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Hunter-gatherer genetic persistence at the onset of megalithism in western Iberia: New mitochondrial evidence from Mesolithic and Neolithic necropolises in central-southern Portugal

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ABSTRACT

Despite its strategic importance at the furthermost edge of the Neolithic expansion in Europe, archaeogenetic data from Mesolithic and Neolithic human remains from Portugal are still very limited. Here we present ancient mtDNA evidence (mostly unpublished) to fill the gap and discuss the pattern of "genetic resurgence" of hunter-gatherer (Mesolithic) ancestry, widely reported elsewhere in Europe, among the first megalith builders (Middle Neolithic) of western Iberia.

A total of 11 Mesolithic and Neolithic necropolises located in the central and southern regions of Portugal dated to *ca.* 6200–3000 BC were studied. These sites comprise all Mesolithic–Neolithic cultural stages and include several funerary architectures and spaces. Reproducible mtDNA HVRI haplotypes were obtained from 23 individuals from six different archaeological sites spread across a >3000-year transect, from the Late Mesolithic to the Late Neolithic.

Our results support a three-stage explanatory demographic and populational model: *i)* local hunter-gatherer populations constituted a highly homogeneous genetic pool; *ii)* the first farming practices were introduced by human groups carrying new, extraneous haplogroups and exhibiting the signature of admixture events occurring at the time of first contact with local hunter-gatherers; *iii)* the genetic pattern detected among the megalith-building populations, showing hunter-gatherer along with farming ancestry, may be explained by the segmentary principles, and attendant endogamic practices, that structured Neolithic societies.

1. Introduction

Located at the western end of continental Europe, the Atlantic façade of the Iberian Peninsula—i.e., modern-day Portugal and Galicia—is an ideal laboratory for studying the Mesolithic−Neolithic transition and ensuing developments, of which the beginning of megalith building has attracted most attention. Indeed, a recurrent research topic in Archaeology has been the very nature of these processes of cultural change, with debates focusing on whether they resulted from the impact of human migration events or were due to autonomous, local developments.

When debating the origins of megalithism, most 20th-century Iberian scholars (e.g., Martínez Santa-Olalla, 1946; Pericot, 1950) adhered to a migrationist approach. This stance was widely shared elsewhere in Europe, namely by authors such as Childe (1925, 1940) or Daniel (1962). Based on comparative stratigraphy and typology, megalithism

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was perceived as imported to Iberia from the Eastern Mediterranean or the Near East, via human migration, through seafaring, and to thenceforth have spread along the Atlantic coasts of Europe. One of the main arguments to support this view was the formal similarity between the Aegean vaulted-chamber tombs (*tholoi*) and some Iberian megaliths. This view prevailed in peninsular archaeology from the late 19th century to the end of the 1960s (e.g., Savory, 1968).

The radiocarbon dating of European megaliths, the application of which became widespread in the 1960s, provided an alternative hypothesis. As first put forward by Renfrew (1973), it was postulated that the early emergence of megaliths in the Atlantic seaboard was due to local processes. Indeed, as demonstrated by absolute chronology, the Atlantic megaliths were considerably older than their supposed eastern prototypes, thus invalidating diffusionist theories and redirecting research towards the economic and social conditions of local Early Neolithic communities. According to Renfrew's hypothesis, expanding farming groups met pre-existing hunter-gatherer populations and as the pressure on arable land—essential for the new economic system—grew, the Neolithic groups began building imposing stone tombs and standing stones, which marked the possession of lands, and the physical presence of ancestors' remains.

This shift in perspective also took place in Portugal during the 1970s, especially after Whittle and Arnaud's (1975) pioneer project dated by thermo-luminescence pottery samples from Neolithic and Chalcolithic tombs in the Alentejo and Estremadura provinces. This study confirmed the antiquity of Portuguese megalithic monuments and, thus, suggested a local origin of the phenomenon. It should be stressed, though, that some scholars had already supported an Iberian origin of megalithism, or even specifically a Portuguese one, before these dating techniques were developed. This is particularly the case of Bosch-Gimpera (1956, 1966) or Heleno (unpublished; see Rocha, 2009/10; Cardoso, 2013), especially after the interpretative change that followed the excavation in the 1940s of the Portuguese dolmens of Comenda and Farisoa, in the Reguengos de Monsaraz megalithic area of Alentejo. In these complex monuments, *tholoi* were juxtaposed to previously existing dolmens under the same mound structure, thus supporting "the possibility of an indigenous evolution of megalithic tombs in Portugal, a problem for the solution of which the Reguengos dolmens have shed new light" (Leisner and Leisner, 1951: 173; Portuguese original).

In Bosch-Gimpera's (1966: 282–3; Spanish original) view, the thesis of a Portuguese origin for megalithism went as far as defining what he called the "Portuguese megalithic culture". This culture would have been in "full development in the fourth millennium", a period during which passage tombs were built and "the megalithic idea had begun to be introduced to Brittany and the British Isles." This author also followed Heleno's thesis that the earliest megalith builders of the country were direct descendants of Muge Mesolithic hunter-gatherers who migrated from the Tagus valley to the interior areas of central-southern Portugal. Such would be the case with the builders of the Montemor-o-Novo megalithic area in the central plains of Alentejo (Heleno,

