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Ancient mitochondrial lineages support the prehistoric maternal root of Basques in Northern Iberian Peninsula

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The Basque population inhabits the Franco-Cantabrian region in southwest Europe where Palaeolithic human groups took refuge during the Last Glacial Maximum. Basques have been an isolated population, largely considered as one of the most ancient European populations and it is possible that they maintained some pre-Neolithic genetic characteristics. This work shows the results of mitochondrial DNA analysis of seven ancient human remains from the Cave of Santimamiñe in the Basque Country dated from Mesolithic to the Late Roman period. In addition, we compared these data with those obtained from a modern sample of Basque population, 158 individuals that nowadays inhabits next to the cave. The results support the hypothesis that Iberians might have been less affected by the Neolithic mitochondrial lineages carried from the Near East than populations of Central Europe and revealed the unexpected presence of prehistoric maternal lineages such as U5a2a and U3a in the Basque region. Comparison between ancient and current population samples upholds the hypothesis of continuity of the maternal lineages in the area of the Franco-Cantabrian region.

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INTRODUCTION

During the last years genetic markers of uniparental lineages, such as Y-chromosome and mitochondrial DNA (mtDNA), arose as suitable tools for tracking population migrations.¹ Specifically, mtDNA is often used for the challenging genetic analysis of prehistoric remains.^{1,2} DNA analysis of ancient remains has evidenced the arrival of some human groups from the Near East to the European continent during the Neolithic period.^{3,4} Nonetheless, the effect of these humans on the pre-existing European genetic pool and how their culture expanded remains unclear.^{5,6} Based on genetic data of current populations, the Franco-Cantabrian region (south-western Europe) appears as a key-stone in the post-glacial settlement of the European continent.^{1,7–9} Even though human remains of pre-Neolithic period are very scarce, archaeological data from this region, such as abundant cultural records of Palaeolithic hunter-gatherer human groups living during the Last Glacial Maximum, suggest that this was the most densely populated European region along the Upper Palaeolithic.¹⁰

The Cave of Santimamiñe is located at the oriental side of the Oka river basin and very close to its estuary, <10 km from the current shore of Cantabrian sea, within the area of settlement of the Franco-Cantabrian refuge (Figure 1). It is a key archaeological site, well known by its valuable Palaeolithic cave art and selected as a World Heritage Site by UNESCO. Evidences of continued human occupation from the Lower Magdalenian (ca. 15 500 calibrated years before Common Era, cal BCE) to the Bronze Age (ca. 1500 cal BCE), including some sporadic occupation along Late Roman period, have been found in this cave.¹¹ Moreover, several human remains from different historical periods have been found in the post-Palaeolithic stratigraphic levels of

the Cave of Santimamiñe, the oldest one belonging to Mesolithic period.^{11,12}

The analysis of the DNA of human remains could contribute to unravel genetic composition of the Palaeolithic population and the human evolution of this region. In contrast with several studies focused on the analysis of the current autochthonous population, there are only a few reports on pre-Neolithic samples from the Franco-Cantabrian region.^{13,14} These studies are grounded in the supposedly genetic continuity between the Palaeolithic hunter-gatherers and the autochthonous Basque population.^{15,16}

Herein we report a study on ancient maternal lineages of the Franco-Cantabrian region and their persistence in modern populations. It is based on the combination of ancient and modern mitochondrial DNA data. We analysed and compared mtDNA from ancient remains found in the Cave of Santimamiñe and from modern autochthonous Basque individuals (from four villages surrounding the Cave, into the region of Busturialdea, Biscay, Spain).

MATERIALS AND METHODS

Ancient human remains selected for this study were found in the Cave of Santimamiñe throughout numerous archaeological dig campaigns (1918–1926, 1960–1962).¹¹ We selected seven teeth attached to the jawbone without visible signs of damage (such as crown abrasion, cracks or cavities). Isotopic analyses were performed by Beta Analytic (Miami, FL, USA). Age dates provided in this study were 2σ calibrated (95% probability). Every step involving ancient samples was carried out in laboratories exclusively dedicated to ancient DNA analysis. DNA extraction was performed by adsorption to silica gel membrane, following a protocol modified from Marshall *et al.*¹⁷ Hypervariable segments HVS-I and HVS-II of the mtDNA control region were amplified by PCR using five overlapping fragments of ~175 base pairs (bp) each one (Supplementary Table S1). Samples were subjected to different additional analyses to confirm

