitochondrial Haplogroup Assignment

Due to the level of degradation in the samples, multiple processes were used to assign mtDNA haplogroups. An initial haplogroup assignment was made by uploading the VCF files generated from the Eager pipeline to Haplogrep3 (Schönherr et al., 2023). This tool assigned haplogroups to the samples based on the Phylotree mtDNA tree Build 17 (Van Oven and Kayser, 2009).

Haplogroups were also checked using the Haplocheck tool version 1.3.3, which can also be used for assessing contamination in samples (Weissensteiner et al., 2021). Using the mtDNA-Server version 2.0.3 tool and default parameters, new VCF files including variant call information were generated (Weissensteiner et al., 2021, 2016). Based on analysis of these files, haplocheck then assigns a major haplogroup as well as a minor haplogroup if one exists. Samples that contain high levels of contamination would be expected to have two haplogroups reported.

The BAM files were also looked through manually for a more accurate identification of true variants. This was done using the IGV software, which allowed for easy viewing of aligned read positions and mutations from the samples in comparison to the rCRS reference genome (Robinson et al., 2023, 2011). Expected SNPs under the initial Haplogrep3 haplogroup assignment were checked along with the entire mitogenomes, and all polymorphisms present as well as their coverage were noted.

Based on this analysis, new VCF files were manually created. These files were then used as inputs for Haplogrep3 for a more accurate haplogroup assignment.

3.10 Kinship Analysis

Kinship analysis was only conducted for samples from the site of La Sagrera in an attempt to understand deposition patterns in the burial clusters at the site. Kinship analysis in this study only utilised data from the mtDNA of the individuals, meaning only matrilineal relationship could be observed. Due to the level of DNA degradation in the samples, any mutations that were found in the author's mtDNA were discarded before analysis.

Due to the small size of the mitochondrial genome and the level of degradation in the samples, it was feasible to manually assign and annotate haplotypes for each of the individuals using IGV. A list of positions where mutations occurred in any of the individuals was compiled. Bases at each of these positions for each of the La Sagrera samples was noted and compared. Individuals that are matrilineally related would be expected to have the same mutations. If there was a mutation present in one individual but not in another and where the coverage at that position was at least 2, it could be concluded that these individuals were not matrilineally related.

3.11 Population Analysis

Population analysis in this study utilised mitochondrial haplogroup data. A comparative database was built using individuals from the Allen Ancient DNA Resource (AADR) version 62 (Mallick et al., 2024). Only individuals with reliable mitochondrial haplogroup data with at least 1X coverage were considered. The database was first searched for individuals matching the specific haplogroups of the individuals analysed in this study to contextualise the haplogroup results. Maps of the distribution of individuals in the database were created using ArcGIS Pro version 3.0.6. In some cases, closely related haplogroups were also searched to better contextualise the data.

The second aspect of population analysis involved splitting the database into population groups. For this analysis, only Iberian individuals were considered. Groups were created based on geographic clusters. These groups were designated Central, North, Northeast, Southeast, and West, following Szécsényi-Nagy et al. (2017), and further split based on archaeological period: Mesolithic (Meso), Early Neolithic (EN), Middle to Late Neolithic (MLN), Chalcolithic (C), Early Bronze Age (EBA), and Middle to Late Bronze Age (BA). Middle and Late Neolithic were combined, as previously published large scale ancient DNA studies do not distinguish between these periods (Olalde et al., 2019). A map of the clusters and archaeological periods was generated

using ArcGIS Pro version 3.0.6. During analysis, all Mesolithic Iberian individuals were considered as one group, as there was not sufficient data to split the individuals into regional groups. This was also the case for EN individuals. This combining of groups across geographical regions was chosen as previous ancient DNA studies have shown that Mesolithic and EN Iberian individuals represent genetic groups distinct from other archaeological periods (Olalde et al., 2019, 2018; Szécsényi-Nagy et al., 2017). In the Central, West, and Northeast regions, EBA and BA individuals were grouped together as they also did not have sufficient data. In contrast to the Mesolithic and EN group combinations, these groups were chosen as previous studies suggest more genetic similarity between EBA and BA individuals in a specific region than Bronze Age individuals across different regions (Olalde et al., 2019, 2018; Szécsényi-Nagy et al., 2017). The database of comparative individuals used in this study can be seen in [Supplementary Table S4](#).

3.12 Metagenomics

Although teeth of human individuals were sampled in this study, DNA from microbes and other organisms could also be found within the samples. These organisms could come from many sources, including the soil the sample was buried in, the oral microbiome of the individual while they were alive, or from pathogens infecting the individuals during life. The DNA of these organisms was extracted, amplified, and sequenced along with the human DNA in each sample. While aligning to the human reference genome filters out these reads, metagenomic study of this ‘exogenous’ DNA material can provide incredible archaeological insight and is becoming an increasingly used tool for archaeological analysis.

The ancient metagenomic pipeline ‘aMeta’ was used in this study to identify organisms present in each sample (Pochon et al., 2023). Sample fastq files that were generated after the ‘adapter removal’ step of the nf-core Eager pipeline were used as the input files for the aMeta pipeline. The files first ran through KrakenUniq, which manages the data by using k-mers, or sub-sequences of the DNA reads of 31 base pairs (bp) length (Breitwieser et al., 2018). These k-mers were then assigned a classification to a taxonomic group based on competitive alignment to a pre-built database. For this study, the prebuilt KrakenUniq database based on microbial as well as human and complete eukaryotic reference genomes, which was made available as part of aMeta, was used (Pochon et al., 2023). KrakenUniq also identified how many unique k-mers had been assigned to each of the identified taxonomic groups. The number of unique k-mers can be used as a good indicator of breadth of coverage (Breitwieser et al., 2018). A higher number of unique k-mers indicates a more even breadth of coverage, while a

lower number could indicate stacking, which happens when reads are misaligned to a reference genome of an organism that they don't come from. Taxonomic classifications were filtered out by KrakenUniq if they did not reach a threshold of 1000 unique k-mers and 50 individual reads. 1000 unique k-mers, which is the pipeline's default, was chosen because it has been shown to be a good threshold for filtering out most false positives while retaining true positives (Bergfeldt et al., 2024; Breitwieser et al., 2018; Pochon et al., 2023). The 50 individual reads threshold was chosen as opposed to the suggested 200 individual reads (Pochon et al., 2023). This was done because the DNA in many of the samples was heavily degraded, and 200 would filter out too many potential true positives in this dataset (Pochon et al., 2023). The amount of unique k-mers is the most important metric for filtering out false positives, and lowering the individual reads threshold has been found to make little difference (Pochon et al., 2023). Any false positives that did make it past this filter could still be identified by the Malt authentication outputs downstream.

Organisms that were present in the samples and which passed the filter criteria were then used downstream to create a database of reference genomes for competitive alignment. The 'Malt' tool built this database and then used a latest common ancestry-based alignment technique to align each individual read (Herbig et al., 2016). This type of alignment was used because some reads may align with equal statistical strength to two different species. Instead of discarding this information, this type of alignment assigned the reads to a higher taxonomic level based on the latest common ancestor (Pochon et al., 2023). The main outputs of Malt included 'abundance quantification' and 'validation of authentication'. Abundance quantification gave information about how many reads are assigned to taxa in each specific sample, and validation of authentication provided information on damage and other metrics associated with aDNA (Herbig et al., 2016). These outputs were then used to determine which organisms were truly ancient and present in each sample. The presence of certain microbes and pathogens in these samples provide valuable information on what health issues the individuals studied were living with, and potentially what could have caused their death.

4. Results

4.1 Screening of endogenous DNA content

The results of the MultiQC reports generated by the nf-core Eager pipeline for the whole genome shotgun sequencing and mtDNA capture results can be seen in Table 16 and Table 17, respectively. The extraction and library building dates for each sample can be seen in the third and fourth column of Table 17.

The first thing to note is that there is a high percent of endogenous DNA in the extraction blank from 9/9/24 and the first library blank from 4/10/24 in the MultiQC report for the whole genome shotgun sequencing results (Table 16). This indicates that there was some level of contamination that could have also affected the samples processed at the same time as these blanks. The median length of reads in the blanks range from 44 to 57 base pairs, similar to the read length in the samples, suggesting possible carry-over from other samples rather than modern DNA contamination. The damage patterns of these reads, however, tell a different story. While damage is similar to ancient samples in EB17/9/24 (7.3% 5' C>T 1st base, 4.7% 3' G>A 1st base) and 2LB4/10/24 (0% 5' C>T 1st base, 50% 3' G>A 1st base but with only 14 reads), damage was less on both 5' and 3' ends for other blanks when compared to all ancient samples (less than 2.9% 5' C>T 1st base, less than 3.5% 3' G>A 1st base). This could indicate contamination from a modern source.

Whole genome results were poor in general, with mean coverage of 0.0X for all samples. The endogenous human DNA percentage was between 0.01 and 1.3 for all samples with the exception of BAR3, which had 4.58% endogenous DNA. The endogenous amount likely reflects true ancient DNA, as the characteristic short fragment length and deamination patterns can be observed in all samples. Median fragment length for all sample was between 44 and 64. Deamination ranged between 2.9%-20.5% for 5' 1st base C>T transition and 3.5%-19.9% for 3' 1st base G>A transitions in samples, matching the expected ranges for partially UDG treated samples (Rohland et al., 2015). While coverage was inadequate for reliable whole genome analysis, use of the Twist Bioscience Ancient DNA Panel for capture of 1350K SNPs or deeper shotgun sequencing at more reads on these samples in the future may yield good results (Rohland et al., 2022).

Due to mitochondrial DNA enrichment using an *in solution* capture approach, and to the higher number of copies of the mitochondrial genome, the mitochondrial coverage was higher for most samples. For La Sagrera samples, mean coverage was below 1.2X in all samples. Sample SA44 had the highest mitochondrial mean coverage of 1.1X, while all other La Sagrera samples had a mean coverage of 0.6X or less. Post deduplication mapped reads were also low for La Sagrera samples. SA44 had the highest with 313 reads, while all other La Sagrera samples each had less than 250. The lack of coverage and reads in these samples made it difficult to complete reliable downstream analysis. Samples from Casais da Mureta were of similarly low quality, with the best sample CMR1 having just 258 post deduplication mapped reads and a mean coverage of 0.8X. This was expected, as previous PCR-based genetic analyses on these samples yielded no results (see section [2.4](#)).

Samples from Convento do Carmo and Algar do Barrão yielded better results. Only samples CVC2 and CVC5 had a mean coverage of less than 1.0X, with samples CVC8 and BAR3 having exceptional coverage of 13.6X and 18.3X respectively. CVC2 and CVC5 had a low number of post deduplication mapped reads with 49 and 126 respectively. All other samples from these two sites had between 324 and 5573 post deduplication mapped reads.

Site	Sample	Nr. Reads Into Mapping	Nr. Mapped Reads	Endogenous DNA (%)	Nr. Mapped Reads Passed Post-Filter	Post-DeDup Mapped Reads	DeDup ClusterFactor	SG Endogenous DNA Post (%)	5 Prime C>T 1st base	3 Prime G>A 1st base	Median read length	Nr. Dedup. Mapped Reads	Mean cov	≥ 1X	≥ 5X	% GC Dedup. Mapped Reads
La Sagrera	1SA4	17,752,120	6,275	0.04	4,598	4,470	1.03	0.03	2.90%	3.60%	44	4,470	0.0X	0.00%	0.00%	59%
	1SA18	42,470,419	52,332	0.12	38,763	37,581	1.03	0.09	18.20%	18.00%	44	37,581	0.0X	0.00%	0.00%	45%
	1SA26	44,963,670	5,840	0.01	4,021	3,831	1.05	0.01	4.20%	3.50%	54	3,831	0.0X	0.00%	0.00%	60%
	1SA43	40,291,876	47,698	0.12	35,830	34,182	1.05	0.09	20.40%	19.90%	44	34,182	0.0X	0.00%	0.00%	45%
	1SA44	44,431,978	73,314	0.17	53,776	51,408	1.04	0.12	20.50%	19.80%	44	51,408	0.0X	0.10%	0.00%	44%
	1SA45	43,841,333	14,905	0.03	11,215	10,773	1.04	0.03	8.60%	8.90%	44	10,773	0.0X	0.00%	0.00%	61%
	1SA46	42,535,105	17,162	0.04	13,424	12,927	1.04	0.03	8.10%	8.40%	44	12,927	0.0X	0.00%	0.00%	57%
	1SA53	31,223,684	11,639	0.04	8,790	8,326	1.05	0.03	12.20%	11.20%	54	8,326	0.0X	0.00%	0.00%	48%
	1SA56	38,354,910	213,934	0.56	158,362	148,113	1.06	0.41	19.20%	19.10%	44	148,113	0.0X	0.20%	0.00%	40%
	1SA68	28,364,144	102,816	0.36	77,850	73,426	1.06	0.27	8.50%	8.70%	44	73,426	0.0X	0.10%	0.00%	42%
Convento do Carmo	1CVC1	40,286,065	65,429	0.16	49,693	38,935	1.22	0.12	4.70%	4.20%	54	38,935	0.0X	0.10%	0.00%	49%
	1CVC2	35,906,987	10,519	0.03	8,054	6,811	1.15	0.02	4.30%	5.30%	54	6,811	0.0X	0.00%	0.00%	52%
	2CVC3	34,332,299	28,252	0.08	20,984	20,087	1.04	0.06	12.10%	12.30%	44	20,087	0.0X	0.00%	0.00%	45%
	1CVC4	23,531,439	206,803	0.88	156,328	129,126	1.17	0.66	9.10%	8.60%	54	129,126	0.0X	0.20%	0.00%	41%
	1CVC5	30,410,101	25,804	0.08	19,044	17,973	1.06	0.06	12.90%	12.80%	44	17,973	0.0X	0.00%	0.00%	44%
	1CVC6	44,410,370	82,383	0.19	60,495	58,657	1.03	0.14	8.00%	8.80%	44	58,657	0.0X	0.10%	0.00%	42%
	2CVC7	34,884,663	598,287	1.72	451,388	434,650	1.04	1.29	16.50%	16.00%	44	434,650	0.0X	0.60%	0.00%	40%
	1CVC8	79,348,498	81,758	0.1	60,679	53,906	1.11	0.08	11.40%	11.30%	44	53,906	0.0X	0.10%	0.00%	45%
	1CVC9	40,112,987	39,912	0.1	29,929	26,471	1.12	0.07	16.60%	16.50%	64	26,471	0.0X	0.00%	0.00%	44%
Algar do Barrão	1BAR3	34,565,926	1,998,178	5.78	1,583,685	1,416,101	1.11	4.58	14.20%	14.10%	54	1,416,101	0.0X	2.70%	0.00%	43%
	1BAR4	42,426,863	31,112	0.07	22,973	21,698	1.06	0.05	10.00%	11.00%	54	21,698	0.0X	0.00%	0.00%	50%
	1BAR5	34,276,839	78,179	0.23	60,440	56,958	1.06	0.18	11.20%	10.70%	44	56,958	0.0X	0.10%	0.00%	44%
Casais da Mureta	1CMR1	38,360,215	25,328	0.07	18,684	17,413	1.07	0.05	16.10%	16.20%	44	17,413	0.0X	0.00%	0.00%	45%
	1CMR2	67,018,439	11,815	0.02	8,434	7,716	1.09	0.01	5.80%	7.00%	44	7,716	0.0X	0.00%	0.00%	61%
	1CMR3	47,869,066	13,835	0.03	9,646	9,306	1.04	0.02	12.00%	12.00%	54	9,306	0.0X	0.00%	0.00%	54%
Extraction Blanks	EB9/9/24	155,984	49,355	31.64	36,764	22,268	1.39	23.57	0.30%	0.40%	44	22,268	0.0X	0.00%	0.00%	50%
	EB17/9/24	172,346	729	0.42	529	350	1.34	0.31	7.30%	4.70%	54	350	0.0X	0.00%	0.00%	46%
	EB30/9/24	62,220	110	0.18	77	42	1.45	0.12	0.00%	0.00%	44	42	0.0X	0.00%	0.00%	48%
Library Blanks	LB23/9/24	22,520	648	2.88	454	259	1.43	2.02	0.00%	0.00%	44	259	0.0X	0.00%	0.00%	42%
	1LB4/10/24	7,800	4,490	57.56	2,477	1,327	1.46	31.76	1.60%	0.60%	54	1,327	0.0X	0.00%	0.00%	52%
	2LB4/10/24	960	16	1.67	14	9	1.36	1.46	0.00%	50.00%	57	9	0.0X	0.00%	0.00%	53%

Table 16: Summary of MultiQC report from the genomic run on samples and blanks.

Site	Sample	Extraction Date	Library Date	Nr. Reads Into Mapping	Nr. Mapped Reads	Endogenous DNA Mitochondrial (%)	Nr. Mapped Reads Passed Post-Filter	Post-DeDup Mapped Reads	Cluster Factor	Endogenous DNA Post (%)	5 Prime C>T 1st base	3 Prime G>A 1st base	Median read length	Mean cov	≥ 1X	≥ 5X	% GC Dedup. Mapped Reads
La Sagrera	SA4	09/09/2024	23/09/2024	17,983,116	34	0	29	28	1.03	0	0.429	0.333	44 bp	0.1X	0.14	0	0.42
	SA18	09/09/2024	04/10/2024	42,487,652	48	0	36	34	1.06	0	0	0	44 bp	0.1X	0.09	0	0.44
	SA26	17/9/2024	04/10/2024	44,986,292	63	0	42	42	1	0	0.333	0.143	54 bp	0.1X	0.1	0	0.44
	SA43	17/9/2024	04/10/2024	40,306,604	57	0	43	36	1.16	0	0.167	0.5	44 bp	0.1X	0.1	0	0.42
	SA44	17/9/2024	04/10/2024	44,450,510	436	0	385	313	1.19	0	0.232	0.262	44 bp	1.1X	0.62	0.026	0.44
	SA45	17/9/2024	04/10/2024	43,855,925	50	0	40	33	1.18	0	0.333	0.111	44 bp	0.1X	0.11	0	0.46
	SA46	17/9/2024	04/10/2024	42,591,209	18	0	7	6	1.14	0	0	0	44 bp	0.0X	0.02	0	0.44
	SA53	17/9/2024	04/10/2024	31,291,999	139	0	89	83	1.07	0	0.278	0.333	54 bp	0.2X	0.18	0	0.43
	SA56	17/9/2024	04/10/2024	38,435,758	241	0	142	128	1.1	0	0.455	0.227	44 bp	0.4X	0.29	0.001	0.42
Convento do Carmo	SA68	17/9/2024	04/10/2024	28,373,258	396	0	261	237	1.09	0	0.133	0.06	44 bp	0.6X	0.49	0	0.43
	CVC1	09/09/2024	23/09/2024	40,293,312	1895	0	1319	1041	1.21	0	0.104	0.176	54 bp	3.0X	0.95	0.195	0.44
	CVC2	09/09/2024	23/09/2024	35,915,571	93	0	52	49	1.06	0	0.3	0.3	54 bp	0.1X	0.12	0	0.44
	CVC3	09/09/2024	23/09/2024	34,440,273	1187	0	856	819	1.04	0	0.103	0.138	44 bp	2.3X	0.91	0.087	0.44
	CVC4	09/09/2024	23/09/2024	23,536,905	721	0	461	363	1.21	0	0.145	0.152	54 bp	1.0X	0.6	0.007	0.42
	CVC5	09/09/2024	23/09/2024	30,418,987	201	0	131	126	1.04	0	0.292	0.28	44 bp	0.3X	0.29	0	0.44
	CVC6	09/09/2024	23/09/2024	44,445,905	1853	0	1619	698	1.57	0	0.123	0.151	44 bp	2.1X	0.85	0.072	0.44
	CVC7	09/09/2024	23/09/2024	34,900,330	1634	0	1103	1059	1.04	0	0.204	0.186	44 bp	3.0X	0.94	0.212	0.44
	CVC8	09/09/2024	23/09/2024	79,390,936	5944	0.01	4643	4353	1.06	0.01	0.155	0.152	44 bp	13.6X	1	0.995	0.44
Algar do Barrão	BAR4	30/9/2024	04/10/2024	40,122,861	2384	0.01	1747	1513	1.13	0	0.21	0.164	64 bp	4.3X	0.97	0.42	0.44
	BAR3	30/9/2024	04/10/2024	34,577,679	8374	0.02	6987	5573	1.2	0.02	0.149	0.152	54 bp	18.3X	1	0.999	0.44
	BAR5	30/9/2024	04/10/2024	42,439,499	689	0	596	324	1.46	0	0.125	0.137	54 bp	1.1X	0.63	0.014	0.45
	BAR4	30/9/2024	04/10/2024	34,309,679	2253	0.01	1904	1749	1.08	0.01	0.126	0.168	44 bp	6.2X	0.99	0.697	0.45
Casais da Mureta	CMR1	30/9/2024	04/10/2024	38,437,603	382	0	306	258	1.16	0	0.259	0.275	54 bp	0.8X	0.51	0.001	0.43
	CMR2	30/9/2024	04/10/2024	67,148,718	171	0	117	97	1.17	0	0.19	0.158	54 bp	0.3X	0.23	0	0.44
	CMR3	30/9/2024	04/10/2024	47,882,829	114	0	71	70	1.01	0	0.333	0.25	54 bp	0.2X	0.17	0	0.44
Extraction Blanks	EB9/9/24	09/09/2024	23/09/2024	157,501	80	0.05	73	36	1.51	0.05	0	0	54 bp	0.1X	0.12	0	0.48
	EB17/9/24	17/09/2024	04/10/2024	172,560	6	0	6	4	1.33	0	0	0	54 bp	0.0X	0.01	0	0.42
	EB30/9/24	30/09/2024	04/10/2024	62,244	0	0	0	0	0	0	0	0	44 bp				
Library Blanks	LB23/9/24		23/09/2024	23,830	0	0	0	0	0	0	0	0	54 bp				
	1LB4/10/24		04/10/2024	7,819	62	0.79	47	23	1.51	0.6	0	0	54 bp	0.1X	0.07	0	0.46
	2LB4/10/24		04/10/2024	963	1	0.1	1	1	1	0.1	0	0	57 bp	0.0X	0	0	0.51

Table 17: Summary MultiQC report from mitochondrial run on samples and blanks as well as dates of extraction and library preparation.

4.2 Analysis of Author's Own mtDNA

The results from the Sanger sequencing of the author's own HVR1 of the mitochondrial DNA can be seen in Table 18 below. A length heteroplasmy in the mtDNA-HV1 poly-C tract (positions 16193.1C and 16193.2C) was detected in the chromatograms, resulting from a T to C transition in position 16189. When analysed in Haplogrep3, the highest haplogroup assignment quality score was 88% for 13 different sub haplogroups within U5b. Samples belonging to the U5b major haplogroup and matching the author's haplotype or without coverage at the sites of the author's mutations can be assumed to be contaminated. This was not the case for any samples. Samples within U5b with coverage of HVRI and a haplotype different from the authors' can safely be assumed not to be contaminated. These can be seen in Table 18 below.

Range	Consensus Haplotype	Possible Haplogroups	Score	Expected Mutations	Local Private Mutation	Hotspots
16016-16351	16093C 16189C 16193.1C 16193.2C 16270T	U5b1b1d U5b1b1f U5b1+16189+@16192 U5b1e U5b1e1 U5b1e1a U5b1h U5b2a+@16192 U5b2a4 U5b2a4a U5b2a6 U5b2b4 U5b2b4a	88	16189C 16192T! 16270T	16093	16193.1C 16193.2C

Table 18: Results of analysis on the author's own mtDNA HVRI.

4.3 Mitochondrial Haplotype and Haplogroup Analysis

Mitochondrial haplotype and haplogroup identifications from the direct analysis of the sequencing results of the mitochondrial capture with Haplogrep3 (Schönherr et al., 2023) can be seen in Table 19. While haplogroups with a score of 94-97% could be assigned to CVC8, CVC9, BAR3, and BAR5, other samples only had haplogroup scores below 88%. This original haplogroup assignment included 16 different haplogroups, only 4 of which received a score over 90% (CVC8: H3r, 94%; CVC9: U5b1c, 95%; BAR3: U5b2b*, 95%; BAR5: U5b1c, 97%). In 8 samples, no mutations from rCRS were detected, so these samples were reported as belonging to haplogroup H2a2a1, as this is the haplogroup that contains the rCRS reference genome (Andrews et al., 1999). Visual inspection of each BAM file in IGV revealed mutations that were previously not called. The results from this visual inspection can be seen in Table 19. These results were then used to generate new VCF input files that were analysed using Haplogrep3. The results from this second analysis were much more successful, with haplogroup assignments for ten individuals receiving a score greater than 90, including a score of 100 for CVC1. Individuals CVC2 and BAR4 received scores of 87 and 81 respectively, while the remaining 13 samples received scores lower than 80. Although major haplogroup diagnostic mutations may have been present in these samples, the low coverage and the possibility of contamination meant that these results could not be considered reliable and were therefore not considered during population analysis.

Site	Sample	Results of Manual Haplotype Assignment	Haplogroup from first analysis	Score	New Haplogroup	New Score
La Sagrera	1SA4	4769G(1) 10398G(1) 14796C(1)	H2a2a1	50	J1c	55
	1SA18	3091T(1) 310C(1) 1135T(1) 7034T(1) 15326G(1)	H2a2a1	50	U4a2a	54
	1SA26	15326G(1)	H2a2a1	50	H2a2	67
	1SA43	750G(2) 6734A(1) 6746T(1) 6750T(1) 6885A(1) 15326G(1)	H2a2a1	50	H10c	67
	1SA44	185A(1) 263G(1) 462T(1) 489C(1) 3010A(1) 4216C(6) 4769G(3) 5596T(1) 7028T(1) 8860G(2) 8959A(1) 10398G(2) 11251G(1) 14798C(8) 15326G(4) 16093C(1)	H1bm	59	J1c	76
	1SA45	15326G(2) 16311C(2)	H2a2a1	50	H2+152+16311	62
	1SA46	1811G(2) 14639G(1) 14766T(1) 15326G(2) 16519C(1)	H2a2a1	50	U234789	60
	1SA53	73G(1) 185A(1) 263G(1) 462T(1) 750G(1) 3394C(3) 3497T(1) 13107T(1) 15452A(1)	M9a/b	51	H2a2a	57
	1SA56	225A(1) 226C(1) 263G(1) 1438G(1) 2706G(1) 8860G(2) 11403T(1) 13708A(1) 14470C(1) 15541C(1) 16129A(1) 16278T(1)	H2a2a1	50	X2b4*	60
	1SA68	263G(2) 750G(1) 1438G(2) 4769G(1) 8860G(1) 15326G(3) 16298C(1)	H	75	H	95
Convento do Carmo	1CVC1	73G(2) 150T(3) 263G(6) 750G(8) 1438G(3) 1811G(7) 2294G(3) 2706G(1/2) 3010A(8) 4703C(2) 4769G(3) 6518T(4) 7028T(3) 8572A(4/5) 8860G(3) 9266A(5) 10506G(5) 11467G(12) 11719A(7) 12308G(13) 12372A(1) 13934T(4/5) 14139G(4) 14766T(4) 15326G(7) 15454C(2) 16209C(6) 16214T(6) 16343G(2) 16390A(3) 16519C(8)	U3a1	87	U3a1	100
	1CVC2	263G(1) 750G(2) 8705C(1) 8860G(2) 15326G(1) 15555T(1) 16255A(1)	H2a2a1	50	H2a	87
	1CVC3	73G(2) 263G(1) 750G(1) 1438G(1) 1811G(1) 2294G(1) 2706G(1) 3010A(4) 4703C(5) 6518T(4) 7028T(1) 8131T(4) 8860G(4) 9266A(2) 10506G(3) 11467G(4) 11719A(3/4) 12308G(3) 12372A(3)	U3a	68	U3a1	97
	1CVC4	13934T(2) 14139G(1) 14766T(3) 15326G(1) 15454C(1) 16343G(6) 16390A(3/4) 16519C(3)	H2a2a1g	53	V23	99
	1CVC5	72C(3) 263G(3) 750G(3) 1438G(3) 2706G(1) 2865A(1) 4580A(2/3) 4769G(1) 5560T(1) 6734A(5/6) 7028T(1) 8860G(4) 10654A(1) 15326G(3) 15904T(1) 16093C(1) 16298C(3)	U5a	55	U5a1c1	75
	1CVC6	750G(1) 2706G(1) 5818A(1) 8860G(1) 9450T(1) 9477A(1) 10353T(1) 10587A(1) 10999A(1) 11719A(1) 12308G(3) 13802T(2) 14766T(2) 14793G(2) 15218G(1) 16192T(1) 16270T(1) 16543A(2)	T2+150	73	T2e1	95
	1CVC7	411T(1) 73G(1) 150T(2) 263G(4) 310C(2) 709A(3) 750G(3) 1888A(1) 2706G(1) 4216C(1) 4769G(1) 4917G(2) 7028T(1) 8697A(3) 8860G(1) 10463C(2) 11251G(9) 11719A(3) 11812G(5) 13368A(2) 14233G(1) 14766T(2) 14905A(1) 15326G(6) 15452A(1) 15607G(2/3) 15928A(3) 16126C(1) 16153A(2) 16294T(2)	J1c3	84	J1c3	96
	1CVC8	73G(1) 185A(2) 228A(2) 263G(5) 295T(4) 462T(2/3) 489C(6) 723G(7) 750G(3) 1438G(4) 2706G(3) 3010A(5) 4216C(6/7) 4769G(8) 7028T(4) 8860G(1) 9242G(2) 10398G(5) 11251G(3) 11719A(7) 12612G(2) 13708A(7) 13934T(3) 14766T(4) 14798C(4) 15326G(8) 15452A(6) 16069T(2/3) 16126C(6/7)	H3r	94	H3r	94
	1CVC9	263G(11) 750G(17) 961C(8) 1438G(16) 4769G(8) 6776C(13) 7824C(14) 8860G(14/15) 15326G(44) 16311C(14) 16519C(13)	U5b1c	95	U5b1c	98
		73G(3) 150T(6) 263G(7) 750G(4) 1438G(10) 2706G(10) 3197C(3) 4769G(3) 5656G(10) 7028T(9) 7768G(9) 8860G(5) 9477A(11) 11467G(3) 11719A(6) 12308G(5) 12372A(3) 13617C(7) 14182C(9) 14766T(10) 15191C(6) 15326G(11/12) 16189C(2) 16192T(1) 16270T(9) 16311C(2)	U5b1c	95	U5b2b*	99
Algar do Barrão	1BAR3	73G(19) 150T(23) 189G(30) 263G(25) 750G(26) 1438G(16) 1721T(18) 2706G(22) 3197C(16) 4769G(19) 7028T(25) 7768G(21) 8860G(25) 9477A(46) 11434T(14) 11467G(14) 11653G(22) 11719A(27) 12308G(23) 12372A(17) 12634G(18) 13617C(17) 13630G(13) 13637G(11) 13759A(15) 14182C(16) 14766T(18) 15299C(32) 15326G(42) 16270T(10)	U5b2b*	95	U5b2b*	99
	1BAR4	73G(3) 1141A(4) 263G(4) 497T(1) 1438G(3) 1811G(2) 2706G(3) 3480G(1) 4769G(3) 9698C(1) 9986A(1) 10398G(3) 10550G(3) 11059T(2) 11299C(1) 11467G(2) 11470G(2) 11719A(3) 12308G(3) 14040A(3) 14167T(2) 14166T(1) 14798C(1) 15326G(4) 16093C(2) 16129A(2) 16311C(1)	K1a1	64	K1a1b1	81
	1BAR5	73G(6) 150T(6) 263G(5) 310C(4) 750G(8) 1438G(7) 2706G(11) 3197C(6) 4769G(6) 5656G(6) 7028T(9) 7768G(6) 8860G(8) 9477A(11) 11467G(10) 11719A(7) 12308G(3) 12372A(4) 13617C(11) 14182C(11) 14766T(9) 15191C(6) 15326G(10) 16189C(12) 16192T(13/14) 16270T(10) 16311C(6)	U5b1c	97	U5b1c	98
Casais da Mureta	1CMR1	72C(1) 750G(2) 1438G(1) 1770A(1) 2706G(1) 4580A(2) 4769G(3) 7028T(1) 12762G(1) 12792T(1)	V+@72	59	V+@16298	74
	1CMR2	750G(1) 1438G(1) 2706G(1) 3470G(1) 4580A(1) 7028T(2) 12242G(1) 12792T(2)	B4a1c*	52	V+@72	65
	1CMR3	4831A(1) 5452T(1) 5457C(1) 9157A(1) 9477A(1) 11340C(1) 11467G(1) 11653G(1) 12308G(1) 12451G(1) 13121T(1) 14182C(1) 15326G(2) 15758G(1)	H2a2a1	50	H2a2a1	50
Extraction Blanks	EB9/8/24	7028T(1) 7385G(1) 10978G(1) 12372A(1) 13617C(1) 15326G(1)			U5b1b1+@16192	52
	EB17/9/24	4769G(1)			H2	59
	EB30/9/24					
Library Blanks	LB23/9/24					
	1LB4/10/24	73G(1) 5441G(1) 6308T(1) 16111T(1)			H2a2a1	50
	2LB4/10/24					

Table 19: Haplotype and haplogroup assignments of studied individuals. Number next to mutation refers to coverage at that position. Mutations in red have less than 5X coverage, mutations in black have at least 5X coverage, and mutations in blue have a read without the mutation present (number of reads with mutation/number of total reads).

4.4 Kinship Analysis

Although the quality of the DNA recovered in the samples from La Sagrera was low, kinship analysis could still be completed. Due to the low and uneven mtDNA coverage in the samples, haplotype comparisons could not be used to infer maternal relationships. Instead, the analysis focused on shared individual polymorphisms in positions with good coverage assuming that if different mutations were present, it demonstrated a lack of matrilineal relation between them. Positions with mutations that were deemed possible contamination DNA were not considered in this analysis.

As seen in Table 20 below, many individuals did not have different mutations in positions where both individuals had coverage, which means that they could possibly be related. While many comparisons between individuals only provided one or two differences, some relations provided stronger evidence of no matrilineal kinship. Individuals SA44 and SA56 had different mutations at 12 positions. Individuals SA53 and SA68 had 5 positions with different mutations. Four contrasting mutations were found between the pairs of individuals SA44 and SA53, SA44 and SA68, SA53 and SA18, and SA56 and SA68 each.

Cluster	Individual	SA26	SA43	SA44	SA45	SA46	SA53	SA18	SA56	SA68	SA4
a	SA26										
	SA43	6885(1)(1)									
	SA44										
	SA45		16311(1)(2)	8959(1)(1)							
	SA46										
	SA53		3394(1)(3) 6885(1)(2)	3394(6)(3) 3497(1)(1) 13107(2)(1)							
b	SA18		1135(1)(1) 6885(1)(1)	462(1)(1) 1135(1)(1) 7034(1)(1)	7034(1)(1)		462(1)(1) 489(1)(1) 1135(1)(1) 13107(1)(1)				
	SA56			225(1)(1) 226(1)(1) 3010(1)(1) 4216(6)(2) 5596(1)(1) 10398(2)(1) 11404(1)(1) 13708(1)(1) 14470(1)(1) 15541(3)(1) 16129(1)(1) 16278(4)(1)							
					16278(2)(1)	14639(1)(1)	3394(3)(1) 3497(1)(2)	16278(1)(1)			
	SA68		6885(1)(1)	462(1)(2) 3010(1)(1) 8959(1)(2) 10398(2)(1)	16298(1)(1)	1811(2)(3) 14639(1)(2)	462(1)(2) 489(1)(1) 3394(3)(1) 3497(1)(1)	1135(1)(3)	225(1)(2) 226(1)(2) 13708(1)(1)		
							15452(1)(1)		16278(1)(1)		
c	SA4			10398G(1)					10398(1)(1)	10398(1)(1) 14798(1)(1)	

Table 20: Comparative base positions between individuals at La Sagrera. Yellow boxes indicate no positions with mutations that had coverage in both individuals. Red boxes indicate at least one contrasting base position. The green box indicates the individuals that had no different mutations in positions with coverage in both individuals and has a shared mutation. Notation is: Position(number of reads at position in x-axis individual)(number of reads at position in y-axis individual)

4.5 Haplogroup Comparative Analysis

Individuals with a final haplogroup assignment score greater than 80 from Haplogrep3 and which had passed previous quality filters were considered for this analysis.

4.5.1 Haplogroup distribution within ancient samples

The first aspect of the analysis involved identifying individuals that share the same haplogroups as the studied samples in the Allan Ancient DNA Resource database (AADR) (Mallick et al., 2024). This is the most comprehensible compilation of published results from NGS sequencing studies of ancient individuals. Maps showing the distribution of these haplogroups across space and time can also be seen in section [4.5.1](#).

Individual CVC4 from Convento do Carmo was assigned to haplogroup V23. Only one individual with haplogroup V23 was present in the database. This individual was found at the site of Casterley Camp hillfort in Wiltshire, England, and dated to 342-351 cal BC (Patterson et al., 2022). Other subgroups within haplogroup V are common in the Neolithic and later periods throughout Europe.

Haplogroup H3r was identified in individual CVC8. This haplogroup along with any of its sub lineages were not found in the database (Mallick et al., 2024). Other H3 lineages are found across the entirety of Europe in the Neolithic and Later periods, with the first evidence of H3 coming from the site of Lepenski-Vir in Serbia (~6150 cal BC)(Marchi et al., 2022). In Iberia, the first instance of H3 is in a Middle Neolithic

individual at the site of Lugar do Canto in Portugal (~4000 cal BC)(Martiniano et al., 2017).

Two individuals from Convento do Carmo, CVC1 and CVC3, were assigned to haplogroup U3a1. This haplogroup is found in 29 individuals in the AADR. The distribution of these individuals can be seen in Figure 15 below. The earlier reports of this haplogroup correspond to two individuals from the Linear Bandkeramik Neolithic site of Asparn Schletz in Austria dated back to 7100 years BP (Gelabert et al., 2025). This haplogroup is then present in other central and eastern European sites from the Neolithic to the Medieval period in modern day Hungary, Romania, and Czechia (Gelabert et al., 2025; Papac et al., 2021; Penske et al., 2023). In the Iberian Peninsula, the earliest report of the haplogroup corresponds to a Middle/Late Neolithic person of the third phase at the site of Cova de Els Trocs in the Spanish Pyrenees (Villalba-Mouco et al., 2019). This haplogroup is also present in Middle and Late Neolithic sites throughout Western Europe, before becoming less common in the Bronze Age and later periods (Fowler et al., 2022; Mathieson et al., 2015a; Olalde et al., 2018). Other instances in Iberia include one individual at the Chalcolithic site of El Mirador Cave in Sierra de Atapuerca, Burgos, Spain (Mathieson et al., 2015a) and one from the Middle Iron Age site of Els Estrets de la Rata near Vilafamés in Castellón de La Plana, Spain (Olalde et al., 2019). This haplogroup has not been found so far in any other studied ancient individuals within Portugal. In addition, no individuals within any other sub-haplogroups within U3 have been reported in Iberia.

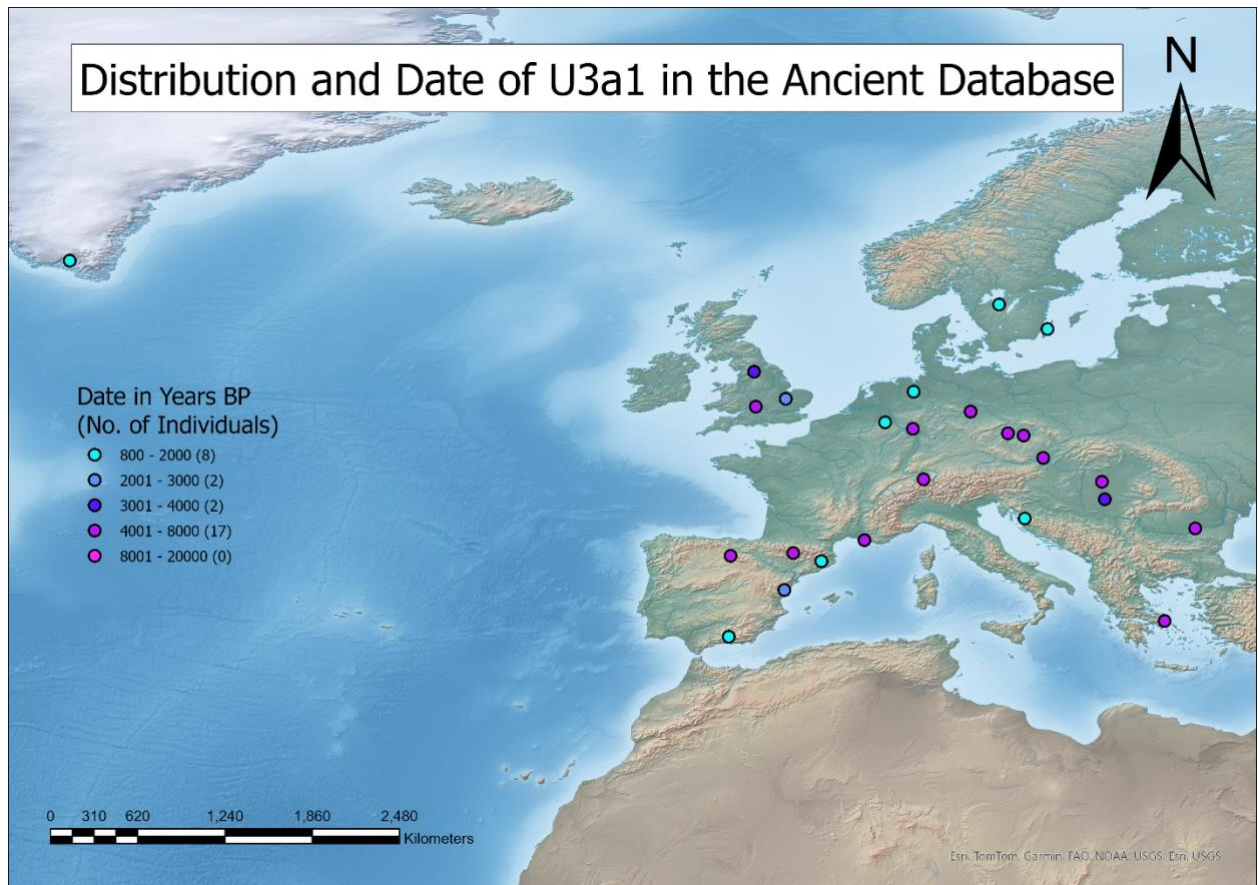


Figure 15: Distribution of individuals of the mitochondrial haplogroup U3a1. Points are coloured based on the mean radiocarbon and contextual dating results in years BP. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6. Detailed information on samples represented in this map can be found in [Supplementary Table S5](#).