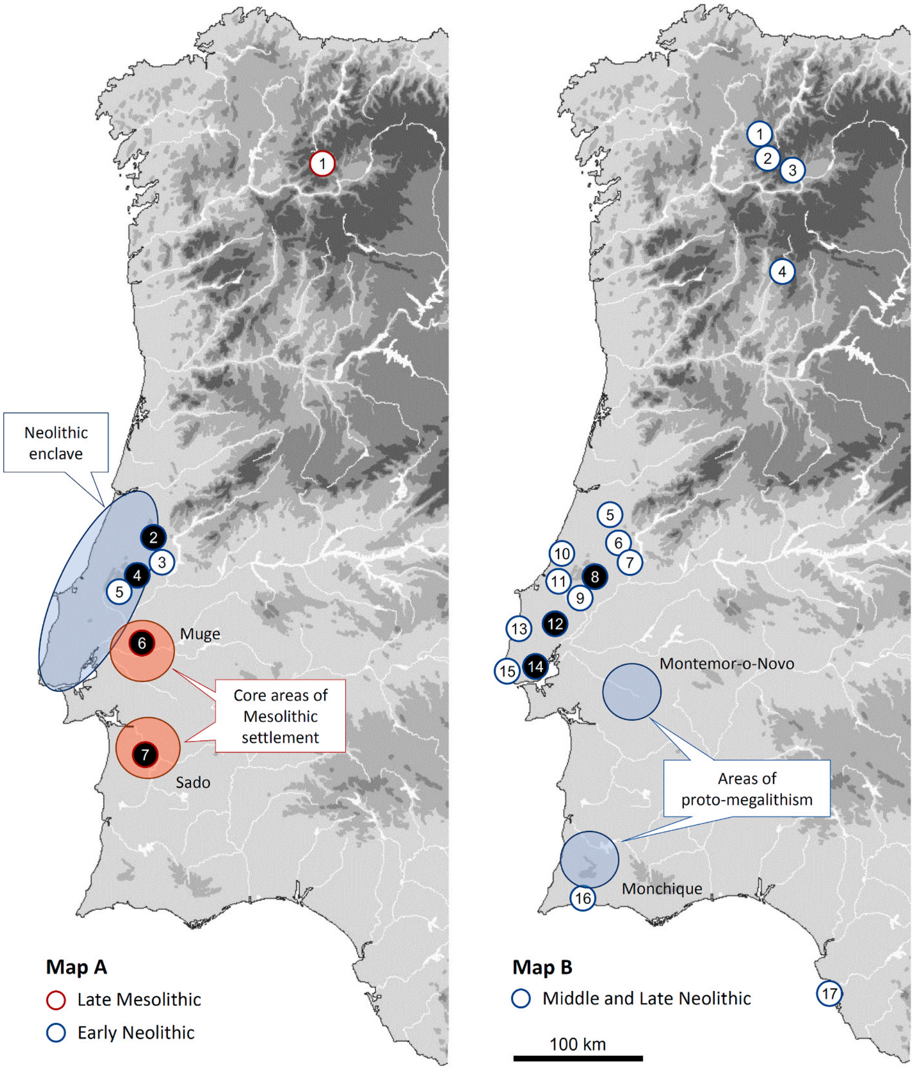

Fig. 1. Late Mesolithic to Neolithic cemeteries in Western Iberia with aDNA results (sites analysed in this study: black dot in maps and bold in caption below).
Map A) Late Mesolithic and Early Neolithic: 1 - Chan do Lindeiro, 2 - **Caldeirão**, 3 - Nossa Senhora das Lapas, 4 - **Picoto**, 5 - Galeria da Cisterna, 6 - **Moita do Sebastião**, 7 - **Amoreiras**.
Map B) Middle and Late Neolithic: 1 - Valdavara, 2 - Eirós, 3 - Pala da Vella, 4 - Lorga de Dine, 5 - Dólmen of Ansião, 6 - Ossos, 7 - Cadaval, 8 - **Barrão**, 9 - Lugar do Canto, 10 - Cova das Lapas, 11 - Alcobertas, 12 - **Bom Santo**, 13 - Cabeço da Arruda, 14 - **Correio-Mor**, 15 - Poço Velho, 16 - Monte Canelas, 17 - Campo de Hockey.

unpublished; see Gonçalves and Andrade, 2020; Rocha, 2009/10), to which similar tombs of the Monchique mountain range in Algarve (Formosinho et al., 1953) would be added later (Fig. 1B).

According to this view, the abundant number of small, cist-type graves built with stone slabs for individual burial known in those regions bore witness to the process and represented the first funerary architectures of the Neolithic. As a rule, pottery is scarce or absent in these inhumation cists, which only yielded few, and "archaic" grave goods: polished stone tools, flint bladelets, and microliths. Trapezoidal microliths were seen as evidence of a direct cultural link with the Mesolithic shell-middens. The later and larger tombs with passages and polygonal chambers would have evolved out of these architectonically simpler stone structures. Indeed, a number of authors still envisage these small tombs as being simultaneously a late manifestation of autochthonous Mesolithic groups and the earliest stage of the megalithic sequence—hence the term "proto-megalithism" (e.g., Silva and Soares, 2000).

However, when Heleno formulated his thesis in the mid-20th century, little was known about the Early Neolithic. As widely acknowledged, a first definition of the period in Portugal is due to Guilaine and Ferreira (1970), who established in firm bases—though only chrono-typological—the existence of an intermediate stage between the last Mesolithic hunter-gatherers and the first megalith builders of the country. According to these authors, as elsewhere in Iberia, the Early Neolithic was culturally part of the west Mediterranean Cardial and comprised two successive phases—the earliest, a pure Cardial, followed by a post-Cardial or Epicardial.