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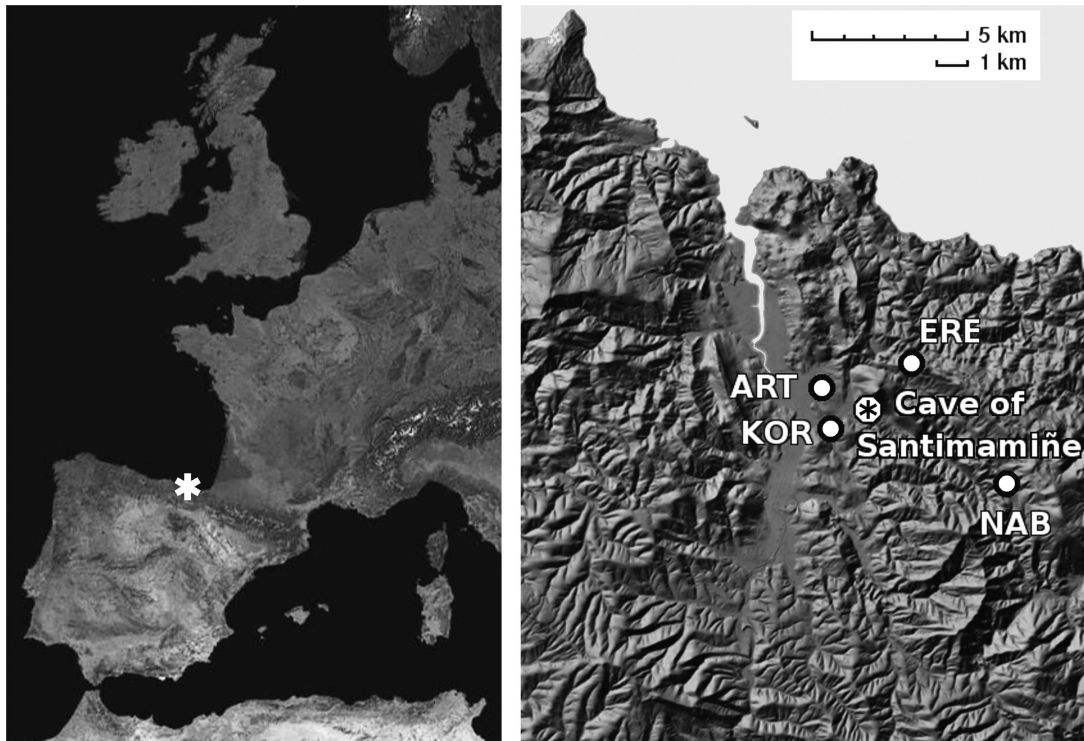


Figure 1 Location of the Cave of Santimamiñe in the North of the Iberian Peninsula. The Basque villages around the cave, where samples of the current autochthonous population were taken, are marked by white dots. ART, Gautegiz-Arteaga; ERE, Ereño; KOR, Kortezubi; NAB, Nabarniz.

the results and to probe their authenticity, such as cloning amplified sequences, analysing haplogroup diagnostic polymorphisms located in the mtDNA coding region by different techniques, and the independent analysis of some samples by an external laboratory of Strasbourg (France).

Saliva samples were collected with mouthwash from 158 autochthonous Basques living in four villages surrounding the area of the Cave of Santimamiñe (Figure 1), a region known as Busturialdea (Biscay, Spain), following procedures in accordance with the ethical standards of the Declaration of Helsinki. DNA was extracted from saliva samples following a standard phenol–chloroform protocol.¹⁸ The entire control region of mtDNA was amplified and sequenced as described by Cardoso *et al.*¹⁹ In some samples coding region polymorphism determinant of H1j1 haplogroup was analysed following the same procedure, using primers shown in Supplementary Table S1. All the control region sequences of the population of Busturialdea are available online at GenBank under accession numbers KR697593–KR697750. Likewise, haplotypes have been deposited into EMPOP (<http://empop.online>)²⁰ under accession number EMP00668.