Only 14 individuals belonging to haplogroup T2e1, found in sample CVC6 from Convento do Carmo, are present in the database. The distribution of these individuals can be seen in Figure 16 below. The T2e1 haplogroup first appears in the Neolithic in 5 individuals in Britain, including 4 from the site of Hazelton North in Gloucestershire, dated to around 3932-3350 cal BC (Fowler et al., 2022) and one from The Tullochs of Assery in Caithness, Scotland, dated to around 3768-3638 cal BC (Olalde et al., 2018). A Bell-Beaker context individual near Kornice, Poland, dated to 2455-2147 Cal BC was also found to belong to the T2e1 haplogroup (Olalde et al., 2018). The distribution of individuals belonging to this haplogroup seems to be concentrated in Northern Europe, with just one individual from the Early Bronze Age site of Bray Cave in Gibraltar, dated to around 1900-1400 cal BC (Olalde et al., 2019) falling outside of this region. In addition, although T2e appears in 60 individuals throughout Europe in the ancient database, only one other instance of this haplogroup is found in ancient Iberia: one individual from another Early Bronze Age site also on the southern part of the Iberian Peninsula, at the site of Loma del Puerco (Cádiz) in modern day Spain (Olalde et al., 2019). No other individuals harbouring haplogroup T2e are found in France, creating a large geographic divide between the two individuals in Southern Iberia and the rest of the

individuals in the database, which are found throughout Central and Eastern Europe, Italy, Ireland, and the United Kingdom (Mallick et al., 2024).

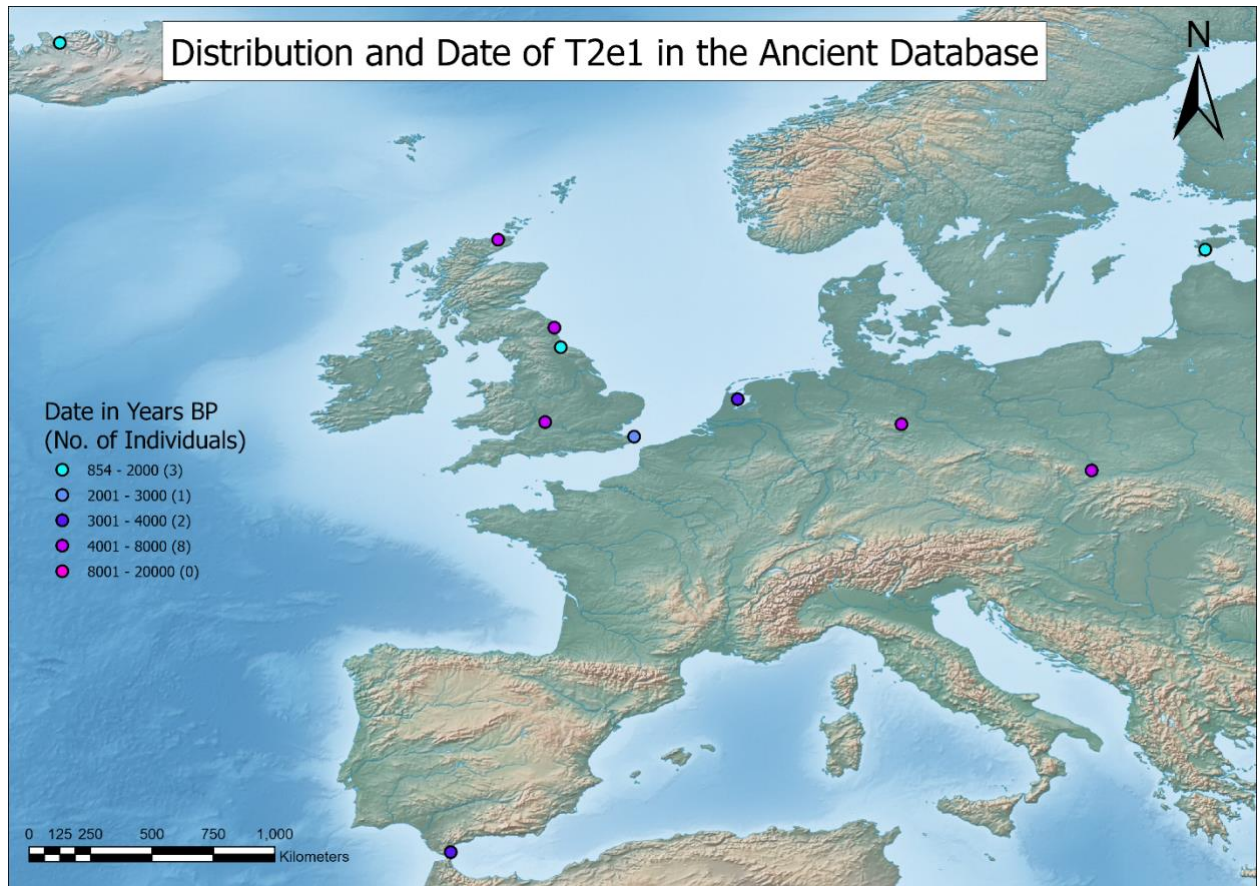


Figure 16: Distribution of individuals of the mitochondrial haplogroup T2e1. Points are coloured based on the mean radiocarbon and contextual dating results in years BP. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6. Detailed information on samples represented in this map can be found in [Supplementary Table S6](#).

Haplogroup J1c3, which was determined to be the haplogroup of individual CVC7 in this study, was also found in 45 studied individuals in the AADR database seen in Figure 17. The earliest of these individuals was found at the Early Neolithic site of Pendimoun on the southeast coast of France, dated to around 5479-5331 cal BC (Rivollat et al., 2020). An individual at the Early Neolithic Spanish Pyrenees site of Cova de Els Trocs is of a slightly later date of around 5312-5212 cal BC (Mathieson et al., 2015a). Other Iberian individuals harbouring this haplogroup include three from the Chalcolithic, one from the Early Bronze Age and one from the Late Bronze Age, all located in the eastern side. Haplogroup J1c3 is also found in other Central and South European Neolithic individuals and in two individuals from Ireland (Cassidy et al.,

2020). This haplogroup appears more broadly distributed throughout Europe from the Bronze Age.

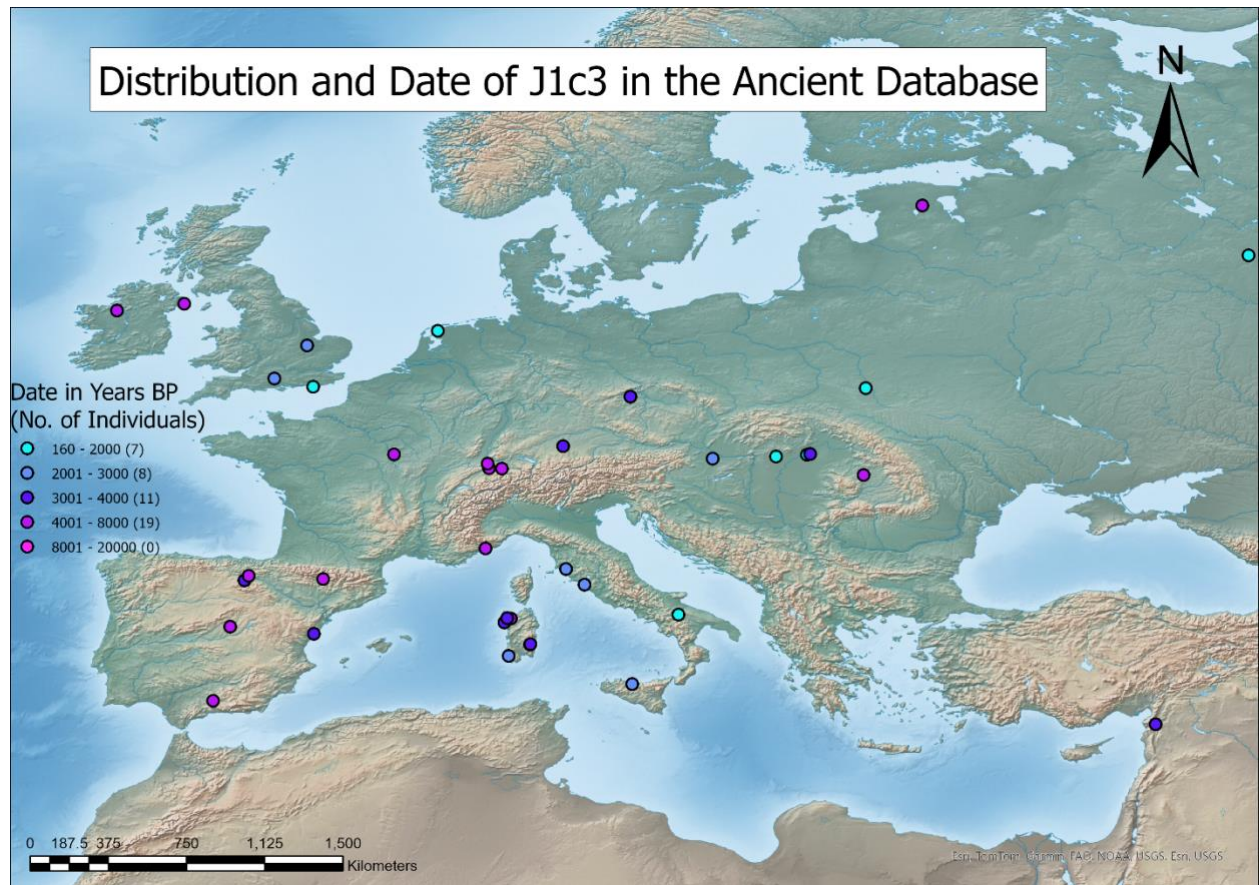


Figure 17: Distribution of individuals of the mitochondrial haplogroup J1c3. Points are coloured based on the mean radiocarbon and contextual dating results in years BP. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6. Detailed information on samples represented in this map can be found in [Supplementary Table S7](#).

Haplogroup U5b2b*, a subclade of U5b2b, was found in BAR3 but does not appear in any other individuals from the AADR database. U5b2b* is basal to U5b2b sub-lineages and distinguished from U5b2b by the additional mutations 189G 13759A 15299C and 16261T. U5b2b is present in 49 individuals, the distribution of which can be seen in Figure 18. This haplogroup first appears in two Epigravettian individuals from San Teodoro cave on the island of Sicily, dating from between 17171-15091 cal BC (Scorrano et al., 2022). The haplogroup is also found in Central and Eastern Europe in Epigravettian and Mesolithic individuals, being found at sites in Italy, France, Croatia, Serbia, and Ukraine (Allentoft et al., 2024; Fu et al., 2016; Mathieson et al., 2018; Scorrano et al., 2022; Yu et al., 2022). The earliest appearance of this haplogroup in Iberia comes from an individual at the Mesolithic site of Cueva de la Cocina in Valencia, dated to around 6064-5926 cal BC (Olalde et al., 2019). Other Iberian individuals with this haplogroup include a Middle to Late Neolithic one from the Spanish site of Les

Llometes (Olalde et al., 2019), two Chalcolithic ones from the Spanish sites of Alto de la Huesera (Olalde et al., 2019) and Arroyal I (Olalde et al., 2018), and another Chalcolithic one from the Portuguese site of Bolores (Olalde et al., 2019). U5b2b becomes significantly less common in Europe after the Neolithic, with only 7 individuals dated between 4000-500 years BP assigned to this haplogroup.

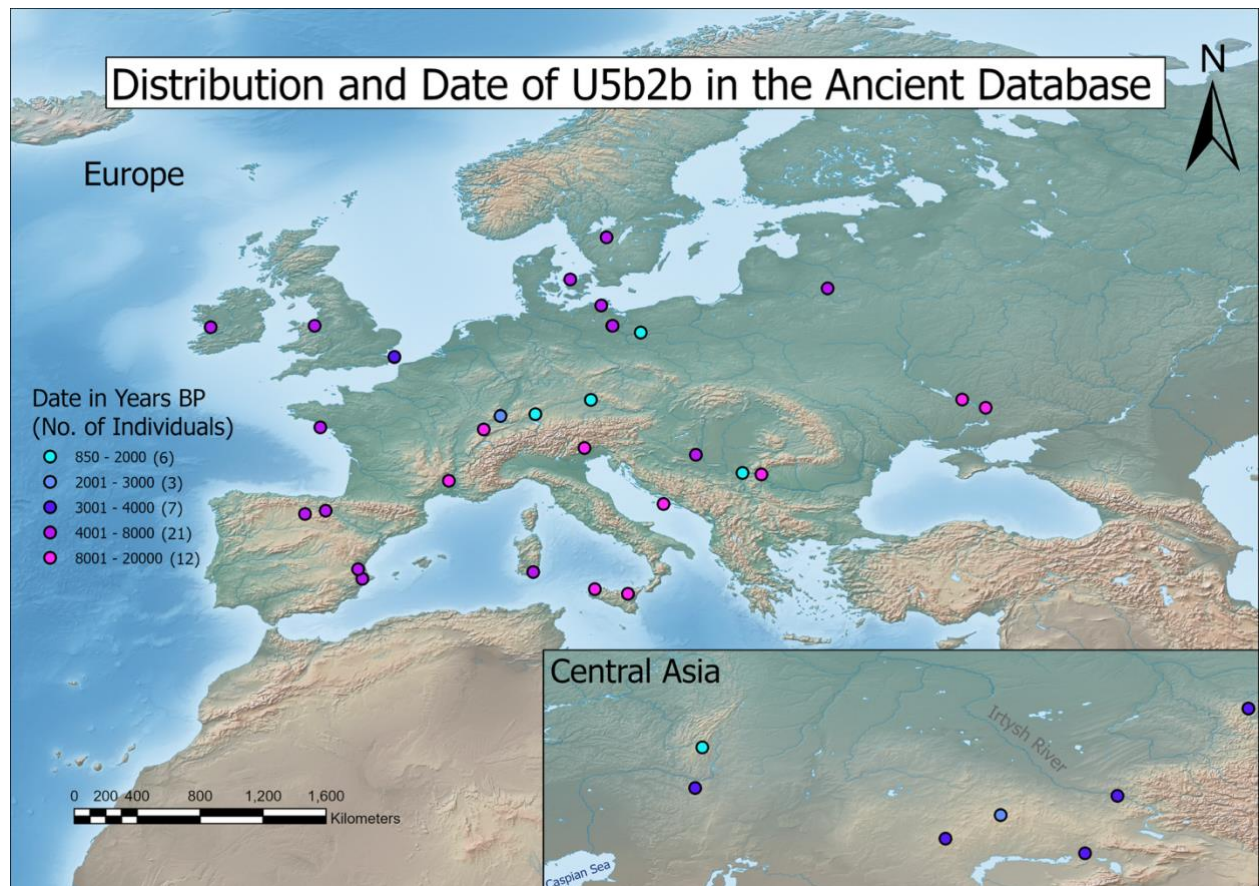


Figure 18: Distribution of individuals of the mitochondrial haplogroup U5b2b. Points are coloured based on the mean radiocarbon and contextual dating results in years BP. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6. Detailed information on samples represented in this map can be found in [Supplementary Table S8](#).

The haplogroup U5b1c from samples CVC9 and BAR5 was only present in 12 individuals in the AADR database, seen in Figure 19. No pre-Neolithic individuals have been found with this haplogroup. The earliest report corresponds to one individual from the Middle Neolithic site of Gurgy Les Noisats in France, dated to 4699-4537 cal BC (Rivollat et al., 2023), and it is also present among Neolithic individuals from Ireland and Scotland. In Iberia, two individuals have been reported as belonging to U5b1c: one from the Chalcolithic site of Camino de las Yeseras dated to 2130-1896 cal BC (Olalde

et al., 2018) and one from the Early Bronze Age site of Can Roquetes dated to 2000-1400 cal BC (Olalde et al., 2019).

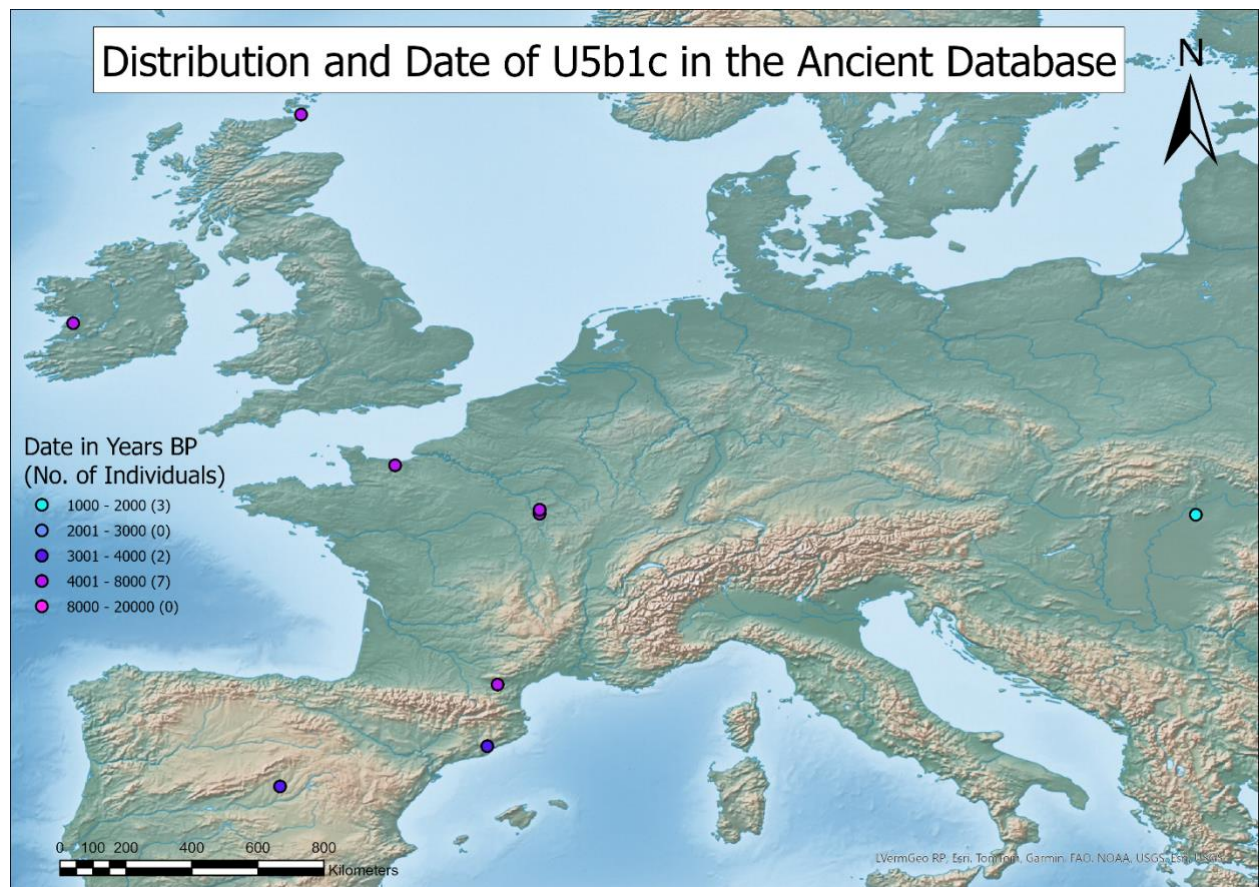


Figure 19: Distribution of individuals of the mitochondrial haplogroup U5b1c. Points are coloured based on the mean radiocarbon and contextual dating results in years BP. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6. Detailed information on samples represented in this map can be found in [Supplementary Table S9](#).

K1a1b1, the haplogroup of individual BAR4 in this study, was found in 27 individuals of the AADR database (Figure 20). The earliest individual was from the site of Pendimoun in France, dated to 5478-5331 cal BC (Rivollat et al., 2020). This haplogroup appears frequently throughout the Neolithic and Chalcolithic in Western Europe, where it is present in 20 individuals, alongside one individual in Ukraine. It decreases in later periods, being only found in 3 Bronze Age and 4 Iron Age individuals. In Iberia, the earliest report of this haplogroup corresponds to one individual from the Middle to Late Neolithic site of Les Llometes, dated to 3952-3713 cal BC (Olalde et al., 2019). It is then present in another Middle to Late Neolithic individual from the site of La Mina (Mathieson et al., 2015a), and then in 5 Chalcolithic individuals in two sites in Spain and one site in Sardinia.

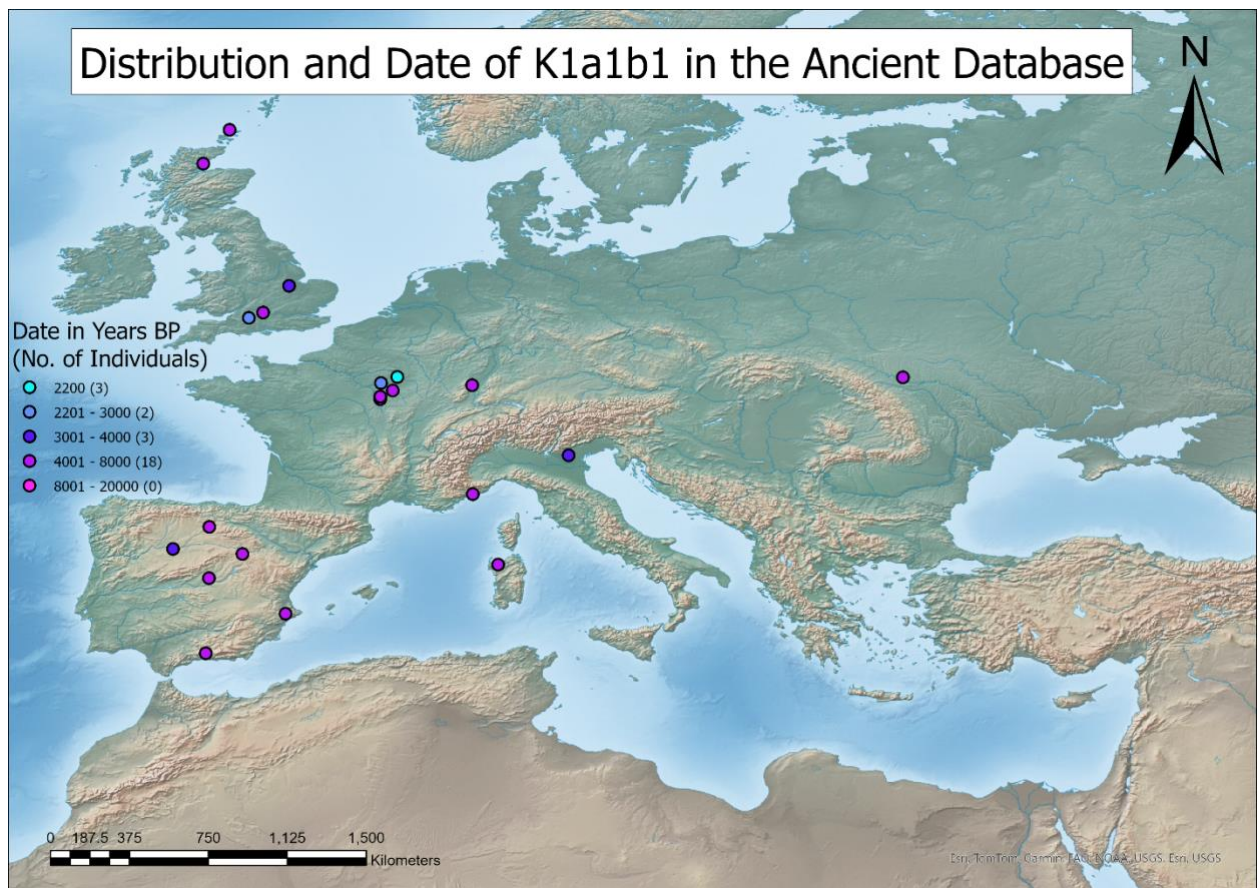


Figure 20: Distribution of individuals of the mitochondrial haplogroup K1a1b1. Points are coloured based on the mean radiocarbon and contextual dating results in years BP. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6. Detailed information on samples represented in this map can be found in [Supplementary Table S10](#).

4.5.2. Population haplogroup frequency comparative analysis

A map of the locations and archaeological period of each of the sites included in the comparative analysis can be seen in Figure 21 below. Archaeological and genetic data from the comparative individuals can be found in [Supplementary Table S4](#). Individuals were assigned to archaeological periods based on the information in the AADR resource, and to geographic regions based on the longitude and latitude coordinates reported (Mallick et al., 2024). This project added 10 new individuals to the published dataset. These were individuals CVC1, CVC3, CVC4, CVC6, CVC7, CVC8, CVC9, BAR3, BAR4, and BAR5. The three individuals from Algar do Barrão were added to the Middle to Late Neolithic West group. The 8 Convento do Carmo individuals with Haplogrep3 scores of over 80 were added to the Chalcolithic West group. Percentages mentioned in this section are rounded to the nearest whole number.

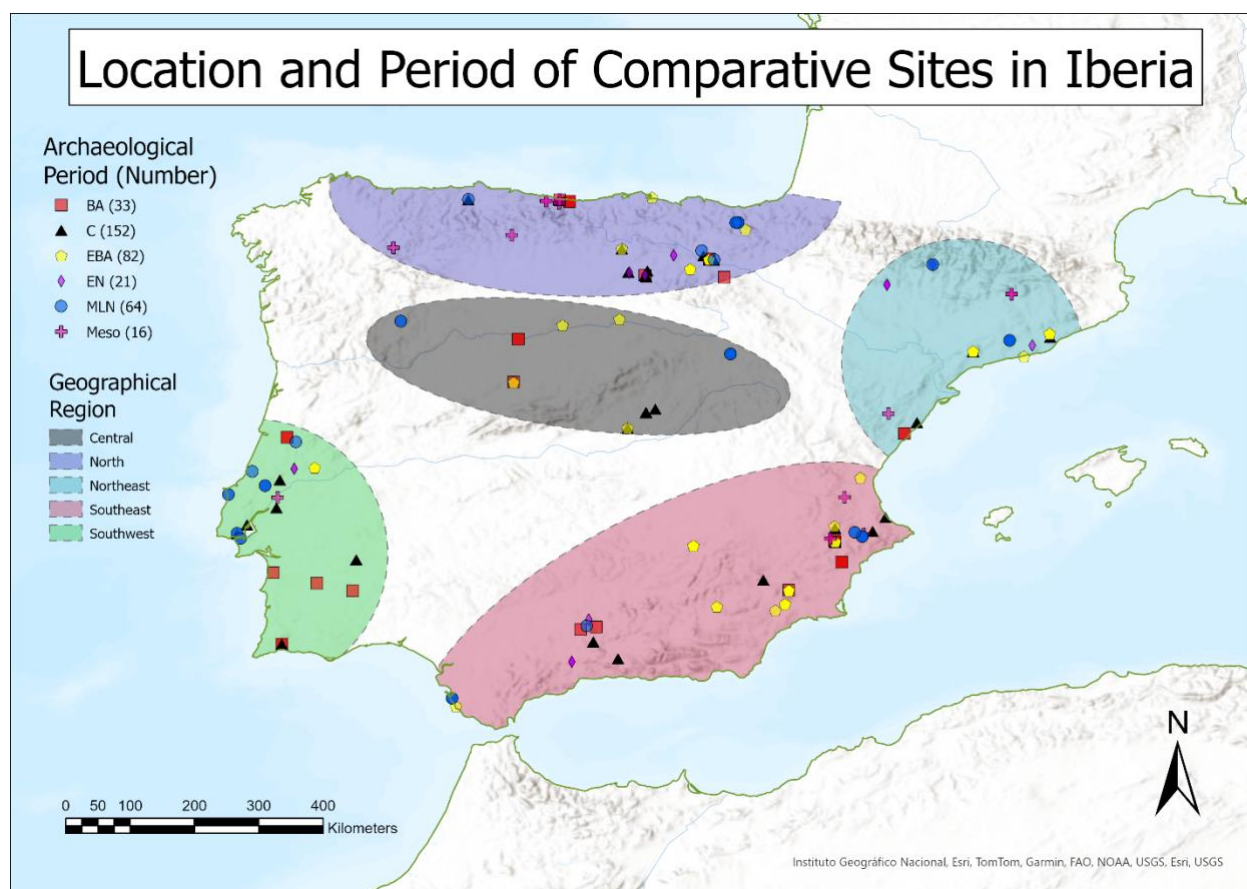


Figure 21: Location and Period of Comparative Sites in Iberia. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6.

Mesolithic

The haplogroup assemblage of published Mesolithic Iberian individuals is composed entirely of U haplogroups. As seen in Figure 22, 63% of individuals belong to the major haplogroup U5b. Of the remaining, four representing 25% belong to haplogroup U5a while two (12%) were classified as part of the U2'3'4'7'8'9 group.

Early Neolithic

The haplogroup composition of Iberia changed dramatically in the Early Neolithic. As seen in Figure 23, only one (5%) studied individual belongs to the U major haplogroup that dominated the Mesolithic period. Three (15%) Early Neolithic individuals belong to the major R0 branch of the mtDNA phylogenetic tree, including one H1 (5%), 1 HV0 (5%) and 1 V (5%). Eight (37%) are part of the JT branch, including 2 J2b (9%), 2 J1c (9%) and 4 T2 (19%) individuals. The most frequent major haplogroup during this period was K1a, with 7 individuals (33%) belonging to this group.

Additionally, one individual (5%) from haplogroup N1 and one (5%) from X2 are also present.

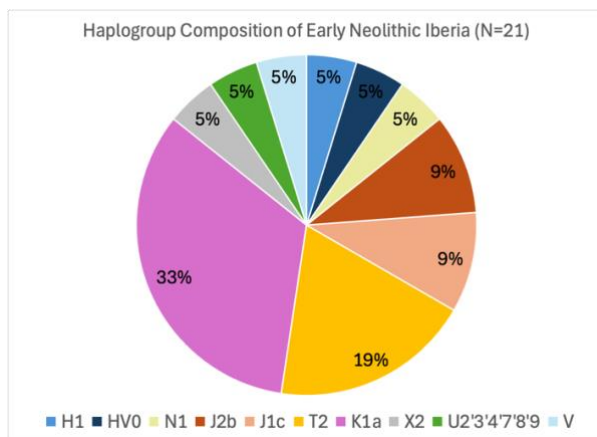
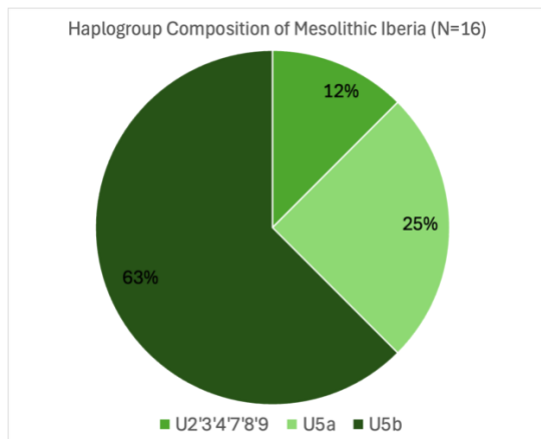


Figure 22 (Top): Frequency pie chart of major haplogroups from published Mesolithic Iberian individuals. A total of 16 individuals are represented in this figure.

Figure 23 (Bottom): Frequency pie chart of major haplogroups of published Early Neolithic Iberian individuals. A total of 21 individuals are represented in this figure.

Middle and Late Neolithic

The Middle to Late Neolithic period had more published mtDNA data available than previous periods. This data was organised by region to provide a more nuanced picture of the genetic composition of Iberia during this period. A map including the haplogroup compositions of the regions can be seen in Figure 24.

U haplogroups (in green) make up a larger percentage (24%) during the Middle to Late Neolithic compared to the Early Neolithic period (5%). Two of 12 individuals (17%) in the North, 6 of 16 individuals (38%) in the Northeast, 5 of 14 individuals (36%) in the West, 2 of 6 individuals (34%) in the Centre, and 4 of 20 individuals (20%) in the Southeast harbour U haplogroups. The most common U haplogroup is U5b, which appears in every population group.

Members of the R0 macro-haplogroups (in blue), which was previously discovered in 3 Early Neolithic individuals (15%), appear here also in every Middle to Late Neolithic population group. The most common of these lineages is H1, which also appears in every group. Unclassified H haplogroups are found in 2 individuals (13%) from the Northeast group, 1 (5%) from the Southeast group and 1 (7%) from the West group. Three H3 individuals are present in this period, with one each in the North, Central, and West groups. HV0 is present in 2 individuals (10%) from the Southeast group and 1 (7%) from the West group. Two V individuals are present, one (7%) in the West group and one (8%) in the North group. Overall, these haplogroups appear more prominently in the North, Central, and West groups, and in lower percentages in the Northeast and Southeast groups.

Individuals with haplogroup assignments with the JT branch of the mtDNA phylogenetic tree only appear in the North (33%), Northeast (31%), and Southeast (15%) groups. They are completely absent from the Central and West groups. T2 only appears in 2 individuals, one (6%) from the Northeast group and one (5%) from the Southeast group. J1c appears in 3 samples (19%) from the Northeast group and 1 (8%) in the North group. J2b appears in 3 individuals, with 2 (10%) from the Southeast group and 1 (6%) from the Northeast. Interestingly, J2a only appears in the North group, and makes up a significant percentage (25%) of that population group.

Haplogroup X2 is also only found in the North (1, 8%), Northeast (1, 6%), and Southeast (3, 15%) groups during the Middle to Late Neolithic period in Iberia.

K1 sub-haplogroups, the most common in Early Neolithic Iberia, appear in significant proportions in the West, Central, and Southeast groups. Two individuals, one K1a (17%) and one K1b (17%), could be found in the Central group. In the West population, 3 K1a (22%) and 1 K1b (7%) were present. The Southeast group included 4 K1a (20%) and 2 K1b (10%) members. In contrast to the other geographical groups, only one member of K1a is present in each of the North (8%) and Northeast (6%) groups.

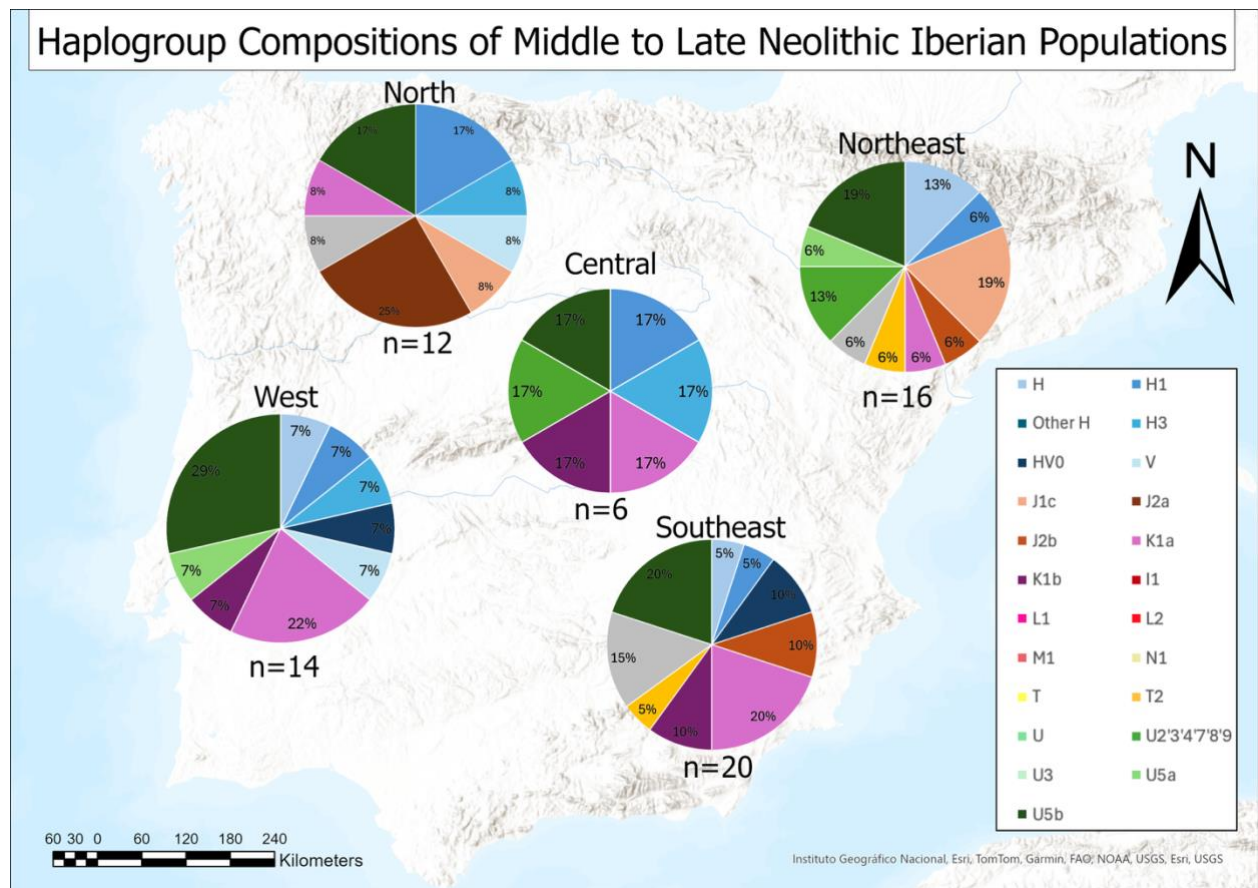


Figure 24: Map of haplogroup compositions of Middle to Late Neolithic Iberian populations based on groupings seen in Figure 21. Numbers on graphs represent number of individuals. n= total individuals in the population group. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6.

Chalcolithic

The Chalcolithic period in Iberia is the most well represented in the ancient DNA literature, with a total of 159 individuals across the five population groups. After increasing in frequency between the Early and Middle to Late Neolithic periods as seen in Figure 23 and Figure 24 respectively, U haplogroups become less frequent during the Chalcolithic in every group besides the Central one (See Figure 25). In this, 33%(n=10) of analysed samples are of the U5b haplogroup. Two members from the Northeast group (17%), 6 (11%) from the North group, 5 (11%) from the Southeast group, and 3 (17%) from the West group belong also to U5b. Before this study, only two U5b individuals were present in the West group. This study added another U5b individual as well as two U3 individuals, a haplogroup which was previously not found in this population group. This raises the frequency of U3 from 0% to 11% in the Chalcolithic West Iberia population. U3 is also present in one individual (2%) from the North group. U5a is the only other U haplogroup found in individuals from the Chalcolithic period, with 3 (6%) examples in the North group and 1 (2%) in the Southeast group.

The frequency of haplogroups within the R0 branch remains similar between the Middle to Late Neolithic and Chalcolithic periods except for the Northeast group. The frequency of R0 haplogroups in this group increased from 19% in the Middle to Late Neolithic to 58% in the Chalcolithic period. The R0 haplogroups in the Chalcolithic Northeast are predominantly H1, with 5 individuals (41%) belonging to this haplogroup. One H (8%) and one H5 (8%) are also present in this group. H1 is also the predominant R0 haplogroup in the other population groups apart from the North. The North group contains 10 H3 members (19%), and only 3 H1 members (6%). H3 also presents in one individual in each of the Central, West, and Southeast groups. A collection of diverse H haplogroups have been discovered in the Chalcolithic period and are referred to in Figure 25 as 'Other H' for simplicity. These include H5 in the Northeast (n=1, 8%), H4 in the Central (n=1, 3%) and Southeast (n=2, 5%), H2 in the West (n=1, 5%), and H45 in the North (n=1, 2%).

Individuals belonging to haplogroups within the JT branch of the mtDNA phylogenetic tree appear in every population group except for the Northeast in the Chalcolithic period. J1c is again the most common haplogroup, appearing in 6 North (11%), 2 Central (7%), 1 West (5%) and 6 Southeast (13%) individuals. J2a is present in 2 individuals from the North group (4%), 1 (3%) from the Central group, and 1 (2%) from the Southeast group. J2b is observed in 1 member (2%) of the North group, 2 (11%) of the West group, and 7 (15%) of the Southeast group. Haplogroup T2 is not present in the Northeast in the Chalcolithic, but appears in 1 North (2%), 1 West (6%), 3 Central (10%), and 2 Southeast (5%) individuals. One T haplogroup is also present in the Southeast, comprising 2% of the regional population.

Haplogroup X2 is again present in a small number of samples from across Iberia. This includes 1 West (6%), 1 Northeast (8%), and 4 North (7%) individuals. Two individuals with haplogroups from branches that were not observed in previous periods are found in the Chalcolithic, both in the Central group. These individuals belong to haplogroups L1 (3%) and M1 (3%).

K haplogroups are slightly less common in the Chalcolithic than they were in the Neolithic. K1a is again the most common, with 2 Northeast (17%), 4 Central (14%), 9 Southeast (20%), 1 West (6%), and 13 North (24%) samples belonging to this haplogroup. Only 3 K1b individuals appear in the Chalcolithic period; 1 (2%) in the North group, 1 (6%) in the West group, and 1 (2%) in the Southeast group. Surprisingly, the two groups that contained the smallest proportion of K haplogroups in the Middle to Late Neolithic period, the North and Northeast, had an increased proportion of K haplogroups in the Chalcolithic, rising from 8% (n=1) to 26% (n=14) in the North and 6% (n=1) to 17% (n=2) in the Northeast. The other population groups experienced a decrease in the K haplogroup proportion, with the most notable being the West group, which dropped from 29% (n=4) to 12% (n=2).