Later research gave rise to two principal, opposite views, still debated today, on the nature of the neolithization process itself. One perspective sees a process structurally characterised by developments taking place along lines of strong cultural and population continuity, with Neolithic novelties (polished stone, pottery, domesticates) being introduced piecemeal through exchange networks. This model postulates dominant indigenous processes (e.g., Silva and Soares, 2007; Soares, 2020) and, in this view, the Cardial is seen as one type of impressed ware among others, with no particular cultural significance. The other view emphasizes the intrusive nature of the earliest Neolithic sites in Estremadura and western Algarve—which are understood as fully Cardial in cultural terms—and the completeness of the "Neolithic package" whenever agricultural sites and favourable preservation conditions are met. Contrasting with the former, this model argues for migratory processes to have triggered the transition, and for differential demographic growth to underpin the gradual assimilation, through cultural interaction and mating exchange, of hunter-gatherer groups persisting alongside the first farming communities (e.g., Zilhão, 1993, 2001; Isern et al., 2017).

Mixed perspectives claim that, depending on the specific ecogeographical conditions and, above all, the demography, historical trajectory, and socio-economic conditions of the human communities involved both processes played a role in the formation of the first farming societies of western Iberia (e.g., Cardoso, 2007; Carvalho, 2008). Indeed, although the process is less clear in Galicia (Fábregas et al., 2019), available archaeological data for Portugal indicate the presence of different transition processes taking place in these heterogeneous regions, from migration and colonisation events to local, indigenous processes—for a recent, brief synthesis see Carvalho (2022).

Current knowledge on the chronology and geography of the neolithisation process in western Iberia indicates that farming economies were established along the coastal areas of Estremadura and western Algarve by 5500 BC. From there, farming spread to the interior and the north, where its presence is documented by 5100 BC (according to the currently available radiocarbon chronologies for central-northern Portugal and Galicia). Megalith building started around, or a little after 4000 BC (Table 1). During at least the first couple of centuries of the *ca.* 1500-years duration of the Early Neolithic, farmers coexisted with Mesolithic hunter-gatherers in the lower Tagus and Sado valleys and the Alentejo coast (Fig. 1A) before spreading to regions sparsely, if at all occupied by the latter.

Table 1
Chronology and geography of the Early and Middle Neolithic in Western Iberia.

Periods	Approx. chronologies (cal BC)	Geography	Main characteristics and funerary practices
Late Mesolithic	6200–5000	Tagus and Sado lower valleys and SW coast	Hunter-gatherer lifeways (imp. of marine/estuarine foods). Cemeteries in shell-middens. Individual burials.
Cardial/Initial Early Neolithic	5500–5100	Portuguese Estremadura and Western Algarve	Introduction of farming. First ceramics (Cardial complex). Cemeteries in caves and pits in open air sites. Individual burials.
Epicardial/Evolved Early Neolithic	5100–4500	Spread to inner and northern Portugal, and Galicia	Regionalisation of ceramic styles. Cemeteries in caves and pits in open air sites. Individual burials.
Initial Middle Neolithic	4500–3900	Occupation of the whole territory	Reduction in ceramic decoration. Cemeteries in caves and pits in open-air sites; proto-megalithic cists? Individual burials.
Middle Neolithic	3900–3300	Occupation of the whole territory	Plain, dolmenic ceramics. Cemeteries in caves, pits in open air sites, hypogea, and megaliths. Collective burials.

In sum, the above is the broad cultural framework that phenomena of change and continuity took place within, and it should be taken into account when explaining the population and demographic structures of the region's first megalith builders—i.e., the Middle Neolithic farmers (Table 1).

Over the last 20 years, the study of ancient human DNA from the last Mesolithic hunter-gatherers and the first Neolithic farmers has made major contributions to the understanding of the processes of biological interaction between them. The development of new high-throughput sequencing (HTS) technologies in the mid-2000s (Margulies et al., 2005) and the implementation of strategies to "capture" and enrich particular positions in the genome (Fu et al., 2015; Haak et al., 2015; Mathieson et al., 2015) have facilitated the recovery of thousands of phylogenetic informative positions from ancient genomes at affordable costs. Moreover, in 2014 it was first documented that one particular skeletal element, the *pars petrosa* of the temporal bone of the skull, is particularly resilient to DNA degradation (Gamba et al., 2014). Taken together, these developments have been a turning point in the study of the Mesolithic–Neolithic transition in Eurasia, leading to the sequencing of hundreds of genomes from different locations.

Mitochondrial DNA (mtDNA) and whole genome sequencing (WGS) ancient DNA studies have shown that the last hunter-gatherers and first farmers in Europe had a very different genetic make-up (Bramanti et al., 2009; Brandt et al., 2013; Haak et al., 2015; Mathieson et al., 2015; Lazaridis et al., 2016). Most of the ancestry (*ca.* 70–90%) of the first farming groups in Europe can be traced back to Anatolian Neolithic Farmers (ANF), thus indicating that a substantial amount of gene flow from the Near East into different parts of Europe took place in the early stages of the Neolithic (Lazaridis et al., 2016; Mathieson et al., 2018). The genomes of these early farmers also carry a signature of local Western Hunter-Gatherer ancestry (WHG), which argues for a certain degree of local admixture between incoming farmers and indigenous hunter-gatherer groups (Lipson et al., 2017). The proportion of WHG ancestry in Early European Farmers (EEF) varies depending on the region, the site, or even the studied individual. Overall, it has been found to be higher in Early Neolithic groups from the Danube Gorge in the Balkans and in Mediterranean groups associated with the Impresso-Cardial cultural complex (ICC) than in Central European groups associated with the Linearbandkeramik (LBK) cultural complex (Mathieson et al., 2018; Rivollat et al., 2020), but regional differences have also been observed.