Mitochondrial sequences of ancient and modern samples were compared to the revised Cambridge Reference Sequence,²¹ (rCRS, RefSeq NC_012920.1). Haplogroup assignment was performed according to PhyloTree Build 17 (<http://www.phylotree.org>).²² Multivariate analysis of ancient European populations through Principal Component Analysis (PCA) was performed using PAST v3.01.²³ Population statistics were calculated with Arlequin v3.5.²⁴ Software SplitsTree v4²⁵ was used to draw the neighbour-joining tree which represented the relation between the haplotypes of modern and ancient samples, and the median-joining network was performed with software Network v5 (<http://www.fluxus-engineering.com>),²⁶ which also calculated the time elapsed between ancient and modern lineages.

A more detailed explanation of the methods employed in this study can be found on Appendix (Supplementary Appendix).

RESULTS AND DISCUSSION

Isotopic analysis of ancient samples from Santimamiñe

Seven human remains from the Cave of Santimamiñe were studied. In order to establish their antiquity and obtain information about their diet

these remains were radiocarbon dated and subjected to the analysis of stable isotopes of carbon $^{13}\text{C}/^{12}\text{C}$ ($\delta^{13}\text{C}$) and nitrogen $^{15}\text{N}/^{14}\text{N}$ ($\delta^{15}\text{N}$; Table 1, Supplementary Dataset S1 and Supplementary Appendix).

The analysis of the ^{14}C radioactive isotope dated the antiquity of these remains in a wide period of time, ranging from 5210 cal BCE to 390 calibrated years of Common Era (cal CE). Data from four of these individuals were comprised in a short time period, between 1740 and 1320 cal BCE.

The oldest sample found in the Cave of Santimamiñe (S.12N) was dated in the transition between the 5th and 6th millennia cal BCE, which is a critical time period due to the controversy that exists around the arrival of Neolithic period to the Franco-Cantabrian region.^{27,28} In this case, a more thorough analysis of the stable isotopes of carbon and nitrogen, combined with ^{14}C , made possible to infer the cultural period to which individual S.12N belongs (Figure 2). Values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of sample S.12N were consistent with a marine diet mainly based on invertebrates (Figure 2a), which was characteristic of Mesolithic groups living close to the littoral (Figure 2b). Thus, although the presence of Neolithic groups along Franco-Cantabrian region at the same time could not be discarded, the oldest individual from the Cave of Santimamiñe showed a Mesolithic diet, probably indicating the persistence of this subsistence way of life in the area at least until 5210–4950 cal BCE.

On the contrary, the values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of the remaining samples of Santimamiñe matched with the type of diets that would be supposed to the cultural periods in which each sample was dated by ^{14}C (Figure 2).

Authenticity of ancient DNA results

Beside the laboratory strict conditions and precautions followed during the manipulation and analysis of these samples, the genetic

Table 1 Isotopic data of the samples from the Cave of Santimamiñe analysed in this study, the cultural period to which they presumably belong and their corresponding mtDNA haplotypes and haplogroups

Sample ^b	Stable isotopes		¹⁴ C Dating	Period	HVS-I (16 159–16 406)	Haplotype ^a		Haplogroup
	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	2 σ Calibration			HVS-II (12–408)		
S.15I	-16.1	10.6	240–390 cal CE	Late Roman	rCRS	214G, 263G, 315.1C		H
S.16G	-19.4	8.9	1450–1380 cal BCE, 1340–1320 cal BCE	Bronze Age	16343G, 16390A	73G, 150T, 263G, 309.1C, 315.1C		U3a
S.I.147	-19.9	8.8	1600–1570 cal BCE, 1560–1550 cal BCE, 1540–1440 cal BCE	Bronze Age	16299G, 16311C	263G, 309.1C, 309.2C, 315.1C		R0
S.7J	-19.4	8.6	1740–1610 cal BCE	Bronze Age	16192T, 16270T	73G, 146C, 150T, 263G, 309.1C, 315.1C		U5b
S.11N	-19.8	9.2	1740–1610 cal BCE	Bronze Age	rCRS	263G, 309.1C, 315.1C		H1
S.17G	-18.8	10.4	2200–2010 cal BCE, 2000–1980 cal BCE	Chalcolithic	16185T, 16294T, 16296T, 16304C, 16311C, 16366T	Not analysed		T2b
S.12N	-16.3	10.4	5210–4950 cal BCE	Mesolithic	(16114A) ^c , 16192T, 16256T, 16270T, 16294T	54A, 73G, 263G, 309.1C, 315.1C		U5a2a

Abbreviations: BCE, years before Common Era; mtDNA, mitochondrial DNA; rCRS, revised Cambridge Reference Sequence.