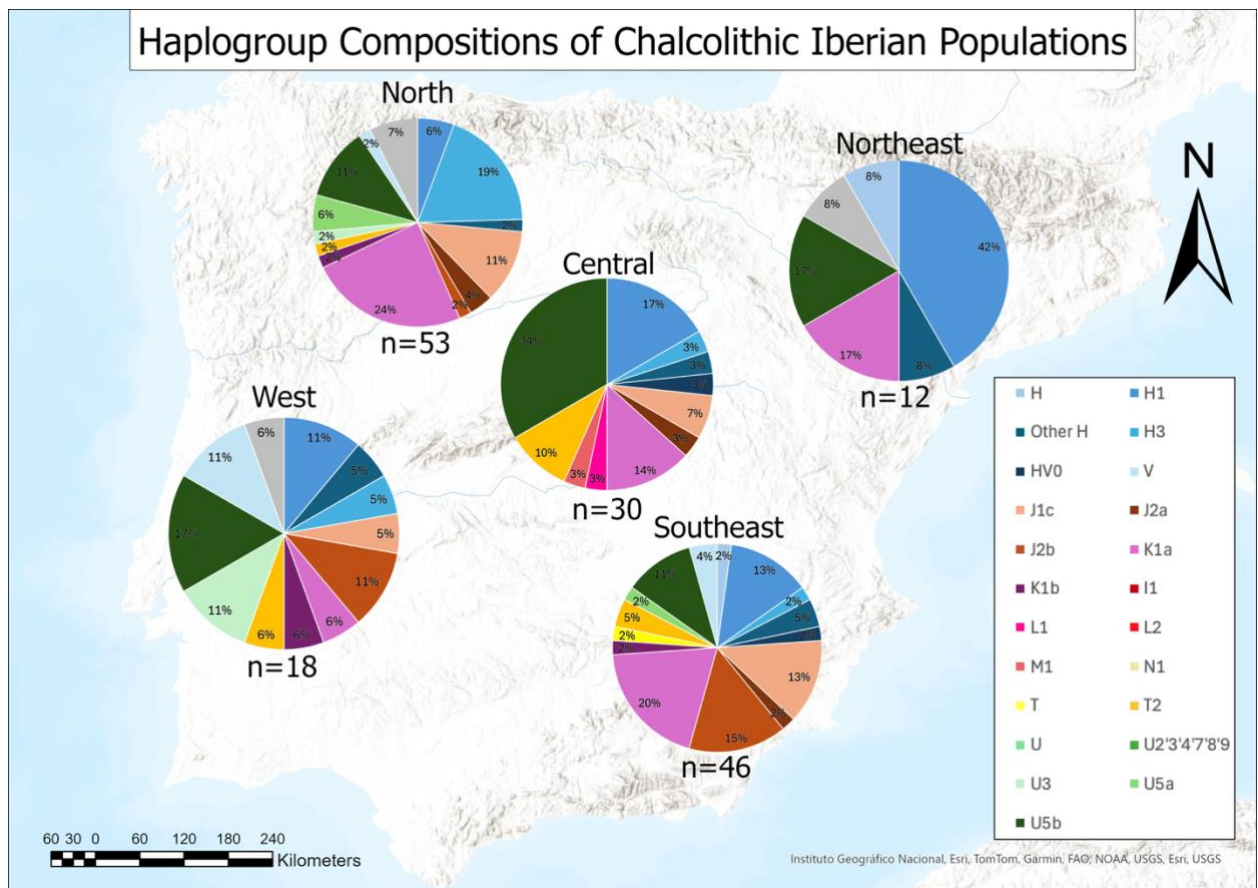


Figure 25: Map of haplogroup compositions of Chalcolithic Iberian populations based on groupings seen in Figure 21. Numbers on graphs represent number of individuals. n= total individuals in the population group. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6.

Bronze Age

Enough published data was available from the Bronze Age period to split the North and Southeast regions into two time periods: Early Bronze Age and Middle to Late Bronze Age. Two maps were generated to display the haplogroup compositions in Bronze Age Iberian populations. Figure 26 displays the Early Bronze Age haplogroup compositions of North and Southeast Iberia, as well as the complete Bronze Age composition of the West, Central, and Northeast regions. Figure 27 displays the Middle to Late Bronze Age haplogroup compositions of the North and Southeast regions, and the complete Bronze Age compositions of the other regions.

The haplogroup composition of the North and Southeast Iberian population groups in the Early Bronze Age are similar to those of the Chalcolithic. U5b appears in two members (18%) of the North group and 8 (15%) of the Southeast group. U5a is also present in 1 (2%) Southeast sample. J1c is present in 2 individuals (19%) in the North and 3 (6%) in the Southeast groups. R0 haplogroups observed in the North group are H3 (9%) and HV0 (9%). The Southeast again has a very diverse range of R0 haplogroups. These include 1 H (2%), 12 H1 (22%), 1 H3 (2%), 1 HV0 (2%), 3 V (6%), and 4 'Other H'

(7%), which is comprised of 1 H105 (2%), 1 H4 (2%), 1 H5 (2%), and 1 H6 (2%) individual. J2b is observed in 2 individuals in the North group (18%) and 1 in the Southeast group (2%). Haplogroup T2 presents in 3 individuals (6%) from the Southeast group. Two individuals from the North (18%) and Southeast (4%) groups belong to X2 haplogroup. K1a haplogroup is only observed in one member of the North group (9%), while 11 K1a (21%) and 2 K1b (4%) members are found in the Southeast group.

The haplogroup composition of the North and Southeast population groups changes slightly in the Middle to Late Bronze Age. U haplogroup composition and frequency remains similar, with 1 (10%) in the North and 2 (18%) individuals in the Southeast group, all U5b. R0 haplogroups become more frequent in the North group, with 1 H3 (10%), 1 HV0 (10%), and 3 H1 (30%) individuals. In the Southeast group, R0 haplogroup frequency decreases, with only 3 members being present. These belong to haplogroups H1 (9%), H3 (9%), and V (9%). No members of the JT branch are observed in the Southeast group, and only 2 J2b (20%) are present in the North group. K1a appears in 2 individuals (20%) in the North group and 4 (37%) in the Southeast group. Two members of the Southeast group have haplogroups not seen in the periods previously discussed. These are haplogroups L2 (9%) and I1 (9%).

The remaining groups, Northeast, Central, and West, only have enough published data to create population groups encompassing the entirety of the Bronze Age period. U haplogroups again play a role in all three groups. U5b appears in 4 West (40%), 1 Northeast (8%), and 2 Central (29%) samples. An undifferentiated U haplogroup individual is also present in the Northeast group (8%). R0 haplogroup frequency increases in the Central and West groups but decreases in the Northeast group between the Chalcolithic and Bronze Age periods. H1 is found in 1 (7%) of the Northeast group, 2 (29%) of the Central group, and 2 (20%) of the West group. H3 is present in only 1 individual (10%) in the West group. Haplogroup V is found in 2 samples, 1 (10%) from the West group and 1 (8%) from the Northeast group. HV0 is also found in 2 individuals in the Northeast (8%) and Central (14%) groups. Two (15%) of the Northeast group were reported to be of undefined H haplogroups. The J1c haplogroup is found in 2 individuals (15%) from the Northeast group and 1 individual (10%) in the West group, while 1 individual (8%) of the haplogroup J2b is present in the Northeast group. No J haplogroups are found in the Central group, but one individual was found belonging to the T2 haplogroup (14%). Only 1 individual belonging to X2 is observed between the three groups and is found in the West group (10%). In turn, The K1a Haplogroup is present in 3 individuals (23%) from the Northeast and 1 (14%) from the Central group. There is a notable absence of K haplogroup samples in the West group.

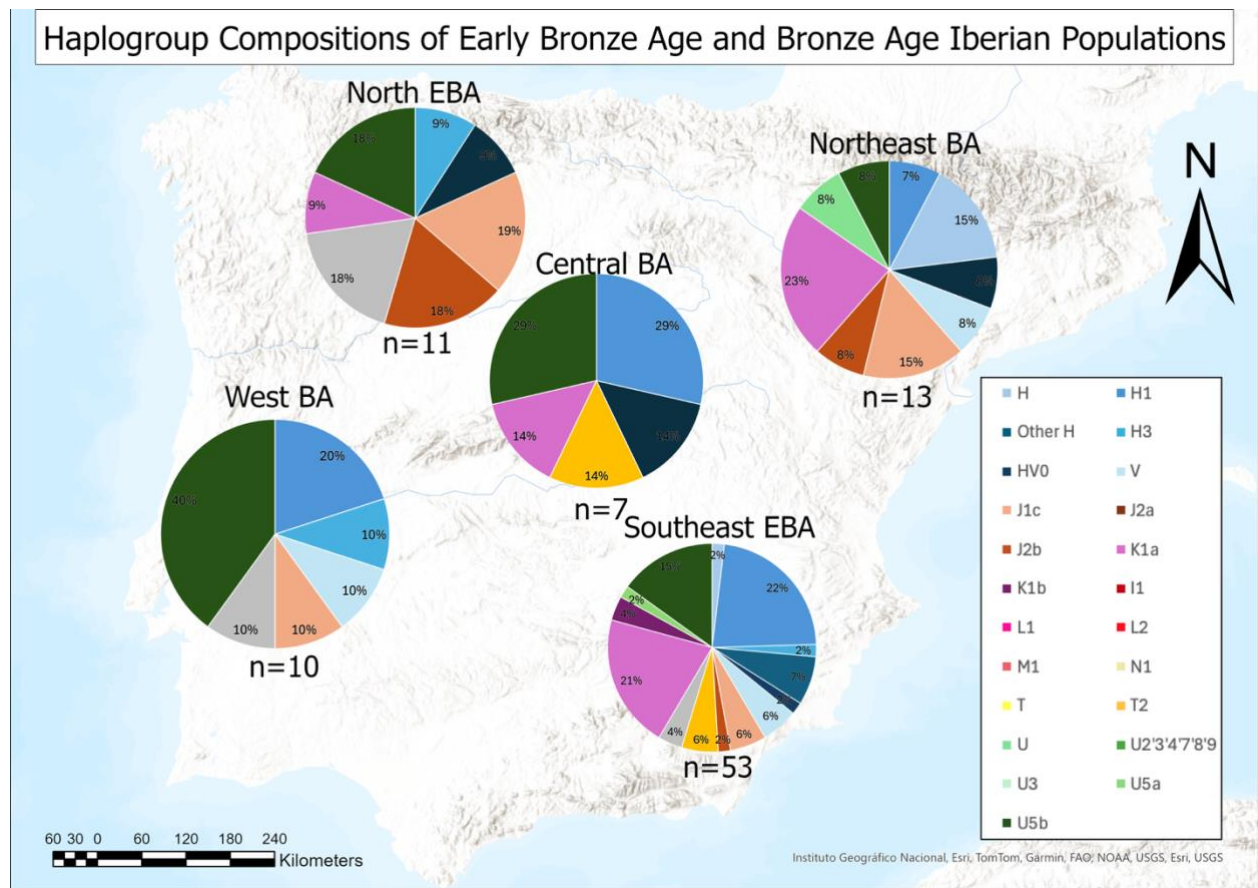


Figure 26: Map of haplogroup compositions of Bronze Age West, Central, and Northeast Iberian populations and Early Bronze Age North and Southeast populations based on groupings seen in Figure 21. Numbers on graphs represent number of individuals. n= total individuals in the population group. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6.

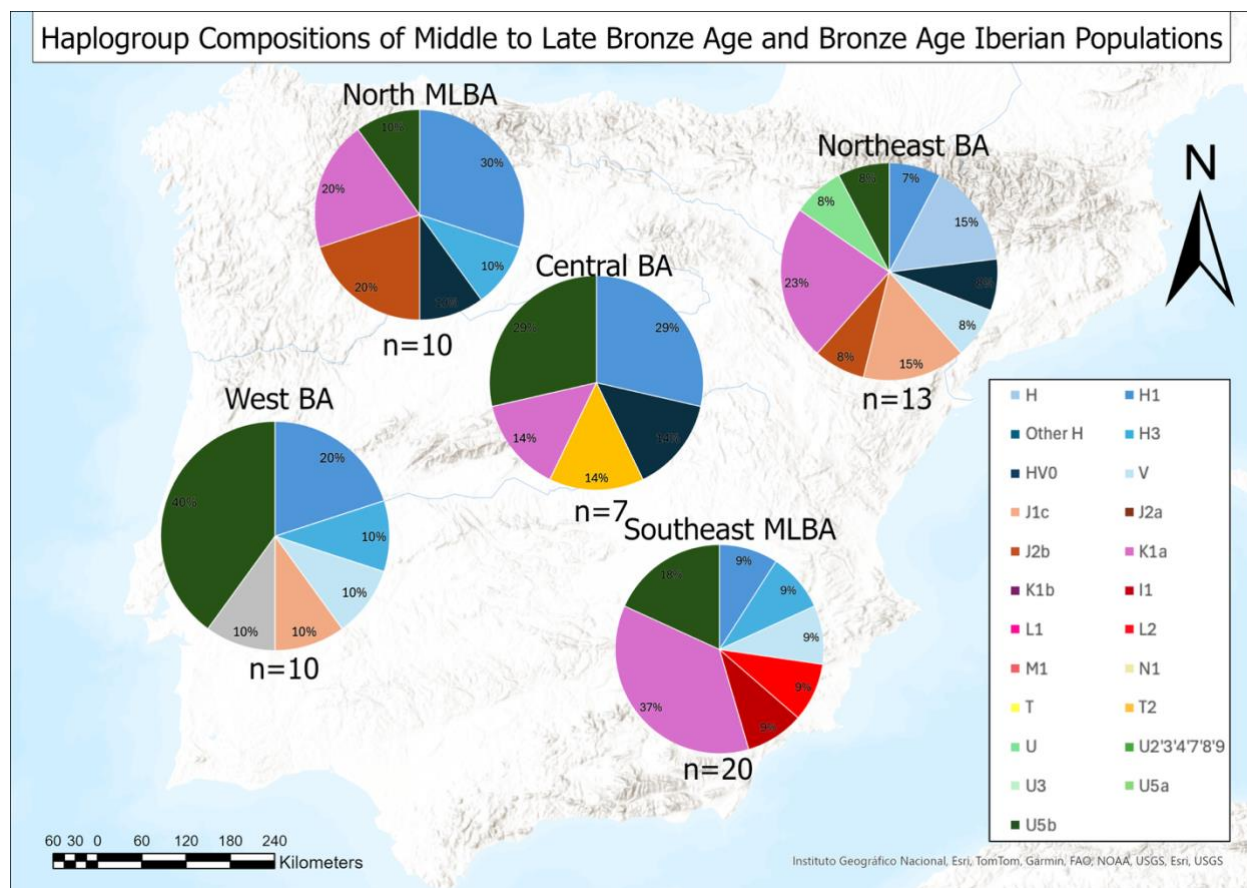


Figure 27: Map of haplogroup compositions of Bronze Age West, Central, and Northeast Iberian populations and Middle to Late Bronze Age North and Southeast populations based on groupings seen in Figure 21. Numbers on graphs represent number of individuals. n= total individuals in the population group. Based on data from the AADR resource (Mallick et al., 2024). Created using ArcGIS Pro v3.0.6.

4.6 Metagenomic Analysis

A full table of all organisms found in each sample and the quality score of their true presence in each sample can be found in the [supplementary material](#). The quality scores are calculated by aMeta, and are based on edit distance for all reads and ancient reads, evenness of coverage, deamination patterns, read length distribution, PMD scores, average nucleotide identity (ANI) and number of assigned reads (Pochon et al., 2023). Most of the organisms identified in all La Sagrera samples using the aMeta pipeline were either common soil microbes or organisms typically associated with a healthy human oral microbiota (Pochon et al., 2023). Overall, four organism that are known to cause disease in humans and are not common soil or oral microbiota were identified in individuals from this and the other two studied sites (Table 21).

The first, *Neisseria meningitidis*, was found in individuals CVC5 (Figure 28) and CVC6 (Figure 29), with authenticity scores of 6 and 5 respectively. This organism can cause Meningococcal disease (Hollingshead and Tang, 2019). This bacterial infection is usually transmitted through the spread of saliva from kissing, coughing, and sneezing. Invasive Meningococcal disease has been shown to have an 80% fatality rate in

humans without modern treatment, although many strains are less deadly (Parikh et al., 2020).

Tannerella forsythia was found in samples SA18 (Figure 30), SA46 (Figure 31), SA68 (Figure 32), and CVC6 (Figure 33). This organism is a member of the ‘red complex’ of bacteria shown to cause periodontal diseases such as gingivitis and periodontitis (Assandri et al., 2023). While not usually life threatening, these diseases can cause chronic pain and tooth loss (Assandri et al., 2023). *Tannerella forsythia* has been identified in ancient individuals from pre- and post-colonial Central America, Bronze Age Ireland, and throughout Europe (Jackson et al., 2024).

Bordetella bronchialis was found in four samples, including CVC9 (Figure 34) and all three samples from the site of Algar do Barrão (Figure 35, Figure 36, and Figure 37). While not much has been published on this specific species, other *Bordetella* species cause respiratory diseases in humans and animals (Vandamme et al., 2015). *Bordetella bronchialis* has not been identified in ancient samples before.

Finally, *Spirometra erinaceieuropaei* was found in one sample, BAR3. This organism, which is a species of tapeworm, can infect humans through unclean water or eating meat from intermediate host animals, usually frogs and snakes (Tang et al., 2017). The organism migrates through human tissues, which can lead to tissue swelling, muscle damage, and nervous system damage leading to seizures in severe cases. As seen in Table 21, this organism received an authenticity score of 7. Figure 38 displays that the *Spirometra erinaceieuropaei* reads covered 21.08% of the genome. Edit distance, deamination, read length distribution were all also as expected for an authentic identification (Pochon et al., 2023; Tang et al., 2017). This organism has not been identified in ancient samples before.

Site	Individual	Organism	Score	Number of Reads
La Sagrera	SA18	Tannerella Forsythia	2	11
	SA46	Tannerella Forsythia	5	122
	SA68	Tannerella Forsythia	2	22
Convento do Carmo	CVC5	Neisseria meningitidis	6	418
	CVC6	Neisseria meningitidis	5	444
		Tannerella Forsythia	2	10
	CVC9	Bordetella bronchialis	6	3365
Algar do Barrão	BAR3	Spirometra erinaceieuropaei	7	566
		Bordetella bronchialis	7	2765
	BAR4	Bordetella bronchialis	6	4804
	BAR5	Bordetella bronchialis	7	3769

Table 21: Quality scores and number of reads for pathogenic organisms identified by aMeta.

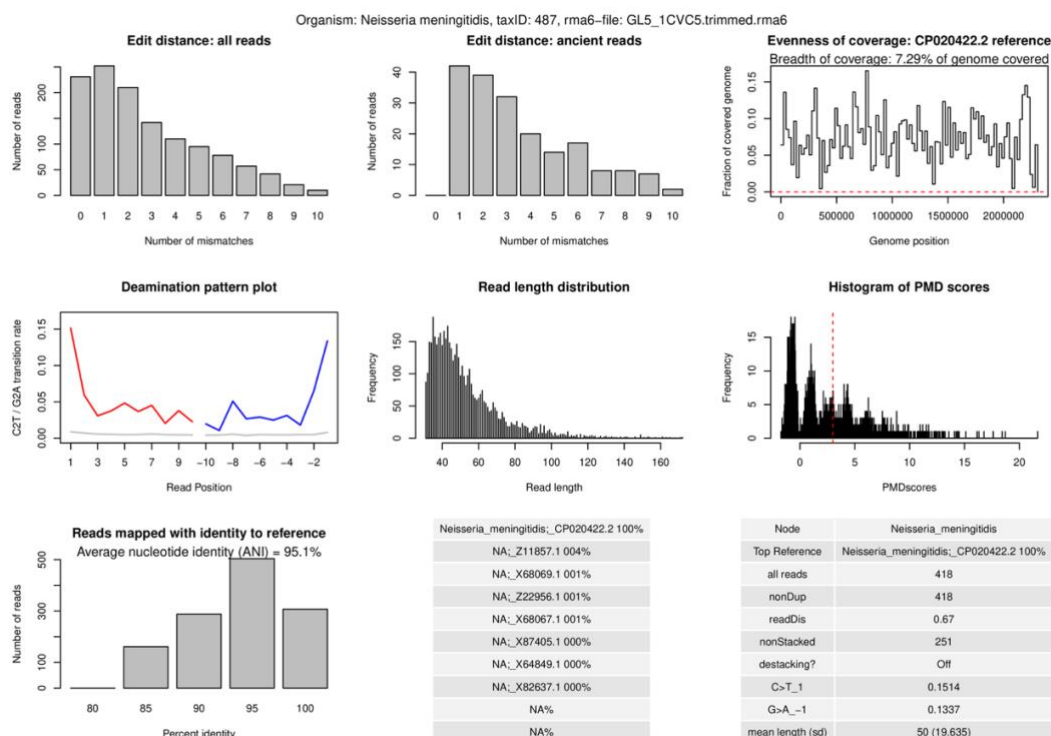


Figure 28: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Neisseria meningitidis* in the sample CVC5.

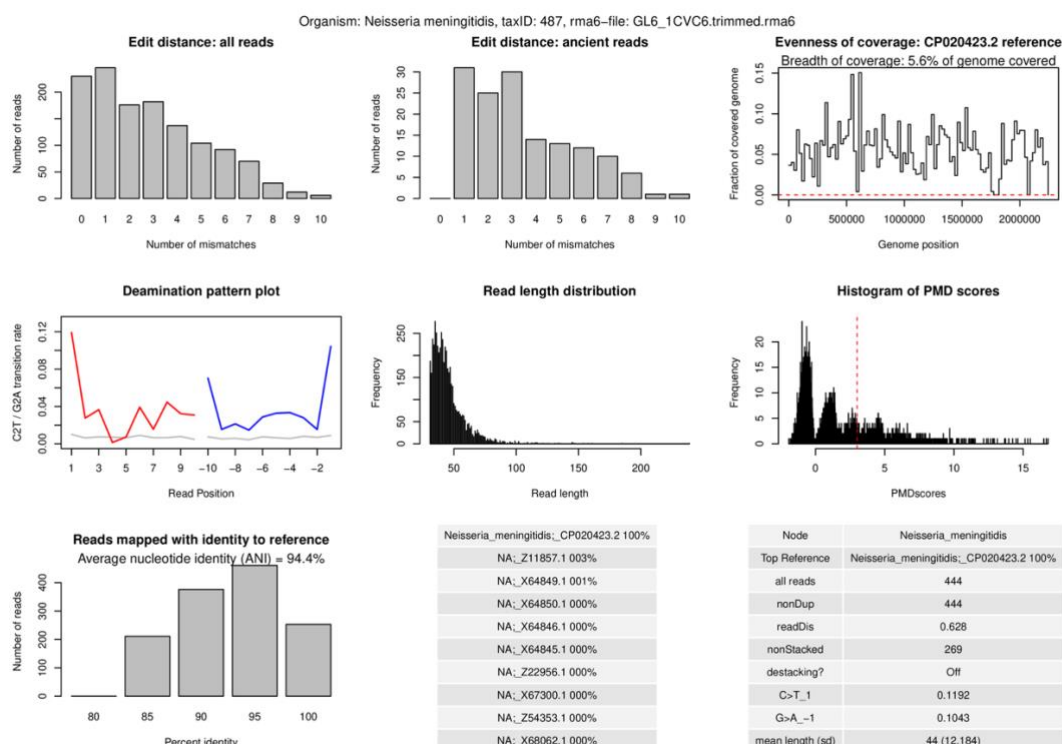


Figure 29: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Neisseria meningitidis* in the sample CVC6.

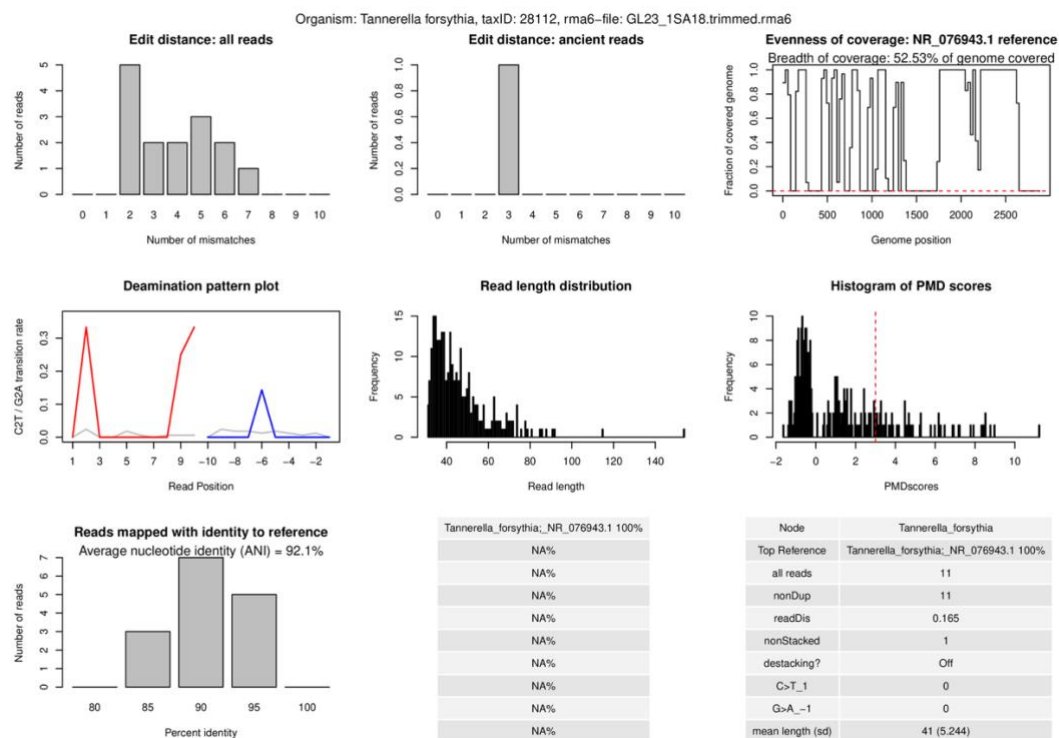


Figure 30: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Tannerella forsythia* in the sample SA18.

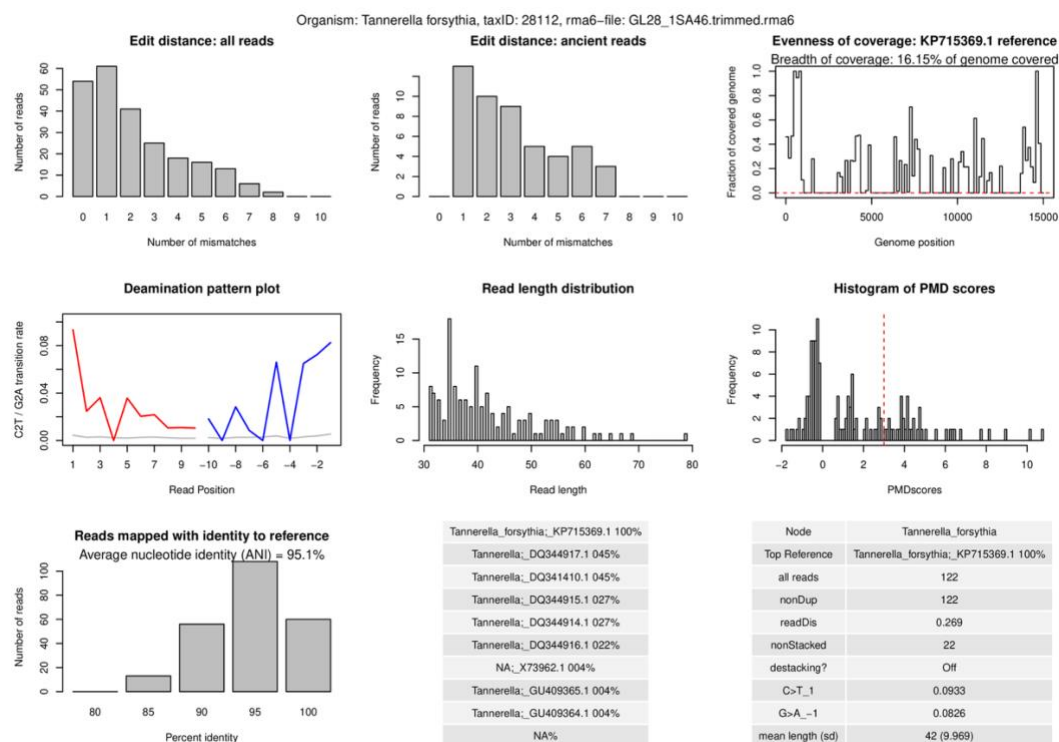


Figure 31: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Tannerella forsythia* in the sample SA46.

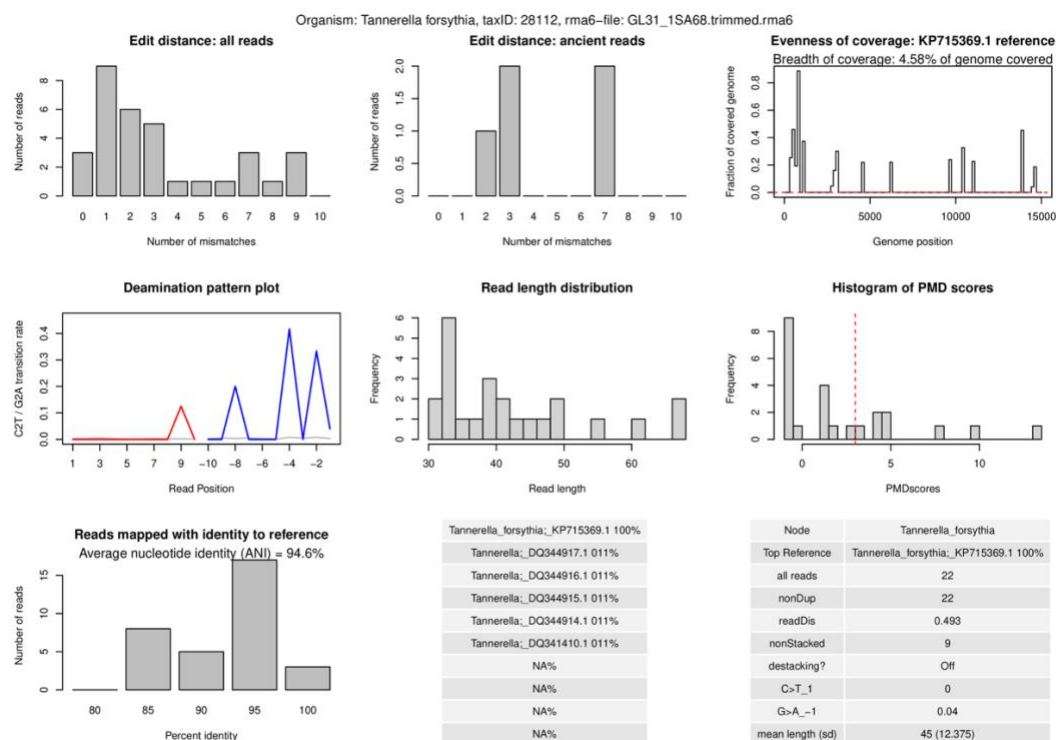


Figure 32: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Tannerella forsythia* in the sample SA68.

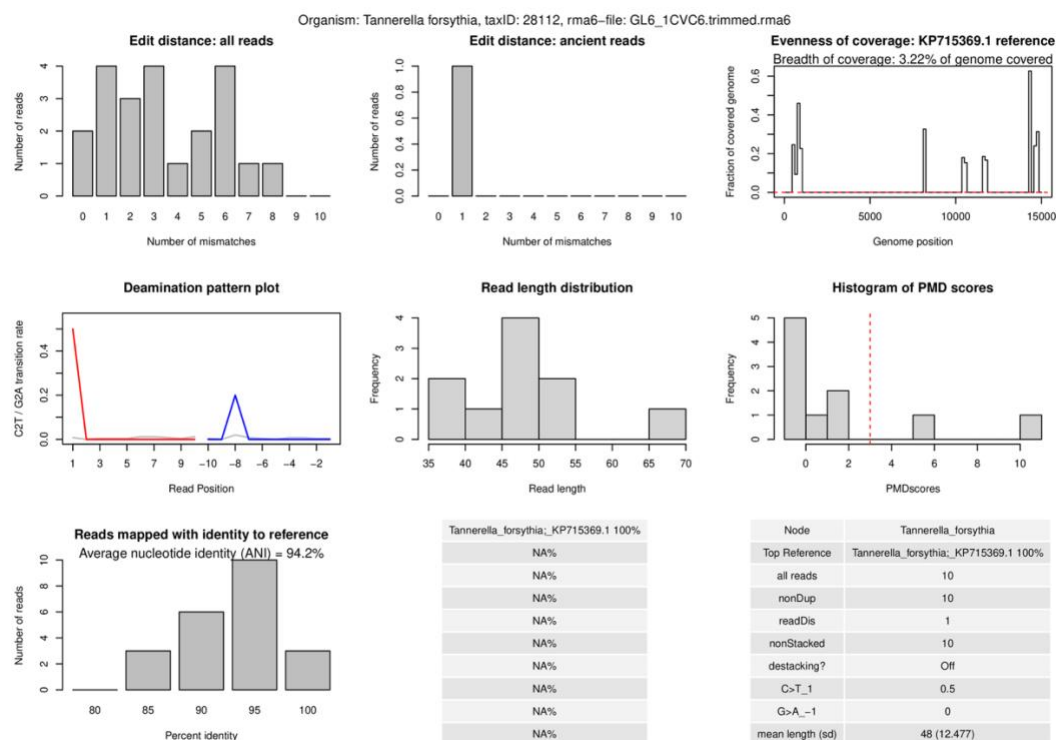


Figure 33: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Tannerella forsythia* in the sample CVC6.

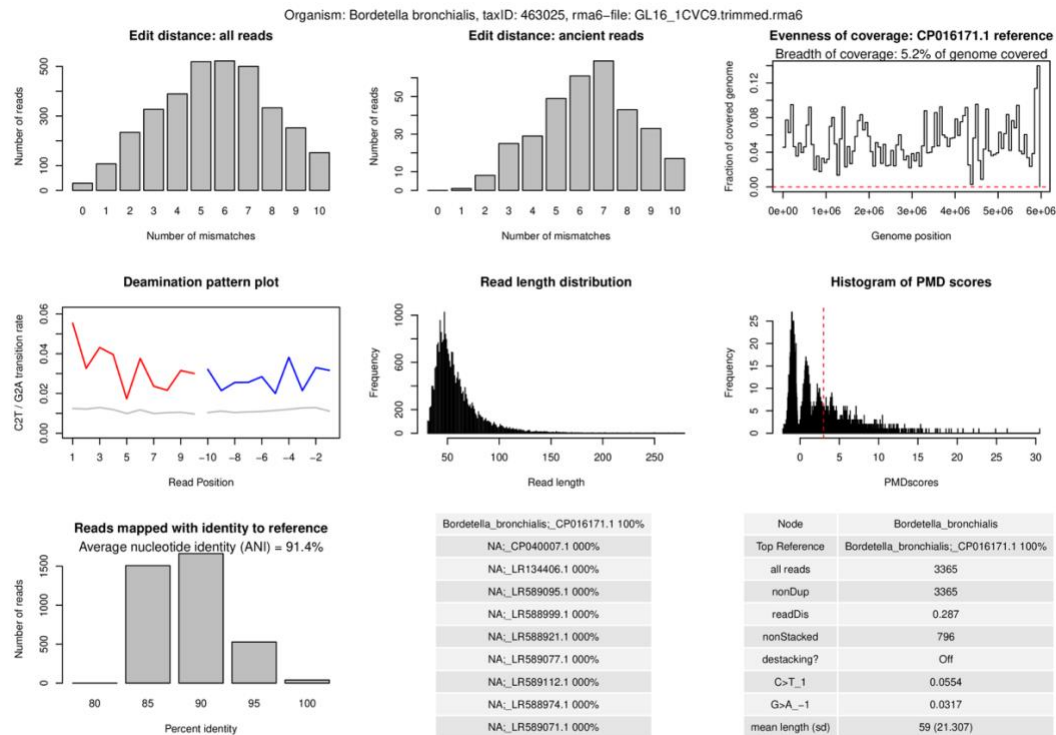


Figure 34: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Bordetella bronchialis* in the sample CVC9.

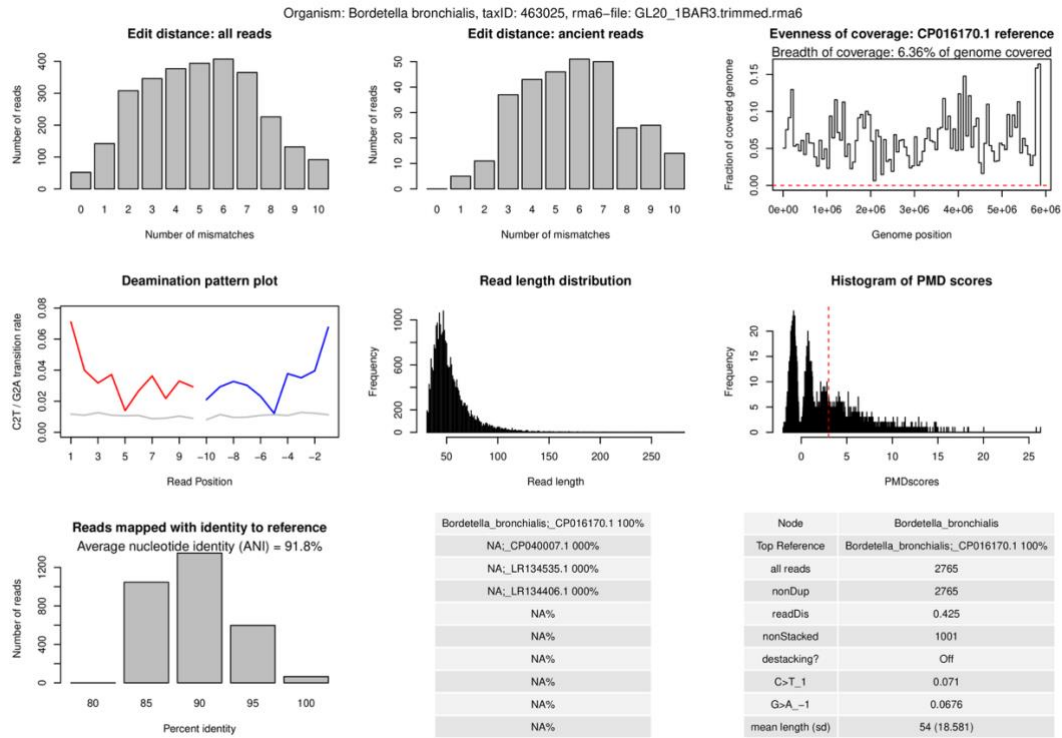


Figure 35: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Bordetella bronchialis*

in the sample BAR3.

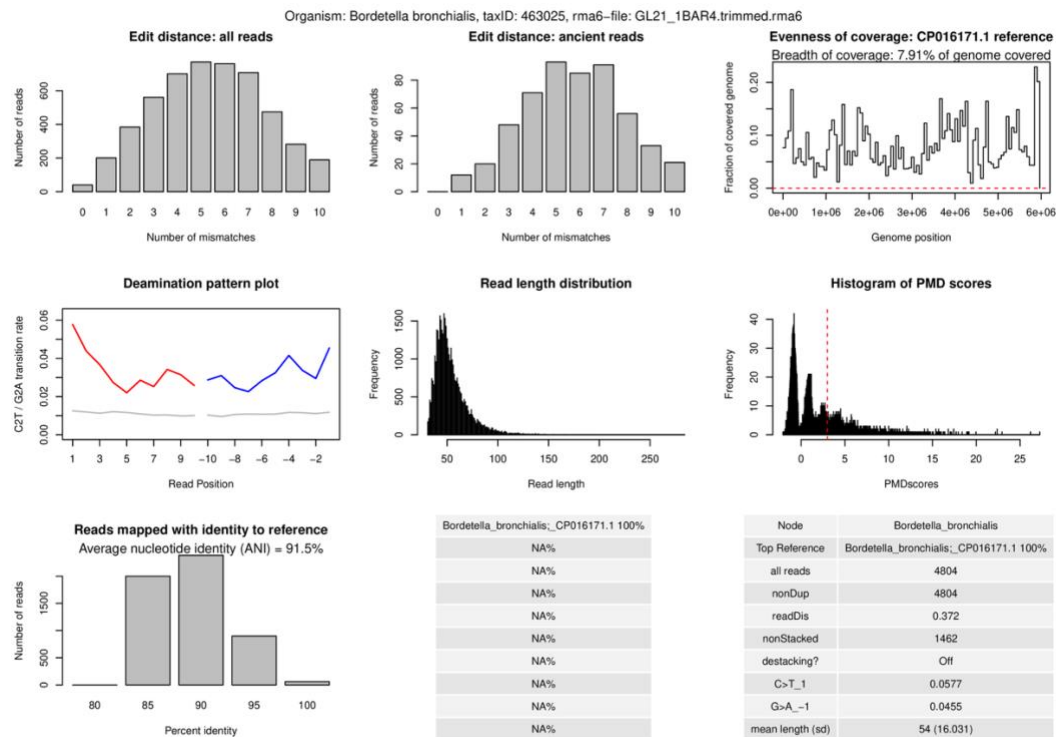


Figure 36: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Bordetella bronchialis* in the sample BAR4.

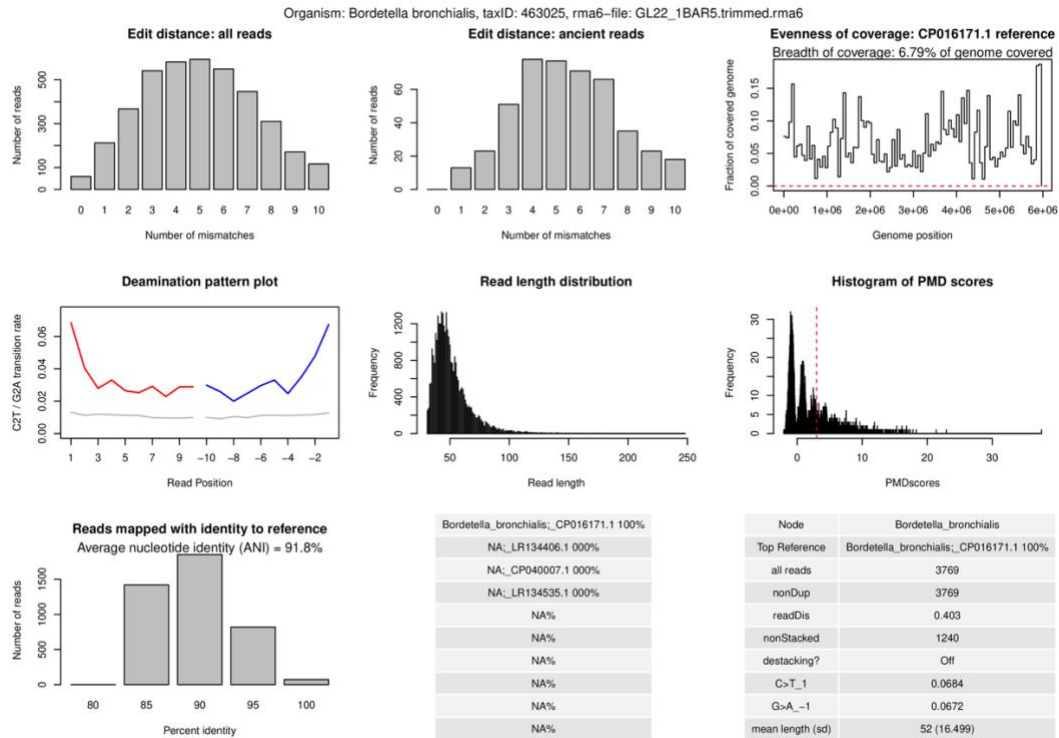


Figure 37: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Bordetella bronchialis* in the sample BAR5.