In Iberia, the differences in WHG ancestry between early farmer groups from the East and the West are notable: while the few Cardial and Epicardial studied individuals from Eastern Iberia show a maximum of 10–20% WHG ancestry (Haak et al., 2015; Olalde et al., 2015, 2019; Villalba-Mouco et al., 2019), a recent work, still in pre-print (Allentoft et al., 2022), identified a 27–43% WHG ancestry in two Cardial Neolithic individuals from the cemetery cave of Caldeirão in Portugal, in agreement with Isern et al.'s (2017) modelling of the Iberian neolithization process. This pattern suggests different dynamics of interaction between foragers and farmers in each region. An increase in WHG ancestry is observed at a pan-European level during the Middle and Late Neolithic, several millennia after the establishment of the first farming groups in the continent, suggesting recurrent and sustained gene-flow with groups bearing high levels of hunter-gatherer ancestry (Brandt et al., 2013; Lipson et al., 2017). This "resurgence" of WHG ancestry is also present in individuals buried in megalithic structures (Sánchez-Quinto et al., 2019).

Despite its strategic location at the furthermost edge of the Neolithic expansion in continental Europe and the documented concentration of hunter-gatherer groups—particularly in the estuaries of the rivers Tagus and Sado—that coexisted with farming groups for up to 500 years, archaeogenetic data from Mesolithic and Neolithic human remains from Portugal remain rather limited. This article aims to fill this gap in knowledge by presenting ancient mtDNA evidence, mostly unpublished (but see Carvalho et al., 2016), retrieved from Mesolithic and Neolithic cemeteries located in the central and southern regions of Portugal (Fig. 1). The addition of this new dataset is crucial for a refined understanding of the nature of the biological relationships between the human groups that once inhabited this region, which, in turn, can provide information on more complex questions of human interaction patterns, demographic trends, and processes of technological transfer and economic shift.

The results we have obtained are discussed against the background of the available palaeogenetic evidence to assess their impact in current population models for western Iberia. While also discussing what happened in this region during the Early Neolithic, special emphasis will be put on the pattern of "genetic resurgence" of hunter-gatherer (Mesolithic) ancestry among the first megalith builders (Middle Neolithic).

2. Material and methods

2.1. Sampled Mesolithic and Neolithic cemetery-sites

The research presented in this work results from four projects successively carried out between 2006 and 2016 in Madrid, Manchester, Faro, Liverpool, and Durham (see acknowledgements below). Overall, 11 cemeteries in Portugal, individually described in the Supplementary Material and Methods, were sampled (Fig. 1): four Mesolithic shell-middens (Moita do Sebastião, Cabeço da Arruda, Arapouco, and Amoreiras), two Early Neolithic burial caves (Caldeirão and Picoto), and five Middle and/or Late Neolithic cemeteries (the caves of Barrão, Casais da Mureta, Bom Santo, and Correio-Mor, a megalithic tomb, the Dolmen 4 of Cabeceira, and the burial-pits of Castelo Belinho). As can be seen, this sample spans all Mesolithic–Neolithic cultural stages and encompasses all types of funerary sites used throughout.

For ancient DNA analysis, 91 skeletal samples were selected from 52 individuals spread across a >3000-year transect, from the Mesolithic to the Late Neolithic (Table 2). This sample spans all the cultural stages of the interval and encompasses all the types of funerary sites used throughout.

Regarding the representativity of the sampled individuals, it should be emphasized that the use of human samples from burial caves in the limestone massifs of Estremadura (where human remains are well preserved) to make inferences on populations from megaliths located in the acidic granite and schistose soils of Alentejo (where organic matter is seldomly preserved) is appropriate. This is because these Middle and Late Neolithic populations were coeval and shared the same material culture, rituals, and funerary practices.

2.2. Ancient DNA procedures

All samples were documented photographically on arrival. Ancient DNA analyses were conducted in four different aDNA laboratories at Liverpool John Moores University, UK (LJMU), Durham University, UK (DU), Complutense University of Madrid, Spain (UCM) and the Manchester Institute of Biotechnology, UK (MIB).

Samples were externally cleaned, UVed for 30 min per side and either ground in a freezer mill or sampled with a drill. Ancient DNA was extracted from 250 to 500 mg of tooth/bone powder with a modification of the protocols of Rohland and Hofreiter (2007); Rohland et al. (2010) or from 50 mg of tooth/bone powder according to Dabney et al. (2013). One extraction blank without powdered sample for every seven samples was processed in parallel to detect contamination during the extraction process. A fragment of 305 base pairs (bp) (np 16,095–16,399) of the mtDNA Hypervariable Region I (mtDNA-HVRI) was amplified in two overlapping fragments following Fernández et al. (2014). Specific coding-region haplogroup diagnostic SNPs were also amplified according to Fernández et al. (2014) (see Supplementary Material and Methods). PCR reactions were set up in a final volume of 25 μl using the Multiplex PCR Kit (Qiagen) (5 μl of DNA extract, 1X Multiplex PCR Kit (Qiagen) and 0.2 μM of each outer primer). Cycling conditions consisted of 40 cycles of 30 s at 95 °C, 90 s at 54 °C and 60 s at 72 °C, with a previous activation cycle of 15 min at 95 °C and a final extension cycle of 10 min at 72 °C. Amplicons were visualized in a 2% agarose gel stained with GelRed and purification was performed directly from the

Table 2
Sampled cemeteries and analysed individuals. For location in map, see Fig. 1. For project full name and funding body, see acknowledgements.