^aThe polymorphisms showed are changes referred to rCRS.²¹

^bSample names noted in this table, as well as those used in all the text, are the shortened names used in our laboratory. In Supplementary Table S1 the relation between each laboratory sample name and its archaeological label is shown.

^cThe polymorphism in brackets was revealed through the authentication analyses performed in the laboratory of Strasbourg (France), since it is out of the region analysed in our laboratory.

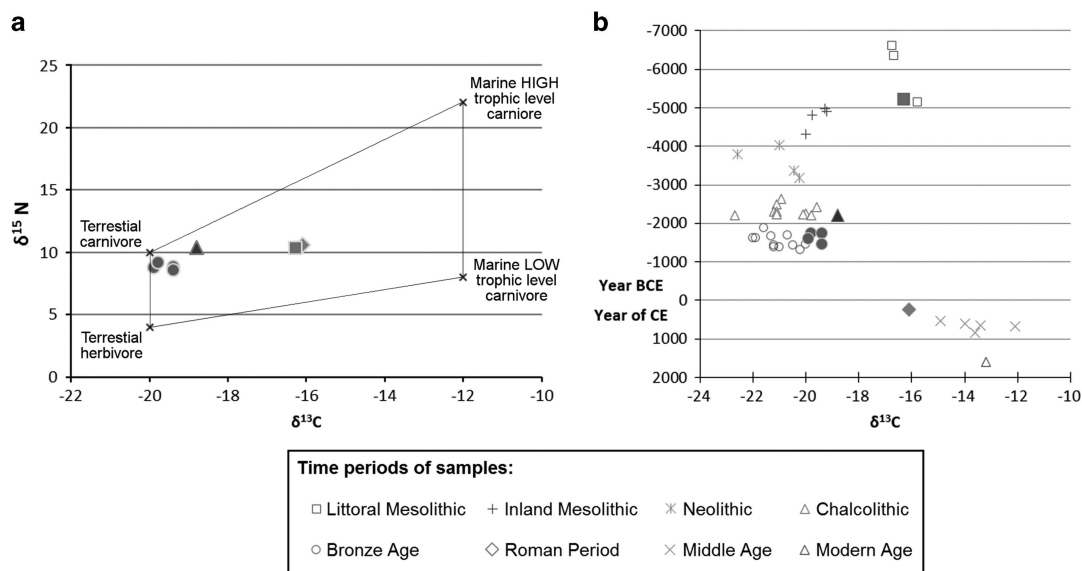


Figure 2 Ratios of carbon and nitrogen stable isotopes of the ancient remains from the Cave of Santimamiñe. (a) Distribution of the samples into the four (theoretical) extreme human dietary types as Richards and Hedges⁴⁶ based on their stable isotope content. (b) Evolution of $\delta^{13}\text{C}$ content in human remains from the Cantabrian region over time (age of the samples is based on ¹⁴C dating, without calibration) modified from Arias⁴⁷ to include samples from our study.

results obtained from the ancient remains of Santimamiñe were authenticated through diverse analyses (Supplementary Dataset S1). Samples with sufficient tooth powder (57.1%) were subjected to two independent DNA extractions, and the concordance of the control region sequences obtained was confirmed. Cloning of the amplified products and duplicated analysis by an external laboratory was performed over almost a third of the samples as proposed by Cooper and Poinar.²⁹ Finally, the analysis of coding region SNPs, through different methodologies (direct sequencing, minisequencing or MALDI-TOF mass spectrometry analysis), was carried out to corroborate the haplogroup obtained by control region analysis on 71.4% of the samples.

Altogether, the results of all samples, except for S.17G, were confirmed by one or more additional analyses. Moreover, results of the external laboratory were consistent with our results, and showed no trace of contamination. In the same way, none of the haplotypes obtained from the human remains of Santimamiñe was coincident with the haplotypes of the researchers, except for the sample S.11N, which shared mitochondrial lineage with one researcher. However, bearing in mind that it is the most common haplotype among the current European population and that the other authentication analyses corroborated the result of this ancient sample, contamination along the analysis process was discarded.