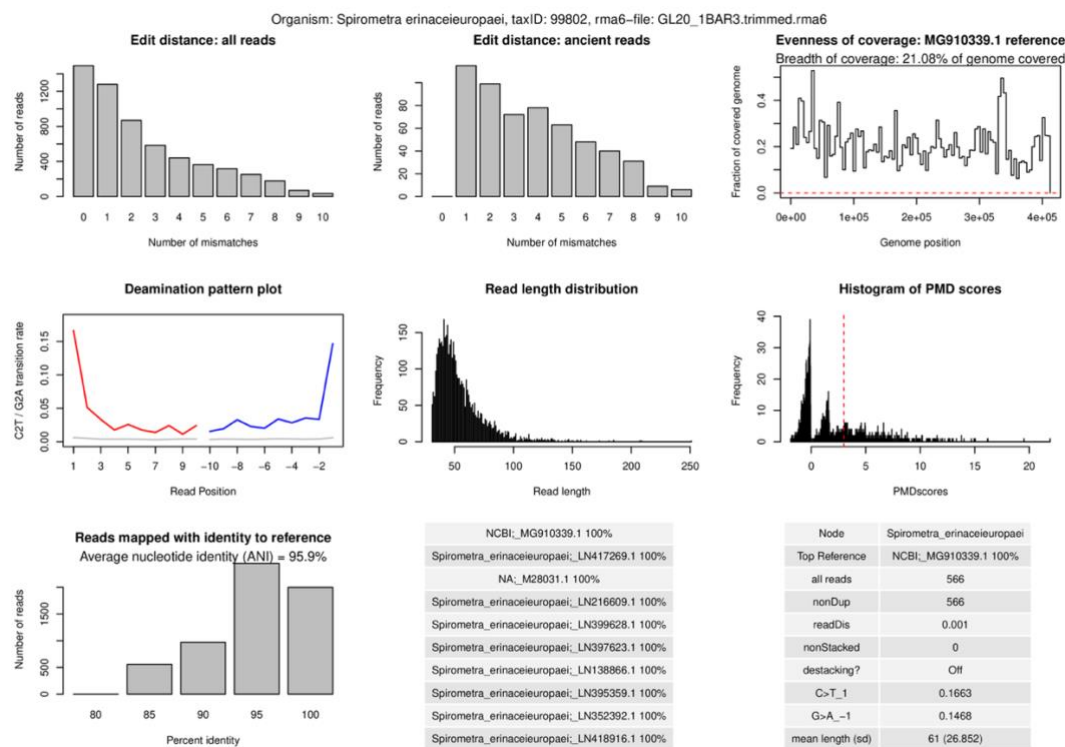


Figure 38: Detailed metrics of edit distance, breadth of coverage, deamination plot, read length distribution, PMD scores, percent identity of reads of *Spirometra erinaceieuropaei* in the sample BAR3.

5. Discussion

5.1 Preservation and Authentication of Results

When studying ancient DNA, where samples can often have limited DNA preservation and the possibility of contamination causing false results is high, it is important to ensure that results are authentic and reliable. The first steps in reducing and controlling contamination risk were the sampling, extraction and library preparation of samples in a dedicated ancient DNA clean lab as described in section 3.2. Further, extraction and library blanks were included during the experimental process to monitor any contamination. While most blanks contained negligible amounts of endogenous DNA (2.02-0.12%, see Table 16), the extraction blank from 9/9/24 and library blank 1 from 4/10/24 contained high endogenous DNA percentages (23.57% and 31.76% respectively, see Table 16). The median read length in blanks between 44 and 57 base pairs (within the 44-64bp range of the samples) and the low amount of deamination in the reads (<1.60% 5' C>T 1st base, <0.60% 3' G>A 1st base, except for EB17/9/24 and 2LB4/10/24, see section 4.1) provide contrasting evidence as to whether contamination was from ancient *carry-over* or a modern source. The most likely source of modern contamination is the researcher carrying out the studies, which in this case was the author. Results of the analysis of the author's own mtDNA, seen in section 4.2, revealed that they belong to the U5b major haplogroup. The author's haplotype and haplogroup was taken into consideration when discussing authenticity. The haplotype and haplogroup of previous researchers who handled the samples, which is information curated by and obtained from the Durham Archaeo-DNA Lab, were also taken into consideration. The author's complete HVRI haplotype and top haplogroup hits (see Table 18) were not detected in any of the samples or blanks. Comparison with previously published results was also important in determining the validity of the results in this study. Ideally, samples from the affected batches would have been reextracted to verify results in this study, but due to time and budget constraints this was impractical. The results from the sites of Algar do Barrão and Convento do Carmo (excluding CVC2 and CVC5) can still be considered authentic due to the lack of detection of the author's DNA profile and characteristic aDNA damage pattern in the reads. While *carry-over* between samples is possible, the strong assignment of diverse haplogroups in these samples suggest any *carry-over* did not impact results.

5.1.1 La Sagrera

DNA preservation in samples from La Sagrera was generally low (between 0.41-0.01% endogenous DNA). Positions with at least 1X coverage in the mitochondrial

genome were between 62% and 2%, and only two samples had positions with 5X coverage (SA44, 2.6%; SA56, 0.1%). Macroscopic preservation in teeth analysed from La Sagrera was good in SA43, SA44, SA45, SA46, and SA56, while SA4, SA18, SA26, SA53, and SA68 had broken roots and a brown discolouration (see [supplementary table S1](#))(Robinson, 2018). Age, temperature, and other environmental factors have also been shown to impact DNA preservation (Gilbert et al., 2003; Higgins et al., 2015; Höss et al., 1996; Lindahl, 1993b; Poinar et al., 1996). In the case of La Sagrera, the site was determined to be an open burial. This exposure to the elements, combined with the heat and fluctuating temperatures of the region, likely contributed to the poor preservation in the samples. The Northeast region of Iberia is typically subject to temperatures of over 30°C in the summer months and below 0°C in the winter months (Mohammed et al., 2018), as well as an average annual rainfall of ~200-800mm (Claro et al., 2023).

Although samples from caves in the region have produced good results (Gamba et al., 2012; Mathieson et al., 2015b; Olalde et al., 2019; Villalba-Mouco et al., 2019) due to their ability to keep a stable temperature year-round and protect from rainfall (De Torres et al., 2002; Hedges and Millard, 1995), genetic studies of samples from burial sites outside of caves from the Neolithic period in the Northeast region of Iberia are absent from the AADR database (Mallick et al., 2024). A study using a PCR approach to analyse Neolithic individuals in the Northeast region found that cave sites had higher success rates (42-100%) for mitochondrial analysis than open air sites (29%)(Gamba et al., 2012). Genetic data from two individuals (related, both mtDNA haplogroup K1a4a1) from the Middle Neolithic context of the site Mas d'en Boixos, has been recovered recently (100% success rate in the Middle Neolithic context) (Roca-Rada et al., 2025), although these individuals come from an inhumation burial and not an open pit grave. No other successful whole genome analysis from open air sites during the Neolithic in Northeast Iberia has been published.

Previous genetic studies were able to obtain some results despite the preservation issues presented by the context (Robinson, 2018). It is unlikely that the DNA in these samples degraded much between the previous study in 2018 and the current study. Sample powder was held in -20° freezers for the entire period, a method which has shown to be effective in preserving DNA in samples for long periods of time (Burger et al., 1999; Iyavoo et al., 2019). The reason for the discrepancy in results between both studies could be due to several other factors, such as inefficient DNA extraction or amplification. Extraction in this study utilised the Dabney et al (2013) extraction protocol, whereas the previous study used the Rohland and Hofreiter (2010) protocol. Although the Dabney et al (2013) protocol has been shown to be more efficient in general (Dabney et al., 2013a; Rohland et al., 2018), the use of a different binding buffer (5M GuSCN, 300mM sodium acetate, pH 5.2) in the Rohland and Hofreiter (2010) protocol may have been more efficient for the La Sagrera samples.

PCR studies are more sensitive to minute amounts of DNA in samples (McDonald et al., 2024; Rizzi et al., 2012), which could be another explanation for why the previous study was able to obtain results where this study was not. While this characteristic of the PCR approach means it can be useful for poorly preserved samples, it also makes it more susceptible to contamination. Any amount of contaminant DNA that enters the sample will be amplified and sequenced alongside the targeted DNA.

The author mentions that contamination may be the case in sample SA18, as the PCR and clone sequences for this sample had several differences (Robinson, 2018). Although the mutations found in SA18 in the previous study did not match the mutation of any of the researchers associated with the samples in that study (Robinson, 2018), there is still the possibility that the sample was contaminated through *carry-over* from other samples. NGS methods of library preparation and capture result in the loss of a lot of DNA, which reduces contamination but can also reduce the effectiveness of studies with very little preserved DNA, possibly explaining the discrepancy of results between the two studies.

5.1.2 Casais da Mureta

Preservation of DNA in samples at Casais da Mureta was very low (between 0.05-0.01% endogenous DNA). All three samples from this site had coverage at less than 52% of positions in the mitochondrial genome. Coverage was also 5.0X in less than 0.1% of position in each sample. This meant that any further mtDNA analysis on these samples was uninformative, so they were not considered. Although there was optimism that these samples may produce better results with the newer sequencing techniques utilized in this study, the poor quality of results was not unexpected. No mtDNA results were obtained using a PCR approach in a previous study (Robinson, 2018). Conditions for preservation were also not ideal, as it was suggested that the remains could have been deposited on the surface, although the destruction of the site made it impossible to confirm this fact (Carvalho et al., 2019). Macroscopic preservation of the remains from Casais da Mureta was poor, including the sampled teeth. The damage included chipped enamel in CMR1, broken enamel in CMR2, and a crack in the tooth of CMR3 (Robinson, 2018). The severity of the macroscopic damage in these teeth roughly correlated to the quality of DNA recovered. CMR1, with only a chipped enamel, had the best preservation of the three with 0.05% endogenous DNA. CMR2 and CMR3, which had more severe damage, had 0.01% and 0.02% endogenous DNA respectively. This is consistent with previous studies showing that macroscopic damage can provide a loose estimate for DNA preservation, as cracks and breaks open the interior of the teeth to environmental and microscopic elements that cause DNA degradation (Sosa et al., 2013).

5.1.3 Algar do Barrão

Samples from Algar do Barrão had also been previously analysed alongside samples from Casais da Mureta and La Sagrera using PCR (Robinson, 2018). Previous analyses in Barrão were successful, demonstrating that the preservation of DNA in these samples was good. In this study, the samples from Algar do Barrão showed the best preservation of any site, with endogenous DNA percent between 0.5 and 4.58%. The percentage of covered mitochondrial genome at $\geq 1X$ was 100% in BAR3, 63% in BAR4, and 99% in BAR5, while mitogenome coverage of at least 5X was 99.9% in BAR3, 1.4% in BAR4, and 69.7% in BAR5. The exceptional state of preservation was likely due to the cave environment in which these samples were found. Caves provide protection from the environment as well as a stable and usually cool environment throughout the year, making them a good environment for DNA preservation (Burger et al., 1999; Gamba et al., 2012).

Although both BAR3 and BAR5 belonged to the same U5b haplogroup as the author of this study, the author's specific four HVRI mutations were not present in the samples with coverage in those positions. In BAR3, the only mutations in common with the author were 16270T and 16192T!. In BAR5, 16093C and the back mutation 16192T! were not present. The mutations shared with the author had similar coverage to those of the other mutations in the samples, suggesting that they were likely from the same authentic source. Read length (median 44-54bp) and C to T deamination patterns (10.00-14.20% 5' C>T 1st base, 10.70-14.10% 3' G>A 1st base) suggest the reads in these samples were truly ancient, and not from a modern contaminant. Moreover, previous mtDNA major haplogroup estimations in the individuals of Algar do Barrão (Robinson, 2018), seen in Table 11 match the ones in this study. MtDNA haplotypes also coincide in the regions covered in both studies (HVRI, positions 16105-16280 for BAR3; 16105-16399 for BAR4; 16232-16399 for BAR5), further supporting the authenticity of these results.

Considering the state of preservation, the lack of clear evidence for contamination, and the replication of previous results, the results from the three Algar do Barrão samples can be considered reliable.

5.1.4 Convento do Carmo

Convento do Carmo samples had good DNA preservation generally, with endogenous DNA percentages between 0.02 and 1.29 (see Table 16). Coverage at $\geq 1X$ of the mitochondrial genome was between 59.7-100% (0.7-99.5% $\geq 5X$), except for samples CVC2 and CVC5 (see Table 17). With only 12% (CVC2) and 29% (CVC5) of $\geq 1X$ mtDNA coverage and no coverage at $\geq 5X$, any further analysis of the results from these

samples would have been uninformative and potentially unreliable. Macroscopic preservation in CVC2 was noted by the archaeologist as being the poorest of any studied sample from Convento do Carmo with high fragmentation and exposure of dentine on the occlusal surface, likely contributing to DNA degradation and lack of results (Granja, 2019). Sample CVC5 was noted as having good macroscopic preservation with a complete mandible only missing tooth 38, although the tooth sampled for DNA had a long vertical fissure that would have exposed the inner dentine that was accessed at sampling (Granja, 2019).

Previous studies obtained partial mtDNA profiles from samples CVC4, CVC6, and CVC7, that were validated through independent DNA extractions and PCR amplifications (Fernández-Domínguez et al., 2019; Neff, 2016). These focused on the HVRI of the mtDNA (positions 16095-16399), and all other samples either gave no results or partial haplotypes (positions 16126-16261 for CVC3; 16252-16369 for CVC1, CVC2, and CVC8) which could not be used to determine haplogroup. The increased success rate of the current study is due to the use of in solution capture of targeting the entire mitochondrial genome followed by Next Generation Sequencing. Although the HVRI in most samples had no coverage, the analysis of mutations in the coding region of the mtDNA allowed for haplogroup identification to be performed on all samples except CVC2 and CVC5.

The haplogroup analysis of Convento do Carmo individuals in this study did not coincide with the previously published results based on PCR amplification of the mtDNA-HVRI. As seen in Table 9, individuals CVC4, CVC6, and CVC7 were all previously assigned to the K major haplogroup, contrasting with the assignments of V, T2, and J1c major haplogroups in this study (Fernández-Domínguez et al., 2019). Haplotypes of the mtDNA-HVRI were also different in both studies, and none of the mutations previously reported in these three individuals were found in this study. Conversely, mutations that appeared in the HVRI of the three individuals in this study were not present in Fernández-Domínguez et al., 2019.

In the first study of these samples, it was determined that besides CVC6, for which a consensus haplotype could not be established, all the individuals had haplotype 16311C (positions 16108-16339 for CVC1; 16108-16397 for CVC2; 16111-16329 for CVC3; 16235-16316 for CVC4; 16107-16336 for CVC5; 16235-16328 for CVC7; 16108-16340 for CVC8), and were assigned to haplogroup H2a with a confidence of 100% (Neff, 2016). It is important to note that the researcher that conducted the analyses (MN) also harboured the same mutation 16311C, but contamination was originally excluded as the blanks in the study were deemed uncontaminated (Neff, 2016). A reanalysis of these samples, including an independent extraction and amplification, reported again 16311C in all samples except CVC3 and CVC5 (Fernández-Domínguez et al., 2019). This study also found the mutation 16362C in

CVC1, CVC2, CVC4, CVC7, and CVC8 (Fernández-Domínguez et al., 2019). This mutation was also present in the archaeologist's mtDNA profile.

The results from the current study determined that CVC9 belonged to the same major haplogroup as the author (U5b). When looking at the haplotypes, however, CVC9 does not have the mutations 16093C and 16192T that are present in the author's haplotype. Furthermore, CVC9 also has mutation 16311C, which does not appear in the author's DNA sequence. This makes it unlikely that contamination from the author's DNA was responsible for the results from CVC9. Samples CVC1 and CVC3 were both assigned to haplogroup U3a1 and could also have been impacted by contamination from the author's mtDNA, as U3a1 and U5b share the mutations 73G, 150T, 263G, 750G, 1438G, 2706G, 4769G, 7028T, 8860G, 11467G, 11719A, 12308G, 12372A, 14766T, and 15326G (Van Oven and Kayser, 2009). These samples have similar levels of coverage between potential contaminant mutations and mutations not expected in the author's mtDNA profile but found in the samples. This strengthens the case that the results from these samples are authentic.

Because of the smaller region amplified in Neff (2016) and Fernández-Domínguez et al (2018), the possibility of contamination from one of the researcher and the archaeologist, and the higher risk of contamination of PCR versus NGS workflows (Rizzi et al., 2012), it is likely that the results of the previous studies were affected by contamination rather than the results of this study. Further, as there is no evidence of contamination from the author, the metrics from the MultiQC report suggest true ancient DNA (44-64bp read length; 4.30-16.60% 5' C>T 1st base; 4.20-16.50% 3' G>A 1st base), and the haplogroups and haplotypes present in the sample are diverse, the results from the Convento do Carmo individuals in this study are likely authentic.

5.2 Kinship Analysis

5.2.1 La Sagrera

Kinship analysis of the individuals at La Sagrera was complicated by the low mitochondrial genome coverage of samples, which oscillated between 1.6% and 62% ($\geq 1X$). As previously discussed in section 5.1.1, possibility of contamination and the lack of comparable positions meant that low coverage samples, with coverage below 62.5%, were unreliable. Possible matrilineal relationship as mentioned in Table 20 could be a by-product of poor coverage, which led to a lack of comparable positions. Only in one case, between individuals SA44 and SA4, was there a shared mutation and no instances of different mutations in sites with coverage in both samples. This was not enough to determine if these individuals were matrilineally related.

Although it was not possible to determine if any individuals were matrilineally related, it was possible to rule out matrilineal kinship in many cases. It is clear from Table 20 that individuals within burial clusters were mostly unrelated, with some individuals being possibly related. Comparison of individuals between clusters A and B showed that there was no matrilineal relationship between individuals. The individual from cluster C, SA4, was not related to individuals SA56 or SA68 from cluster B. Matrilineal kinship could not be ruled out between SA4 and individuals in cluster A. Assuming that there was no contamination of the samples, there appears to be no pattern between matrilineal relation and burial practice at La Sagrera. Unfortunately, the possibility of contamination is too great to say with any certainty that these results are reliable.

A lack of matrilineal relation between the individuals at La Sagrera would contrast with other Late Neolithic hypogeum sites. Sites that exhibit clear clusters of individuals in these sites are typically arranged based on kinship (Alt et al., 2016; Haak et al., 2008). If there is an absence of kinship at La Sagrera, it could indicate that the event that led to the mass burial deposition at La Sagrera affected a larger diverse population, rather than just a single-family unit. It should also be noted that without the use of Y-chromosome analysis, patrilineal kinship between the individuals at La Sagrera cannot be discounted.

5.2.2 Convento do Carmo

Although kinship analysis was not conducted on Convento do Carmo individuals, it should be noted that previous studies have suggested it was a homogeneous population. This was based on results of three individuals all within major haplogroup K, two of which shared a haplotype (Fernández-Domínguez et al., 2019). Partial haplotypes of individuals CVC1, CVC2, and CVC8 with the mutations 16311C and 16362C also suggested this may be the case (Fernández-Domínguez et al., 2019; Neff, 2016). This was not supported in the results of this study, however. Only two individuals, CVC1 and CVC3, shared a haplogroup in this study, and no individuals shared a haplotype. Based on these results, it can be said that the population at Convento do Carmo was 100% mitochondrially heterogeneous (haplotypes present/total number of individuals), similar to other Bell Beaker Culture Chalcolithic sites in Portugal such as Cova da Moura (100% heterogeneity), Verdelha dos Ruivos (100% heterogeneity), and Bolores (100% heterogeneity) (Martiniano et al., 2017; Olalde et al., 2019, 2018).

5.3 Contextualisation of Individual Results

5.3.1 Algar do Barrão

The results of the three individuals from Algar do Barrão display a mix of both West Hunter Gatherer (WHG) and European Early Farmer (EEF) maternal lineages at the site. Individuals BAR3 and BAR5 belong to the major haplogroup U5b associated with WHG groups, while BAR4 is of the K1a major haplogroup, associated with EEF and Early Neolithic Near East populations (Kılınç et al., 2016). Isotopic $^{87}\text{Sr}/^{86}\text{Sr}$ results place all these individuals within the local bioavailable Sr range (Carvalho et al., 2019), meaning BAR4 was not likely a first generation migrant, and WHG and EEF populations had already admixed in the region by ~3,250 BC.

BAR3, which is dated to a slightly earlier period (~3700 BC) than the other two samples (Carvalho et al., 2019), belongs to a WHG haplogroup as well as having higher percentages of marine protein as determined from carbon and nitrogen stable isotope analysis (Carvalho et al., 2019). Individual #01 from Algar do Barrão (dated to ~3690 cal BC), which was not selected for genetic studies, had a similar diet of 11% marine protein and 21% aquatic protein (Carvalho et al., 2019). These individuals could potentially indicate a continuation of local hunter-gatherer subsistence traditions at the site, as typical Neolithic individuals have a more terrestrial based diet, including the other individuals at Algar do Barrão (Carvalho et al., 2019; Richards et al., 2003; Richards and Schulting, 2006; Salazar-García et al., 2017). At the site of Blätterhöhle, Germany, Bollongino et al (2013) found a correlation between Neolithic individuals with U haplogroups and a diet high in freshwater fish. A similar pattern may have occurred in the Portuguese Neolithic, with certain cultural practices such as marine based subsistence and burial traditions being maintained within groups with hunter-gatherer lineages.

Although uncommon, other sites in Iberia have shown evidence of cultural continuity between the Mesolithic and Neolithic periods. Cueva de la Cocina in Valencia (García-Puchol et al., 2025) shows evidence of burial reuse associated with the Cardial pottery tradition around 5,250 cal BC, ~200 years after the last use by Mesolithic populations (García-Puchol et al., 2018; Molina-Balaguer et al., 2023), although modern disturbance and removal of sediment caused loss and mixing of layers (García-Puchol et al., 2025). Within the same geographical Tagus River valley region as Algar do Barrão, Bicho et al (2013) concludes that after the end of the Mesolithic period in the region (~5450 cal BC), Neolithic groups were reusing Mesolithic burial sites.

Bicho et al (2013) also suggests that the reuse of sites may be related to mixing and integration of Mesolithic and Neolithic people in the region. The genetic evidence

extracted from the AADR database (Mallick et al., 2024) supports this, as 29% of Middle to Late Neolithic individuals from Portugal carry the U5b WHG associated mtDNA lineage, while the rest carry EEF associated haplogroups. The increased presence of WHG ancestry in Portugal during the Middle Neolithic when compared to other Iberian populations both through mtDNA (29% U5b in MLN Portugal, 17-20% in other Iberian populations, see Figure 24) and whole nuclear genome (Roca-Rada et al., 2024) could suggest that Mesolithic populations played a greater role in the development of cultural practices in the region during the Neolithic, continuing to use local burial sites. Further studies taking an interdisciplinary holistic approach combining carbon and nitrogen dietary isotopic data with genomic data of Mesolithic and Neolithic populations in Portugal would be able to determine more confidently if any correlations between genetic lineage, diet, and burial site reuse exist in Early to Middle Neolithic groups in the region.

The distribution of U2b2b sub-haplogroup, to which individual BAR3's lineage U5b2b* belongs (see section [4.5.1](#) and Figure 18) in the Ancient Allen Database Resource (AADR)(Mallick et al., 2024) used for result contextualisation covers a wide area from Western Europe to Central Asia (see Figure 18). Chronological and phylogenetic evidence described in section [4.5.1](#) confirms U5b2b as a WHG lineage which possibly originated or increased in frequency in Epigravettian populations in the Italian and Southeastern European refugia during the last glacial maximum (Översti and Palo, 2022; Posth et al., 2016). U5b2b is estimated to have diverged from U5b2 ~26,700 years ago at the earliest, while the earliest known sub lineage of U5b2b, U5b2b-a, diverged from U5b ~8,400 years ago, meaning U5b2b* likely diverged from U5b2b in the period between 26,700-8400 years ago (Översti and Palo, 2022).

The U5b2b lineage then appears to have spread throughout Northern and Western Europe in the Mesolithic alongside the expansion of WHG populations from the southern refugia as the glaciers retreated. The lack of evidence of the U5b2b haplogroup in Iberia prior to the Mesolithic could be a signature of divergent genetic profiles between the Iberian and the Southeast European refugia during the Palaeolithic, but the small sample size of only 11 individuals from 7 Palaeolithic sites in Iberia between 26000-10000 cal BC leaves doubt about whether this haplogroup perhaps has not been observed yet. As the U5b2b haplogroup has been reported during the Mesolithic in Iberia (1 individual from Cueva de la Cocina, ~6000 cal BC), individual BAR3 likely represents evidence of mitochondrial continuity of WHG lineages between the Mesolithic and Neolithic in Portugal. The number of individuals found within this haplogroup and the appearance of the haplogroup throughout Europe and Central Asia in Chalcolithic through Iron Age periods suggest it was a common WHG lineage and a major part of the WHG genetic contribution to later populations. Sub lineages of U5b2b (U5b2b1-U5b2b5) follow the same general pattern of spread, and cannot be associated with any particular regions (Mallick et al., 2024).

Haplogroup U5b1c from BAR5 has been found in only 12 individuals in the AADR database, all from Western Europe with the exception of 3 Early Medieval individuals from Hungary. This haplogroup diverged from U5b1 ~15,800 years ago (Översti and Palo, 2022). The appearance of U5b1c exclusively in Western Europe up until the Early Medieval period could mean the divergence occurred in this region or from a Mesolithic group which expanded throughout this region after the last glacial maximum. Although it has not been reported yet before the Neolithic period, its place within the U5b major haplogroup still identifies it as a WHG lineage, and it is possible it existed much earlier in Western Europe. Individual BAR5 marks the earliest appearance of this haplogroup in Iberia, about 1,300 years before the Chalcolithic individual at Camino de las Yeseras based on radiocarbon dating (Carvalho et al., 2019; Olalde et al., 2018). This is important, as it means the appearance of this haplogroup alone cannot be used as a sign of migration into Northern Iberia from Central Europe during the Late Neolithic or Chalcolithic.

The EEF associated haplogroup K1a1b1, found in individual BAR4, is mostly found in Western Europe. Interestingly, no instances of this specific haplogroup have been uncovered in the Near East or Eastern Mediterranean regions. This could be evidence that K1a1b1 diverged from K1a1b in Europe after the initial Neolithic spread, although the generally poor preservation in the region means that specific haplogroup classifications cannot often be made and it is possible the divergence occurred earlier in the Near East. It has previously been reported that haplogroup K1a1b1 is one of the founder Ashkenazi Jew lineages that was assimilated within Europe, and K1a1 lineages had likely spread to Europe during the Late Glacial period between ~18-12 kya before the coalescence of the K1a1b1 haplogroup ~11,700 years ago (Costa et al., 2013). This, however, was based on phylogenetic analysis of modern data. The present study finds no evidence of any K1a lineages in Europe before the Neolithic in the AADR (Mallick et al., 2024), and therefore refutes the claim that K1a1b1 lineages represent pre Neolithic Western European ancestry. The earliest instance of K1a1 appears in the database in a transitional Early Neolithic individual from the site of Lepenski Vir, dated to ~6,000 cal BC (Marchi et al., 2022). This lends credence to the theory that K1a1 lineages could have existed in Southeast Europe before the arrival of the Neolithic, likely due to bidirectional movement of people between Southeast Europe and the Near East prior to the Neolithic expansion, and were assimilated into the Neolithic genetic population that then spread throughout the rest of Europe via the Central European route as previously proposed (Marchi et al., 2022; Mathieson et al., 2018). The lack of appearance of the K1a1b1 lineage before the Early Neolithic site of Pendimoun in France (~5400 cal BC) (Rivollat et al., 2020), suggests it likely followed a similar pattern to other K1a1 lineages, existing in the Near East and Southeast Europe before the Neolithic before spreading to the rest of Europe in the Early Neolithic (Costa et al., 2013; Marchi et al., 2022). In any case, the appearance of the K1a1b1 haplogroup in Europe only after the Mesolithic

period solidifies it as an EEF lineage. In the Chalcolithic through Iron Age periods, it is less common and found only in Western Europe.

Overall, the mitochondrial genetic evidence from the site of Algar do Barrão provides interesting insight into the Middle Neolithic period of West Iberia as well as Iberia as a whole. The Early Neolithic period in Portugal saw the arrival of the Cardial pottery tradition alongside agricultural practices (Antolín, 2016; Defranould, 2021). Unlike other areas of Early Neolithic Europe, where HG ancestry rarely accounted for >10% of whole genome ancestry, Portuguese Early Neolithic individuals were reported as having 27-43% local HG ancestry (Allentoft et al., 2024). This increased percentage of HG ancestry persists into the Middle and Late Neolithic (reported as ~28.25%) (Roca-Rada et al., 2024). Along with this, reuse of Mesolithic sites and potentially subsistence techniques indicate a greater contribution of HG groups to the Neolithic of Portugal when compared to the rest of Iberia and Western Europe (Bicho et al., 2013; Olalde et al., 2019; Roca-Rada et al., 2024). The combination of HG and EEF lineages at the site of Algar do Barrão exemplify the admixture of populations during this period, as well as the increased proportion of HG lineages (66.6%) previously supported by the literature.

The possibility of a shift in diet from the earlier period of the site to the later period makes this site a great candidate for future studies attempting to understand how the shift between Mesolithic and Neolithic diets correlated with migration events. The site of Algar do Bom Santo offers a close comparison to Algar do Barrão, both in period of use (3800-3380 cal BC), geographical region (Tagus River valley) and bioarchaeological results (Carvalho et al., 2019, 2016). Of the nine individuals successfully analysed using a PCR approach, 2 (22.2%) had U5 haplogroups, while 5 (77.7%) had EEF related haplogroups (T2, J, HV0, H10, K1a, H1/R8), showing a similarly admixed - although more EEF-heavy population – as that of Barrão (Carvalho et al., 2016). Individuals at the site also had a wide range of marine (2-14%) and aquatic (6-42%) protein in their diets, although the individuals with higher values do not correlate with earlier radiocarbon dates as is the case with Algar do Barrão (Carvalho et al., 2019). It is likely, then, that this correlation seen in Barrão is a result of small sample size rather than a genuine shift in dietary patterns. In both this study and the previous study of these samples (Robinson, 2018), preservation has been shown to be exceptional. Future analysis on the remaining 4 mandibles recovered from Algar do Barrão, specifically individual BAR1 who was of the earlier period of the site and had a high marine protein diet similar to BAR3, will likely yield positive and interesting results. Genomic analysis of all Algar do Barrão individuals will also be able to reveal more nuanced ancestry patterns.

5.3.2 Convento do Carmo

The Chalcolithic Bell Beaker context at the site of Convento do Carmo provided samples of relatively good preservation, and haplogroup assignments were able to be made for seven of the nine individuals studied.

CVC1 and CVC3:

Individuals CVC1 and CVC3 from Convento do Carmo are so far the only two studied archaeological individuals from Portugal belonging to the mitochondrial haplogroup U3a1, which branched off from U3a ~4-7 kya (Derenko et al., 2013). Previous studies have confirmed the spread of this haplogroup from Central Europe into Northern Iberia during the Neolithic period (Villalba-Mouco et al., 2019). Based on the results of this study, it can now be said that this haplogroup spread further into Iberia, reaching the Estremadura region of Portugal by 2460-2290 Cal BC. The presence of no other U3 sub haplogroups in Iberia suggests that the U3a1 haplogroup likely diverged from U3 outside of Iberia before its arrival in the MLN. This is supported by the fact that the earliest examples of U3 come from 6 individuals at two sites in modern day Türkiye between around 10190 and 8085 years BP (Feldman et al., 2019; Kılınç et al., 2016; Lazaridis et al., 2022; Mathieson et al., 2015b) and one individual in Romania from around 7700 years BP (Lazaridis et al., 2022). These are then followed by the first instances of U3a1 in the LBK site of Asparn Schletz in Austria (Gelabert et al., 2025) and the expansion of the haplogroup throughout Central and Western Europe (Fowler et al., 2022; Gelabert et al., 2025; Mathieson et al., 2015b; Papac et al., 2021; Penske et al., 2023). This suggests that the U3a1 haplogroup spread across Central Europe throughout the Neolithic, reaching the Pyrenees in the MLN and eventually Central Portugal in the Chalcolithic. It could also be the case, however, that the haplogroup was present in Neolithic Portugal and has not yet been detected.

The existence of U3a1 in Iberia predates the emergence of the Bell-Beaker Culture. In contrast to the rest of Europe, previous genetic evidence suggests that there was relative continuity in the genetic composition of Iberia between the MLN and the Chalcolithic (Olalde et al., 2018). It is therefore likely that the two individuals with U3a1 haplogroups in this study represent MLN Iberian lineages that continued into the Chalcolithic and adopted the Bell-Beaker Culture. As U3 haplogroups are found at Pre Pottery Neolithic and Late Neolithic sites in Anatolia, as well as in Early Neolithic sites in Europe, these haplogroups could signify early farmer migrations from Anatolia into Europe. Association of U3a and U3a1 to Linear Bandkeramik sites in Austria and Germany associate these haplogroups more with the Neolithic expansion into Southeast Europe rather than the Neolithic expansion along the Mediterranean coastline, although data from Cardial and Impressa contexts is not complete enough to

exclude the Mediterranean route as a possibility. Connectivity and gene flow between populations of these two Neolithic expansions into Europe in the Middle and Late Neolithic could have led to the appearance of U3a haplogroups in Iberia.

CVC4:

Individual CVC4 was determined to belong to haplogroup V23. Its presence in only one other ancient individual in the entire database suggests that this haplogroup was exceptionally rare in ancient populations. Although no reliable coalescence date for this haplogroup exists in the current literature, YFull gives an estimate of ~2400 years BP (“YTree,” 2025). Interestingly, individual CVC4 (dated to ~3900 years BP by context, see section 2.2) predates the estimated formation of the V23 haplogroup by ~1500 years (“YTree,” 2025).

The commonality of V sub haplogroups throughout Neolithic Europe and the absence of V haplogroups before the Neolithic suggest V haplogroups are associated with EEF ancestry, which is supported by the current literature (Brandt et al., 2013; Haak et al., 2010). Haplogroup V diverged from HV0 between ~16-11.5 kya (Brandstätter et al., 2008). No V or HV0 haplogroup individuals from the Near East were found in the database, suggesting this could be a case of an EEF lineage that diverged in Europe and was assimilated into the EEF population during the initial migration event from the Near East. V haplogroups are associated with the LBK neolithic culture, and may therefore potentially represent Neolithic spread through the Central European route as discussed with haplogroup U3a1 previously (Brandt et al., 2013; Haak et al., 2010).

V haplogroups had reached Iberia by at least the Early Neolithic, as an individual within the Early Neolithic context of Cova de Els Trocs has been found to be of haplogroup V (Mathieson et al., 2015b). The $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of individual CVC4 is outside of the established local range (Fernández-Domínguez et al., 2019). As V haplogroups have been established to exist in Portugal in similar frequencies in the Middle to Late Neolithic (7%) and Chalcolithic (11%)(see section 4.5.2), the most probable provenance of individual CVC4 remains the nearby Beira region suggested by the previous publication (See section 2.2)(Fernández-Domínguez et al., 2019).

The only other appearance of haplogroup V23 in the database corresponds to an individual from the site of Casterley Camp Iron Age hillfort in Wiltshire, which could suggest gene flow or a migration event between West Iberia and Southwest England or vice versa at some point between the Chalcolithic and Iron Age. More likely, however, the haplogroup followed a similar dispersal pattern to other V sub-haplogroups like V1 and V7, and its rarity in ancient populations has caused it to only be found in two individuals to date. As V23 is distinguished from V only by the mutation 6734A (Van

Oven and Kayser, 2009), it is also possible that a lack of resolution is responsible for the scarce appearances of the haplogroup in the database.

CVC6:

T2e1 was determined to be the haplogroup of individual CVC6. As described in section 4.5.1, the 14 individuals from the database with this sub-haplogroup were distributed almost exclusively in Northern Europe. T2 as a major haplogroup appears to have originated in the Near East ~21.3 kya (Pala et al., 2012), and is first found in 8 individuals from the Neolithic sites of NevalıÇori (N=1, 8281-7849 cal BC), Tepe-Abdul-Hosein (N=1, 8201-7751 cal BC), Aşıklı-Höyük (N=2, 8198-7657 cal BC), Boncuklu (N=1, 10483-4554 cal BC), and Çatalhöyük (N=3, 7100-6000 cal BC) in Türkiye and Iran (Altınışik et al., 2022; Broushaki et al., 2016; Wang et al., 2023; Yaka et al., 2021), and is associated with EEF ancestry (Fernández-Domínguez et al., 2017). More specifically, the T2e haplogroup, which is estimated to have diverged from T2 ~11 kya (Pala et al., 2012), is first reported in the Late Neolithic site of Çatalhöyük in Anatolia (Yaka et al., 2021) before appearing in other Early Neolithic sites in Southeast Europe (Gelabert et al., 2025; Marchi et al., 2022; Mathieson et al., 2018), and eventually Germany (Childebayeva et al., 2022; Mathieson et al., 2015b).

The earliest appearances of T2e1 in five Neolithic individuals in Britain, 4 from the site of Hazelton North (~3930-3350 cal BC)(Fowler et al., 2022) and 1 from the site of Caithness (~3768-3638 cal BC)(Olalde et al., 2018), could indicate that this lineage diverged from T2e in Northern Europe – as T2e is found at the sites of Stuttgart-Mühlhausen-2 (5500-4850 cal BC)(Mathieson et al., 2015b) and Derenburg-Meeranstieg-2 (5211-4993 cal BC)(Childebayeva et al., 2022) in Germany - and gene flow between Britain, Northern Europe, and Iberia caused it to eventually reach the sites of Convento do Carmo and Bray Cave in Gibraltar (Olalde et al., 2019). The estimated time of divergence of T2e1 from T2e is ~9 kya (Pala et al., 2012), which predates the Neolithic expansion into Europe. Similar to haplogroup V23, discussed in the previous section, another possibility is that haplogroup T2e1 followed a similar distribution into Western Europe as other T2 and T2e haplogroups, matching the expansion of the Neolithic, and its lack of appearances in the database is due to its specific rarity in the ancient populations or lack of resolution in ancient DNA haplogroup analysis.

CVC7:

The individual CVC7 harboured haplogroup J1c3. The earliest individuals with haplogroup J1c, which originated ~16 kya (Pala et al., 2012), have been found at the Late Neolithic site of Barcın-Höyük in Northwestern Anatolia (Lazaridis et al., 2022;

Mathieson et al., 2015b). Although previous studies have lacked the resolution to determine specific sub lineages, J haplogroups have been associated with the spread of the Neolithic throughout Europe in the Early Neolithic, specifically with the LBK culture (Brandt et al., 2013; Fernández-Domínguez et al., 2017; Haak et al., 2010; Szécsényi-Nagy et al., 2015).

J1c3 splits off from the J1c lineage around 11 kya (Pala et al., 2012), before the spread of the Neolithic into Europe. The concentration of J1c3 in Western Europe and its oldest appearance at the site of Pendimoun on the Southeast coast of France (5479-5331 cal BC) (Rivollat et al., 2020) could indicate that the haplogroup represents a lineage from a group that spread to Western Europe along the Mediterranean coastline as proposed by Fernández-Domínguez et al (2017), and was present in the spread of Neolithic populations from these regions into the rest of Western and Atlantic Europe. Although Pendimoun is the oldest appearance of J1c3, the distribution of J1c3 sub lineages throughout Central and Eastern Europe (in chronological order from ~5300-3025 cal BC: 1 in Romania, 1 in East Central France, 1 in Hungary, 2 in Denmark, and 2 in Germany) in the Neolithic more closely resembles the pattern of Neolithic spread through Central Europe (Marchi et al., 2022; Mathieson et al., 2018).

CVC8:

Haplogroup H3 was formed ~10.2 kya (Brandstätter et al., 2008), with haplogroup H3r diverging an estimated ~10.2 kya according to YFull (“YTree,” 2025). This haplogroup, found in individual CVC8 in this study, was not present in any individuals in the ancient database, suggesting it was rare in ancient populations.

It was initially suggested based on the coalescence date and the concentration of H3 in modern Iberian populations that H3 was formed in the Iberian refugium during the Last Glacial Maximum (Torroni et al., 2006). The distribution of H3 and its sub-haplogroups in the ancient database (Mallick et al., 2024), with earliest occurrences in Serbia (Lepenski-Vir, ~6150 cal BC) (Marchi et al., 2022) and France (Hungeheuer-Hoelzle, ~5100 cal BC) (Brunel et al., 2020) in the Early Neolithic followed by a widespread distribution throughout all of Europe in later periods, suggests that H3 is more likely associated with EEF ancestry. This is supported by the association of undefined and other H lineages with a migration of early farmers from Anatolia (Brotherton et al., 2013; Fernández-Domínguez et al., 2017). Brotherton et al (2013) suggests that H3 and sub lineages may have diverged in Iberia during the Late Glacial Maximum, and later Middle to Late Neolithic demographic changes as well as the expansion of the Bell Beaker Culture are responsible for the expansion of H3 from Iberia to the rest of Europe. The identification of H3r in the Bell Beaker individual CVC8 in this study supports the idea that H3 can be associated with Bell Beaker populations but

does not provide insight into how H3 originally reached Iberia. A previous PCR approach study identified H haplogroups in Iberia before the Neolithic (Hervella et al., 2012), but this has not been confirmed by whole mitogenome studies (see section [4.5.2](#)).

Ultimately, the lack of H3 being found in any pre–Neolithic European populations or ancient Anatolian or Near East populations (which may be a result of a lack of resolution due to poor coverage caused by low preservation in the region), as well as a lack of basal H haplogroups in Iberia before the Neolithic, leaves the debate about the exact provenance and spread of H3 lineages still open. It is impossible to say without further evidence any more about the spread of the H3r lineage specifically, and whether it followed a similar expansion to other H3 haplogroups or are unique to West Iberia or Iberia as a whole.

CVC9:

Individual CVC9, as BAR5 from Algar do Barrão, belonged to haplogroup U5b1c, and its distribution and possible origins were discussed in section [5.3.1](#). The appearance of this haplogroup at the site of Convento do Carmo further confirms the existence of this haplogroup in West Iberia and its presence in the Chalcolithic period. Similar to the haplogroups of other individuals at Convento do Carmo that have been discussed, its presence in the Middle Neolithic and the Chalcolithic could be explained as evidence of the genetic continuity in mitochondrial lineages between these periods.