Site	Region and municipality	Site type	Period	Project acronym	Date (cal BC)	N Ind	N samples
Caldeirão	Estremadura, Tomar	Cave	Cardial Neolithic	NeolithicGenes	5500–5200	2	3
Picoto	Estremadura, Torres Novas	Cave	Epicardial Neolithic	NeolithicGenes	*ca.* 5000	2	6
Barrão	Estremadura, Alcanena	Cave	Middle Neolithic	mDNA	3700–3100	3	6
Casais da Mureta	Estremadura, Alcanena	Cave	Middle Neolithic	mDNA	3220–3000	3	6
Moita do Sebastião	Tagus Valley (Muge), Salvaterra de Magos	Shell-midden	Late Mesolithic	NeolithicGenes	5900–5700	10	16
Cabeco da Arruda	Tagus Valley (Muge), Salvaterra de Magos	Shell-midden	Late Mesolithic	NeolithicGenes	6100–5000	1	1
Bom Santo	Estremadura, Alenquer	Cave	Middle Neolithic	ABS	3800–3400	14	29
Dolmen 4 of Cabeceira	Alentejo, Mora	Dolmen	Middle Neolithic	mDNA	3650–3500	1	2
Correio-Mor	Estremadura, Loures	Cave	Late Neolithic	mDNA	3200–2900	2	2
Arapouco	Sado Valley, Alcácer do Sal	Shell-midden	Late Mesolithic	MesolithicGenes	*ca.* 6000	1	1
Amoreiras	Sado Valley, Alcácer do Sal	Shell-midden	Late Mesolithic	MesolithicGenes	6150–5300	4	5
Castelo Belinho	Algarve, Silves	Burial-pits	Initial Middle Neolithic	mDNA	4500–4100	8	14

amplification reaction using the Qiagen PCR purification Kit or the Nucleospin® Gel and PCR Clean-up Kit according to the manufacturer's instructions. Purified PCR products were sequenced in an ABI Prism 3700 Genetic Analyzer (Applied Biosystems, Life Technologies).

Amplifications yielding reproducible HVRI profiles from at least two independent extractions of the same individual were cloned using the TOPO-TA Cloning kit K4575-01 (Invitrogen) following the manufacturer's instructions. Between 10 and 20 clones with insert were selected and DNA was directly purified from the bacterial colonies using the Jetquick Plasmid Miniprep Spin Kit (Genycell), the Qiagen Miniprep Kit (Qiagen) or the Nucleospin Plasmid Miniprep Kit (Fisher Scientific). Cloned DNA was sequenced with universal primer SP6 or T7 as described above.

Strict ancient DNA criteria of authenticity were observed during all the experimental process. These included, among others, the use of dedicated aDNA facilities, the intra and inter-laboratory replication of extraction and amplification procedures, the bacterial cloning of PCR amplifications and sequencing of multiple clones, the identification of characteristic aDNA damage patterns and the disclosure of all potential sources of contamination. A detailed description of the laboratory facilities and validation procedures used can be found in Supplementary Material and Methods. Clone sequence alignments are presented in Table S1.

Direct and clone sequences were aligned to the revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999) (Table S1) and differences were computed using Mutation Surveyor (Demo version 3.24, SoftGenetics, LLC) and/or Sequencher (Gene Codes Corporation). Consensus haplotypes were established from clone alignments as follows: *i)* Only reproducible haplotypes from independent extractions and amplifications, ideally from separate samples, were considered authentic endogenous sequences; *ii)* The complete haplotype should make phylogenetic sense; and *iii)* Miscoding lesions identified by non-replication in the cloned sequences were discounted (Fernández et al., 2014). Mitochondrial haplogroup prediction was based on diagnostic SNPs in the HVRI and coding mtDNA regions according to the rCRS oriented version of Phylotree Build 17. Haplogroup assignment was conducted with Haplogrep vs. 2 (van Oven and Kayser, 2009; Kloss-Brandstätter et al., 2011).

A database containing mtDNA and Y chromosome data retrieved from Mesolithic to Late Neolithic/Chalcolithic Iberian sites, published in peer-reviewed journals or pre-print archives and following standard methods of authentication, was built for comparative and interpretative purposes (Table S3). Only data with reported coverage >10 was used, if known. The data was organised in population groups using chronological and geographical criteria and our recovered mtDNA data was merged with published mtDNA data.

3. Results and discussion

Reproducible mtDNA HVRI profiles could be obtained from 23 individuals from six different archaeological sites, covering a >3000-year time transect spanning the Late Mesolithic to Late Neolithic interval (Table 3). From these, 17 cover a fragment of 244 bp (positions 16126–16379) and the remaining seven shorter fragments of 133bp (positions 16126–16261) or 148bp (16232–16379). Sequence alignments of cloned amplicons presented in Table S1 display characteristic *post-mortem* aDNA modifications, mainly recognisable as an excess of C-T/G-A resulting from cytosine deamination. Other described aDNA miscoding lesions were also detected (Gilbert et al., 2003, 2007). This pattern, alongside the reproducibility of the reported haplotypes across different samples, extractions, and PCRs, some of them performed in different laboratories, strengthens the authenticity of the recovered mtDNA profiles.