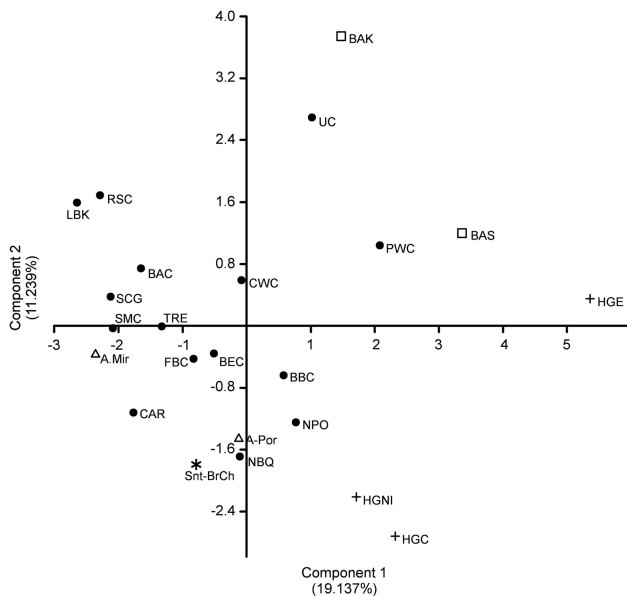


Figure 3 Graphic representation of the PCA of European prehistoric populations based on their haplogroup frequencies. Each population is represented by different symbols. The symbol refers to the cultural period being cross for hunter-gatherers and pre-Neolithic groups; circle for Neolithic populations; triangle for Chalcolithic period and square for Bronze Age. The group of samples from Santimamiñe of Chalcolithic and Bronze Age is represented by an asterisk. Data are shown in Supplementary Dataset S3.

Mitochondrial diversity in the ancient remains from the Cave of Santimamiñe

First one human jawbone was selected for a pilot analysis (S.17G) with the aim of determining the inhibition effect on the DNA extracted from Santimamiñe remains and their suitability for genetic analyses. As positive results were obtained, six additional human remains were analysed.

The quantification by qPCR of the DNAs extracted showed very low amounts of nuclear DNA (Supplementary Dataset S1), even though, it was possible to amplify mtDNA in all the samples.

Sequencing of 645 bp of mtDNA control region was carried out with five overlapping fragments: two fragments for HVS-I and three fragments for HVS-II. The unique exception was sample S.17G, where only HVS-I was sequenced, due to the scarce DNA recovered from this remain and the previous tests carried out in the pilot analysis. The analysis of HVS-II usually does not go beyond 73 A/G position in ancient DNA studies, but in our study, the analysis of the whole segment provided valuable information about each sample. The analysis of these sequences resulted in seven different mitochondrial haplotypes, showing that all the remains included in this study belonged to different maternally unrelated individuals (Table 1).

The diversity of haplotypes is also shown at the haplogroup level. A complete analysis of HVS-II allowed detecting some private polymorphisms of the haplotypes that, not only made possible to differentiate the maternal lineage of all the samples, but, in some cases, it was also helpful to make a more accurate phylogenetic classification. Most of the lineages belonged to R0 and U macrohaplogroups, with three individuals each, being T2b the only lineage of JT macrohaplogroup found in the cave.

Overall, genomic and isotopic data highlight that the most ancient haplogroup found in the Cave of Santimamiñe is U5a2a, belonging to the individual S.12N. This finding pointed to the presence of this

haplogroup in the northern fringe of the current Basque Country at least 7000 years ago.

Phylogenetic history

Lineages H1, T2b and U5b were observed in Santimamiñe between 2200 and 1610 cal BCE. All of them have been found widely distributed along the European prehistory, with several human remains scattered through this continent.^{2,30–32} These three subhaplogroups have the highest frequencies in the extant European populations. Strikingly, with the exception of T2b, whose frequency peak is located in the North of Italy,³³ subhaplogroups H1 and U5b reach their highest frequencies in the North of the Iberian Peninsula, specifically in the region where the Franco-Cantabrian refuge was settled during the Upper Palaeolithic.^{8,34,35}

Two subhaplogroups found in Santimamiñe, U5a2a and U3a, are scarce in prehistoric remains and virtually absent in current European populations. However, these haplogroups of the samples S.12N and S.16G are of great interest because they are the most ancient evidence of the presence of subhaplogroups U5a2a and U3a found to date in Western Europe (Supplementary Appendix).