Bringing together the analysis of the haplogroups of the Convento do Carmo individuals, the site seems to represent a population of mostly EEF associated mitochondrial lineages with a small persistence of HG lineages (in this case individual CVC9). This mitochondrial composition is similar to other Bell Beaker contexts in Iberia, such as at Alto de la Huesera in the Basque Region (3150-2650 cal BC; 28.5% HG, 71.5% EEF) (Olalde et al., 2019), Camino de las Yeseras in Madrid (2622-1700 cal BC; 44% HG, 25% EEF, 31% other)(Olalde et al., 2018), and Camino del Molino in Murcia (Olalde et al., 2018).

The site of Camino del Molino provides an especially interesting comparison to Convento do Carmo. Although the true size of the Convento do Carmo hypogeum could not be determined, the hypogeum of Camino del Molino was likely much more populated, with a minimum number of 1348 individuals (Díaz-Navarro et al., 2023). Of the nine individuals successfully analysed from this site, two were of major haplogroup H1, two of J1c, one of J2a, one of V, one of K1a, one of H3, and one of U5b (Olalde et al., 2018; Villalba-Mouco et al., 2021). The similarity of these sites is also reflected in the archaeological context. As mentioned, both sites were hypogeums associated with the Chalcolithic period. Radiocarbon dating of the nine Camino del Molino individuals gives a range between 3300-2100 cal BC (Díaz-Navarro et al., 2023), which includes

Convento do Carmo's range of 2550-2290 cal BC (Petchey, 2019). The site had a relatively equal osteological sex ratio (27.9% female, 29.7% male, 4.6% probable female, 4.6% probably male, 33.2% undetermined) (Díaz-Navarro et al., 2023) similar to that of Convento do Carmo (Granja, 2019). A small number of grave goods at Camino del Molino associate it also with the Bell Beaker culture (Lomba-Maurandi et al., 2009), however, more recent research concludes that most of the occupation of the site predates the Bell Beaker culture, although there is still some degree of chronological overlap (Díaz-Navarro et al., 2023). The populations at Convento do Carmo and Camino del Molino differ in size and age at death (Díaz-Navarro et al., 2023), likely meaning the circumstances around the deaths of these groups were different. Despite this, the mitochondrial genetic evidence and artefactual evidence point to similar population composition at the sites.

The Chalcolithic Bell Beaker context population at the site of Convento do Carmo can be described as a typical Iberian Bell Beaker population. The appearance of only one HG associated mitochondrial lineage and the greater percentage of EEF associated haplogroups matches the overall population trends of Bell Beaker groups in Iberia. Richards and Fernández-Domínguez (2022) propose haplogroups U5a, U4, T1, and T2 as lineages assimilated in the steppe and matrilineal signifiers of the spread of steppe ancestry throughout Europe in the Chalcolithic and Early Bronze Age. This spread of steppe ancestry has previously been associated with the spread of the Bell Beaker culture across Atlantic and Central Europe (Olalde et al., 2018). Of these haplogroups, only T2 was found at Convento do Carmo, in individual CVC6. In all cases, the haplogroups or closely related haplogroup lineages of the individuals at Convento do Carmo were present in Iberia already in the Neolithic period (see section 4.5.2). Results from Convento do Carmo in this study therefore show no particular association to steppe ancestry and are more likely to represent genetic continuity between the Middle to Late Neolithic/Chalcolithic in the region. This is in line with genomic results from Olalde (2018), which determined that steppe ancestry was less prevalent in the Bell Beaker populations in Iberia (~0-30% of ancestry) when compared to the rest of Europe (~25-75% of ancestry).

The mitochondrial genetic evidence does not provide much more insight into the provenance of the two individuals that were outside the expected local $^{87}\text{Sr}/^{86}\text{Sr}$ ratio range. The V23 haplogroup assigned to CVC4 was likely present in Iberia before the Chalcolithic and therefore cannot be associated with a population movement during the Chalcolithic period. Unfortunately, the only other individual outside the expected local $^{87}\text{Sr}/^{86}\text{Sr}$ ratio range, CVC2, did not produce reliable results. No patterns between mitochondrial genetic lineage and burial group were observed in the site, as the only WHG lineage individual was found in Ossuary 1 along with four individuals with EEF associated haplogroups.

5.4 Population Structure in Mesolithic to Bronze Age Iberia

Figure 39 below provides a temporal depiction of haplogroup frequencies based on their association to HG or EEF populations presented in section 1.4.

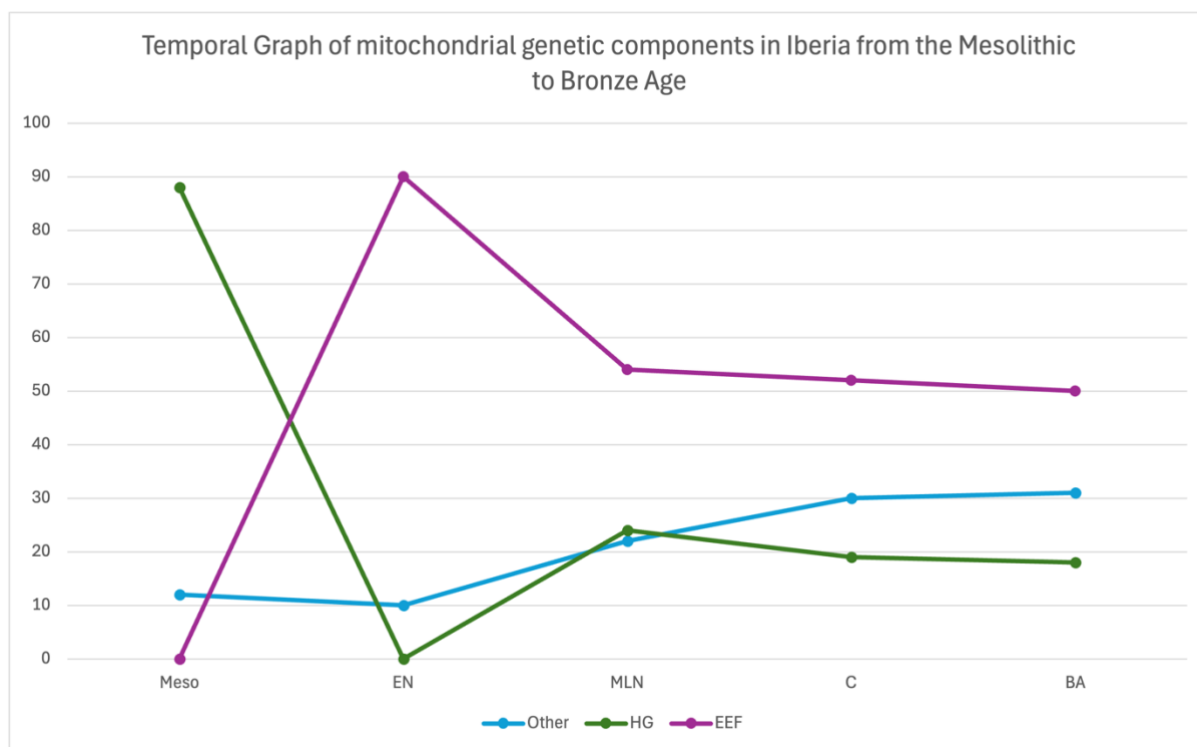


Figure 39: Temporal graph of the percentage of Hunter Gatherer (HG)(U5a, U5b), Early European Farmer (EEF)(K, T, J, HV, V, N), and other (H, other U, M, L, I) associated mitochondrial haplogroups in Mesolithic to Bronze Age populations in Iberia based on analysis in this study and previously published material in the AADR (Mallick et al., 2024). All Bronze Age individuals are combined into one population.

5.4.1 Mesolithic Iberian Haplogroup Frequency

Although there is only a small sample size of 14 Mesolithic individuals for the entirety of Iberia in the AADR database (see [Supplementary Table S4](#) for sites and detailed chronology) (Mallick et al., 2024), the results show a clear difference in haplogroup lineages in relation to later periods. The presence of exclusively U haplogroups in Mesolithic Iberia matches with Mesolithic populations in other parts of Western Europe, supporting the idea of a genetic bottleneck during the Late Glacial Maximum in Europe creating a relatively homogeneous population (Fu et al., 2016; Pala et al., 2012; Posth et al., 2023; Simões et al., 2024). Posth et al (2016) propose that this bottleneck event led to U5 lineages becoming the predominant Mesolithic HG lineages

in Europe. Although there is some debate about other sub haplogroups within U (U3, U4, U6, and U7 all appear outside of Europe before the Neolithic)(Mallick et al., 2024), U5 offers a clear mtDNA signal that can be used to identify local hunter-gatherer ancestry in later periods (Pala et al., 2012; Posth et al., 2023; Simões et al., 2024). This genetic signal will be referred to as Hunter-Gatherer, or HG.

Studies of genomic HG ancestry in Iberia during the Mesolithic have identified two distinct sources (Posth et al., 2023; Villalba-Mouco et al., 2019). The ‘GoyetQ2’ ancestral component - named after the ~17 ky old Upper Palaeolithic individual GoyetQ-2 from the site of Troisième caverne of Goyet in Belgium - is associated with the Magdalenian culture observed from Iberia to Eastern Europe ~19-14 kya (Allentoft et al., 2024; Fu et al., 2016; Villalba-Mouco et al., 2019). The ‘Villabruna’ ancestral component – named for the ~14 ky old Upper Palaeolithic individual Villabruna 1 from the site of Ripari Villabruna in Northern Italy – is associated with the Epigravettian culture which spread from the Southeast refugium throughout Europe after the last glacial maximum (~14600 years bp) (Fu et al., 2016; Montoya and Peresani, 2005). This ancestral component replaced GoyetQ2 ancestry in Mesolithic populations throughout most of Europe (Villalba-Mouco et al., 2019). However, at some point in the Palaeolithic, these ancestries admixed, creating a distinct Mesolithic HG genomic signature primarily found in Iberia (Olalde et al., 2019, 2018; Posth et al., 2023; Villalba-Mouco et al., 2019), often referred to as ‘La Braña 1’(named for the ~7030 year old Mesolithic individual at La Braña in León, Spain)(Olalde et al., 2019, 2018; Villalba-Mouco et al., 2019). Although the exact time and location of this admixture cannot be discerned by the current data, the first instance of this admixed ancestry observed in an individual at the site of El Mirón demonstrates that the admixture occurred by at least ~18700 years ago (Villalba-Mouco et al., 2019). Interestingly, the earliest Mesolithic Iberian individual, Chan, from Northwest Iberia (~7995 bp) was more similar to the El Mirón individual than La Braña 1 in Cantabrian Region, which in turn was more similar to Central European HGs (González-Fortes et al., 2017; Olalde et al., 2019). More genomic data from the Mesolithic is needed before any temporal or geographical patterns related to the different levels of these ancestries in the Mesolithic can be established (Olalde et al., 2019).

5.4.2 Early Neolithic

The Neolithic arrived in Iberia by at least 5500 cal BC (Martins et al., 2015) alongside the Cardial pottery tradition along the Mediterranean coast, which was followed by the development of the Epicardial in the Central region around ~5200 cal BC (Cámara Manzaneda et al., 2022) (see section [1.3](#)). Archaeological observations have shown the Neolithisation of Central Iberia occurred chronologically later than in coastal regions of Iberia, suggesting that maritime travel was a major factor in Neolithic spread

in Iberia (Davison et al., 2006; Fernández et al., 2014; Paschou et al., 2014). In this theory, migration of farmers of Anatolian Neolithic descent across the Mediterranean and along the Iberian coasts was followed by the spread of the Neolithic to the inner regions of Iberia through a combination of cultural diffusion and integration into HG groups (Alday et al., 2024; Szécsényi-Nagy et al., 2017; Villalba-Mouco et al., 2019).

Genetically, the Early Neolithic population in Iberia is represented in the AADR database by published mtDNA data from 21 individuals (Mallick et al., 2024). Of these, only two are from Portugal, both of the Cardial context at the site of Gruta do Caldeirão (5474-5063 cal BC; haplogroups T2, J2b) (Allentoft et al., 2024). In Spain, the first Neolithic mitochondrial evidence comes from an individual at the Cardial site of Cova Bonica (haplogroup K1a, dated to ~5400 cal BC) (Olalde et al., 2015). This is followed by five individuals from the first phase of mass inhumation at the site of Cova de Els Trocs, dated to between 5312-5051 cal BC (haplogroups J1c, N1, V, K1a, T2) (Mathieson et al., 2015b; Tejedor-Rodríguez et al., 2021). An individual from Cueva de los Murciélagos (J1c) (Valdiosera et al., 2018) and three individuals from the Cardial site of Cueva de Chaves (HV0, 2 K1a) (Villalba-Mouco et al., 2019) are of similar dates (5308-4991 cal BC). Two individuals from the sites of El Portalón Cave (J2b) (Valdiosera et al., 2018) and El Prado de Pancorbo (H1) (Lipson et al., 2017) both fall chronologically between 5214-5015 cal BC, and are associated with the Cardial pottery tradition (Alday et al., 2019; Lipson et al., 2017). From a Neolithic context of the primarily Chalcolithic site of Fuente Celada, one individual (haplogroup X2) dated to 5209-4947 cal BC is present in the database (Szécsényi-Nagy et al., 2017; Villalba-Mouco et al., 2019). Four individuals (2 K1a, 2 T2) from the Cardial site of El Toro between 5100-4800 cal BC (Fregel et al., 2018) are next in the chronological sequence, followed by another individual of a slightly later date from El Prado de Pancorbo (Lipson et al., 2017). The most recent individual (U4, 4545-4367 cal BC) is from the previously mentioned site of Cueva de Chaves (Villalba-Mouco et al., 2019). For more detailed genomic information and chronologies of these individuals, see [Supplementary Table S4](#).

Despite the small sample size, the appearance of haplogroups different from those present during the Mesolithic and the presence of just one individual harbouring a U haplogroup (U4, Cueva de Chaves, ~4450 cal BC), clearly differentiates the Neolithic population from the Mesolithic one. This new genetic signature has been referred to as Early European Farmer, or EEF (Mathieson et al., 2015b). While mitochondrial data suggests a near complete genetic turnover (see Figure 39), whole nuclear genome analysis of Early Neolithic Iberians uncovers a more nuanced story. HG ancestry levels in Cardial and Epicardial individuals from eastern Iberia have been reported up to 20% (Carvalho et al., 2023; Olalde et al., 2019; Villalba-Mouco et al., 2019). This ancestry is also closer to Iberian Mesolithic ancestry rather than other European HG ancestry (Allentoft et al., 2024), demonstrating that local Iberian HG peoples did admix with EEF populations. In Portugal, Caldeirão individuals (~5474-5063 cal BC) had higher levels of

27-43% HG ancestry (Allentoft et al., 2024), indicating a greater incorporation of local HG and early farming groups in this region, supported by the reuse of Mesolithic burial sites in the Neolithic (Bicho et al., 2013). The small sample size of only 2 individuals should be noted, as this may not be an accurate representation of the contemporary population.

The appearance of new haplogroups and EEF genomic ancestry in the Early Neolithic period of Iberia suggests a large-scale migration event. Previous genetic studies have placed the origin of this migration in Late Neolithic Northwest Anatolia (Fernández et al., 2014; Mathieson et al., 2018; Szécsényi-Nagy et al., 2015). Nuclear genomic studies have determined that the non-HG related ancestry in Neolithic Iberian individuals is most closely related to that of the Anatolian Neolithic (Fregel et al., 2018; Olalde et al., 2019; Valdiosera et al., 2018). When analysing mtDNA types specifically, the haplogroup frequencies of Neolithic Near Eastern populations are similar to those observed in Early Neolithic Iberia in this study. K, R0 (which includes H, HV, and V), H haplogroups have all been found in Syrian populations before the arrival of farming in Europe (Fernández et al., 2014). JT haplogroups are also prevalent in the Iberian Early Neolithic. Haplogroups of the JT branch appear in pre and Early Neolithic sites from Anatolia (Mathieson et al., 2015b). K, JT, and R0 haplogroups do not appear in Iberia until the Early Neolithic, where they comprise ~66% of the population (see section [4.5.2](#)). The seemingly almost complete genetic turnover between the Mesolithic and Early Neolithic periods in Iberia and the comparison to Near East haplogroup frequencies supports the theory of migration though maritime pioneering colonisation through the Mediterranean (Fernández et al., 2014; Gamba et al., 2012; Zilhão, 2001b) or by land via a Central European route (Mathieson et al., 2018; Szécsényi-Nagy et al., 2015) rather than cultural diffusion for the spread of the Neolithic from the Near East. Genomic analysis of Neolithic populations in the Mediterranean coastline during the earliest Neolithic phases and an increase in the number of studied individuals from Cardial levels may be able to determine which of these routes is more likely the source of EEF ancestry in Iberia.

Although the published archaeogenomic mitochondrial data suggests HG lineages persisted in very minor percentages during the Early Neolithic in Iberia, this may not be the case. Another explanation could be that HG groups did not interact with EEF groups, and HG lineages continued to exist in the Early Neolithic. In this case, these lineages then don't appear in the database due to archaeological biases. This could be due to the sites being easier to find or having better preservation. Very few Mesolithic sites have been uncovered in Iberia, most of which do not have human remains (Aguirre-Uribesalgo et al., 2024; Arias et al., 2009). There are cases of hunter-gatherer sites dating to the Neolithic period that have not had genetic studies done (Alday et al., 2024; Carvalho et al., 2023). Analysis at these sites could potentially have a great

impact on the understanding on the origins of the Early Neolithic genetic stock in Iberian populations.

As the database only includes 21 individuals from this period, it could also be the case that the small sample size does not accurately represent the real population composition, and HG lineages in Early Neolithic sites were more frequent. Whether or not the data is a completely accurate portrayal of the real population, the genetic evidence points to a significant migration period in the Early Neolithic of Iberia.

5.4.3 Middle to Late Neolithic

The Middle to Late Neolithic period in Iberia (~4500-3200 cal BC) saw the emergence of new funerary practices, such as megalithic tombs in the West, potentially linked to later megalithic structures along the Atlantic seaboard (Alt et al., 2016; Cassidy et al., 2020; Rojo and Garrido-Pena, 2012). The first evidence at ~4300 cal BC corresponds to the site of Campo de Hockey, from which 6 individuals were included in the Southeast group in this study (Vijande-Vila et al., 2022). Pit and stone box burials, also found in South France and Italy, also appear in the Northeast of Iberia by ~4770-4230 cal BC (Albizuri et al., 2019; Morell Rovira et al., 2018). There is also a persistence of cave burials in the whole territory (Morell Rovira et al., 2018). This period also saw a shift in agricultural techniques, such as an increased focus on stockbreeding practices and wheat production (Antolín, 2016), and a shift to open countryside settlements (Albizuri et al., 2019).

While other regions have more diverse sites, genetic evidence from the Northeast region in this period comes exclusively from the cave sites of Cova de Els Trocs (2 individuals, ~3949-3660 cal BC) and Cova de la Guineu (13 individuals, ~3400-2500 cal BC), due to low levels of preservation outside of caves in the region. This was exemplified by the failure to obtain reliable results from the Late Neolithic hypogeum site of La Sagrera in this study. This lack of material presents a serious issue when discussing this period, as there is a possibility that individuals buried outside of caves could represent a different genetic population. Sites and detailed chronologies of individuals of Middle to Late Neolithic Iberia used for comparison in this study can be found in [Supplementary Table S4](#).

This study finds that all major haplogroups present in the Early Neolithic, with the exception of U4 and N1, were also present in the Middle to Late Neolithic (see section 4.5.2). In addition, haplogroups J2a and H3 appear for the first time in Iberia during this period. The Middle to Late Neolithic period in Iberia also sees a resurgence in U5 haplogroup frequencies, which were common (~88%) in the Mesolithic but absent from the Early Neolithic (see section 4.5.2 and Figure 39). At each site where HG U5 haplogroups were found in the AADR database dated to the Middle to Late Neolithic, at

least one EEF associated haplogroup was found, with the exception of Covas das Lapas in Portugal where only one individual was studied (Olalde et al., 2019). This shows that by the Middle to Late Neolithic across Iberia, populations with HG ancestry had admixed with EEF populations. This admixture may have occurred in the Early Neolithic, but there is no mitochondrial genetic evidence to make that claim. Genomic studies indicate again higher levels of HG ancestry in Portugal (~25-40%)(Roca-Rada et al., 2024) compared to the rest of Iberia (~18-35%) (Lipson et al., 2017; Olalde et al., 2019). This is reflected by the higher proportion of U5 haplogroups in Portugal (36% in Portugal, 17-25% in other Iberian regions) during this period as determined by the current study (see section 4.5.2). The proportion of U5 haplogroups reported in Middle to Late Neolithic Portugal before this study was only ~27% (3 individuals), which is similar to the compositions of other regions in Iberia. This study, therefore, has significantly changed the understanding of the mitochondrial haplogroup composition in the region during this period.

Previous genomic studies have come to the conclusion that the increase in HG ancestry during the Middle to Late Neolithic periods is due to continuous integration of HG and EEF ancestry (Carvalho et al., 2023; Lipson et al., 2017; Villalba-Mouco et al., 2019). This trend of increased HG ancestry in the Middle to Late Neolithic can also be observed in other parts of Europe, such as Germany and Hungary (Brandt et al., 2013; Lipson et al., 2017). Carvalho et al (2023) propose that cultural assimilation of hunter-gatherer groups by pioneering farming groups in the Early Neolithic leading to a demographic imbalance towards local HGs followed by a sharp population increase after the introduction of farming led to “recurrent and sustained gene-flow with groups bearing high levels of hunter-gatherer ancestry” that could explain this trend, but genomic evidence is needed to support this theory. Another possibility is that the lack of genetic data from Early Neolithic sites has resulted in an inaccurate representation of the Early Neolithic population. Future results could determine the Early Neolithic population more closely resembled the Middle and Late Neolithic populations in proportions of HG ancestry.

The lack of J and T2 haplogroups in the Central Iberian group might be explained by the smaller sample size of this group, with only 6 individuals being represented. This is less likely the case for the West Iberian group, which has a sample size of 14. Interestingly, the only two individuals in the database from the Early Neolithic period in West Iberia, both from the site of Gruta do Caldeirão, belonged to haplogroups T2 and J2b (Allentoft et al., 2024). As these are haplogroups associated with EEF populations, it is most likely the case that J and T2 haplogroups continued to exist in the West of Iberia during the Middle to Late Neolithic similarly to other EEF haplogroups, and they don't appear in the database for the West region due to the small sample size.

Ultimately, the Middle to Late Neolithic genetic landscape in Iberia is marked by the continuing existence of EEF lineages with increasing contribution from HG lineages. In comparison to the rest of Europe, Iberia sees a markedly higher contribution of HG genomic ancestry during this period (>22% in Germany, 5-15% in Hungary, and ~20% in Britain vs ~18-40% in Iberia)(Brace et al., 2019; Lipson et al., 2017; Roca-Rada et al., 2024), potentially indicating a separate Neolithization process with increased integration of HG lineages.

5.4.4 Chalcolithic

The genetic evidence from the Chalcolithic period in Iberia covers a time span between ~3516 (El Portalón Cave) and ~1700 cal BC (Camino de las Yeseras). From the Central region, only the sites of Humanejos (~2900-2000 cal BC), Camino de las Yeseras (~2474-1744 cal BC), and La Magdalena (~2500-2000 cal BC) have been studied. The North region offers a more diverse assemblage consisting of 10 sites. These range from ~3516 cal BC at El Portalón Cave to ~1983 cal BC at Virgatal. The data in the Northeast region only consists of samples from three sites: Galls Carboners (~3022-2907 cal BC), Cova dels Diablets (~2872-2626 cal BC), and Paris Street (~2850-2250 cal BC). Individuals from the Southeast group include data from five sites: Cova de Sant Gómcng (3800-2500 cal BC), Camino del Molino (~3000-2100 cal BC), Sima del 3ngel (~2900-2459 cal BC), La Vital (~2600-2356 cal BC), and La Navilla (~2200-2000 cal BC). The West group, which also represents 5 sites, has individuals from Cabeço da Arruda I (~3350-2850 cal BC), Monte Canelas 1 (~3336-3026 cal BC), Perdigões (~3081-2886 cal BC), Verdelha dos Ruivos (~2876-2300 cal BC), and Galeria da Cisterna (~2454-2153 cal BC)(Mallick et al., 2024). Full detailed chronologies and regional assignments of individuals from this period used in this study can be seen in [Supplementary Table S4](#).

Within the Chalcolithic period, the Bell Beaker Culture emerged around ~2750 cal BC, with the first archaeological evidence coming from the sites of Verdelha dos Ruivos and Leceia in the Estremadura region of Portugal (Cardoso, 2014). This cultural group is typically associated with bell-beaker shaped pottery, copper weapons or adornments, single inhumation burials, and occasionally ivory (Gibaja et al., 2012; Linares-Catela and Vera-Rodríguez, 2023; Schuhmacher et al., 2009). Other funerary contexts present in this period include cave burials and hypogea as well as megalithic tombs along the Atlantic coastline (Linares-Catela and Vera-Rodríguez, 2023; Sousa and Gonçalves, 2022).

The slight decrease in HG lineages in the North (17% to 11%), Northeast (19% to 17%), West (29% to 17%), and Southwest (20% to 11%) regions in the Chalcolithic is likely a random effect caused by the sample size of populations analysed. Interestingly,

the Central region maintains an ~34% frequency of HG mitochondrial lineages between the MLN and Chalcolithic periods. Previous genomic studies have also demonstrated that HG lineages provide a greater contribution to Neolithic and Chalcolithic populations in Central Iberia (Lipson et al., 2017; Olalde et al., 2019; Villalba-Mouco et al., 2019). It might be expected in this case that HG haplogroups should account for a greater percentage of the Middle to Late Neolithic Central Iberia population. The frequency of HG haplogroups observed in this group may simply be due its small sample size (N=6), meaning it doesn't accurately represent the entire population, as discussed in the previous section. On the other hand, it could be the case that earlier Neolithic migrations affected the Central region more than Chalcolithic migrations such as the Bell Beaker expansion or the expansion of steppe ancestry. Other shifts observed in haplogroup frequencies of the Central region in the Chalcolithic, such as an increased frequency of J and T2 haplogroups and the appearance of new haplogroups like L1 and M1 make this unlikely. Another possibility is that later migratory groups carried higher levels of HG ancestry, but further research on steppe mitochondrial haplogroup frequencies is necessary before this can be determined.

Although it was initially theorised that the Bell Beaker Culture (BBC) originated in Iberia and spread through population movements to other parts of Europe (Brotherton et al., 2013), a genome wide study concluded that the genetic components associated with Central European BBC ancestry throughout the rest of Europe did not originate in Iberia, but in the Eurasian Steppe, although the levels of this ancestry vary depending on the region of Europe (Olalde et al., 2018). In Iberia, it was determined that Steppe ancestry was rare in the Chalcolithic, and most BBC individuals were genetically similar to previous Neolithic populations (Olalde et al., 2018). As seen in Figure 24 and Figure 25, while minor shifts occur, haplogroup compositions between the MLN and Chalcolithic in Iberia remain similar, supporting the idea of genetic continuity between the periods. As the earliest archaeological evidence of the Bell Beaker Culture is found in Portugal and other areas of Iberia (Cardoso, 2014), and these areas show little evidence of Steppe ancestry, the early expansion of the Bell Beaker Culture likely coincided but was not strictly related to a migration event from the Eurasian Steppe in the third millennium BC.

The only major change in haplogroup compositions during the Chalcolithic period is the increased frequency of H haplogroups, specifically H1, in the Northeast region. These haplogroups also increase in frequency to a lesser extent in the North and Southeast regions. Haplogroups H1 and H3 (derived from H ~12.5 and 10.2 kya respectively) (Brandstätter et al., 2008) have previously been linked to BBC expansion from Iberia (Brotherton et al., 2013), although these haplogroups do not appear in great frequency in the populations seen in Figure 25 and Figure 26 where the BBC is said to originate. It is more probable that the increase in the frequency of these haplogroups is due to genetic drift or geneflow between the rest of Europe and the areas of Iberia

closest to the rest of Europe. Areas such as southern France have shown high frequencies of H mitochondrial haplogroups during the Neolithic in previous studies (Arzelier et al., 2022; Rivollat et al., 2020, 2015). The frequency of H haplogroups in the Northeast region may also be impacted by the relatively small sample size of 12 individuals. Genomic studies have also determined ~25% of Bell Beaker associated individuals in Iberia carried a steppe related ancestral component also found in Bell Beaker contexts in France and Central Europe during this period, supporting the idea of cultural contact and geneflow between these regions during this period (Olalde et al., 2018).

The new appearances of haplogroups L1 and M1 in the Central region stand out. L1 was found in a Bell Beaker context at the site of Camino de las Yeseras (2461-2146 cal BC), and is associated with North African mitochondrial lineages (Olalde et al., 2018). Genome wide analysis of the individual revealed a combination of North African and EEN ancestry (Olalde et al., 2018). The individual harbouring the M1 mitochondrial haplogroup, found at the same site also from a Bell Beaker context, was determined to be of mixed North African and Neolithic farmer ancestry (Olalde et al., 2019). These individuals suggest some geneflow between North Africa and Central Iberia. It is likely that this geneflow existed throughout other regions of Iberia, but in a small enough amount that they have not been reported in the mitochondrial database yet. Outside of the database, a study utilising a PCR approach found evidence of potential African lineages (haplogroup L3) in Iberia at the Chalcolithic sites of Malpaso Cave, Jovellús Sepulchre, and Assud de Villareal on the Mediterranean coast, but the targeting of only the HVRI in this study leaves doubt about the accuracy of these haplogroup assignments (Gamba et al., 2008). Although these North African mitochondrial lineages don't appear until the Chalcolithic, whole genome studies have suggested geneflow from Iberia into North Africa as early as the Early Neolithic period based on the similarity between genomes of Early Neolithic individuals at the site of Kaf Taht el-Gharand (~5400 cal BC) and Late Neolithic individuals at the site of Kehf el Baroud (~3700 cal BC) in the Maghreb to Iberian Neolithic populations (Simões et al., 2023). Archaeological evidence of contact between these regions can also be seen through similar pottery styles in the Neolithic (Linstädter et al., 2012) and the presence of ostrich eggs and ivory in Bell Beaker sites across Iberia in the Chalcolithic (Schuhmacher et al., 2009).

To sum up, the Chalcolithic period in Iberia saw an increase in H haplogroup frequencies in the North, Northeast, and Southeast regions, likely associated with geneflow between Iberia and the rest of Europe (Olalde et al., 2018). Persistence of HG mitochondrial lineages in the Central region, differing frequencies of haplogroups between regions, and appearance of North African mitochondrial lineages suggest a complex social landscape and regional differences in Chalcolithic Iberian populations unrelated to the spread of the Bell Beaker Culture.

5.4.5 Bronze Age

The earliest Bronze Age individual in Iberia with genetic data comes from the site of Castillejo del Bonete (~2343-1770 cal BC) in the Southeast region. Early Bronze Age individuals in this area cover a period of ~2343-1422 cal BC, with the latest site being Cerro de la Virgen (1534-1422 cal BC)(Mallick et al., 2024). The Middle and Late Bronze Age group in this region includes individuals from Puntal de los Carniceros (~1882-1566 cal BC), Covacha del Angel (~1751-1615 cal BC), and La Horna (~29-1441 cal BC)(Mallick et al., 2024). In the North, Early Bronze Age sites cover a span from ~2199-1450 cal BC, with the earliest evidence from the site of La Fragua Cave and the latest from El Sotillo. The Middle and Late Bronze Age group from this region includes individuals from the sites of El Portalón Cave (~1863-1564 cal BC), El Toral-3 (~1601-1425 cal BC), El Sotillo (~1533-813 cal BC), and El Espinoso (~1300-1000 cal BC)(Mallick et al., 2024). Dating for individuals in the Northeast region spanned from ~2000 (at the site of Can Roquetes) to ~1000 cal BC (at the site of Túmulo Mortorum). In the West region, only 10 individuals spanning from ~2200(at Monte da Cabida 3) to ~700 cal BC (at Gruta do Medronhal) were included. Even less genetic data is available from the Central region, with only 7 individuals from the sites of Humanejos (~1880-1691 cal BC), Fuente la Mora (~1850-1150 cal BC), La Horra (~1850-1150 cal BC), Tordillos (~1850-1150), and La Requejada (~1400-1200 cal BC)(Mallick et al., 2024). For full chronologies and geographical groupings, see [Supplementary Table S4](#).

The Bronze Age in Iberia is characterised by mitochondrial genetic continuity from the Chalcolithic period in most regions. In the West region, an increase in HG associated mitochondrial lineages from ~16.6% in the Chalcolithic to 40% in the Bronze Age could suggest groups from elsewhere in Europe or the Eurasian Steppe with higher levels of HG ancestry were admixing with local Bronze Age populations. Genomic studies show HG ancestry continued to decrease in this period in Iberia (Olalde et al., 2019; Villalba-Mouco et al., 2021) and the small sample size of only 10 West Iberia Bronze Age individuals (3 EBA, 5 MBA, 2 LBA) makes it possible that the composition of this group is skewed.

As mitochondrial analysis can only capture changes in maternal lineages of a population, other population changes can be missed. Although mitochondrial lineage proportions show relative continuity from the Chalcolithic, the same cannot be said about Y-chromosome and whole genome ancestry. Y-chromosome studies show a nearly complete turnover between Neolithic and Chalcolithic haplogroups such as H, G2, and I2 to the Steppe associated haplogroup R1b-M269 during the Bronze Age (Olalde et al., 2019). Whole genome studies also point to an increase in steppe related ancestry in Bronze Age Iberian individuals, with greater percentages in individuals in the North and Northeast population groups (Olalde et al., 2019, 2018; Villalba-Mouco et al.,

2021). Steppe mitochondrial ancestry primarily consists of haplogroups U4, U5a, T1, and T2 (Richards and Fernández-Domínguez, 2022). Haplogroups U5a and T2 are observed in Bronze Age Iberian populations, however, both haplogroups have also been observed in Neolithic Iberian individuals, and therefore could represent either genetic continuity or steppe ancestry. The lack of a clear Steppe mitochondrial influence and the dominance of Steppe Y-chromosome ancestry suggest a sex biased admixture between local and Steppe ancestry populations in the Late Chalcolithic and Early Bronze Age.

An interesting difference between the Early and Middle to Late Bronze Age is the appearance of two individuals of the Haplogroups L2 and I1 in the Southeast group. These haplogroups are not found anywhere else in Iberia in the database between the Mesolithic and Bronze Age. The individual of haplogroup L2 was found at the site of Covacha del Angel near Córdoba (González-Fortes et al., 2019). This haplogroup is associated with sub-Saharan African populations (González-Fortes et al., 2019; Skoglund et al., 2017). The individual, however, clusters closely with other contemporary Iberian individuals at whole genome level (González-Fortes et al., 2019; Villalba-Mouco et al., 2021), suggesting a distant sub-saharan maternal legacy and providing further evidence of geneflow between Africa and Iberia during the Chalcolithic or Bronze Age.

The individual with an I1 haplogroup was found at the site of Puntal de los Carniceros (Villalba-Mouco et al., 2021). Although not found anywhere else in Iberia in the AADR database, an individual at the Neolithic site of Paternanbidea in Navarre was found to also belong to haplogroup I in a study using Sanger Sequencing (Hervella et al., 2012). Another study also found members of this haplogroup in Early Neolithic populations from Southwest Asia (Lazaridis et al., 2016). These findings suggest that this lineage could have also been a part of the EEF genetic profile, although with less frequency than other EEF associated haplogroups, meaning they are rarely found in ancient samples. Alternatively, I haplogroups may be a marker of steppe ancestry that expanded across Europe in the Chalcolithic, as proposed by Brandt et al (2013) and supported through cross-reference of mitochondrial and genomic results by Haak et al (2015).

5.5 Metagenomic Analysis

Potential pathogen DNA was found in samples from 3 of the 4 sites analysed in this study. *Tannerella forsythia* was found in three individuals from La Sagrera and one individual from Convento do Carmo. Two samples from Convento do Carmo also showed evidence of the presence of *Neisseria meningitidis*. *Bordetella bronchialis* was also detected in a sample from Convento do Carmo as well as in all three samples from

Algar do Barrão. Finally, *Spirometra erinaceieuropaei*, a species of tapeworm, was detected in individual BAR3 from the same site.

At the site of La Sagrera, although the appearance of *T. forsythia* in individuals SA18, SA46, and SA68 could be useful for understanding the oral hygiene of the individuals, as it is associated with gingivitis and periodontitis, there is no indication of pathogens present in any of the samples that could explain the reason for the mass simultaneous burial event at the site. It should be noted that the low authenticity scores *T. forsythia* received by aMeta in all cases (see Table 21) means that it cannot be confidently said that this organism was truly present in the skeletal remains. Individual SA46 shows the most likely case, but the amount of stacking of the reads, observed in the evenness of coverage plot in Figure 32 for this organism, suggest they may have been misaligned. Stacking occurs when reads from an organism are misaligned to a specific section of DNA in the reference of another organism due to conserved regions of the genome or adapters in the reference sequence, creating a stack-like pattern of reads in one area of the genome rather than displaying an even pattern of coverage across the whole genome expected from true reads (Pochon et al., 2023). Similar to La Sagrera samples, the appearance of *T. Forsythia* in sample CVC6 received a quality score of 2 and only had 10 reads assigned, so it was likely an artifact.

The aMeta pipeline is conservative in order to avoid false positive identifications. It was therefore possible that there was indeed pathogen DNA present in samples from La Sagrera, but not well preserved enough to be detected or validated through the KrakenUnique filter of 1000 unique kmers and 50 individual reads. The poor preservation of DNA witnessed through the mitochondrial analysis makes this a reasonable possibility. Moreover, it is also likely that pathogens were not responsible for the demise of the individuals in the hypogeum, as the dietary isotopic results suggested.

Samples CVC5 and CVC6 may have had *Neisseria meningitidis* present. Scores of 6 and 5 leave some doubt about these classifications, but the number of reads (>400 in both samples) as well as C to T deamination plots and breadth of coverage (see Figure 29 and Figure 30) make the classifications likely authentic. This organism can cause Meningococcal disease, which is potentially fatal. However, *Neisseria meningitidis* is reported to exist in approximately 10% of modern individuals, and only rarely causes Invasive Meningococcal disease (Parikh et al., 2020).

The organism *Bordetella bronchialis* appears in all three samples analysed from Algar do Barrão as well as sample CVC9 from Convento do Carmo. Further research into this species in the medical and microbiological field is needed before the effect it had on these ancient individuals can be truly understood, as very little research has been done on this species (Spilker et al., 2016; Vandamme et al., 2015). It is likely that it could have contributed to minor respiratory disease (Vandamme et al., 2015). A high number of reads (>2700 for each sample) as well as evenness of coverage and deamination patterns (see Figure 35, Figure 36, Figure 37, and Figure 38) suggest the organism is truly ancient and present in all samples. Edit distance, however, is ≥ 3 in more than half of the reads in each sample, which could indicate that the reads are misaligned, and actually represent a different but closely related organism.

The appearance of *Spirometra erinaceieuropaei* in sample BAR3 stands out as the only multicellular organism besides *Homo sapiens* identified in any sample. As this

organism infects humans most commonly through consumption of frogs (Tang et al., 2017), the appearance of the organism in BAR3 could indicate that this individual was consuming frogs or other amphibians as part of their diet. This is supported by the high amounts of aquatic protein as determined by the carbon and nitrogen isotopic analysis (Carvalho et al., 2019), although it is possible the individual was infected simply by drinking unclean water. The presence of this organism has been shown to have varying effects on infected individuals (Tang et al., 2017). Migration of the organism through tissue can cause swelling, while migration through brain tissue and the central nervous system can cause seizures, numbness, and death (Tang et al., 2017). In other cases, individuals show no symptoms until years or decades after infection (Greninger and Glaser, 2017). In this context, it is impossible to speculate on the actual impact the *Spirometra erinaceieuropaei* organism had on individual BAR3.

6. Conclusion

The reason behind the existence and funerary organisation in the Late Neolithic hypogeum of La Sagrera remains a mystery, as no mitochondrial haplogroups were able to be reliably assigned, and kinship analysis was severely inhibited by poor coverage. No evidence of pathogens that could result in a mass fatality were found, although this could be due to a lack of preservation. A mass fatality caused by famine or disease remains the most likely reason for the mass simultaneous inhumation event. Future analysis of sulphur isotope ratios from incremental dentine samples may uncover patterns consistent with famine.

All three individuals from the site of Algar do Barrão were successfully analysed. Two individuals had mitochondrial lineages relating to HG ancestry, while the third had a lineage relating to EEF ancestry. Previous genetic studies have solidified a model of migration and admixture with local hunter-gatherers for the spread of the Neolithic into Europe and eventually Iberia, and the population of Algar do Barrão fits within that narrative. Further genomic analysis of these and other individuals from the site of Algar do Barrão is highly recommended due to the promising state of preservation in the samples analysed here and the general lack of information from this region during the Middle Neolithic. This gap in available data can somewhat be attributed to lack of preservation in the region, a problem that is highlighted in this study by the unfortunate failure to obtain reliable results from the nearby site of Casais da Mureta. This stresses the importance to pursue further study on materials that have shown to be well preserved like those of Barrão. Genomic analysis would provide a more nuanced picture of the state of admixture between EEF and HG ancestry within this site, which provides an interesting case due to the archaeological evidence of dense Mesolithic population in the surrounding Tagus River valley.

Seven individuals from the Bell Beaker context of Convento do Carmo could also be successfully analysed. The Bell Beaker population at Convento do Carmo displayed a mixture of both HG and EEF lineages typical of other Bell Beaker contexts in the region. Genomic studies have recently uncovered gene-flow of steppe-related ancestry into Iberia that is less pronounced in Portugal in the contemporary period. Without a strong mitochondrial signal for steppe-related ancestry, and the possibility of a sex-biased migration event, mitochondrial analysis alone was not enough to determine if and how steppe-related ancestry appeared at the site. Future work targeting the whole genome of these individuals will be able to provide a better understanding of the site and region within the steppe-migration narrative. Research focussed on establishing a more definitive understanding of the steppe-related mitochondrial composition will offer the opportunity for a reinterpretation of the results of this study in the future.

The tapeworm DNA identified in individual BAR3 marks the oldest tapeworm ancient DNA identified and the only known case of *Spirometra erinaceieuropaei* from an ancient sample to date. Despite the convincing metrics, the novelty of this discovery and its presence in a tooth sample demands future analysis mapping the identified reads to the reference genome and potentially a second extraction to confirm the presence of this organism in the sample.