Using, in most cases, a combination of HVRI and mtDNA coding region SNPs, it was possible to assign 21 out of these 23 individuals to mtDNA haplogroups, with high confidence, i.e., with Haplogrep scores higher than 0.8 (Table 3 and Table S2). Haplogroup assignment for the mtDNA profiles from the Middle Neolithic necropolis of Bom Santo cave, previously reported in Carvalho et al. (2016), was refined here using coding-region SNPs. Further details about haplogroup assignment can

Table 3
Reproducible mtDNA HVRI profiles and mtDNA haplogroup assignments. Haplotypes shared between individuals from this dataset highlighted in bold, italics or underlined.

Period	Site	Individual	Recovered positions	Haplotype HVRI	Coding region SNPs	Haplogroup
Late Mesolithic	Moita do Sebastião	MS2	16126–16379	16192T 16270T 16355T	–	U5
Late Mesolithic	Moita do Sebastião	MS4	16126–16261	16195C	–	–
Late Mesolithic	Moita do Sebastião	MS7	16126–16261	CRS	–	–
Late Mesolithic	Moita do Sebastião	MS17	16126–16379	***16270T 16311C***	7028T	U5b1d1a
Late Mesolithic	Amoreiras	AM6	16126–16379	**16270T**	–	U5b/H1ba
Late Mesolithic	Amoreiras	AM7	16126–16379	16189C 16270T	–	U5b1b1+@16192
Late Mesolithic	Amoreiras	AM8	16252–16379	**16270T**	–	U5b/H1ba
Cardial Neolithic	Caldeirão	CA7	16126–16379	16304C	7028T 14766T	HV
Epicardial Neolithic	Picoto	2AL	16126–16379	16223T 16362C	7028T 10398A, 10400C 12378T	N
Middle Neolithic	Barrão	BAR3	16252–16379	**16270T**	3197C 7028T 10873T 12308G	U5a′b
Middle Neolithic	Barrão	BAR4	16126–16379	16129A 16224C 16311C	–	K1a11
Middle Neolithic	Barrão	BAR5	16252–16379	***16270T 16311C***	–	U5b1c
Middle Neolithic	Bom Santo	BS1	16126–16379	16270T 16296T	3197C	U5b2b5
Middle Neolithic	Bom Santo	BS2	16126–16379	16126C 16294T 16304C	–	T2b
Middle Neolithic	Bom Santo	BS4	16126–16379	16126C	13708A	J
Middle Neolithic	Bom Santo	BS6	16252–16379	16298C	–	HV0
Middle Neolithic	Bom Santo	BS7	16126–16261	16221T	–	H7b1*
Middle Neolithic	Bom Santo	BS9	16126–16379	16224C 16311C	–	K1a2a1
Middle Neolithic	Bom Santo	BS10	16126–16379	16126C 16196A 16259T	13708A	J
Middle Neolithic	Bom Santo	BS12	16126–16379	16239T 16292T	–	H1+16239
Middle Neolithic	Bom Santo	BS14	16126–16379	16256T 16270T	–	U5a1+@16192
Late Neolithic	Correio-Mor	1 GC	16126–16379	16129C 16183C 16189C 16270T 16311C	3197C	U5b2a5
Late Neolithic	Correio-Mor	2 GC	16126–16379	16224C 16311C	10238T 10550G 11947A	K

be found in Table S2.

The five studied Mesolithic individuals in which it was possible to confidently determine a mtDNA haplogroup belong to haplogroup U5, the predominant lineage in western European Mesolithic groups (Table S3; Posth et al., 2016). Among these, three individuals could be sub-classified, and all belonged to subgroup U5b, the only branch of U5 that has been detected to date in Iberia (Table S3; Sánchez-Quinto et al., 2012; Olalde et al., 2019; Villalba-Mouco et al., 2019). In contrast, the two Early Neolithic individuals from Caldeirão and Picoto were assigned to major haplogroup branches HV and N, both previously detected among Anatolian, Levantine and European farmers (Brandt et al., 2013; Fernández et al., 2014; Szécsényi-Nagy et al., 2015; Feldman et al., 2019). Sub-groups within these major branches could not be resolved with the examined mtDNA positions. Middle and Late Neolithic studied samples exhibit a mixture of typically pre-Neolithic (U5) and Near Eastern Neolithic (K, T, J, H) haplogroups. An increase in hunter-gatherer ancestry from the Middle Neolithic onwards, peaking during the Late Neolithic, is documented elsewhere in Europe, where it has been described as a “resurgence” (Lipson et al., 2017).

As for the rest of Iberia and most of western Europe, our results for Moita do Sebastião and Amoreiras (Fig. 1A) confirm that mtDNA haplogroup U5 was also prevalent among Late Mesolithic Portuguese groups, expanding the available Portuguese Mesolithic mtDNA haplogroup data from 1 to 6 individuals (Table S3).