Therefore, these findings show that subhaplogroup U3a, which probably arose in the Near East and entered the European continent with Neolithic migrations,³⁶ had already reached the western area of the European continent in the Bronze Age, about 3300 years ago. On the other hand, U5a2a was present in a Mesolithic population from Western Europe 7000 years ago, around its first appearance in Central Europe,³ even though nowadays U5a2a is only found at very low frequencies in central and northern Europe.^{37–39}

Prehistoric European genetic context

The contextualisation of the human remains of the Cave of Santimamiñe into the European prehistory is not a simple issue due to the different antiquity of these samples. Therefore, four individuals of Santimamiñe, which dated into a relatively narrow period of time, ranging from Chalcolithic to Bronze Age, were grouped together (Snt-BrCh) to study their phylogenetic link with other prehistoric European remains (Supplementary Dataset S3). On the other hand, Mesolithic sample from Santimamiñe, S.12N, was joined to previously studied pre-Neolithic/hunter-gatherer remains from the northern Iberian Peninsula (HGNI).^{13,14} A PCA was performed with haplogroup frequencies of European populations from different prehistoric periods (Figure 3, Supplementary Dataset S3).

The graphic shows that pre-Neolithic individuals of the Northern Iberian Peninsula (HGNI), including S.12N, spread similarly to hunter-gatherers of Central Europe (HGC). The group formed by Chalcolithic and Bronze Age remains of Santimamiñe appeared closer to other Iberian populations, and specially, to those from the North of the Iberian Peninsula, such as the Neolithic group of Basque Country and Navarre (NBQ) and the Chalcolithic group of El Portalón in Atapuerca (A-Por). However, remaining population groups of Central and Eastern Europe showed scarce genetic relationship with Chalcolithic and Bronze Age individuals from Santimamiñe. Indeed, every group from the Iberian Peninsula spreads similarly, close to the negative axis of the second component, except for El Mirador from Atapuerca (A-Mir), which shows quite different haplogroup frequencies, and is placed near to other Central European population groups.

Overall, pre-Neolithic group of North Iberia showed a close relation with most of the chronologically later groups of the Iberian Peninsula, while, on the contrary, on Central Europe hunter-gatherers appeared more distant from Neolithic and following Central European

populations. Biplot PCA (Supplementary Figure S1) showed that this proximity between populations of different periods of Northern and Western Iberia was due to a higher persistence of U and U5b haplogroups in this region even after the arrival of Neolithic culture. This could confirm that Iberian populations, especially those distant from Mediterranean coastal regions, were less affected by the Neolithic mitochondrial lineages carried from Near East than population groups of Central Europe, as it was previously pointed by Brand *et al.*⁵

Current population of Busturialdea

The phylogeographic nature of mtDNA is very useful to trace the movements of human groups based on the frequency distribution of haplogroups in current populations and to infer their origin. However, to find the place of origin of more precise subhaplogroups is not easy. Nevertheless, there are some exceptions on lineages that have remained in isolated populations or in populations from regions that have not been influenced by great migration waves through the history, and thus, they have preserved their characteristic lineages over time. Autochthonous Basque population of Franco-Cantabrian region is one of these exceptional populations. It preserves autochthonous subhaplogroups rarely found out of this region, some of which have been described in the last years.^{15,16,34,40}

With the aim of studying the presence of the ancient mitochondrial lineages from past times in the present, the control region of mtDNA was analysed in a sample of 158 autochthonous Basque individuals belonging to the population inhabiting the vicinity of the Cave of Santimamiñe, the region of Busturialdea (Figure 1, Supplementary Appendix). MtDNA haplotypes (Supplementary Dataset S4), haplogroup frequencies (Supplementary Table S2) and descriptive statistics (Supplementary Table S3) of this population can be accessed in the Supplementary Material.

In this population the most remarkable lineages found were those of subhaplogroups H1j1 and U5b1f1a. The two most frequent haplotypes belong to these subhaplogroups, which have been suggested as autochthonous lineages of the Franco-Cantabrian region. Subhaplogroup H1j1, which only presents the polymorphism 16129A in the control region, was described by Behar *et al.*¹⁵ as an autochthonous haplogroup of the Franco-Cantabrian region.^{15,16} This haplotype has been found with a remarkable frequency among numerous populations of the North of the Iberian Peninsula, especially in the Basque area, or in populations of Basque ancestry.^{15,35,41,42} In this population sample of Busturialdea 18 individuals (11.39%) were classified into H1j1 subhaplogroup by the analysis of the coding region polymorphism T4733C.