Bibliography:

- Aguirre-Uribesalgo, A., Álvarez-Fernández, E., Saña, M., 2024. Feet on the ground: Marine archaeomalacology resources during the Mesolithic-Neolithic transition in the Iberian Peninsula. *Quat. Int.* 680, 17–35. <https://doi.org/10.1016/j.quaint.2023.11.005>
- Alaeddini, R., Walsh, S.J., Abbas, A., 2010. Forensic implications of genetic analyses from degraded DNA—A review. *Forensic Sci. Int. Genet.* 4, 148–157. <https://doi.org/10.1016/j.fsigen.2009.09.007>
- Albizuri, S., Nadal, J., Martín, P., Gibaja, J.F., Cóllica, A.M., Esteve, X., Oms, X., Martí, M., Pou, R., López-Onaindia, D., Subirà, M.E., 2019. Dogs in funerary contexts during the Middle Neolithic in the northeastern Iberian Peninsula (5th–early 4th millennium BCE). *J. Archaeol. Sci. Rep.* 24, 198–207. <https://doi.org/10.1016/j.jasrep.2019.01.004>
- Alday, A., Pérez-Romero, A., Carretero, J.-M., Galindo-Pellicena, M.Á., Adán, G., Arsuaga, J.-L., 2015. Proofs of Long-Distance Relations between Central Europe and Inland Iberian Peninsula during Neolithic and Bronze Age. Evidences from the Material Culture of the Site of El Portalón (Sierra de Atapuerca, Burgos, Spain). *Adv. Anthropol.* 5, 294–323. <https://doi.org/10.4236/aa.2015.54023>
- Alday, A., Pérez-Romero, A., Iriarte, E., Francés-Negro, M., Arsuaga, J.-L., Carretero, J.-M., 2019. Pottery with ramiform-anthropomorphic decoration from El Portalón de Cueva Mayor site (Sierra de Atapuerca, Burgos) and the globalized symbolic world of the first Neolithic. *Quat. Int.* 515, 125–137. <https://doi.org/10.1016/j.quaint.2017.10.044>
- Alday, A., Rodríguez-Lejarza, A., Soto, A., Montes, L., 2024. The land of the last hunter-gatherer groups in the Ebro basin: Forgers of their own destiny. *J. Anthropol. Archaeol.* 76, 101626. <https://doi.org/10.1016/j.jaa.2024.101626>
- Allard, M.W., Young, D., Huyen, Y., 1995. Detecting Dinosaur DNA. *Science* 268, 1192–1192. <https://doi.org/10.1126/science.7761840>
- Allentoft, M.E., Sikora, M., Refoyo-Martínez, A., Irving-Pease, E.K., Fischer, A., Barrie, W., Ingason, A., Stenderup, J., Sjögren, K.-G., Pearson, A., Sousa da Mota, B., Schulz Paulsson, B., Halgren, A., Macleod, R., Jørkov, M.L.S., Demeter, F., Sørensen, L., Nielsen, P.O., Henriksen, R.A., Vimala, T., McColl, H., Margaryan, A., Ilardo, M., Vaughn, A., Fischer Mortensen, M., Nielsen, A.B., Ulfeldt Hede, M., Johannsen, N.N., Rasmussen, P., Vinner, L., Renaud, G., Stern, A., Jensen, T.Z.T., Scorrano, G., Schroeder, H., Lysdahl, P., Ramsøe, A.D., Skorobogatov, A., Schork, A.J., Rosengren, A., Ruter, A., Outram, A., Timoshenko, A.A., Buzhilova, A., Coppa, A., Zubova, A., Silva, A.M., Hansen, A.J., Gromov, A., Logvin, A., Gotfredsen, A.B., Henning Nielsen, B., González-Rabanal, B., Lalueza-Fox, C., McKenzie, C.J., Gaunitz, C., Blasco, C., Liesau, C., Martinez-Labarga, C., Pozdnyakov, D.V., Cuenca-Solana, D., Lordkipanidze, D.O., En'shin, D., Salazar-García, D.C., Price, T.D., Borić, D., Kostyleva, E., Veselovskaya, E.V., Usmanova, E.R., Cappellini, E., Brinch Petersen, E., Kannegaard, E., Radina, F., Eylem Yediay, F., Duday, H., Gutiérrez-Zugasti, I., Merts, I., Potekhina, I., Shevnina, I., Altinkaya, I., Guilaine, J., Hansen, J., Aura Tortosa, J.E., Zilhão, J., Vega, J., Buck Pedersen, K., Tunia, K., Zhao, L., Mylnikova, L.N., Larsson, L., Metz, L., Yepiskoposyan, L., Pedersen, L., Sarti, L., Orlando, L., Slimak, L., Klassen, L., Blank, M., González-Morales, M.,

- Silvestrini, M., Vretemark, M., Nesterova, M.S., Rykun, M., Rolfo, M.F., Szmyt, M., Przybyła, M., Calattini, M., Sablin, M., Dobisíková, M., Meldgaard, M., Johansen, M., Berezina, N., Card, N., Saveliev, N.A., Poshekhonova, O., Rickards, O., Lozovskaya, O.V., Gábor, O., Uldum, O.C., Aurino, P., Kosintsev, P., Courtaud, P., Ríos, P., Mortensen, P., Lotz, P., Persson, P., Bangsgaard, P., de Barros Damgaard, P., Vang Petersen, P., Martinez, P.P., Włodarczak, P., Smolyaninov, R.V., Maring, R., Menduiña, R., Badalyan, R., Iversen, R., Turin, R., Vasilyev, S., Wåhlin, S., Borutskaya, S., Skochina, S., Sørensen, S.A., Andersen, S.H., Jørgensen, T., Serikov, Y.B., Molodin, V.I., Smrcka, V., Merts, V., Appadurai, V., Moiseyev, V., Magnusson, Y., Kjær, K.H., Lynnerup, N., Lawson, D.J., Sudmant, P.H., Rasmussen, S., Korneliussen, T.S., Durbin, R., Nielsen, R., Delaneau, O., Werge, T., Racimo, F., Kristiansen, K., Willerslev, E., 2024. Population genomics of post-glacial western Eurasia. *Nature* 625, 301–311. <https://doi.org/10.1038/s41586-023-06865-0>
- Alt, K.W., Zesch, S., Garrido-Pena, R., Knipper, C., Szécsényi-Nagy, A., Roth, C., Tejedor-Rodríguez, C., Held, P., García-Martínez-de-Lagrán, Í., Navitainuck, D., Magallón, H.A., Rojo-Guerra, M.A., 2016. A Community in Life and Death: The Late Neolithic Megalithic Tomb at Alto de Reinoso (Burgos, Spain). *PLOS ONE* 11, e0146176. <https://doi.org/10.1371/journal.pone.0146176>
- Altınışık, N.E., Kazancı, D.D., Aydoğan, A., Gemici, H.C., Erdal, Ö.D., Sarialtun, S., Vural, K.B., Koptekin, D., Gürün, K., Sağlıcan, E., Fernandes, D., Çakan, G., Koruyucu, M.M., Lagerholm, V.K., Karamurat, C., Özkan, M., Kılınç, G.M., Sevkari, A., Sürer, E., Götherström, A., Atakuman, Ç., Erdal, Y.S., Özer, F., Erim Özdoğan, A., Somel, M., 2022. A genomic snapshot of demographic and cultural dynamism in Upper Mesopotamia during the Neolithic Transition. *Sci. Adv.* 8, eabo3609. <https://doi.org/10.1126/sciadv.abo3609>
- Ammerman, A.J., Cavalli-Sforza, L.L., 1984. *The Neolithic Transition and the Genetics of Populations in Europe*. Princeton University Press.
- Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., Young, I.G., 1981. Sequence and organization of the human mitochondrial genome. *Nature* 290, 457–465. <https://doi.org/10.1038/290457a0>
- Andrews, R.M., Kubacka, I., Chinnery, P.F., Lightowlers, R.N., Turnbull, D.M., Howell, N., 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nat. Genet.* 23, 147–147. <https://doi.org/10.1038/13779>
- Antolín, F., 2016. Local, intensive and diverse?: Early farmers and plant economy in the North-East of the Iberian Peninsula (5500-2300 cal BC). *Barkhuis*. <https://doi.org/10.2307/j.ctt20p56hs>
- Arias, P., Armendáriz, A., Balbín, R., Fano Martínez, M.Á., Esteban Álvarez, F., Fernández-Tresguerres, J.A., González Morales, M.R., Iriarte, M.J., Ontañón, R., 2009. Burials in the cave: New evidence on mortuary practices during the Mesolithic of Cantabrian Spain, in: *Burials in the Cave: New Evidence on Mortuary Practices during the Mesolithic of Cantabrian Spain*. En *Mesolithic Horizons, Vol. II, Papers Presented at the Seventh International Conference on the Mesolithic in Europe (Belfast 2005)*. Presented at the Burials in the cave: New

- evidence on mortuary practices during the Mesolithic of Cantabrian Spain". En *Mesolithic Horizons*, vol. II, Papers presented at the Seventh International Conference on the Mesolithic in Europe (Belfast 2005), Oxbow Books, pp. 650–656.
- Árnadóttir, E.R., Moore, K.H.S., Guðmundsdóttir, V.B., Ebenesersdóttir, S.S., Guity, K., Jónsson, H., Stefánsson, K., Helgason, A., 2024. The rate and nature of mitochondrial DNA mutations in human pedigrees. *Cell* 187, 3904–3918.e8. <https://doi.org/10.1016/j.cell.2024.05.022>
- Arzelier, A., Rivollat, M., De Belvalet, H., Pemonge, M.-H., Binder, D., Convertini, F., Dудay, H., Gandelin, M., Guilaine, J., Haak, W., Deguilloux, M.-F., Pruvost, M., 2022. Neolithic genomic data from southern France showcase intensified interactions with hunter-gatherer communities. *iScience* 25, 105387. <https://doi.org/10.1016/j.isci.2022.105387>
- Assandri, M.H., Malamud, M., Trejo, F.M., Serradell, M. de los A., 2023. S-layer proteins as immune players: Tales from pathogenic and non-pathogenic bacteria. *Curr. Res. Microb. Sci.* 4, 100187. <https://doi.org/10.1016/j.crmicr.2023.100187>
- Balaguer, P., 2016. Les agrupacions poblacionals del segon moment d'ús de l'hipogeu funerari de la Sagrera. Estudi antropològic. Servei d'Arqueològica. Ajuntament de Barcelona, Barcelona.
- Balaguer, P., García, P., Tenza, A., Antequera, F., 2013. L'hipogeu funerari de la Sagrera (Barcelona) Resultats preliminars. *Rev. Arqueol.* Ponent.
- Bandelt, H.-J., Kloss-Brandstätter, A., Richards, M.B., Yao, Y.-G., Logan, I., 2014. The case for the continuing use of the revised Cambridge Reference Sequence (rCRS) and the standardization of notation in human mitochondrial DNA studies. *J. Hum. Genet.* 59, 66–77. <https://doi.org/10.1038/jhg.2013.120>
- Bär, W., Kratzer, A., Mächler, M., Schmid, W., 1988. Postmortem stability of DNA. *Forensic Sci. Int.* 39, 59–70. [https://doi.org/10.1016/0379-0738\(88\)90118-1](https://doi.org/10.1016/0379-0738(88)90118-1)
- Behar, D.M., van Oven, M., Rosset, S., Metspalu, M., Loogväli, E.-L., Silva, N.M., Kivisild, T., Torroni, A., Villems, R., 2012. A “Copernican” Reassessment of the Human Mitochondrial DNA Tree from its Root. *Am. J. Hum. Genet.* 90, 675–684. <https://doi.org/10.1016/j.ajhg.2012.03.002>
- Behar, D.M., Villems, R., Soodyall, H., Blue-Smith, J., Pereira, L., Metspalu, E., Scozzari, R., Makkan, H., Tzur, S., Comas, D., Bertranpetit, J., Quintana-Murci, L., Tyler-Smith, C., Wells, R.S., Rosset, S., 2008. The Dawn of Human Matrilineal Diversity. *Am. J. Hum. Genet.* 82, 1130–1140. <https://doi.org/10.1016/j.ajhg.2008.04.002>
- Bicho, N., Cascalheira, J., Marreiros, J., Gonçalves, C., Pereira, T., Dias, R., 2013. Chronology of the Mesolithic occupation of the Muge valley, central Portugal: The case of Cabeço da Amoreira. *Quat. Int., Geoarchaeology: a toolbox of approaches applied in a multidisciplinary research discipline* 308–309, 130–139. <https://doi.org/10.1016/j.quaint.2012.10.049>
- Blanco-González, A., Lillios, K.T., López-Sáez, J.A., Drake, B.L., 2018. Cultural, Demographic and Environmental Dynamics of the Copper and Early Bronze Age in Iberia (3300–1500 BC): Towards an Interregional Multiproxy Comparison at the Time of the 4.2 ky BP Event. *J. World Prehistory* 31, 1–79. <https://doi.org/10.1007/s10963-018-9113-3>
- Brace, S., Diekmann, Y., Booth, T.J., van Dorp, L., Faltyskova, Z., Rohland, N., Mallick, S., Olalde, I., Ferry, M., Michel, M., Oppenheimer, J., Broomandkhoshbacht, N.,

- Stewardson, K., Martiniano, R., Walsh, S., Kayser, M., Charlton, S., Hellenthal, G., Armit, I., Schulting, R., Craig, O.E., Sheridan, A., Parker Pearson, M., Stringer, C., Reich, D., Thomas, M.G., Barnes, I., 2019. Ancient genomes indicate population replacement in Early Neolithic Britain. *Nat. Ecol. Evol.* 3, 765–771. <https://doi.org/10.1038/s41559-019-0871-9>
- Bramanti, B., Thomas, M.G., Haak, W., Unterlaender, M., Jores, P., Tambets, K., Antanaitis-Jacobs, I., Haidle, M.N., Jankauskas, R., Kind, C.-J., Lueth, F., Terberger, T., Hiller, J., Matsumura, S., Forster, P., Burger, J., 2009. Genetic Discontinuity Between Local Hunter-Gatherers and Central Europe's First Farmers. *Science* 326, 137–140. <https://doi.org/10.1126/science.1176869>
- Bramanti, B., Zedda, N., Rinaldo, N., Gualdi-Russo, E., 2018. A critical review of anthropological studies on skeletons from European plague pits of different epochs. *Sci. Rep.* 8, 17655. <https://doi.org/10.1038/s41598-018-36201-w>
- Brandstätter, A., Zimmermann, B., Wagner, J., Göbel, T., Röck, A.W., Salas, A., Carracedo, A., Parson, W., 2008. Timing and deciphering mitochondrial DNA macro-haplogroup R0 variability in Central Europe and Middle East. *BMC Evol. Biol.* 8, 191. <https://doi.org/10.1186/1471-2148-8-191>
- Brandt, G., Haak, W., Adler, C.J., Roth, C., Szécsényi-Nagy, A., Karimnia, S., Möller-Rieker, S., Meller, H., Ganslmeier, R., Friederich, S., Dresely, V., Nicklisch, N., Pickrell, J.K., Sirocko, F., Reich, D., Cooper, A., Alt, K.W., The Genographic Consortium, 2013. Ancient DNA Reveals Key Stages in the Formation of Central European Mitochondrial Genetic Diversity. *Science* 342, 257–261. <https://doi.org/10.1126/science.1241844>
- Briggs, A.W., Stenzel, U., Johnson, P.L.F., Green, R.E., Kelso, J., Prüfer, K., Meyer, M., Krause, J., Ronan, M.T., Lachmann, M., Pääbo, S., 2007. Patterns of damage in genomic DNA sequences from a Neandertal. *Proc. Natl. Acad. Sci.* 104, 14616–14621. <https://doi.org/10.1073/pnas.0704665104>
- Brotherton, P., Endicott, P., Sanchez, J.J., Beaumont, M., Barnett, R., Austin, J., Cooper, A., 2007. Novel high-resolution characterization of ancient DNA reveals C > U-type base modification events as the sole cause of post mortem miscoding lesions. *Nucleic Acids Res.* 35, 5717–5728. <https://doi.org/10.1093/nar/gkm588>
- Brotherton, P., Haak, W., Templeton, J., Brandt, G., Soubrier, J., Jane Adler, C., Richards, S.M., Sarkissian, C.D., Ganslmeier, R., Friederich, S., Dresely, V., van Oven, M., Kenyon, R., Van der Hoek, M.B., Korfach, J., Luong, K., Ho, S.Y.W., Quintana-Murci, L., Behar, D.M., Meller, H., Alt, K.W., Cooper, A., 2013. Neolithic mitochondrial haplogroup H genomes and the genetic origins of Europeans. *Nat. Commun.* 4, 1764. <https://doi.org/10.1038/ncomms2656>
- Broushaki, F., Thomas, M.G., Link, V., López, S., van Dorp, L., Kirsanow, K., Hofmanová, Z., Diekmann, Y., Cassidy, L.M., Díez-del-Molino, D., Kousathanas, A., Sell, C., Robson, H.K., Martiniano, R., Blöcher, J., Scheu, A., Kreutzer, S., Bollongino, R., Bobo, D., Davoudi, H., Munoz, O., Currat, M., Abdi, K., Biglari, F., Craig, O.E., Bradley, D.G., Shennan, S., Veeramah, K.R., Mashkour, M., Wegmann, D., Hellenthal, G., Burger, J., 2016. Early Neolithic genomes from the eastern Fertile Crescent. *Science* 353, 499–503. <https://doi.org/10.1126/science.aaf7943>
- Brunel, S., Bennett, E.A., Cardin, L., Garraud, D., Barrand Emam, H., Beylier, A., Boulestin, B., Chenal, F., Ciesielski, E., Convertini, F., Dedet, B., Desbrosse-Degobertiere, S., Desenne, S., Dubouloz, J., Duday, H., Escalon, G., Fabre, V.,

- Gaillardet, E., Gandelin, M., Gleize, Y., Goepfert, S., Guilaine, J., Hachem, L., Ilett, M., Lambach, F., Maziere, F., Perrin, B., Plouin, S., Pinard, E., Praud, I., Richard, I., Riquier, V., Roure, R., Sendra, B., Thevenet, C., Thiol, S., Vauquelin, E., Vergnaud, L., Grange, T., Geigl, E.-M., Pruvost, M., 2020. Ancient genomes from present-day France unveil 7,000 years of its demographic history. *Proc. Natl. Acad. Sci.* 117, 12791–12798. <https://doi.org/10.1073/pnas.1918034117>
- Burger, J., Hummel, S., Herrmann, B., Henke, W., 1999. DNA preservation: A microsatellite-DNA study on ancient skeletal remains. *ELECTROPHORESIS* 20, 1722–1728. [https://doi.org/10.1002/\(SICI\)1522-2683\(19990101\)20:8%253C1722::AID-ELPS1722%253E3.0.CO;2-4](https://doi.org/10.1002/(SICI)1522-2683(19990101)20:8%253C1722::AID-ELPS1722%253E3.0.CO;2-4)
- Cámara Manzaneda, J., Clop García, X., García Rosselló, J., Martín Colliga, A., 2022. Pottery forming of the Cardial and Epicardial Neolithic wares: Analysis and systematisation of technological traces from the ceramic productions of Cova del Frare (NE Iberian Peninsula, 5200–4800 BCE). *J. Archaeol. Sci. Rep.* 43, 103457. <https://doi.org/10.1016/j.jasrep.2022.103457>
- Cann, R.L., Stoneking, M., Wilson, A.C., 1987. Mitochondrial DNA and human evolution. *Nature* 325, 31–36. <https://doi.org/10.1038/325031a0>
- Cano, R.J., Poinar, H.N., Pieniazek, N.J., Acra, A., Poinar, G.O., 1993. Amplification and sequencing of DNA from a 120–135-million-year-old weevil. *Nature* 363, 536–538. <https://doi.org/10.1038/363536a0>
- Cardoso, J.L., 2014. Cronología absoluta del fenómeno campaniforme al Norte del estuario del Tago: implicaciones demográficas y sociales. *Trab. Prehist.* 71, 56–75. <https://doi.org/10.3989/tp.2014.12124>
- Carolina Azevedo de Sá, L., 2021. Mitochondrial DNA phylogeography of African lineages. Universidade do Minho.
- Carracedo, A., Bär, W., Lincoln, P., Mayr, W., Morling, N., Olaisen, B., Schneider, P., Budowle, B., Brinkmann, B., Gill, P., Holland, M., Tully, G., Wilson, M., 2000. DNA Commission of the International Society for Forensic Genetics: guidelines for mitochondrial DNA typing. *Forensic Sci. Int.* 79–85.
- Carvalho, A., Cardoso, J., 2014. Ideas sobre transformaciones en las dinámicas de utilización de cementerios neolíticos y calcolíticos del sur de Portugal. Las dataciones radiocarbónicas de la cueva de Lugar do Canto (Santarém). *SPAL Rev. Prehist. Arqueol. Univ. Sevilla* 35–53. <https://doi.org/10.12795/spal.2015i24.02>
- Carvalho, A., Zilhão, J., 1996. O Neolítico do Maciço Calcário Estremenho. *Crono-estratigrafia e povoamento*. p. 659–671.
- Carvalho, A.F., 2019. Arquitetura e oferendas funerárias, in: *Hipogeum Campaniforme Do Convento Do Carmo (Torres Novas)*. Município de Torres Novas, Portugal, pp. 117–130.
- Carvalho, A.F., Alves-Cardoso, F., Gonçalves, D., Granja, R., Cardoso, J.L., Dean, R.M., Gibaja, J.F., Masucci, M.A., Arroyo-Pardo, E., Fernández-Domínguez, E., Petchey, F., Price, T.D., Mateus, J.E., Queiroz, P.F., Callapez, P., Pimenta, C., Regala, F.T., 2016. The Bom Santo Cave (Lisbon, Portugal): Catchment, Diet, and Patterns of Mobility of a Middle Neolithic Population. *Eur. J. Archaeol.* 19, 187–214. <https://doi.org/10.1179/1461957115Y.0000000014>
- Carvalho, A.F., Antunes-Ferreira, N., Valente, M.J., 2003. A gruta-necrópole neolítica do Algar do Barrão (Monsanto, Alcanena) 6.

- Carvalho, A.F., Fernández-Domínguez, E., Arroyo-Pardo, E., Robinson, C., Cardoso, J.L., Zilhão, J., Gomes, M.V., 2023. Hunter-gatherer genetic persistence at the onset of megalithism in western Iberia: New mitochondrial evidence from Mesolithic and Neolithic necropolises in central-southern Portugal. *Quat. Int., The Last Hunter-Gatherers on the Iberian Peninsula: An Integrative Evolutionary and Multiscalar Approach from Cueva de la Cocina (Western Mediterranean)* 677–678, 111–120. <https://doi.org/10.1016/j.quaint.2023.03.015>
- Carvalho, A.F., Gonçalves, D., Díaz-Zorita Bonilla, M., Valente, M.J., 2019. Multi-isotope approaches to the Neolithic cemetery-cave of Bom Santo (Lisbon): new data and comparisons with fourth millennium BC populations from central–southern Portugal. *Archaeol. Anthropol. Sci.* 11, 6141–6159. <https://doi.org/10.1007/s12520-019-00908-2>
- Carvalho, A.F., Petchey, F., 2013. Stable Isotope Evidence of Neolithic Palaeodiets in the Coastal Regions of Southern Portugal. *J. Isl. Coast. Archaeol.* 8, 361–383. <https://doi.org/10.1080/15564894.2013.811447>
- Cassidy, L.M., Maoldúin, R.Ó., Kador, T., Lynch, A., Jones, C., Woodman, P.C., Murphy, E., Ramsey, G., Dowd, M., Noonan, A., Campbell, C., Jones, E.R., Mattiangeli, V., Bradley, D.G., 2020. A dynastic elite in monumental Neolithic society. *Nature* 582, 384–388. <https://doi.org/10.1038/s41586-020-2378-6>
- Childebayeva, A., Rohrlach, A.B., Barquera, R., Rivollat, M., Aron, F., Szolek, A., Kohlbacher, O., Nicklisch, N., Alt, K.W., Gronenborn, D., Meller, H., Friederich, S., Prüfer, K., Deguilloux, M.-F., Krause, J., Haak, W., 2022. Population Genetics and Signatures of Selection in Early Neolithic European Farmers. *Mol. Biol. Evol.* 39, msac108. <https://doi.org/10.1093/molbev/msac108>
- Çilingiroğlu, Ç., 2005. The concept of “Neolithic package”: considering its meaning and applicability. *Doc. Praehist.* 32, 1–13. <https://doi.org/10.4312/dp.32.1>
- Claro, A.M., Fonseca, A., Fraga, H., Santos, J.A., 2023. Susceptibility of Iberia to Extreme Precipitation and Aridity: A New High-Resolution Analysis over an Extended Historical Period. *Water* 15, 3840. <https://doi.org/10.3390/w15213840>
- Cooper, A., Poinar, H.N., 2000. Ancient DNA: Do It Right or Not at All. *Science* 289, 1139–1139. <https://doi.org/10.1126/science.289.5482.1139b>
- Costa, M.D., Pereira, J.B., Pala, M., Fernandes, V., Olivieri, A., Achilli, A., Perego, U.A., Rychkov, S., Naumova, O., Hatina, J., Woodward, S.R., Eng, K.K., Macaulay, V., Carr, M., Soares, P., Pereira, L., Richards, M.B., 2013. A substantial prehistoric European ancestry amongst Ashkenazi maternal lineages. *Nat. Commun.* 4, 2543. <https://doi.org/10.1038/ncomms3543>
- Cubas, M., Peyroteo-Stjerna, R., Fontanals-Coll, M., Llorente-Rodríguez, L., Lucquin, A., Craig, O.E., Colonese, A.C., 2019. Long-term dietary change in Atlantic and Mediterranean Iberia with the introduction of agriculture: a stable isotope perspective. *Archaeol. Anthropol. Sci.* 11, 3825–3836. <https://doi.org/10.1007/s12520-018-0752-1>
- Cucart-Mora, C., Gómez-Puche, M., Romano, V., de Pablo, J.F.-L., Lozano, S., 2022. Reconstructing Mesolithic social networks on the Iberian Peninsula using ornaments. *Archaeol. Anthropol. Sci.* 14, 174. <https://doi.org/10.1007/s12520-022-01641-z>
- Dabney, J., Knapp, M., Glocke, I., Gansauge, M.-T., Weihmann, A., Nickel, B., Valdiosera, C., García, N., Pääbo, S., Arsuaga, J.-L., Meyer, M., 2013a. Complete

- mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. *Proc. Natl. Acad. Sci.* 110, 15758–15763. <https://doi.org/10.1073/pnas.1314445110>
- Dabney, J., Meyer, M., Pääbo, S., 2013b. Ancient DNA Damage. *Cold Spring Harb. Perspect. Biol.* 5, a012567. <https://doi.org/10.1101/cshperspect.a012567>
- Damgaard, P.B., Margaryan, A., Schroeder, H., Orlando, L., Willerslev, E., Allentoft, M.E., 2015. Improving access to endogenous DNA in ancient bones and teeth. *Sci. Rep.* 5, 11184. <https://doi.org/10.1038/srep11184>
- Davidson, R., Ravishankar, S., Souilmi, Y., Roca-Rada, X., Sobek, C., Taufik, L., Evans, S., Purnomo, G.A., Rohrlach, A.B., Harvey, T., Haarkötter, C., Pfeiler, K., Lewis, D., Llamas, B., Wood, J.R., Pérez, V., 2025. The necessity for authentication of ancient DNA from archaeological artefacts. *J. Archaeol. Sci.* 181, 106317. <https://doi.org/10.1016/j.jas.2025.106317>
- Davison, K., Dolukhanov, P., Sarson, G.R., Shukurov, A., 2006. The role of waterways in the spread of the Neolithic. *J. Archaeol. Sci.* 33, 641–652. <https://doi.org/10.1016/j.jas.2005.09.017>
- Dawid, I.B., Blackler, A.W., 1972. Maternal and cytoplasmic inheritance of mitochondrial DNA in *Xenopus*. *Dev. Biol.* 29, 152–161. [https://doi.org/10.1016/0012-1606\(72\)90052-8](https://doi.org/10.1016/0012-1606(72)90052-8)
- De Torres, T., Ortiz, J.E., Llamas, F.J., Canoira, L., Juliá, R., García-Martínez, M.J., 2002. Bear dentine aspartic acid racemization analysis: a proxy for the dating of Pleistocene cave infills. *Archaeometry* 44, 417–426. <https://doi.org/10.1111/1475-4754.t01-1-00074>
- Defranould, E., 2021. The Cardial–Epicardial Early Neolithic of Lower Rhône Valley (South-Eastern France): A Lithic Perspective. *Open Archaeol.* 7, 939–952. <https://doi.org/10.1515/opar-2020-0182>
- Dennell, R., 1983. *European Economic Prehistory: A New Approach*. Academic Press.
- Derenko, M., Malyarchuk, B., Bahmanimehr, A., Denisova, G., Perkova, M., Farjadian, S., Yepiskoposyan, L., 2013. Complete Mitochondrial DNA Diversity in Iranians. *PLOS ONE* 8, e80673. <https://doi.org/10.1371/journal.pone.0080673>
- DeSalle, R., Gatesy, J., Wheeler, W., Grimaldi, D., 1992. DNA Sequences from a Fossil Termite in Oligo-Miocene Amber and their Phylogenetic Implications. *Science* 257, 1933–1936. <https://doi.org/10.1126/science.1411508>
- Díaz-Navarro, S., Haber Uriarte, M., Tejedor-Rodríguez, C., Lomba Maurandi, J., 2023. Emphasising the community: demographic composition of an exceptional tomb—the Chalcolithic burial site of Camino del Molino, Caravaca de la Cruz, Murcia. *Archaeol. Anthropol. Sci.* 15, 140. <https://doi.org/10.1007/s12520-023-01844-y>
- DiRenno, F., 2016. *Population Dynamics in Neolithic Northeastern Iberia: An Isotopic and Ancient DNA Approach*. Durham University, Durham.
- DiRenno, F., Towers, J., Nowell, G., Molist, M., Montgomery, J., Fernández-Domínguez, E., 2016. *Dying in the Neolithic: isotope analysis of the Late Neolithic mass grave of La Sagrera, Barcelona*. Durham University, Durham.
- Drake, B.L., Blanco-González, A., Lillios, K.T., 2017. Regional Demographic Dynamics in the Neolithic Transition in Iberia: Results from Summed Calibrated Date Analysis. *J. Archaeol. Method Theory* 24, 796–812. <https://doi.org/10.1007/s10816-016-9286-y>

- Eisenmann, S., Bánffy, E., van Dommelen, P., Hofmann, K.P., Maran, J., Lazaridis, I., Mitnick, A., McCormick, M., Krause, J., Reich, D., Stockhammer, P.W., 2018. Reconciling material cultures in archaeology with genetic data: The nomenclature of clusters emerging from archaeogenomic analysis. *Sci. Rep.* 8, 13003. <https://doi.org/10.1038/s41598-018-31123-z>
- España, Á., Palomo-Díez, S., Velasco-Vázquez, J., Delibes, G., Arroyo-Pardo, E., Salazar-García, D.C., 2017. Familiar Kinship? Palaeogenetic and Isotopic Evidence from a Triple Burial of the Cogotas I Archaeological Culture (Bronze Age, Iberian Peninsula). *Oxf. J. Archaeol.* 36, 223–242. <https://doi.org/10.1111/ojoa.12113>
- Fano, M.Á., 2019. The Mesolithic “Asturian” culture (North Iberia), one century on. *Quat. Int.* 515, 159–175. <https://doi.org/10.1016/j.quaint.2017.12.025>
- Feldman, M., Fernández-Domínguez, E., Reynolds, L., Baird, D., Pearson, J., Hershkovitz, I., May, H., Goring-Morris, N., Benz, M., Gresky, J., Bianco, R.A., Fairbairn, A., Mustafaoğlu, G., Stockhammer, P.W., Posth, C., Haak, W., Jeong, C., Krause, J., 2019. Late Pleistocene human genome suggests a local origin for the first farmers of central Anatolia. *Nat. Commun.* 10, 1218. <https://doi.org/10.1038/s41467-019-09209-7>
- Fernández, E., Pérez-Pérez, A., Gamba, C., Prats, E., Cuesta, P., Anfruns, J., Molist, M., Arroyo-Pardo, E., Turbón, D., 2014. Ancient DNA Analysis of 8000 B.C. Near Eastern Farmers Supports an Early Neolithic Pioneer Maritime Colonization of Mainland Europe through Cyprus and the Aegean Islands. *PLOS Genet.* 10, e1004401. <https://doi.org/10.1371/journal.pgen.1004401>
- Fernández-Domínguez, E., Neff, M., Novell, G., Montgomery, J., 2019. Isótopos de estrôncio e análise de ADN mitocondrial: Perspetivas sobre a estrutura populacional, parentesco e padrões de mobilidade, in: *Hipogeum Campaniforme Do Convento Do Carmo (Torres Novas)*. Município de Torres Novas, Portugal.
- Fernández-Domínguez, E., Reynolds, L., Salazar-García, D.C., García-Puchol, O., 2017. The Mesolithic-Neolithic Transition in Europe: A Perspective from Ancient Human DNA, in: *Times of Neolithic Transition along the Western Mediterranean, Fundamental Issues in Archaeology*. Springer International Publishing, Cham, pp. 311–338. https://doi.org/10.1007/978-3-319-52939-4_12
- Fernández-Posse, M.D., Rodríguez Marcos, J.A., Fernández Manzano, J., 2012. Cogotas I: una cultura de la Edad del Bronce en la Península Ibérica : homenaje a Ma Dolores Fernández-Posse, *Serie Arte y arqueología*. Universidad de Valladolid, Valladolid.
- Fowler, C., Olalde, I., Cummings, V., Armit, I., Büster, L., Cuthbert, S., Rohland, N., Cheronet, O., Pinhasi, R., Reich, D., 2022. A high-resolution picture of kinship practices in an Early Neolithic tomb. *Nature* 601, 584–587. <https://doi.org/10.1038/s41586-021-04241-4>
- Fregel, R., Méndez, F.L., Bokbot, Y., Martín-Socas, D., Camalich-Massieu, M.D., Santana, J., Morales, J., Ávila-Arcos, M.C., Underhill, P.A., Shapiro, B., Wojcik, G., Rasmussen, M., Soares, A.E.R., Kapp, J., Sockell, A., Rodríguez-Santos, F.J., Mikdad, A., Trujillo-Mederos, A., Bustamante, C.D., 2018. Ancient genomes from North Africa evidence prehistoric migrations to the Maghreb from both the Levant and Europe. *Proc. Natl. Acad. Sci. U. S. A.* 115, 6774–6779.

- Fu, Q., Posth, C., Hajdinjak, M., Petr, M., Mallick, S., Fernandes, D., Furtwängler, A., Haak, W., Meyer, M., Mitnik, A., Nickel, B., Peltzer, A., Rohland, N., Slon, V., Talamo, S., Lazaridis, I., Lipson, M., Mathieson, I., Schiffels, S., Skoglund, P., Derevianko, A.P., Drozdov, N., Slavinsky, V., Tsybankov, A., Cremonesi, R.G., Mallegni, F., Gély, B., Vacca, E., Morales, M.R.G., Straus, L.G., Neugebauer-Maresch, C., Teschler-Nicola, M., Constantin, S., Moldovan, O.T., Benazzi, S., Peresani, M., Coppola, D., Lari, M., Ricci, S., Ronchitelli, A., Valentin, F., Thevenet, C., Wehrberger, K., Grigorescu, D., Rougier, H., Crevecoeur, I., Flas, D., Semal, P., Mannino, M.A., Cupillard, C., Bocherens, H., Conard, N.J., Harvati, K., Moiseyev, V., Drucker, D.G., Svoboda, J., Richards, M.P., Caramelli, D., Pinhasi, R., Kelso, J., Patterson, N., Krause, J., Pääbo, S., Reich, D., 2016. The genetic history of Ice Age Europe. *Nature* 534, 200–205. <https://doi.org/10.1038/nature17993>
- Fuertes-Prieto, M.N., Ramil-Rego, E., Fernández-Rodríguez, C., Herrero-Alonso, D., González-Gómez de Agüero, E., Neira-Campos, A., 2023. An overview of the Mesolithic in the northwest Atlantic and inland area of the Iberian Peninsula. *Quat. Int., The Last Hunter-Gatherers on the Iberian Peninsula: An Integrative Evolutionary and Multiscalar Approach from Cueva de la Cocina (Western Mediterranean)* 677–678, 95–110. <https://doi.org/10.1016/j.quaint.2023.05.005>
- Gamba, C., Fernández, E., Oliver, A., Tirado, M., Baeza, C., López-Parra, A.M., Arroyo-Pardo, E., 2008. Population genetics and DNA preservation in ancient human remains from Eastern Spain. *Forensic Sci. Int. Genet. Suppl. Ser., Progress in Forensic Genetics* 12 1, 462–464. <https://doi.org/10.1016/j.fsigs.2007.10.037>
- Gamba, C., Fernández, E., Tirado, M., Deguilloux, M.F., Pemonge, M.H., Utrilla, P., Edo, M., Molist, M., Rasteiro, R., Chikhi, L., Arroyo-Pardo, E., 2012. Ancient DNA from an Early Neolithic Iberian population supports a pioneer colonization by first farmers. *Mol. Ecol.* 21, 45–56. <https://doi.org/10.1111/j.1365-294X.2011.05361.x>
- García-Artola, A., Stéphan, P., Cearreta, A., Kopp, R.E., Khan, N.S., Horton, B.P., 2018. Holocene sea-level database from the Atlantic coast of Europe. *Quat. Sci. Rev.* 196, 177–192. <https://doi.org/10.1016/j.quascirev.2018.07.031>
- García-Puchol, O., McClure, S.B., Juan-Cabanilles, J., Díez-Castillo, A.A., Bernabeu-Aubán, J., Martí-Oliver, B., Pardo-Gordó, S., Pascual-Benito, J.L., Pérez-Ripoll, M., Molina-Balaguer, L., Kennett, D.J., 2018. Cocina cave revisited: Bayesian radiocarbon chronology for the last hunter-gatherers and first farmers in Eastern Iberia. *Quat. Int., The Neolithisation of the Western Mediterranean: New Debates About an Old Issue* 472, 259–271. <https://doi.org/10.1016/j.quaint.2016.10.037>
- García-Puchol, O., Pardo-Gordó, S., Ángel Díez Castillo, A., Cortell Nicolau, A., Juan-Cabanilles, J., McClure, S., Ramacciotti, M., 2025. Actuación Arqueológica en los depósitos mesolíticos de Cueva de la Cocina (Dos Aguas, Valencia): valoración preliminar. *ResearchGate*. <https://doi.org/10.7203/SAGVNTVM.50.13273>
- Gelabert, P., Bickle, P., Hofmann, D., Teschler-Nicola, M., Anders, A., Huang, X., Hämmerle, M., Olalde, I., Fournier, R., Ringbauer, H., Akbari, A., Cheronet, O., Lazaridis, I., Broomandkhoshbacht, N., Fernandes, D.M., Buttinger, K., Callan, K., Candilio, F., Bravo Morante, G., Curtis, E., Ferry, M., Keating, D., Freilich, S., Kearns, A., Harney, É., Lawson, A.M., Mandl, K., Michel, M., Oberreiter, V., Zagorc, B., Oppenheimer, J., Sawyer, S., Schattke, C., Özdoğan, K.T., Qiu, L.,

- Workman, J.N., Zalzal, F., Mallick, S., Mah, M., Micco, A., Pieler, F., Pavuk, J., Šefčáková, A., Lazar, C., Starović, A., Djuric, M., Krznarić Škrivanko, M., Šlaus, M., Bedić, Ž., Novotny, F., D. Szabó, L., Cserpák-Laczi, O., Hága, T., Szolnoki, L., Hajdú, Z., Mirea, P., Nagy, E.G., Virág, Z.M., Horváth M., A., Horváth, L.A., T. Biró, K., Domboróczki, L., Szeniczey, T., Jakucs, J., Szelekovszky, M., Zoltán, F., Sztáncsuj, S.J., Tóth, K., Csengeri, P., Pap, I., Patay, R., Putica, A., Vasov, B., Havasi, B., Sebők, K., Raczky, P., Lovász, G., Tvrđý, Z., Rohland, N., Novak, M., Ruttkay, M., Krošlák, M., Batora, J., Paluch, T., Borić, D., Dani, J., Kuhlmann, M., Palamara, P.F., Hajdu, T., Pinhasi, R., Reich, D., 2025. Social and genetic diversity in first farmers of central Europe. *Nat. Hum. Behav.* 9, 53–64.
<https://doi.org/10.1038/s41562-024-02034-z>
- Gibaja Bao, J.F., Carvalho, A.F. de, Chambon, P., 2012. Funerary practices in the Iberian peninsula from the Mesolithic to the Chalcolithic, BAR international series. British Archaeological Reports, Oxford.
<https://doi.org/10.30861/9781407310152>
- Gibaja, J.F., Carvalho, A.F., Chambon, P., 2012. Funerary Practices in the Iberian Peninsula from the Mesolithic to the Chalcolithic. BAR Publishing.
<https://doi.org/10.30861/9781407310152>
- Gilbert, M.T.P., Binladen, J., Miller, W., Wiuf, C., Willerslev, E., Poinar, H., Carlson, J.E., Leebens-Mack, J.H., Schuster, S.C., 2007a. Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. *Nucleic Acids Res.* 35, 1–10. <https://doi.org/10.1093/nar/gkl483>
- Gilbert, M.T.P., Tomsho, L.P., Rendulic, S., Packard, M., Drautz, D.I., Sher, A., Tikhonov, A., Dalén, L., Kuznetsova, T., Kosintsev, P., Campos, P.F., Higham, T., Collins, M.J., Wilson, A.S., Shidlovskiy, F., Buigues, B., Ericson, P.G.P., Germonpré, M., Götherström, A., Iacumin, P., Nikolaev, V., Nowak-Kemp, M., Willerslev, E., Knight, J.R., Irzyk, G.P., Perbost, C.S., Fredrikson, K.M., Harkins, T.T., Sheridan, S., Miller, W., Schuster, S.C., 2007b. Whole-Genome Shotgun Sequencing of Mitochondria from Ancient Hair Shafts. *Science* 317, 1927–1930.
<https://doi.org/10.1126/science.1146971>
- Gilbert, M.T.P., Willerslev, E., Hansen, A.J., Barnes, I., Rudbeck, L., Lynnerup, N., Cooper, A., 2003. Distribution Patterns of Postmortem Damage in Human Mitochondrial DNA. *Am. J. Hum. Genet.* 72, 32–47. <https://doi.org/10.1086/345378>
- Goldberg, A., Günther, T., Rosenberg, N.A., Jakobsson, M., 2017. Reply to Lazaridis and Reich: Robust model-based inference of male-biased admixture during Bronze Age migration from the Pontic-Caspian Steppe. *Proc. Natl. Acad. Sci.* 114, E3875–E3877. <https://doi.org/10.1073/pnas.1704442114>
- Golenberg, E.M., Giannasi, D.E., Clegg, M.T., Smiley, C.J., Durbin, M., Henderson, D., Zurawski, G., 1990. Chloroplast DNA sequence from a Miocene *Magnolia* species. *Nature* 344, 656–658. <https://doi.org/10.1038/344656a0>
- Gomart, L., Weiner, A., Gabriele, M., Durrenmath, G., Sorin, S., Angeli, L., Colombo, M., Fabbri, C., Maggi, R., Panelli, C., Pisani, D.F., Radi, G., Tozzi, C., Binder, D., 2017. Spiralled patchwork in pottery manufacture and the introduction of farming to Southern Europe. *Antiquity* 91, 1501–1514.
<https://doi.org/10.15184/aqy.2017.187>
- González-Fortes, G., Jones, E.R., Lightfoot, E., Bonsall, C., Lazar, C., Grandal-d'Anglade, A., Garraalda, M.D., Drak, L., Siska, V., Simalcsek, A., Boroneanț, A., Romaní,