Genetic data from Portuguese Early Neolithic groups is also scarce, and limited until now to four individuals, from Caldeirão and Galeria da Cisterna, belonging to mtDNA haplogroups J2, T2, H3 and H4 (Olalde et al., 2015; Allentoft et al., 2022). Our study adds two extra data points to this time transect, with an additional individual from Caldeirão (mtDNA haplogroup HV) and a new one from Picoto (mtDNA haplogroup N) (Table 3 and Fig. 1A). When considering our mtDNA data in conjunction with previously published mtDNA datasets from Iberia, it becomes evident that with the arrival of the Neolithic to the Peninsula, mtDNA Mesolithic haplogroups were replaced by new ones, non-local and characteristic of Neolithic Near Eastern populations. While in Portugal there is no evidence of mtDNA continuity between the Mesolithic and the Neolithic, probably due to the small available sample size from both periods, in Spain the replacement was not complete as indicated by the presence of mtDNA haplogroups U, U4 and U5 in a small percentage (8.5%) of Early Neolithic individuals (Table 4 and Fig. 2) from north-eastern and central Spain (Can Sadurní and Avellaner caves in Catalonia, Chaves cave in Aragón, and Fuente Celada in Burgos) (Table S3).

Previously published Y chromosome data from Portugal provide, however, evidence of Mesolithic-Neolithic continuity through the paternal line. Despite carrying typically “farmer” mtDNA haplogroups, the Y chromosome of two individuals from the Cardial Neolithic site of Caldeirão studied by Allentoft et al. (2022) belong to haplogroup I2a, characteristic of pre-Neolithic populations. Whole genome analysis of these two individuals also revealed that they harboured respectively 27% and 43% hunter-gatherer ancestry of Iberian origin (Allentoft et al., 2022: extended data figure D and supplementary Table XII), thus suggesting that the recent ancestors of the Caldeirão individuals mixed with pre-farming populations from the area. Even though the sources for hunter-gatherer ancestry used in the publication are from Spain, it is sensible, considering the archaeological evidence for a centuries-long co-existence of foragers and farmers in central Portugal, to assume that the inferred population mixing may have occurred locally.

The difference between the Early Neolithic ancestry patterns revealed by mtDNA and Y chromosome is also apparent in Spain, where mtDNA haplogroups can be mostly traced back to the Near East but one third (3 out of 9) of the Y chromosome haplogroups are typical of hunter-gatherers (lineage I2a). However, contrasting with the two Early Neolithic individuals from Caldeirão in Portugal, the available genome-wide data from Early Neolithic individuals from Spain show a low-level signature of admixture with hunter-gatherers (Olalde et al., 2015, 2019; Villalba-Mouco et al., 2019).

The Middle Neolithic period is represented in our dataset by 11 individuals from the funerary cave sites of Barrão and Bom Santo (Fig. 1B), which almost doubles the number of previously available individuals with DNA data from this period in Portugal (Table S3). A preliminary genetic and isotopic analysis undertaken on a subset of individuals from Bom Santo showed a high degree of mtDNA haplotype and haplogroup diversity (Carvalho et al., 2016). This heterogeneity in mtDNA lineages is also observed in other Middle Neolithic European groups across Europe. Regardless of their funerary practices and architectures (in caves or in megalithic buildings) these groups show a mixture of typically farmer and hunter-gatherer lineages. At whole genome level, the first megalith builders are also indistinguishable from other non-megalithic contemporaneous groups (Sánchez-Quinto et al., 2019). On the Y chromosome though, European megalithic groups are very homogeneous and show recurrent haplotypes suggestive of a patrilineal kin organisation (Sánchez-Quinto et al., 2019; Cassidy et al., 2020). Unfortunately, the lack of DNA preservation in the only megalithic and burial-pit sites examined in this study (Dolmen 4 of Cabeceira and Castelo Belinho, respectively), and the lack of Y chromosome results in Barrão and Bom Santo caves, prevent us from making further inferences regarding the period’s genomic composition and Y chromosome lineage organisation.

When our Early and Middle Neolithic Portuguese mtDNA results are combined with previously published data for these time periods in Iberia, an increase in the frequency (*ca.* 30%) of maternal hunter-gatherer lineages in the transition from the Early to the Middle Neolithic (*ca.* 3900 BC), peaking at *ca.* 3900–3500 BC, can be observed. This interval coincides with the consensus chronology for the onset of megalithism along western Iberian regions (Schulz-Paulsson, 2019). In contrast, pre-Neolithic Y chromosome lineages, I2a in particular, seem to be prevalent in Iberia through the whole of the Neolithic, with an average frequency of 40–60% according to published data (Fig. 2 and Table 3). This persistence of hunter-gatherer ancestry inferred from uniparental markers is also evident in whole genome analyses, with Middle Neolithic populations from Iberia and elsewhere in Europe falling in a cline of admixture between ANF and WHG (Lipson et al., 2017; Martiniano et al., 2017; Olalde et al., 2019).

The steep increase in hunter-gatherer ancestry observed elsewhere in Europe during the Middle Neolithic has been explained as a result of sustained gene-flow after the initial contact between hunter-gatherers and farmers, suggesting that this type of genetic ancestry was preserved in different regions even after the disappearance of hunter-gatherer groups (Lipson et al., 2017; Villalba-Mouco et al., 2019). It has been also suggested, based on differences in WHG and EAF ancestry proportions in the X chromosome, that there was a sex bias in the direction of gene flow during the latest stages of the Neolithic, and that males mainly drove the resurgence of hunter-gatherer ancestry, particularly in Central Europe and Iberia (Mathieson et al., 2018). This agrees with the higher proportion of characteristic hunter-gatherer Y chromosome lineages observed in both Portugal and Spain during the Middle and the Late Neolithic.