Subhaplogroup U5b1f1a, with a frequency of 5.06% in Busturialdea, is also remarkable. It represents 10% of the mitochondrial diversity of the current populations of the Franco-Cantabrian region, reaching a maximum frequency of ~24% in the populations next to the Pyrenees and decreasing to the West.¹⁶ Due to its frequency distribution pattern and its absence in populations out of the Franco-Cantabrian region, U5b1f1a was proposed as an autochthonous lineage of the Basque population, which could have originated during the Younger Dryas (12 800–11 500 years ago), with a splitting age of 11 985 years.¹⁶

Comparison between ancient individuals of Santimamiñe and current population of Busturialdea

To determine the presence of any kinship between past and present populations from Busturialdea, haplotypes of ancient samples from the Cave of Santimamiñe and current population of the area were studied all together by means of a neighbour-joining tree (Supplementary Figure S3). Even if they do not share the same haplotypes, the tree confirmed that these populations are not completely unlinked since

their lineages gather similarly, belonging to common phylogenetic branches. Indeed, the comparison at the haplogroup level between samples of our study showed that most of the subhaplogroups found in the population that nowadays inhabits the vicinity of Santimamiñe were already present in the populations that passed by the cave in prehistoric times, to be named, H1, T2b and U5b.

The difficulty of finding the same haplotypes of the ancient human remains in current populations has been addressed in previous studies of ancient DNA.³⁰ This fact could be due to the extinction of the ancient lineages throughout history, but also, to the high mutation rate that characterises mtDNA, and specially the control region.⁴³ This high ability to change could modify the ancient haplotype with new polymorphisms or through back mutation of the previous ones, doing improbable to find an exact match between ancient and modern lineages. According to the mutation rate of regions HVSI and HVSI calculated by Rieux *et al.*,⁴⁴ which is calibrated with aDNA samples, the time elapsed would suppose the appearance of up to 1.7 mutations on the lineages of Santimamiñe until today.

By this reason, an alignment analysis was performed with the haplotypes from the Cave of Santimamiñe that belong to the same phylogenetic branch but had incomplete match with lineages of current population of Busturialdea, such as R0 (without HV0), U5a, U5b and T2 (Supplementary Dataset S5). Almost 80% of the samples from Busturialdea included in this alignment presented between one and three differences against those of Santimamiñe (Supplementary Figure S2). According to the mutation rate previously mentioned, direct descendants could present up to two differences, which is fulfilled in more than 50% of them.

Moreover, a median-joining network was performed (Supplementary Figure S4a) including samples from the Cave of Santimamiñe, the modern population of Busturialdea and pre-Neolithic individuals from the Northern Iberian Peninsula.^{13,14} The samples selected for this analysis were those belonging to the phylogenetic branch of haplogroup U5, since it is the most represented on pre-Neolithic period and some of its subhaplogroups are highly frequent in current Basque population, what has been seen as a sign of genetic continuity in this region.^{16,41,45} The estimated time between the selected different ancestors and their respective descendants is close to the time elapsed between these dated individuals (Supplementary Figure S4), supporting the possible genetic link among ancient and current populations (Supplementary Appendix).

Thus, even if ancient remains analysed in this study cannot be considered as a population in itself and the phylogenetic link between ancient and current population has been probably diluted over time, the findings of this study might uphold the hypothesis of continuity.

In conclusion, the uncovering of lineages that inhabited this region in the prehistory, and especially before the arrival of Neolithic, shed light over its ancient populations and brings us closer to the disentanglement of the population that sheltered in the Franco-Cantabrian region. This study revealed the presence of a new mitochondrial haplotype on the pre-Neolithic northern Iberian Peninsula. In addition, DNA analysis of Chalcolithic and Bronze Age samples of Santimamiñe contributed to place the temporal context of the Near Eastern mitochondrial haplogroups that arrived to the Northern Iberian Peninsula with the Neolithic wave, and their effect over previous populations.

On the other hand, the results obtained from the comparison between the ancient samples from Santimamiñe and current autochthonous Basque population from Busturialdea are in line with the existence of continuity of the maternal lineages in the area of the Franco-Cantabrian region.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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