- J.R.V., Rodríguez, M.V., Arias, P., Pinhasi, R., Manica, A., Hofreiter, M., 2017. Paleogenomic Evidence for Multi-generational Mixing between Neolithic Farmers and Mesolithic Hunter-Gatherers in the Lower Danube Basin. *Curr. Biol.* 27, 1801-1810.e10. <https://doi.org/10.1016/j.cub.2017.05.023>
- González-Fortes, G., Tassi, F., Trucchi, E., Henneberger, K., Pajmans, J.L.A., Díez-del-Molino, D., Schroeder, H., Susca, R.R., Barroso-Ruiz, C., Bermudez, F.J., Barroso-Medina, C., Bettencourt, A.M.S., Sampaio, H.A., Grandal-d'Anglade, A., Salas, A., de Lombera-Hermida, A., Fabregas Valcarce, R., Vaquero, M., Alonso, S., Lozano, M., Rodríguez-Alvarez, X.P., Fernández-Rodríguez, C., Manica, A., Hofreiter, M., Barbujani, G., 2019. A western route of prehistoric human migration from Africa into the Iberian Peninsula. *Proc. R. Soc. B Biol. Sci.* 286, 20182288. <https://doi.org/10.1098/rspb.2018.2288>
- Granja, R., 2019. A população: Caracterização bioantropologia, in: *Hipogeu Campaniforme Do Convento Do Carmo (Torres Novas)*. Município de Torres Novas, Portugal, pp. 67–86.
- Granja, R., Carvalho, A.F., 2019. Práticas e rituais funerários, in: *Hipogeu Campaniforme Do Convento Do Carmo (Torres Novas)*. Município de Torres Novas, Portugal, pp. 87–93.
- Greninger, A.L., Glaser, C.A., 2017. 116 - Fungal, Rickettsial, and Parasitic Diseases of the Nervous System, in: Swaiman, K.F., Ashwal, S., Ferriero, D.M., Schor, N.F., Finkel, R.S., Gropman, A.L., Pearl, P.L., Shevell, M.I. (Eds.), *Swaiman's Pediatric Neurology (Sixth Edition)*. Elsevier, pp. 907–917. <https://doi.org/10.1016/B978-0-323-37101-8.00116-8>
- Haak, W., Balanovsky, O., Sanchez, J.J., Koshel, S., Zaporozhchenko, V., Adler, C.J., Sarkissian, C.S.I.D., Brandt, G., Schwarz, C., Nicklisch, N., Dresely, V., Fritsch, B., Balanovska, E., VILLEMS, R., Meller, H., Alt, K.W., Cooper, A., Consortium, the G., 2010. Ancient DNA from European Early Neolithic Farmers Reveals Their Near Eastern Affinities. *PLOS Biol.* 8, e1000536. <https://doi.org/10.1371/journal.pbio.1000536>
- Haak, W., Brandt, G., Jong, H.N. de, Meyer, C., Ganslmeier, R., Heyd, V., Hawkesworth, C., Pike, A.W.G., Meller, H., Alt, K.W., 2008. Ancient DNA, Strontium isotopes, and osteological analyses shed light on social and kinship organization of the Later Stone Age. *Proc. Natl. Acad. Sci.* 105, 18226–18231. <https://doi.org/10.1073/pnas.0807592105>
- Haak, W., Lazaridis, I., Patterson, N., Rohland, N., Mallick, S., Llamas, B., Brandt, G., Nordenfelt, S., Harney, E., Stewardson, K., Fu, Q., Mittnik, A., Bánffy, E., Economou, C., Francken, M., Friederich, S., Pena, R.G., Hallgren, F., Khartanovich, V., Khokhlov, A., Kunst, M., Kuznetsov, P., Meller, H., Mochalov, O., Moiseyev, V., Nicklisch, N., Pichler, S.L., Risch, R., Rojo Guerra, M.A., Roth, C., Szécsényi-Nagy, A., Wahl, J., Meyer, M., Krause, J., Brown, D., Anthony, D., Cooper, A., Alt, K.W., Reich, D., 2015a. Massive migration from the steppe was a source for Indo-European languages in Europe. *Nature* 522, 207–211. <https://doi.org/10.1038/nature14317>
- Haak, W., Lazaridis, I., Patterson, N., Rohland, N., Mallick, S., Llamas, B., Brandt, G., Nordenfelt, S., Harney, E., Stewardson, K., Fu, Q., Mittnik, A., Bánffy, E., Economou, C., Francken, M., Friederich, S., Pena, R.G., Hallgren, F., Khartanovich, V., Khokhlov, A., Kunst, M., Kuznetsov, P., Meller, H., Mochalov, O.,

- Moiseyev, V., Nicklisch, N., Pichler, S.L., Risch, R., Rojo Guerra, M.A., Roth, C., Szécsényi-Nagy, A., Wahl, J., Meyer, M., Krause, J., Brown, D., Anthony, D., Cooper, A., Alt, K.W., Reich, D., 2015b. Massive migration from the steppe was a source for Indo-European languages in Europe. *Nature* 522, 207–211. <https://doi.org/10.1038/nature14317>
- Hansen, A.J., Mitchell, D.L., Wiuf, C., Paniker, L., Brand, T.B., Binladen, J., Gilichinsky, D.A., Rønn, R., Willerslev, E., 2006. Crosslinks Rather Than Strand Breaks Determine Access to Ancient DNA Sequences From Frozen Sediments. *Genetics* 173, 1175–1179. <https://doi.org/10.1534/genetics.106.057349>
- Harrison, R.J., 1980. *The Beaker Folk: copper age archaeology in Western Europe, Ancient peoples and places*. Thames and Hudson, London.
- Hedges, R.E.M., Millard, A.R., 1995. Bones and Groundwater: Towards the Modelling of Diagenetic Processes. *J. Archaeol. Sci.* 22, 155–164. <https://doi.org/10.1006/jasc.1995.0017>
- Hedges, S.B., Schweitzer, M.H., 1995. Detecting Dinosaur DNA. *Science* 268, 1191–1192. <https://doi.org/10.1126/science.7761839>
- Herbig, A., Maixner, F., Bos, K.I., Zink, A., Krause, J., Huson, D.H., 2016. MALT: Fast alignment and analysis of metagenomic DNA sequence data applied to the Tyrolean Iceman. <https://doi.org/10.1101/050559>
- Herrero-Corral, A.M., Garrido-Pena, R., Rebay-Salisbury, K., Flores Fernández, R., Paulos-Bravo, R., Wolf, M., Kanz, F., 2025. Gendered burial practices in Copper Age non-adults of Iberia: Evidence from peptide-based sex identification at Humanejos (Madrid, Spain). *J. Archaeol. Sci. Rep.* 66, 105290. <https://doi.org/10.1016/j.jasrep.2025.105290>
- Hervella, M., Izagirre, N., Alonso, S., Fregel, R., Alonso, A., Cabrera, V.M., Rúa, C. de la, 2012. Ancient DNA from Hunter-Gatherer and Farmer Groups from Northern Spain Supports a Random Dispersion Model for the Neolithic Expansion into Europe. *PLOS ONE* 7, e34417. <https://doi.org/10.1371/journal.pone.0034417>
- Higgins, D., Rohrlach, A.B., Kaidonis, J., Townsend, G., Austin, J.J., 2015. Differential Nuclear and Mitochondrial DNA Preservation in Post-Mortem Teeth with Implications for Forensic and Ancient DNA Studies. *PLOS ONE* 10, e0126935. <https://doi.org/10.1371/journal.pone.0126935>
- Higuchi, R., Bowman, B., Freiberger, M., Ryder, O.A., Wilson, A.C., 1984. DNA sequences from the quagga, an extinct member of the horse family. *Nature* 312, 282–284. <https://doi.org/10.1038/312282a0>
- Hofreiter, M., Jaenicke, V., Serre, D., Haeseler, A. von, Pääbo, S., 2001a. DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Res.* 29, 4793–4799. <https://doi.org/10.1093/nar/29.23.4793>
- Hofreiter, M., Serre, D., Poinar, H.N., Kuch, M., Pääbo, S., 2001b. Ancient DNA. *Nat. Rev. Genet.* 2, 353–359. <https://doi.org/10.1038/35072071>
- Hollingshead, S., Tang, C.M., 2019. An Overview of *Neisseria meningitidis*, in: Seib, K.L., Peak, I.R. (Eds.), *Neisseria Meningitidis: Methods and Protocols*. Springer, New York, NY, pp. 1–16. https://doi.org/10.1007/978-1-4939-9202-7_1
- Höss, M., Jaruga, P., Zastawny, T.H., Dizdaroglu, M., Paabo, S., 1996. DNA Damage and DNA Sequence Retrieval from Ancient Tissues. *Nucleic Acids Res.* 24, 1304–1307. <https://doi.org/10.1093/nar/24.7.1304>

- Iyavoo, S., Hadi, S., Goodwin, W., 2019. Evaluation of five preservation methods for recovery of DNA from bone. *Forensic Sci. Int. Genet. Suppl. Ser.*, The 28th Congress of the International Society for Forensic Genetics 7, 200–202. <https://doi.org/10.1016/j.fsigss.2019.09.077>
- Jackson, I., Woodman, P., Dowd, M., Fibiger, L., Cassidy, L.M., 2024. Ancient Genomes From Bronze Age Remains Reveal Deep Diversity and Recent Adaptive Episodes for Human Oral Pathobionts. *Mol. Biol. Evol.* 41. <https://doi.org/10.1093/molbev/msae017>
- Kemp, B.M., Monroe, C., Judd, K.G., Reams, E., Grier, C., 2014. Evaluation of methods that subdue the effects of polymerase chain reaction inhibitors in the study of ancient and degraded DNA. *J. Archaeol. Sci.* 42, 373–380. <https://doi.org/10.1016/j.jas.2013.11.023>
- Kılınç, G.M., Omrak, A., Özer, F., Günther, T., Büyükkarakaya, A.M., Bıçakçı, E., Baird, D., Dönertaş, H.M., Ghalichi, A., Yaka, R., Koptekin, D., Açıkan, S.C., Parvizi, P., Krzewińska, M., Daskalaki, E.A., Yüncü, E., Dağtaş, N.D., Fairbairn, A., Pearson, J., Mustafaoğlu, G., Erdal, Y.S., Çakan, Y.G., Togan, İ., Somel, M., Storå, J., Jakobsson, M., Götherström, A., 2016. The Demographic Development of the First Farmers in Anatolia. *Curr. Biol.* 26, 2659–2666. <https://doi.org/10.1016/j.cub.2016.07.057>
- Kjær, K.H., Winther Pedersen, M., De Sanctis, B., De Cahsan, B., Korneliussen, T.S., Michelsen, C.S., Sand, K.K., Jelavić, S., Ruter, A.H., Schmidt, A.M.A., Kjeldsen, K.K., Tesakov, A.S., Snowball, I., Gosse, J.C., Alsos, I.G., Wang, Y., Dockter, C., Rasmussen, M., Jørgensen, M.E., Skadhauge, B., Prohaska, A., Kristensen, J.Å., Bjerager, M., Allentoft, M.E., Coissac, E., Rouillard, A., Simakova, A., Fernandez-Guerra, A., Bowler, C., Macias-Fauria, M., Vinner, L., Welch, J.J., Hidy, A.J., Sikora, M., Collins, M.J., Durbin, R., Larsen, N.K., Willerslev, E., 2022. A 2-million-year-old ecosystem in Greenland uncovered by environmental DNA. *Nature* 612, 283–291. <https://doi.org/10.1038/s41586-022-05453-y>
- Knapp, M., Lalueza-Fox, C., Hofreiter, M., 2015. Re-inventing ancient human DNA. *Investig. Genet.* 6, 4. <https://doi.org/10.1186/s13323-015-0020-4>
- Kravanja, P., Golob, A., Concato, M., Leskovic, T., Zupanič Pajnič, I., 2025. Effects of different environmental factors on preservation of DNA in petrous bones: A comparative study of two Slovenian archaeological sites. *Forensic Sci. Int.* 371, 112495. <https://doi.org/10.1016/j.forsciint.2025.112495>
- Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J.P., Miranda, C., Morris, W., Naylor, J., Raymond, Christina, Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J.C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R.H., Wilson, R.K., Hillier, L.W., McPherson, J.D., Marra, M.A., Mardis, E.R., Fulton, L.A., Chinwalla, A.T., Pepin, K.H., Gish, W.R., Chissoe, S.L., Wendl, M.C., Delehaunty, K.D., Miner, T.L.,

Delehaunty, A., Kramer, J.B., Cook, L.L., Fulton, R.S., Johnson, D.L., Minx, P.J., Clifton, S.W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J.-F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R.A., Muzny, D.M., Scherer, S.E., Bouck, J.B., Sodergren, E.J., Worley, K.C., Rives, C.M., Gorrell, J.H., Metzker, M.L., Naylor, S.L., Kucherlapati, R.S., Nelson, D.L., Weinstock, G.M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Smith, D.R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H.M., Dubois, J., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R.W., Federspiel, N.A., Abola, A.P., Proctor, M.J., Roe, B.A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W.R., de la Bastide, M., Dedhia, N., Blöcker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J.A., Bateman, A., Batzoglu, S., Birney, E., Bork, P., Brown, D.G., Burge, C.B., Cerutti, L., Chen, H.-C., Church, D., Clamp, M., Copley, R.R., Doerks, T., Eddy, S.R., Eichler, E.E., Furey, T.S., Galagan, J., Gilbert, J.G.R., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L.S., Jones, T.A., Kasif, S., Kasprzyk, A., Kennedy, S., Kent, W.J., Kitts, P., Koonin, E.V., Korf, I., Kulp, D., Lancet, D., Lowe, T.M., McLysaght, A., Mikkelsen, T., Moran, J.V., Mulder, N., Pollara, V.J., Ponting, C.P., Schuler, G., Schultz, J., Slater, G., Smit, A.F.A., Stupka, E., Szustakowki, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y.I., Wolfe, K.H., Yang, S.-P., Yeh, R.-F., Collins, F., Guyer, M.S., Peterson, J., Felsenfeld, A., Wetterstrand, K.A., Myers, R.M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D.R., Olson, M.V., Kaul, R., Raymond, Christopher, Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G.A., Athanasiou, M., Schultz, R., Patrinos, A., Morgan, M.J., International Human Genome Sequencing Consortium, Whitehead Institute for Biomedical Research, C. for G.R., The Sanger Centre:, Washington University Genome Sequencing Center, US DOE Joint Genome Institute:, Baylor College of Medicine Human Genome Sequencing Center:, RIKEN Genomic Sciences Center:, Genoscope and CNRS UMR-8030:, Department of Genome Analysis, I. of M.B., GTC Sequencing Center:, Beijing Genomics Institute/Human Genome Center:, Multimegabase Sequencing Center, T.I. for S.B., Stanford Genome Technology Center:, University of Oklahoma's Advanced Center for Genome Technology:, Max Planck Institute for Molecular Genetics:, Cold Spring Harbor Laboratory, L.A.H.G.C., GBF—German Research Centre for Biotechnology:, *Genome Analysis Group (listed in alphabetical order, also includes individuals listed under other headings):, Scientific management: National Human Genome Research Institute, U.N.I. of H., Stanford Human Genome Center:, University of Washington Genome Center:, Department of Molecular Biology, K.U.S. of M., University of Texas Southwestern Medical Center at Dallas:, Office of Science, U.D. of E., The Wellcome Trust:, 2001. Initial sequencing and analysis of the human genome. *Nature* 409, 860–921. <https://doi.org/10.1038/35057062>

- Latham, K.E., Miller, J.J., 2019. DNA Recovery and Analysis from Skeletal Material in Modern Forensic Contexts. *Forensic Sci. Res.* 4, 51–59.
<https://doi.org/10.1080/20961790.2018.1515594>
- LAV estructura de l'estació de la Sagrera, 2012. , in: *Anuari d'arqueologia i Patrimoni de Barcelona 2011*. Ajuntament de Barcelona. Institut de Cultura, Barcelona, pp. 80–84.
- Lazaridis, I., Alpaslan-Roodenberg, S., Acar, A., Açıkkol, A., Agelarakis, A., Aghikyan, L., Akyüz, U., Andreeva, D., Andrijašević, G., Antonović, D., Armit, I., Atmaca, A., Avetisyan, P., Aytekin, A.İ., Bacvarov, K., Badalyan, R., Bakardzhiev, S., Balen, J., Bejko, L., Bernardos, R., Bertsatos, A., Biber, H., Bilir, A., Bodružić, M., Bonogofsky, M., Bonsall, C., Borić, D., Borovinić, N., Morante, G.B., Buttinger, K., Callan, K., Candilio, F., Carić, M., Cheronet, O., Chohadzhiev, S., Chovalopoulou, M.-E., Chrysoulaki, S., Ciobanu, I., Čondić, N., Constantinescu, M., Cristiani, E., Culleton, B.J., Curtis, E., Davis, J., Davtyan, R., Demcenco, T.I., Dergachev, V., Derin, Z., Deskaj, S., Devejian, S., Djordjević, V., Carlson, K.S.D., Eccles, L.R., Elenski, N., Engin, A., Erdoğan, N., Erir-Pazarci, S., Fernandes, D.M., Ferry, M., Freilich, S., Frînculeasa, A., Galaty, M.L., Gamarra, B., Gasparyan, B., Gaydarska, B., Genç, E., Gültekin, T., Gündüz, S., Hajdu, T., Heyd, V., Hobosyan, S., Hovhannisyan, N., Iliev, I., Iliev, L., Iliev, S., İvgin, İ., Janković, I., Jovanova, L., Karkanias, P., Kavaz-Kindıgılı, B., Kaya, E.H., Keating, D., Kennett, D.J., Kesici, S.D., Khudaverdyan, A., Kiss, K., Kılıç, S., Klostermann, P., Valdes, S.K.B.N., Kovačević, S., Krenz-Niedbala, M., Škrivanko, M.K., Kurti, R., Kuzman, P., Lawson, A.M., Lazar, C., Leshtakov, K., Levy, T.E., Liritzis, I., Lorentz, K.O., Łukasik, S., Mah, M., Mallick, S., Mandl, K., Martirosyan-Olshansky, K., Matthews, R., Matthews, W., McSweeney, K., Melikyan, V., Micco, A., Michel, M., Milašinović, L., Mitnik, A., Monge, J.M., Nekhrizov, G., Nicholls, R., Nikitin, A.G., Nikolov, V., Novak, M., Olalde, I., Oppenheimer, J., Osterholtz, A., Özdemir, C., Özdoğan, K.T., Öztürk, N., Papadimitriou, N., Papakonstantinou, N., Papathanasiou, A., Paraman, L., Paskary, E.G., Patterson, N., Petrakiev, I., Petrosyan, L., Petrova, V., Philippa-Touchais, A., Piliposyan, A., Kuzman, N.P., Potrebica, H., Preda-Bălănică, B., Premužić, Z., Price, T.D., Qiu, L., Radović, S., Aziz, K.R., Šikanjić, P.R., Raheem, K.R., Razumov, S., Richardson, A., Roodenberg, J., Ruka, R., Russeva, V., Şahin, M., Şarbak, A., Savaş, E., Schattke, C., Schepartz, L., Selçuk, T., Sevim-Erol, A., Shamoony-Pour, M., Shephard, H.M., Sideris, A., Simalcik, A., Simonyan, H., Sinika, V., Sirak, K., Sirbu, G., Šlaus, M., Soficaru, A., Söğüt, B., Sołtysiak, A., Sönmez-Sözer, Ç., Stathi, M., Steskal, M., Stewardson, K., Stocker, S., Suata-Alpaslan, F., Suvarov, A., Szécsényi-Nagy, A., Szeniczey, T., Telnov, N., Temov, S., Todorova, N., Tota, U., Touchais, G., Triantaphyllou, S., Türker, A., Ugarković, M., Valchev, T., Veljanovska, F., Videvski, Z., Virag, C., Wagner, A., Walsh, S., Włodarczak, P., Workman, J.N., Yardumian, A., Yarovsky, E., Yavuz, A.Y., Yılmaz, H., Zalazala, F., Zettl, A., Zhang, Z., Çavuşoğlu, R., Rohland, N., Pinhasi, R., Reich, D., 2022. The genetic history of the Southern Arc: A bridge between West Asia and Europe. *Science*. <https://doi.org/10.1126/science.abm4247>
- Lazaridis, I., Nadel, D., Rollefson, G., Merrett, D.C., Rohland, N., Mallick, S., Fernandes, D., Novak, M., Gamarra, B., Sirak, K., Connell, S., Stewardson, K., Harney, E., Fu, Q., Gonzalez-Fortes, G., Jones, E.R., Roodenberg, S.A., Lengyel, G., Bocquentin, F., Gasparian, B., Monge, J.M., Gregg, M., Eshed, V., Mizrahi, A.-S., Meiklejohn, C.,

- Gerritsen, F., Bejenaru, L., Blüher, M., Campbell, A., Cavalleri, G., Comas, D., Froguel, P., Gilbert, E., Kerr, S.M., Kovacs, P., Krause, J., McGettigan, D., Merrigan, M., Merriwether, D.A., O'Reilly, S., Richards, M.B., Semino, O., Shamoon-Pour, M., Stefanescu, G., Stumvoll, M., Tönjes, A., Torroni, A., Wilson, J.F., Yengo, L., Hovhannisyan, N.A., Patterson, N., Pinhasi, R., Reich, D., 2016. Genomic insights into the origin of farming in the ancient Near East. *Nature* 536, 419–424. <https://doi.org/10.1038/nature19310>
- Lazaridis, I., Reich, D., 2017. Failure to replicate a genetic signal for sex bias in the steppe migration into central Europe. *Proc. Natl. Acad. Sci.* 114, E3873–E3874. <https://doi.org/10.1073/pnas.1704308114>
- Linares-Catela, J.A., Vera-Rodríguez, J.C., 2023. Small Houses of the Dead: A Model of Collective Funerary Activity in the Chalcolithic Tombs of Southwestern Iberia. *La Orden-Seminario Site (Huelva, Spain)*. *Open Archaeol.* 9. <https://doi.org/10.1515/opar-2022-0294>
- Lindahl, T., 1997. Facts and Artifacts of Ancient DNA. *Cell* 90, 1–3. [https://doi.org/10.1016/S0092-8674\(00\)80306-2](https://doi.org/10.1016/S0092-8674(00)80306-2)
- Lindahl, T., 1993a. Instability and decay of the primary structure of DNA. *Nature* 362, 709–715. <https://doi.org/10.1038/362709a0>
- Lindahl, T., 1993b. Instability and decay of the primary structure of DNA. *Nature* 362, 709–715. <https://doi.org/10.1038/362709a0>
- Lipson, M., Szécsényi-Nagy, A., Mallick, S., Pósa, A., Stégmár, B., Keerl, V., Rohland, N., Stewardson, K., Ferry, M., Michel, M., Oppenheimer, J., Broomandkhoshbacht, N., Harney, E., Nordenfelt, S., Llamas, B., Gusztáv Mende, B., Köhler, K., Oross, K., Bondár, M., Marton, T., Osztás, A., Jakucs, J., Paluch, T., Horváth, F., Csengeri, P., Koós, J., Sebők, K., Anders, A., Raczky, P., Regenye, J., Barna, J.P., Fábíán, S., Serlegi, G., Toldi, Z., Gyöngyvér Nagy, E., Dani, J., Molnár, E., Pálfi, G., Márk, L., Meleg, B., Bánfai, Z., Domboróczki, L., Fernández-Eraso, J., Antonio Mújika-Alustiza, J., Alonso Fernández, C., Jiménez Echevarría, J., Bollongino, R., Orschiedt, J., Schierhold, K., Meller, H., Cooper, A., Burger, J., Bánffy, E., Alt, K.W., Lalueza-Fox, C., Haak, W., Reich, D., 2017. Parallel palaeogenomic transects reveal complex genetic history of early European farmers. *Nature* 551, 368–372. <https://doi.org/10.1038/nature24476>
- Lomba-Maurandi, J., López-Martínez, M., Ramos-Martínez, F., Avilés-Fernández, A., 2009. El enterramiento múltiple, calcolítico, de Camino del Molino (Caravaca, Murcia). *Metodología y primeros resultados de un yacimiento excepcional*. *Trab. Prehist.* 66, 143–159. <https://doi.org/10.3989/tp.2009.09025>
- Lull, V., Rihuete-Herrada, C., Risch, R., Bonora, B., Celdrán-Beltrán, E., Fregeiro, M.I., Molero, C., Moreno, A., Oliart, C., Velasco-Felipe, C., Andúgar, L., Haak, W., Villalba-Mouco, V., Micó, R., 2021. Emblems and spaces of power during the Argaric Bronze Age at La Almoloya, Murcia. *Antiquity* 95, 329–348. <https://doi.org/10.15184/aqy.2021.8>
- Mallick, S., Micco, A., Mah, M., Ringbauer, H., Lazaridis, I., Olalde, I., Patterson, N., Reich, D., 2024. The Allen Ancient DNA Resource (AADR) a curated compendium of ancient human genomes. *Sci. Data* 11, 182. <https://doi.org/10.1038/s41597-024-03031-7>
- Marchi, N., Winkelbach, L., Schulz, I., Bami, M., Hofmanová, Z., Blöcher, J., Reyna-Blanco, C.S., Diekmann, Y., Thiéry, A., Kapopoulou, A., Link, V., Piuz, V., Kreutzer,

- S., Figarska, S.M., Ganiatsou, E., Pukaj, A., Struck, T.J., Gutenkunst, R.N., Karul, N., Gerritsen, F., Pechtl, J., Peters, J., Zeeb-Lanz, A., Lenneis, E., Teschler-Nicola, M., Triantaphyllou, S., Stefanović, S., Papageorgopoulou, C., Wegmann, D., Burger, J., Excoffier, L., 2022. The genomic origins of the world's first farmers. *Cell* 185, 1842-1859.e18. <https://doi.org/10.1016/j.cell.2022.04.008>
- Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.-J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L.I., Jarvie, T.P., Jirage, K.B., Kim, J.-B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu, H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P., Begley, R.F., Rothberg, J.M., 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437, 376–380. <https://doi.org/10.1038/nature03959>
- Marom, N., Bar-Oz, G., 2009. Culling profiles: the indeterminacy of archaeozoological data to survivorship curve modelling of sheep and goat herd maintenance strategies. *J. Archaeol. Sci.* 36, 1184–1187. <https://doi.org/10.1016/j.jas.2009.01.007>
- Martiniano, R., Cassidy, L.M., Ó'Maoldúin, R., McLaughlin, R., Silva, N.M., Manco, L., Fidalgo, D., Pereira, T., Coelho, M.J., Serra, M., Burger, J., Parreira, R., Moran, E., Valera, A.C., Porfirio, E., Boaventura, R., Silva, A.M., Bradley, D.G., 2017. The population genomics of archaeological transition in west Iberia: Investigation of ancient substructure using imputation and haplotype-based methods. *PLOS Genet.* 13, e1006852. <https://doi.org/10.1371/journal.pgen.1006852>
- Martins, H., Oms, F.X., Pereira, L., Pike, A.W.G., Rowsell, K., Zilhão, J., 2015. Radiocarbon dating the beginning of the Neolithic in Iberia: new results, new problems. *J. Mediterr. Archaeol.* 28, 105–131. <https://doi.org/10.1558/jmea.v28i1.27503>
- Mathieson, I., Alpaslan-Roodenberg, S., Posth, C., Szécsényi-Nagy, A., Rohland, N., Mallick, S., Olalde, I., Broomandkhoshbacht, N., Candilio, F., Cheronet, O., Fernandes, D., Ferry, M., Gamarra, B., Fortes, G.G., Haak, W., Harney, E., Jones, E., Keating, D., Krause-Kyora, B., Kucukkalipci, I., Michel, M., Mitnik, A., Nägele, K., Novak, M., Oppenheimer, J., Patterson, N., Pfrengle, S., Sirak, K., Stewardson, K., Vai, S., Alexandrov, S., Alt, K.W., Andreescu, R., Antonović, D., Ash, A., Atanassova, N., Bacvarov, K., Gusztáv, M.B., Bocherens, H., Bolus, M., Boroneanț, A., Boyadzhiev, Y., Budnik, A., Burmaz, J., Chohadzhiev, S., Conard, N.J., Cottiaux, R., Čuka, M., Cupillard, C., Drucker, D.G., Elenski, N., Francken, M., Galabova, B., Ganetsovski, G., Gély, B., Hajdu, T., Handzhyska, V., Harvati, K., Higham, T., Iliev, S., Janković, I., Karavanić, I., Kennett, D.J., Komšo, D., Kozak, A., Labuda, D., Lari, M., Lazar, C., Leppek, M., Leshtakov, K., Vetro, D.L., Los, D., Lozanov, I., Malina, M., Martini, F., McSweeney, K., Meller, H., Mengušić, M., Mirea, P., Moiseyev, V., Petrova, V., Price, T.D., Simalcsek, A., Sineo, L., Šlaus, M., Slavchev, V., Stanev, P., Starović, A., Szeniczey, T., Talamo, S., Teschler-Nicola, M., Thevenet, C., Valchev, I., Valentin, F., Vasilyev, S., Veljanovska, F., Venelinova, S., Veselovskaya, E., Viola, B., Virag, C., Zaninović, J., Zäuner, S., Stockhammer,

- P.W., Catalano, G., Krauß, R., Caramelli, D., Zariņa, G., Gaydarska, B., Lillie, M., Nikitin, A.G., Potekhina, I., Papathanasiou, A., Borić, D., Bonsall, C., Krause, J., Pinhasi, R., Reich, D., 2018. The genomic history of southeastern Europe. *Nature* 555, 197–203. <https://doi.org/10.1038/nature25778>
- Mathieson, I., Lazaridis, I., Rohland, N., Mallick, S., Patterson, N., Roodenberg, S.A., Harney, E., Stewardson, K., Fernandes, D., Novak, M., Sirak, K., Gamba, C., Jones, E.R., Llamas, B., Dryomov, S., Pickrell, J., Arsuaga, J.L., de Castro, J.M.B., Carbonell, E., Gerritsen, F., Khokhlov, A., Kuznetsov, P., Lozano, M., Meller, H., Mochalov, O., Moiseyev, V., Guerra, M.A.R., Roodenberg, J., Vergès, J.M., Krause, J., Cooper, A., Alt, K.W., Brown, D., Anthony, D., Lalueza-Fox, C., Haak, W., Pinhasi, R., Reich, D., 2015a. Genome-wide patterns of selection in 230 ancient Eurasians. *Nature* 528, 499–503. <https://doi.org/10.1038/nature16152>
- Mathieson, I., Lazaridis, I., Rohland, N., Mallick, S., Patterson, N., Roodenberg, S.A., Harney, E., Stewardson, K., Fernandes, D., Novak, M., Sirak, K., Gamba, C., Jones, E.R., Llamas, B., Dryomov, S., Pickrell, J., Arsuaga, J.L., de Castro, J.M.B., Carbonell, E., Gerritsen, F., Khokhlov, A., Kuznetsov, P., Lozano, M., Meller, H., Mochalov, O., Moiseyev, V., Guerra, M.A.R., Roodenberg, J., Vergès, J.M., Krause, J., Cooper, A., Alt, K.W., Brown, D., Anthony, D., Lalueza-Fox, C., Haak, W., Pinhasi, R., Reich, D., 2015b. Genome-wide patterns of selection in 230 ancient Eurasians. *Nature* 528, 499–503. <https://doi.org/10.1038/nature16152>
- McDonald, C., Taylor, D., Linacre, A., 2024. PCR in Forensic Science: A Critical Review. *Genes* 15, 438. <https://doi.org/10.3390/genes15040438>
- Meyer, S., Weiss, G., von Haeseler, A., 1999. Pattern of Nucleotide Substitution and Rate Heterogeneity in the Hypervariable Regions I and II of Human mtDNA. *Genetics* 152, 1103–1110. <https://doi.org/10.1093/genetics/152.3.1103>
- Micó, R., 2011. El Argar and the Beginning of the Class Society in the Western Mediterranean. *Sozialarchäologische Perspekt. Ges. Wandel 5000–1500 V Chr Zwischen Atl. Kaukasus* 24, 381–414.
- Mohammed, A.J., Alarcón, M., Pino, D., 2018. Extreme temperature events on the Iberian Peninsula: Statistical trajectory analysis and synoptic patterns. *Int. J. Climatol.* 38, 5305–5322. <https://doi.org/10.1002/joc.5733>
- Molina-Balaguer, L., Escribá-Ruiz, P., Jiménez-Puerto, J., Bernabeu-Aubán, J., 2023. Sequence and context for the Cocina cave neolithic pottery: An approach from social networks analysis. *Quat. Int., The Last Hunter-Gatherers on the Iberian Peninsula: An Integrative Evolutionary and Multiscalar Approach from Cueva de la Cocina (Western Mediterranean)* 677–678, 65–77. <https://doi.org/10.1016/j.quaint.2023.05.006>
- Montgomery, J., Beaumont, J., Jay, M., Keefe, K., Gledhill, A.R., Cook, G.T., Dockrill, S.J., Melton, N.D., 2013. Strategic and sporadic marine consumption at the onset of the Neolithic: increasing temporal resolution in the isotope evidence. *Antiquity* 87, 1060–1072. <https://doi.org/10.1017/S0003598X00049863>
- Montgomery, J., Evans, J.A., Cooper, R.E., 2007. Resolving archaeological populations with Sr-isotope mixing models. *Appl. Geochem.* 22, 1502–1514. <https://doi.org/10.1016/j.apgeochem.2007.02.009>
- Montoya, C., Peresani, M., 2005. Nouveaux éléments de diachronie dans l'Épigravettien récent des Préalpes de la Vénétie [WWW Document]. ResearchGate. URL https://www.researchgate.net/publication/278777727_Nouveaux_elements_de_

- diachronie_dans_l'Epigravettien_recent_des_Prealpes_de_la_Venetie (accessed 10.24.25).
- Morell Rovira, B., Barceló Álvarez, J.A., Oms Arias, X.F., Remolins Zamora, G., Subirà De Galdàcano, M.E., Chambon, P., Gibaja Bao, J.F., 2018. Tracing the chronology of neolithic pit and stone box burials in North-eastern Iberia. *J. Archaeol. Sci. Rep.* 19, 491–504. <https://doi.org/10.1016/j.jasrep.2018.03.024>
- Neff, M., 2016. Genetic and Strontium Isotopic Profiling of Bell Beaker Culture at Convento do Carmo (Lisbon, Portugal). Durham University, Durham.
- Neustupný, E., 1982. Prehistoric migrations by infiltration. *Archeol. Rozhl.* 34, 278–293.
- NGS Workflow Steps | Illumina sequencing workflow [WWW Document], 2025. . Illumina. URL <https://emea.illumina.com/science/technology/next-generation-sequencing/beginners/ngs-workflow.html> (accessed 12.8.25).
- Olalde, I., Brace, S., Allentoft, M.E., Armit, I., Kristiansen, K., Booth, T., Rohland, N., Mallick, S., Szécsényi-Nagy, A., Mitnik, A., Altena, E., Lipson, M., Lazaridis, I., Harper, T.K., Patterson, N., Broomandkhoshbacht, N., Diekmann, Y., Faltyskova, Z., Fernandes, D., Ferry, M., Harney, E., de Knijff, P., Michel, M., Oppenheimer, J., Stewardson, K., Barclay, A., Alt, K.W., Liesau, C., Ríos, P., Blasco, C., Miguel, J.V., García, R.M., Fernández, A.A., Bánffy, E., Bernabò-Brea, M., Billoin, D., Bonsall, C., Bonsall, L., Allen, T., Büster, L., Carver, S., Navarro, L.C., Craig, O.E., Cook, G.T., Cunliffe, B., Denaire, A., Dinwiddy, K.E., Dodwell, N., Ernée, M., Evans, C., Kuchařík, M., Farré, J.F., Fowler, C., Gazenbeek, M., Pena, R.G., Haber-Uriarte, M., Haduch, E., Hey, G., Jowett, N., Knowles, T., Massy, K., Pfrengle, S., Lefranc, P., Lemercier, O., Lefebvre, A., Martínez, C.H., Olmo, V.G., Ramírez, A.B., Loma-Maurandi, J., Majó, T., McKinley, J.I., McSweeney, K., Mende, B.G., Modi, A., Kulcsár, G., Kiss, V., Czene, A., Patay, R., Endrődi, A., Köhler, K., Hajdu, T., Szeniczey, T., Dani, J., Bernert, Z., Hoole, M., Cheronet, O., Keating, D., Velemínský, P., Dobeš, M., Candilio, F., Brown, F., Fernández, R.F., Herrero-Corral, A.-M., Tusa, S., Carnieri, E., Lentini, L., Valenti, A., Zanini, A., Waddington, C., Delibes, G., Guerra-Doce, E., Neil, B., Brittain, M., Luke, M., Mortimer, R., Desideri, J., Besse, M., Brücken, G., Furmanek, M., Hałuszko, A., Mackiewicz, M., Rapiński, A., Leach, S., Soriano, I., Lillios, K.T., Cardoso, J.L., Pearson, M.P., Włodarczak, P., Price, T.D., Prieto, P., Rey, P.-J., Risch, R., Rojo Guerra, M.A., Schmitt, A., Serrallongue, J., Silva, A.M., Smrčka, V., Vergnaud, L., Zilhão, J., Caramelli, D., Higham, T., Thomas, M.G., Kennett, D.J., Fokkens, H., Heyd, V., Sheridan, A., Sjögren, K.-G., Stockhammer, P.W., Krause, J., Pinhasi, R., Haak, W., Barnes, I., Lalueza-Fox, C., Reich, D., 2018. The Beaker phenomenon and the genomic transformation of northwest Europe. *Nature* 555, 190–196. <https://doi.org/10.1038/nature25738>
- Olalde, I., Mallick, S., Patterson, N., Rohland, N., Villalba-Mouco, V., Silva, M., Dulias, K., Edwards, C.J., Gandini, F., Pala, M., Soares, P., Ferrando-Bernal, M., Adamski, N., Broomandkhoshbacht, N., Cheronet, O., Culleton, B.J., Fernandes, D., Lawson, A.M., Mah, M., Oppenheimer, J., Stewardson, K., Zhang, Z., Jiménez Arenas, J.M., Toro Moyano, I.J., Salazar-García, D.C., Castanyer, P., Santos, M., Tremoleda, J., Lozano, M., García Borja, P., Fernández-Eraso, J., Mujika-Alustiza, J.A., Barroso, C., Bermúdez, F.J., Viguera Mínguez, E., Burch, J., Coromina, N., Vivó, D., Cebrià, A., Fullola, J.M., García-Puchol, O., Morales, J.I., Oms, F.X., Majó, T., Vergès, J.M., Díaz-Carvajal, A., Ollich-Castanyer, I., López-Cachero, F.J.,

- Silva, A.M., Alonso-Fernández, C., Delibes De Castro, G., Jiménez Echevarría, J., Moreno-Márquez, A., Pascual Berlanga, G., Ramos-García, P., Ramos-Muñoz, J., Vijande Vila, E., Aguilera Arzo, G., Esparza Arroyo, Á., Lillios, K.T., Mack, J., Velasco-Vázquez, J., Waterman, A., Benítez De Lugo Enrich, L., Benito Sánchez, M., Agustí, B., Codina, F., De Prado, G., Estalrich, A., Fernández Flores, Á., Finlayson, C., Finlayson, G., Finlayson, S., Giles-Guzmán, F., Rosas, A., Barciela González, V., García Atiénzar, G., Hernández Pérez, M.S., Llanos, A., Carrión Marco, Y., Collado Beneyto, I., López-Serrano, D., Sanz Tormo, M., Valera, A.C., Blasco, C., Liesau, C., Ríos, P., Daura, J., De Pedro Michó, M.J., Díez-Castillo, A.A., Flores Fernández, R., Francès Farré, J., Garrido-Pena, R., Gonçalves, V.S., Guerra-Doce, E., Herrero-Corral, A.M., Juan-Cabanilles, J., López-Reyes, D., McClure, S.B., Merino Pérez, M., Oliver Foix, A., Sanz Borràs, M., Sousa, A.C., Vidal Encinas, J.M., Kennett, D.J., Richards, M.B., Werner Alt, K., Haak, W., Pinhasi, R., Lalueza-Fox, C., Reich, D., 2019. The genomic history of the Iberian Peninsula over the past 8000 years. *Science* 363, 1230–1234.
<https://doi.org/10.1126/science.aav4040>
- Olalde, I., Schroeder, H., Sandoval-Velasco, M., Vinner, L., Lobón, I., Ramirez, O., Civit, S., Borja, P.G., Salazar-García, D.C., Talamo, S., Fullola, J.M., Oms, F.X., Pedro, M., Martínez, P., Sanz, M., Daura, J., Zilhão, J., Marquès-Bonet, T., Gilbert, M.T.P., Lalueza-Fox, C., 2015. A common genetic origin for early farmers from mediterranean cardial and central european LBK cultures. *Mol. Biol. Evol.* 32, 3132–3142. <https://doi.org/10.1093/molbev/msv181>
- Översti, S., Palo, J.U., 2022. Variation in the Substitution Rates among the Human Mitochondrial Haplogroup U Sublineages. *Genome Biol. Evol.* 14.
<https://doi.org/10.1093/gbe/evac097>
- Pääbo, S., 1985. Molecular cloning of Ancient Egyptian mummy DNA. *Nature* 314, 644–645. <https://doi.org/10.1038/314644a0>
- Pääbo, S., Poinar, H., Serre, D., Jaenicke-Després, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L., Hofreiter, M., 2004. Genetic Analyses from Ancient DNA. *Annu. Rev. Genet.* 38, 645–679.
<https://doi.org/10.1146/annurev.genet.37.110801.143214>
- Pala, M., Olivieri, A., Achilli, A., Accetturo, M., Metspalu, E., Reidla, M., Tamm, E., Karmin, M., Reisberg, T., Kashani, B.H., Perego, U.A., Carossa, V., Gandini, F., Pereira, J.B., Soares, P., Angerhofer, N., Rychkov, S., Al-Zahery, N., Carelli, V., Sanati, M.H., Houshmand, M., Hatina, J., Macaulay, V., Pereira, L., Woodward, S.R., Davies, W., Gamble, C., Baird, D., Semino, O., Villems, R., Torroni, A., Richards, M.B., 2012. Mitochondrial DNA Signals of Late Glacial Recolonization of Europe from Near Eastern Refugia. *Am. J. Hum. Genet.* 90, 915–924.
<https://doi.org/10.1016/j.ajhg.2012.04.003>
- Palencia-Madrid, L., de Pancorbo, M.M., 2015. Next generation sequencing as a suitable analysis for authentication of degraded and ancient DNA. *Forensic Sci. Int. Genet. Suppl. Ser.* 5, e338–e340. <https://doi.org/10.1016/j.fsigss.2015.09.134>
- Papac, L., Ernée, M., Dobeš, M., Langová, M., Rohrlach, A.B., Aron, F., Neumann, G.U., Spyrou, M.A., Rohland, N., Velemínský, P., Kuna, M., Brzobohatá, H., Culleton, B., Daněček, D., Danielisová, A., Dobisíková, M., Hložek, J., Kennett, D.J., Klementová, J., Kostka, M., Křišťuf, P., Kuchařík, M., Hlavová, J.K., Limburský, P., Malyková, D., Mattiello, L., Pecinová, M., Petříšáková, K., Průchová, E.,

- Stránská, P., Smejtek, L., Špaček, J., Šumberová, R., Švejcar, O., Trefný, M., Vávra, M., Kolář, J., Heyd, V., Krause, J., Pinhasi, R., Reich, D., Schiffels, S., Haak, W., 2021. Dynamic changes in genomic and social structures in third millennium BCE central Europe. *Sci. Adv.* <https://doi.org/10.1126/sciadv.abi6941>
- Parikh, S.R., Campbell, H., Bettinger, J.A., Harrison, L.H., Marshall, H.S., Martinon-Torres, F., Safadi, M.A., Shao, Z., Zhu, B., von Gottberg, A., Borrow, R., Ramsay, M.E., Ladhani, S.N., 2020. The everchanging epidemiology of meningococcal disease worldwide and the potential for prevention through vaccination. *J. Infect.* 81, 483–498. <https://doi.org/10.1016/j.jinf.2020.05.079>
- Paschou, P., Drineas, P., Yannaki, E., Razou, A., Kanaki, K., Tsetsos, F., Padmanabhuni, S.S., Michalodimitrakakis, M., Renda, M.C., Pavlovic, S., Anagnostopoulos, A., Stamatoyannopoulos, J.A., Kidd, K.K., Stamatoyannopoulos, G., 2014. Maritime route of colonization of Europe. *Proc. Natl. Acad. Sci.* 111, 9211–9216. <https://doi.org/10.1073/pnas.1320811111>
- Patterson, N., Isakov, M., Booth, T., Büster, L., Fischer, C.-E., Olalde, I., Ringbauer, H., Akbari, A., Cheronet, O., Bleasdale, M., Adamski, N., Altena, E., Bernardos, R., Brace, S., Broomandkhoshbacht, N., Callan, K., Candilio, F., Culleton, B., Curtis, E., Demetz, L., Carlson, K.S.D., Edwards, C.J., Fernandes, D.M., Foody, M.G.B., Freilich, S., Goodchild, H., Kearns, A., Lawson, A.M., Lazaridis, I., Mah, M., Mallick, S., Mandl, K., Micco, A., Michel, M., Morante, G.B., Oppenheimer, J., Özdoğan, K.T., Qiu, L., Schattke, C., Stewardson, K., Workman, J.N., Zalzal, F., Zhang, Z., Agustí, B., Allen, T., Almássy, K., Amkreutz, L., Ash, A., Baillif-Ducros, C., Barclay, A., Bartosiewicz, L., Baxter, K., Bernert, Z., Blažek, J., Bodružić, M., Boissinot, P., Bonsall, C., Bradley, P., Brittain, M., Brookes, A., Brown, F., Brown, L., Brunning, R., Budd, C., Burmaz, J., Canet, S., Carnicero-Cáceres, S., Čaušević-Bully, M., Chamberlain, A., Chauvin, S., Clough, S., Čondić, N., Coppa, A., Craig, O., Črešnar, M., Cummings, V., Czifra, S., Danielisová, A., Daniels, R., Davies, A., de Jersey, P., Deacon, J., Deminger, C., Ditchfield, P.W., Dizdar, M., Dobeš, M., Dobisíková, M., Domboróczki, L., Drinkall, G., Đukić, A., Ernée, M., Evans, C., Evans, J., Fernández-Götz, M., Filipović, S., Fitzpatrick, A., Fokkens, H., Fowler, C., Fox, A., Gallina, Z., Gamble, M., González Morales, M.R., González-Rabanal, B., Green, A., Gyenesei, K., Habermehl, D., Hajdu, T., Hamilton, D., Harris, J., Hayden, C., Hendriks, J., Hernu, B., Hey, G., Horňák, M., Ilon, G., Istvánovits, E., Jones, A.M., Kavur, M.B., Kazek, K., Kenyon, R.A., Khreisheh, A., Kiss, V., Kleijne, J., Knight, M., Kootker, L.M., Kovács, P.F., Kozubová, A., Kulcsár, G., Kulcsár, V., Le Pennec, C., Legge, M., Leivers, M., Loe, L., López-Costas, O., Lord, T., Los, D., Lyall, J., Marín-Arroyo, A.B., Mason, P., Matošević, D., Maxted, A., McIntyre, L., McKinley, J., McSweeney, K., Meijlink, B., Mende, B.G., Mendušić, M., Metlička, M., Meyer, S., Mihovilić, K., Milasinovic, L., Minnitt, S., Moore, J., Morley, G., Mullan, G., Musilová, M., Neil, B., Nicholls, R., Novak, M., Pala, M., Papworth, M., Paresys, C., Patten, R., Perkić, D., Pesti, K., Petit, A., Petrišćáková, K., Pichon, C., Pickard, C., Pilling, Z., Price, T.D., Radović, S., Redfern, R., Resutík, B., Rhodes, D.T., Richards, M.B., Roberts, A., Roefstra, J., Sankot, P., Šefčáková, A., Sheridan, A., Skae, S., Šmolíková, M., Somogyi, K., Somogyvári, Á., Stephens, M., Szabó, G., Szécsényi-Nagy, A., Szeniczey, T., Tabor, J., Tankó, K., Maria, C.T., Terry, R., Teržan, B., Teschler-Nicola, M., Torres-Martínez, J.F., Trapp, J., Turle, R., Ujvári, F., van der Heiden, M., Veleminsky, P., Veselka, B.,

- Vytlačil, Z., Waddington, C., Ware, P., Wilkinson, P., Wilson, L., Wiseman, R., Young, E., Zaninović, J., Žitňan, A., Lalueza-Fox, C., de Knijff, P., Barnes, I., Halkon, P., Thomas, M.G., Kennett, D.J., Cunliffe, B., Lillie, M., Rohland, N., Pinhasi, R., Armit, I., Reich, D., 2022. Large-scale migration into Britain during the Middle to Late Bronze Age. *Nature* 601, 588–594.
<https://doi.org/10.1038/s41586-021-04287-4>
- Penske, S., Rohrlach, A.B., Childebayeva, A., Gneccchi-Ruscione, G., Schmid, C., Spyrou, M.A., Neumann, G.U., Atanassova, N., Beutler, K., Boyadzhiev, K., Boyadzhiev, Y., Bruyako, I., Chohadzhiev, A., Govedarica, B., Karaucak, M., Krauss, R., Leppek, M., Manzura, I., Privat, K., Ross, S., Slavchev, V., Sobotkova, A., Toderaş, M., Valchev, T., Ringbauer, H., Stockhammer, P.W., Hansen, S., Krause, J., Haak, W., 2023. Early contact between late farming and pastoralist societies in southeastern Europe. *Nature* 620, 358–365. <https://doi.org/10.1038/s41586-023-06334-8>
- Petchey, F., 2019. Datação absoluta e análise paleodietética, in: *Hipogeu Campaniforme Do Convento Do Carmo (Torres Novas)*. Município de Torres Novas, Portugal, pp. 94–98.
- Pinhasi, R., Fernandes, D., Sirak, K., Novak, M., Connell, S., Alpaslan-Roodenberg, S., Gerritsen, F., Moiseyev, V., Gromov, A., Raczky, P., Anders, A., Pietrusewsky, M., Rollefson, G., Jovanovic, M., Trinhhoang, H., Bar-Oz, G., Oxenham, M., Matsumura, H., Hofreiter, M., 2015. Optimal Ancient DNA Yields from the Inner Ear Part of the Human Petrous Bone. *PLOS ONE* 10, e0129102.
<https://doi.org/10.1371/journal.pone.0129102>
- Pochon, Z., Bergfeldt, N., Kirdök, E., Vicente, M., Naidoo, T., van der Valk, T., Altınışık, N.E., Krzewińska, M., Dalén, L., Götherström, A., Mirabello, C., Unneberg, P., Oskolkov, N., 2023. aMeta: an accurate and memory-efficient ancient metagenomic profiling workflow. *Genome Biol.* 24, 242.
<https://doi.org/10.1186/s13059-023-03083-9>
- Poinar, H.N., Cano, R.J., Poinar, G.O., 1993. DNA from an extinct plant. *Nature* 363, 677–677. <https://doi.org/10.1038/363677a0>
- Poinar, H.N., Höss, M., Bada, J.L., Pääbo, S., 1996. Amino Acid Racemization and the Preservation of Ancient DNA. *Science* 272, 864–866.
<https://doi.org/10.1126/science.272.5263.864>
- Posth, C., Renaud, G., Mitnik, A., Drucker, D.G., Rougier, H., Cupillard, C., Valentin, F., Thevenet, C., Furtwängler, A., Wißing, C., Francken, M., Malina, M., Bolus, M., Lari, M., Gigli, E., Capecchi, G., Crevecoeur, I., Beauval, C., Flas, D., Germonpré, M., van der Plicht, J., Cottiaux, R., Gély, B., Ronchitelli, A., Wehrberger, K., Grigorescu, D., Svoboda, J., Semal, P., Caramelli, D., Bocherens, H., Harvati, K., Conard, N.J., Haak, W., Powell, A., Krause, J., 2016. Pleistocene Mitochondrial Genomes Suggest a Single Major Dispersal of Non-Africans and a Late Glacial Population Turnover in Europe. *Curr. Biol.* 26, 827–833.
<https://doi.org/10.1016/j.cub.2016.01.037>
- Posth, C., Yu, H., Ghalichi, A., Rougier, H., Crevecoeur, I., Huang, Y., Ringbauer, H., Rohrlach, A.B., Nägele, K., Villalba-Mouco, V., Radzeviciute, R., Ferraz, T., Stoessel, A., Tukhbatova, R., Drucker, D.G., Lari, M., Modi, A., Vai, S., Saupe, T., Scheib, C.L., Catalano, G., Pagani, L., Talamo, S., Fewlass, H., Klaric, L., Morala, A., Rué, M., Madelaine, S., Crépin, L., Caverne, J.-B., Bocaege, E., Ricci, S.,

- Boschin, F., Bayle, P., Maureille, B., Le Brun-Ricalens, F., Bordes, J.-G., Oxilia, G., Bortolini, E., Bignon-Lau, O., Debout, G., Orliac, M., Zazzo, A., Sparacello, V., Starnini, E., Sineo, L., van der Plicht, J., Pecqueur, L., Merceron, G., Garcia, G., Leuvrey, J.-M., Garcia, C.B., Gómez-Olivencia, A., Połtowicz-Bobak, M., Bobak, D., Le Luyer, M., Storm, P., Hoffmann, C., Kabaciński, J., Filimonova, T., Shnaider, S., Berezina, N., González-Rabanal, B., González Morales, M.R., Marín-Arroyo, A.B., López, B., Alonso-Llamazares, C., Ronchitelli, A., Polet, C., Jadin, I., Cauwe, N., Soler, J., Coromina, N., Rufí, I., Cottiaux, R., Clark, G., Straus, L.G., Julien, M.-A., Renhart, S., Talaa, D., Benazzi, S., Romandini, M., Amkreutz, L., Bocherens, H., Wißing, C., Villotte, S., de Pablo, J.F.-L., Gómez-Puche, M., Esquembre-Bebia, M.A., Bodu, P., Smits, L., Souffi, B., Jankauskas, R., Kozakaitė, J., Cupillard, C., Benthien, H., Wehrberger, K., Schmitz, R.W., Feine, S.C., Schüler, T., Thevenet, C., Grigorescu, D., Lüth, F., Kotula, A., Piezonka, H., Schopper, F., Svoboda, J., Sázelová, S., Chizhevsky, A., Khokhlov, A., Conard, N.J., Valentin, F., Harvati, K., Semal, P., Jungklaus, B., Suvorov, A., Schulting, R., Moiseyev, V., Mannermaa, K., Buzhilova, A., Terberger, T., Caramelli, D., Altena, E., Haak, W., Krause, J., 2023. Palaeogenomics of Upper Palaeolithic to Neolithic European hunter-gatherers. *Nature* 615, 117–126. <https://doi.org/10.1038/s41586-023-05726-0>
- Rasmussen, M., Li, Y., Lindgreen, S., Pedersen, J.S., Albrechtsen, A., Moltke, I., Metspalu, M., Metspalu, E., Kivisild, T., Gupta, R., Bertalan, M., Nielsen, K., Gilbert, M.T.P., Wang, Y., Raghavan, M., Campos, P.F., Kamp, H.M., Wilson, A.S., Gledhill, A., Tridico, S., Bunce, M., Lorenzen, E.D., Binladen, J., Guo, X., Zhao, J., Zhang, X., Zhang, H., Li, Z., Chen, M., Orlando, L., Kristiansen, K., Bak, M., Tommerup, N., Bendixen, C., Pierre, T.L., Grønnow, B., Meldgaard, M., Andreasen, C., Fedorova, S.A., Osipova, L.P., Higham, T.F.G., Ramsey, C.B., Hansen, T. v O., Nielsen, F.C., Crawford, M.H., Brunak, S., Sicheritz-Pontén, T., Villemis, R., Nielsen, R., Krogh, A., Wang, J., Willerslev, E., 2010. Ancient human genome sequence of an extinct Palaeo-Eskimo. *Nature* 463, 757–762. <https://doi.org/10.1038/nature08835>
- Renfrew, C., 1989. *Archaeology and language: the puzzle of Indo-European origins*, Penguin archaeology. Penguin Books, London.
- Richards, M., Fernández-Domínguez, E., n.d. Chasing the “steppe ancestry”: a reassessment of human mobility and interaction during the 3rd millennium BCE in Europe using uniparental markers.
- Richards, M.P., Schulting, R.J., 2006. Touch not the fish: the Mesolithic-Neolithic change of diet and its significance. *Antiquity* 80, 444–456. <https://doi.org/10.1017/S0003598X00093765>
- Richards, M.P., Schulting, R.J., Hedges, R.E.M., 2003. Sharp shift in diet at onset of Neolithic. *Nature* 425, 366–366. <https://doi.org/10.1038/425366a>
- Rivollat, M., Jeong, C., Schiffels, S., Küçükkalipçı, İ., Pemonge, M.-H., Rohrlach, A.B., Alt, K.W., Binder, D., Friederich, S., Ghesquière, E., Gronenborn, D., Laporte, L., Lefranc, P., Meller, H., Réveillas, H., Rosenstock, E., Rottier, S., Scarre, C., Soler, L., Wahl, J., Krause, J., Deguilloux, M.-F., Haak, W., 2020. Ancient genome-wide DNA from France highlights the complexity of interactions between Mesolithic hunter-gatherers and Neolithic farmers. *Sci. Adv.* <https://doi.org/10.1126/sciadv.aaz5344>

- Rivollat, M., Mendisco, F., Pemonge, M.-H., Safi, A., Saint-Marc, D., Brémond, A., Couture-Veschambre, C., Rottier, S., Deguilloux, M.-F., 2015. When the Waves of European Neolithization Met: First Paleogenetic Evidence from Early Farmers in the Southern Paris Basin. *PLOS ONE* 10, e0125521. <https://doi.org/10.1371/journal.pone.0125521>
- Rivollat, M., Rohrlach, A.B., Ringbauer, H., Childebayeva, A., Mendisco, F., Barquera, R., Szolek, A., Le Roy, M., Colleran, H., Tuke, J., Aron, F., Pemonge, M.-H., Späth, E., Télouk, P., Rey, L., Goude, G., Balter, V., Krause, J., Rottier, S., Deguilloux, M.-F., Haak, W., 2023. Extensive pedigrees reveal the social organization of a Neolithic community. *Nature* 620, 600–606. <https://doi.org/10.1038/s41586-023-06350-8>
- Rizzi, E., Lari, M., Gigli, E., De Bellis, G., Caramelli, D., 2012. Ancient DNA studies: new perspectives on old samples. *Genet. Sel. Evol.* 44, 21. <https://doi.org/10.1186/1297-9686-44-21>
- Robinson, C., 2018. Human Mitochondrial DNA from 5th-3rd Millenia BCE: An Analysis of Iberian Populations. LJM U, Liverpool.
- Roca-Rada, X., Cuesta-Aguirre, D.R., Vinueza-Espinosa, D.C., Davidson, R., Ravishankar, S., Taufik, L., Armentano, N., Esteve, X., Souilmi, Y., Teixeira, J.C., Malgosa, A., Llamas, B., Santos, C., 2025. Genetic transitions in the Neolithic and Bronze Age at Mas d'en Boixos (Catalonia, Spain). *iScience* 28, 112871. <https://doi.org/10.1016/j.isci.2025.112871>
- Roca-Rada, X., Davidson, R., Williams, M.P., Ravishankar, S., Collen, E., Haarkötter, C., Taufik, L., Carvalho, A.F., Villalba-Mouco, V., Cuesta-Aguirre, D.R., Tente, C., Calleja, Á.M.M., MacRoberts, R.A., Melo, L., Purnomo, G.A., Souilmi, Y., Tobler, R., Cunha, E., Tereso, S., Matos, V.M.J., Fernandes, T.M., Mauer, A.-F., Silva, A.M., Carvalho, P.C., Llamas, B., Teixeira, J.C., 2024. The genetic history of Portugal over the past 5,000 years. <https://doi.org/10.1101/2024.09.12.612544>
- Rohland, N., Glocke, I., Aximu-Petri, A., Meyer, M., 2018. Extraction of highly degraded DNA from ancient bones, teeth and sediments for high-throughput sequencing. *Nat. Protoc.* 13, 2447–2461. <https://doi.org/10.1038/s41596-018-0050-5>
- Rohland, N., Harney, E., Mallick, S., Nordenfelt, S., Reich, D., 2015. Partial uracil–DNA–glycosylase treatment for screening of ancient DNA. *Philos. Trans. R. Soc. B Biol. Sci.* 370, 20130624. <https://doi.org/10.1098/rstb.2013.0624>
- Rohland, N., Mallick, S., Mah, M., Maier, R., Patterson, N., Reich, D., 2022. Three assays for in-solution enrichment of ancient human DNA at more than a million SNPs. *Genome Res.* 32, 2068–2078. <https://doi.org/10.1101/gr.276728.122>
- Royo, M., Garrido-Pena, R., 2012. From pits to megaliths: Neolithic burials in the interior of iberia, in: *Funerary Practices in the Iberian Peninsula: From the Mesolithic to the Chalcolithic.*, International Series. British Archaeological Reports, pp. 21–28.
- Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim, N., 1985. Enzymatic Amplification of β -Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sick Cell Anemia. *Science* 230, 1350–1354.
- Salazar-García, D.C., Aura, J.E., Olària, C.R., Talamo, S., Morales, J.V., Richards, M.P., 2014. Isotope evidence for the use of marine resources in the Eastern Iberian Mesolithic. *J. Archaeol. Sci.* 42, 231–240. <https://doi.org/10.1016/j.jas.2013.11.006>
- Salazar-García, D.C., Pérez-Ripoll, M., García-Borja, P., Jordá Pardo, J.F., Aura Tortosa, J.E., 2017. A Terrestrial Diet Close to the Coast: A Case Study from the Neolithic

- Levels of Nerja Cave (Málaga, Spain), in: García-Puchol, O., Salazar-García, D.C. (Eds.), *Times of Neolithic Transition along the Western Mediterranean*. Springer International Publishing, Cham, pp. 281–307. https://doi.org/10.1007/978-3-319-52939-4_11
- Sampietro, M. I., Lao, O., Caramelli, D., Lari, M., Pou, R., Martí, M., Bertranpetit, J., Lalueza-Fox, C., 2007. Palaeogenetic evidence supports a dual model of Neolithic spreading into Europe. *Proc. R. Soc. B Biol. Sci.* 274, 2161–2167. <https://doi.org/10.1098/rspb.2007.0465>
- Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74, 5463–5467. <https://doi.org/10.1073/pnas.74.12.5463>
- Scarre, C., 2019. Megalithic tombs in Western Iberia, in: *Megalithic Tombs in Western Iberia*. Oxbow Books. <https://doi.org/10.2307/j.ctv138wsbz.10>
- Schönherr, S., Weissensteiner, H., Kronenberg, F., Forer, L., 2023. Haplogrep 3 - an interactive haplogroup classification and analysis platform. *Nucleic Acids Res.* 51, W263–W268. <https://doi.org/10.1093/nar/gkad284>
- Schuhmacher, T.X., Cardoso, J.L., Banerjee, A., 2009. Sourcing African ivory in Chalcolithic Portugal. *Antiquity* 83, 983–997. <https://doi.org/10.1017/S0003598X00099294>
- Scorrano, G., Nielsen, S.H., Vetro, D.L., Sawafuji, R., Mackie, M., Margaryan, A., Fotakis, A.K., Martínez-Labarga, C., Fabbri, P.F., Allentoft, M.E., Carra, M., Martini, F., Rickards, O., Olsen, J.V., Pedersen, M.W., Cappellini, E., Sikora, M., 2022. Genomic ancestry, diet and microbiomes of Upper Palaeolithic hunter-gatherers from San Teodoro cave. *Commun. Biol.* 5, 1262. <https://doi.org/10.1038/s42003-022-04190-2>
- Sczepanski, J.T., Wong, R.S., McKnight, J.N., Bowman, G.D., Greenberg, M.M., Dervan, P.B., 2010. Rapid DNA—protein cross-linking and strand scission by an abasic site in a nucleosome core particle. *Proc. Natl. Acad. Sci. U. S. A.* 107, 22475–22480.
- Simões, L.G., Günther, T., Martínez-Sánchez, R.M., Vera-Rodríguez, J.C., Iriarte, E., Rodríguez-Varela, R., Bokbot, Y., Valdiosera, C., Jakobsson, M., 2023. Northwest African Neolithic initiated by migrants from Iberia and Levant. *Nature* 618, 550–556. <https://doi.org/10.1038/s41586-023-06166-6>
- Simões, L.G., Peyroteo-Stjerna, R., Marchand, G., Bernhardsson, C., Vialet, A., Chetty, D., Alaçamlı, E., Edlund, H., Bouquin, D., Dina, C., Garmond, N., Günther, T., Jakobsson, M., 2024. Genomic ancestry and social dynamics of the last hunter-gatherers of Atlantic France. *Proc. Natl. Acad. Sci.* 121, e2310545121. <https://doi.org/10.1073/pnas.2310545121>
- Sirak, K., Fernandes, D., Cheronet, O., Harney, E., Mah, M., Mallick, S., Rohland, N., Adamski, N., Broomandkhoshbacht, N., Callan, K., Candilio, F., Lawson, A.M., Mandl, K., Oppenheimer, J., Stewardson, K., Zalzal, F., Anders, A., Bartík, J., Coppa, A., Dashtseveg, T., Évinger, S., Farkaš, Z., Hajdu, T., Bayarsaikhan, J., McIntyre, L., Moiseyev, V., Okumura, M., Pap, I., Pietrusewsky, M., Raczky, P., Šefčáková, A., Soficaru, A., Szeniczey, T., Szőke, B.M., Gerven, D.V., Vasilyev, S., Bell, L., Reich, D., Pinhasi, R., 2020. Human auditory ossicles as an alternative optimal source of ancient DNA. *Genome Res.* 30, 427–436. <https://doi.org/10.1101/gr.260141.119>

- Skoglund, P., Thompson, J.C., Prendergast, M.E., Mitnik, A., Sirak, K., Hajdinjak, M., Salie, T., Rohland, N., Mallick, S., Peltzer, A., Heinze, A., Olalde, I., Ferry, M., Harney, E., Michel, M., Stewardson, K., Cerezo-Román, J.I., Chiumia, C., Crowther, A., Goman-Chindebvu, E., Gidna, A.O., Grillo, K.M., Helenius, I.T., Hellenthal, G., Helm, R., Horton, M., López, S., Mabulla, A.Z.P., Parkington, J., Shipton, C., Thomas, M.G., Tibesasa, R., Welling, M., Hayes, V.M., Kennett, D.J., Ramesar, R., Meyer, M., Pääbo, S., Patterson, N., Morris, A.G., Boivin, N., Pinhasi, R., Krause, J., Reich, D., 2017. Reconstructing Prehistoric African Population Structure. *Cell* 171, 59-71.e21. <https://doi.org/10.1016/j.cell.2017.08.049>
- Soltis, P.S., Soltis, D.E., Smiley, C.J., 1992. An rbcL sequence from a Miocene *Taxodium* (bald cypress). *Proc. Natl. Acad. Sci.* 89, 449–451. <https://doi.org/10.1073/pnas.89.1.449>
- Soriano, I., Herrero-Corral, A.M., Garrido-Pena, R., Majó, T., 2021. Sex/gender system and social hierarchization in Bell Beaker burials from Iberia. *J. Anthropol. Archaeol.* 64, 101335. <https://doi.org/10.1016/j.jaa.2021.101335>
- Sosa, C., Vispe, E., Núñez, C., Baeta, M., Casalod, Y., Bolea, M., Hedges, R. e. m., Martinez-Jarreta, B., 2013. Association between ancient bone preservation and dna yield: A multidisciplinary approach. *Am. J. Phys. Anthropol.* 151, 102–109. <https://doi.org/10.1002/ajpa.22262>
- Sousa, A.C., Gonçalves, V.S., 2022. Changements et permanences des rites funéraires dans les anciennes sociétés paysannes du centre et du sud du Portugal.
- Spilker, T., Darrah, R., LiPuma, J.J., 2016. Complete Genome Sequences of *Bordetella flabilis*, *Bordetella bronchialis*, and “*Bordetella pseudohinzii*.” *Genome Announc.* 4, 10.1128/genomea.01132-16. <https://doi.org/10.1128/genomea.01132-16>
- Stewart, J.R., Stringer, C.B., 2012. Human Evolution Out of Africa: The Role of Refugia and Climate Change. *Science* 335, 1317–1321. <https://doi.org/10.1126/science.1215627>
- Szécsényi-Nagy, A., Brandt, G., Haak, W., Keerl, V., Jakucs, J., Möller-Rieker, S., Köhler, K., Mende, B.G., Oross, K., Marton, T., Osztás, A., Kiss, V., Fecher, M., Pálfi, G., Molnár, E., Sebők, K., Czene, A., Paluch, T., Šlaus, M., Novak, M., Pećina-Šlaus, N., Ósz, B., Voicsek, V., Somogyi, K., Tóth, G., Kromer, B., Bánffy, E., Alt, K.W., 2015. Tracing the genetic origin of Europe’s first farmers reveals insights into their social organization. *Proc. R. Soc. B Biol. Sci.* 282, 20150339. <https://doi.org/10.1098/rspb.2015.0339>
- Szécsényi-Nagy, A., Roth, C., Brandt, G., Rihuete-Herrada, C., Tejedor-Rodríguez, C., Held, P., García-Martínez-de-Lagrán, Í., Arcusa Magallón, H., Zesch, S., Knipper, C., Bánffy, E., Friederich, S., Meller, H., Bueno Ramírez, P., Barroso Bermejo, R., de Balbín Behrmann, R., Herrero-Corral, A.M., Flores Fernández, R., Alonso Fernández, C., Jiménez Echevarria, J., Rindlisbacher, L., Oliart, C., Fregeiro, M.-I., Soriano, I., Vicente, O., Micó, R., Lull, V., Soler Díaz, J., López Padilla, J.A., Roca de Togores Muñoz, C., Hernández Pérez, M.S., Jover Maestre, F.J., Lomba Maurandi, J., Avilés Fernández, A., Lillios, K.T., Silva, A.M., Magalhães Ramalho, M., Oosterbeek, L.M., Cunha, C., Waterman, A.J., Roig Buxó, J., Martínez, A., Ponce Martínez, J., Hunt Ortiz, M., Mejías-García, J.C., Pecero Espín, J.C., Cruz-Auñón Briones, R., Tomé, T., Carmona Ballester, E., Cardoso, J.L., Araújo, A.C., Liesau von Lettow-Vorbeck, C., Blasco Bosqued, C., Ríos Mendoza, P., Pujante, A., Royo-Guillén, J.I., Esquembre Beviá, M.A., Dos Santos Goncalves, V.M.,

- Parreira, R., Morán Hernández, E., Méndez Izquierdo, E., Vega y Miguel, J., Menduiña García, R., Martínez Calvo, V., López Jiménez, O., Krause, J., Pichler, S.L., Garrido-Pena, R., Kunst, M., Risch, R., Rojo-Guerra, M.A., Haak, W., Alt, K.W., 2017. The maternal genetic make-up of the Iberian Peninsula between the Neolithic and the Early Bronze Age. *Sci. Rep.* 7, 15644.
<https://doi.org/10.1038/s41598-017-15480-9>
- Tang, T.H.C., Wong, S.S.Y., Lai, C.K.C., Poon, R.W.S., Chan, H.S.Y., Wu, T.C., Cheung, Y.-F., Poon, T.-L., Tsang, Y.-P., Tang, W.-L., Wu, A.K.L., 2017. Molecular Identification of *Spirometra erinaceieuropaei* Tapeworm in Cases of Human Sparganosis, Hong Kong. *Emerg. Infect. Dis.* 23, 665–668.
<https://doi.org/10.3201/eid2304.160791>
- Tavares da Silva, C., Soares, J., 2000. Protomegalitismo no Sul de Portugal: inauguração das paisagens megalíticas, in: *Muitas antas, pouca gente?: Actas do I Colóquio Internacional sobre Megalitismo*, 2000, ISBN 972-97903-8-8, págs. 117-134. Presented at the *Muitas antas, pouca gente?: Actas do I Colóquio Internacional sobre Megalitismo*, Instituto Português de Arqueologia, pp. 117–134.
- Taylor, R.W., Turnbull, D.M., 2005. Mitochondrial DNA mutations in human disease. *Nat. Rev. Genet.* 6, 389–402. <https://doi.org/10.1038/nrg1606>
- Tejedor-Rodríguez, C., Moreno-García, M., Tornero, C., Hoffmann, A., Lagrán, Í.G.-M. de, Arcusa-Magallón, H., Garrido-Pena, R., Royo-Guillén, J.I., Díaz-Navarro, S., Peña-Chocarro, L., Alt, K.W., Rojo-Guerra, M., 2021. Investigating Neolithic caprine husbandry in the Central Pyrenees: Insights from a multi-proxy study at Els Trocs cave (Bisaurri, Spain). *PLOS ONE* 16, e0244139.
<https://doi.org/10.1371/journal.pone.0244139>
- Torroni, A., Achilli, A., Macaulay, V., Richards, M., Bandelt, H.-J., 2006. Harvesting the fruit of the human mtDNA tree. *Trends Genet.* 22, 339–345.
<https://doi.org/10.1016/j.tig.2006.04.001>
- Valdiosera, C., Günther, T., Vera-Rodríguez, J.C., Ureña, I., Iriarte, E., Rodríguez-Varela, R., Simões, L.G., Martínez-Sánchez, R.M., Svensson, E.M., Malmström, H., Rodríguez, L., de Castro, J.-M.B., Carbonell, E., Alday, A., Vera, J.A.H., Götherström, A., Carretero, J.-M., Arsuaga, J.L., Smith, C.I., Jakobsson, M., 2018. Four millennia of Iberian biomolecular prehistory illustrate the impact of prehistoric migrations at the far end of Eurasia. *Proc. Natl. Acad. Sci. U. S. A.* 115, 3428–3433.
- Valente, M.J., 2019. Meso e macrovertebrados e elementos de adorno em concha, in: *Hipogeu Campaniforme Do Convento Do Carmo (Torres Novas)*. Município de Torres Novas, Portugal, pp. 59–64.
- Valente, M.J., Dias, R., Pereira-Gonçalves, R., Catarino, D., Carvalho, A.F., 2017. Casais da Mureta (Alcanena, Portugal), a cave-necropolis from the Middle/Late Neolithic: first data on its faunal materials.
- Valério, P., Soares, J., Alves, L.C., Silva, R.J.C., Fátima Araújo, M., Tavares da Silva, C., 2023. Chalcolithic copper production and use in the western end of the Iberian Peninsula: The testimony of Castro de Chibanes (Portugal). *J. Archaeol. Sci. Rep.* 48, 103868. <https://doi.org/10.1016/j.jasrep.2023.103868>
- Van Oven, M., Kayser, M., 2009. Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. *Hum. Mutat.* 30, E386–E394.
<https://doi.org/10.1002/humu.20921>

- Vandamme, P.A., Peeters, C., Cnockaert, M., Inganäs, E., Falsen, E., Moore, E.R.B., Nunes, O.C., Manaia, C.M., Spilker, T., LiPuma, J.J., 2015. *Bordetella bronchialis* sp. nov., *Bordetella flabilis* sp. nov. and *Bordetella sputigena* sp. nov., isolated from human respiratory specimens, and reclassification of *Achromobacter sediminum* Zhang et al. 2014 as *Verticia sediminum* gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* 65, 3674–3682. <https://doi.org/10.1099/ijsem.0.000473>
- Vijande-Vila, E., Díaz-Zorita Bonilla, M., Morell-Rovira, B., Olalde, Í., Sánchez-Barba Muñoz, L.P., Domínguez-Bella, S., Emslie, S.D., Becerra-Martín, S., Rubio-Salvador, Á., Fernández-Sánchez, D.S., Cantillo-Duarte, J.J., Alemán-Aguilera, I., Moreno-Márquez, A., Molina-Piernas, E., Ramírez-Amador, J.L., Gómez-Sánchez, M.L., Botella-López, M.C., Rodríguez-Vidal, J., Ramos-Muñoz, J., 2022. At the beginnings of the funerary Megalithism in Iberia at Campo de Hockey necropolis. *Sci. Rep.* 12, 9431. <https://doi.org/10.1038/s41598-022-13014-6>
- Villalba-Mouco, V., Oliart, C., Rihuete-Herrada, C., Childebayeva, A., Rohrlach, A.B., Fregeiro, M.I., Celdrán Beltrán, E., Velasco-Felipe, C., Aron, F., Himmel, M., Freund, C., Alt, K.W., Salazar-García, D.C., García Atiénzar, G., de Miguel Ibáñez, Ma.P., Hernández Pérez, M.S., Barciela, V., Romero, A., Ponce, J., Martínez, A., Lomba, J., Soler, J., Martínez, A.P., Avilés Fernández, A., Haber-Uriarte, M., Roca de Togores Muñoz, C., Olalde, I., Lalueza-Fox, C., Reich, D., Krause, J., García Sanjuán, L., Lull, V., Micó, R., Risch, R., Haak, W., 2021. Genomic transformation and social organization during the Copper Age–Bronze Age transition in southern Iberia. *Sci. Adv.* 7, eabi7038. <https://doi.org/10.1126/sciadv.abi7038>
- Villalba-Mouco, V., van de Loosdrecht, M.S., Posth, C., Mora, R., Martínez-Moreno, J., Rojo-Guerra, M., Salazar-García, D.C., Royo-Guillén, J.I., Kunst, M., Rougier, H., Crevecoeur, I., Arcusa-Magallón, H., Tejedor-Rodríguez, C., García-Martínez de Lagrán, I., Garrido-Pena, R., Alt, K.W., Jeong, C., Schiffels, S., Utrilla, P., Krause, J., Haak, W., 2019. Survival of Late Pleistocene Hunter-Gatherer Ancestry in the Iberian Peninsula. *Curr. Biol.* 29, 1169–1177.e7. <https://doi.org/10.1016/j.cub.2019.02.006>
- Walsh, B., 2001. Estimating the Time to the Most Recent Common Ancestor for the Y chromosome or Mitochondrial DNA for a Pair of Individuals. *Genetics* 158, 897–912. <https://doi.org/10.1093/genetics/158.2.897>
- Wang, X., Skourtanioti, E., Benz, M., Gresky, J., Ilgner, J., Lucas, M., Morsch, M., Peters, J., Pöllath, N., Ringbauer, H., le Roux, P., Schultz, M., Krause, J., Roberts, P., Stockhammer, P.W., 2023. Isotopic and DNA analyses reveal multiscale PPNB mobility and migration across Southeastern Anatolia and the Southern Levant. *Proc. Natl. Acad. Sci.* 120, e2210611120. <https://doi.org/10.1073/pnas.2210611120>
- White, D.J., Wolff, J.N., Pierson, M., Gemmell, N.J., 2008. Revealing the hidden complexities of mtDNA inheritance. *Mol. Ecol.* 17, 4925–4942. <https://doi.org/10.1111/j.1365-294X.2008.03982.x>
- Woodward, S.R., Weyand, N.J., Bunnell, M., 1994. DNA Sequence from Cretaceous Period Bone Fragments. *Science* 266, 1229–1232. <https://doi.org/10.1126/science.7973705>
- Yaka, R., Mapelli, I., Kaptan, D., Doğu, A., Chyleński, M., Erdal, Ö.D., Koptekin, D., Vural, K.B., Bayliss, A., Mazzucato, C., Fer, E., Çokoğlu, S.S., Lagerholm, V.K., Krzewińska, M., Karamurat, C., Gemici, H.C., Sevkar, A., Dağtaş, N.D., Kılınç,

- G.M., Adams, D., Munters, A.R., Sağlıcan, E., Milella, M., Schotsmans, E.M.J., Yurtman, E., Çetin, M., Yorulmaz, S., Altınışik, N.E., Ghalichi, A., Juras, A., Bilgin, C.C., Günther, T., Storå, J., Jakobsson, M., de Kleijn, M., Mustafaoğlu, G., Fairbairn, A., Pearson, J., Togan, İ., Kayacan, N., Marciniak, A., Larsen, C.S., Hodder, I., Atakuman, Ç., Pilloud, M., Sürer, E., Gerritsen, F., Özbal, R., Baird, D., Erdal, Y.S., Duru, G., Özbaşaran, M., Haddow, S.D., Knüsel, C.J., Götherström, A., Özer, F., Somel, M., 2021. Variable kinship patterns in Neolithic Anatolia revealed by ancient genomes. *Curr. Biol.* 31, 2455-2468.e18. <https://doi.org/10.1016/j.cub.2021.03.050>
- YTree [WWW Document], 2025. URL <https://www.yfull.com/tree/> (accessed 12.10.25).
- Yu, H., van de Loosdrecht, M.S., Mannino, M.A., Talamo, S., Rohrlach, A.B., Childebayeva, A., Villalba-Mouco, V., Aron, F., Brandt, G., Burri, M., Freund, C., Radzeviciute, R., Stahl, R., Wissgott, A., Fewlass, H., Tagliacozzo, A., Piperno, M., Tusa, S., Collina, C., Schimmenti, V., Di Salvo, R., Prüfer, K., Posth, C., Hublin, J.-J., Gronenborn, D., Binder, D., Jeong, C., Haak, W., Krause, J., 2022. Genomic and dietary discontinuities during the Mesolithic and Neolithic in Sicily. *iScience* 25, 104244. <https://doi.org/10.1016/j.isci.2022.104244>
- Zhang, J., Rowe, W.L., Clark, A.G., Buetow, K.H., 2003. Genomewide Distribution of High-Frequency, Completely Mismatching SNP Haplotype Pairs Observed To Be Common across Human Populations. *Am. J. Hum. Genet.* 73, 1073–1081. <https://doi.org/10.1086/379154>
- Zhou, J., Teo, Y.-Y., 2016. Estimating time to the most recent common ancestor (TMRCA): comparison and application of eight methods. *Eur. J. Hum. Genet.* 24, 1195–1201. <https://doi.org/10.1038/ejhg.2015.258>
- Zilhão, J., 2001a. Radiocarbon evidence for maritime pioneer colonization at the origins of farming in west Mediterranean Europe. *Proc. Natl. Acad. Sci.* 98, 14180–14185. <https://doi.org/10.1073/pnas.241522898>
- Zilhão, J., 2001b. Radiocarbon evidence for maritime pioneer colonization at the origins of farming in west Mediterranean Europe. *Proc. Natl. Acad. Sci.* 98, 14180–14185. <https://doi.org/10.1073/pnas.241522898>
- Zilhão, J., 1998. On Logical and Empirical Aspects of the Mesolithic-Neolithic Transition in the Iberian Peninsula. *Curr. Anthropol.* 39, 690–698. <https://doi.org/10.1086/204791>
- Zischler, H., Hoss, M., Handt, O., von Haeseler, A., van der Kuyl, A.C., Goudsmit, J., Pääbo, S., 1995. Detecting Dinosaur DNA. *Science* 268, 1192–1193. <https://doi.org/10.1126/science.7605504>
- Zvelebil, M., 2001. The agricultural transition and the origins of Neolithic society in Europe. *Doc. Praehist.* 28, 1–26. <https://doi.org/10.4312/dp.28.1>
- Zvelebil, M., 1996. The agricultural frontier and the transition to farming in the circum-Baltic region, in: *The Origins And Spread Of Agriculture And Pastoralism In Eurasia*. Routledge.