The widespread nature of the resurgence of hunter-gatherer ancestry in Europe and the differential role that males and females could have played on it is poorly understood. In western Iberia, no direct correlations between subsistence and ancestry could be established in Middle Neolithic populations. Quite the contrary, individuals bearing mitochondrial haplogroup U5 (Table 3) show variable isotopic signatures that all remain consistent with a farming-dependent subsistence. This can be seen for example in the percentage of aquatic food sources estimated for individuals BAR3 (15%) and BAR5 (8%) from Barrão, and BS1 (7%) and BS14 (42%) from Bom Santo (for complete isotopic data, see Carvalho et al., 2019: Table 3).

A possible explanation for western Iberia is that hunter-gatherer groups were culturally assimilated during the Cardial and Epicardial stages of the Neolithic (Table 1)—not only through the adoption of the

Table 4

MtDNA and Y chromosome haplogroup frequencies in Iberia by chronological interval (years cal BC) calculated by merging our results with published data (Table S3). Sample size (N) is indicated at the bottom of each table section.

	Hgs	Portugal							Spain								Iberia total
		11-6 BC	6-4.3 BC	4.5-3.9 BC	3.9-3.5 BC	3.5-3 BC	3-2.5 BC	Total	17-11 BC	11-6 BC	6-4.3 BC	4.5-3.9 BC	3.9-3.5 BC	3.5-3 BC	3-2.5 BC	Total	
mtDNA	H	0.00	28.57	25.00	14.29	26.32	9.09	**17.11**	0.00	0.00	14.89	0.00	12.28	32.43	23.03	21.57	**20.85**
	HV	0.00	14.29	0.00	0.00	5.26	0.00	**2.63**	0.00	0.00	0.00	0.00	1.75	0.00	0.00	0.25	**0.64**
	HV0	0.00	0.00	0.00	7.14	5.26	9.09	**5.26**	0.00	0.00	6.38	0.00	7.02	0.90	4.61	3.81	**4.04**
	I	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	0.00	0.90	0.00	0.25	**0.21**
	J	0.00	14.29	25.00	14.29	10.53	22.73	**15.79**	0.00	0.00	10.64	17.65	8.77	19.82	20.39	16.75	**16.60**
	K	0.00	14.29	25.00	28.57	21.05	36.36	**25.00**	0.00	0.00	29.79	29.41	26.32	18.92	26.32	24.11	**24.26**
	N*	0.00	14.29	0.00	0.00	0.00	0.00	**1.32**	0.00	0.00	8.51	0.00	0.00	0.00	0.00	1.02	**1.06**
	N1	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	4.26	0.00	0.00	0.00	0.00	0.51	**0.43**
	T	0.00	14.29	12.50	7.14	15.79	4.55	**9.21**	0.00	0.00	10.64	11.76	12.28	5.41	7.24	7.87	**8.09**
	U	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	2.13	0.00	0.00	10.81	0.00	3.30	**2.77**
	U2′3′4′7′8′9′	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	11.11	0.00	0.00	0.00	0.00	0.00	0.25	**0.21**
	U2	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	1.75	0.00	0.00	0.25	**0.21**
	U3	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	11.76	1.75	0.00	1.32	1.27	**1.06**
	U4	0.00	0.00	0.00	7.14	0.00	0.00	**1.32**	0.00	0.00	2.13	0.00	1.75	0.90	0.66	1.02	**1.06**
	U5	100.00	0.00	12.50	21.43	15.79	18.18	**22.37**	100.00	88.89	4.26	17.65	17.54	6.31	13.16	12.94	**14.47**
	W	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	0.00	0.90	0.00	0.25	**0.21**
	X	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	6.38	11.76	8.77	2.70	3.29	4.57	**3.83**
	N	**6**	**7**	**8**	**14**	**19**	**22**	**76**	**1**	**9**	**47**	**17**	**57**	**111**	**152**	**394**	**470**
Y Chromosome	C1a	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	28.57	0.00	0.00	0.00	0.00	0.00	2.02	1.79	**28.57**
	CT	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	14.29	0.00	0.00	0.00	0.00	0.00	1.01	0.89	**14.29**
	F	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	14.29	0.00	0.00	0.00	0.00	3.39	3.03	2.68	**14.29**
	G2a	0.00	0.00	0.00	0.00	16.67	0.00	**7.69**	0.00	33.33	0.00	0.00	13.33	18.64	16.16	15.18	**0.00**
	GHIJK	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00	0.00	0.00	1.69	1.01	0.89	**0.00**
	H2	0.00	0.00	0.00	0.00	16.67	0.00	**7.69**	0.00	0.00	57.14	0.00	13.33	11.86	13.13	12.50	**0.00**
	I	0.00	0.00	0.00	0.00	0.00	33.33	**7.69**	14.29	0.00	0.00	0.00	0.00	3.39	3.03	3.57	**14.29**
	I2	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	14.29	0.00	0.00	0.00	0.00	5.08	4.04	3.57	**14.29**
	I2a	0.00	100.00	100.00	100.00	50.00	66.67	**69.23**	0.00	33.33	42.86	100.00	66.67	52.54	49.49	51.79	**0.00**
	IJ	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	14.29	0.00	0.00	0.00	0.00	1.69	2.02	1.79	**14.29**
	J2	0.00	0.00	0.00	0.00	16.67	0.00	**7.69**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.89	**0.00**
	R1b	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	33.33	0.00	0.00	6.67	1.69	5.05	4.46	**0.00**
	N	**0**	**2**	**1**	**1**	**6**	**3**	**13**	**0**	**7**	**9**	**7**	**2**	**15**	**59**	**99**	**112**

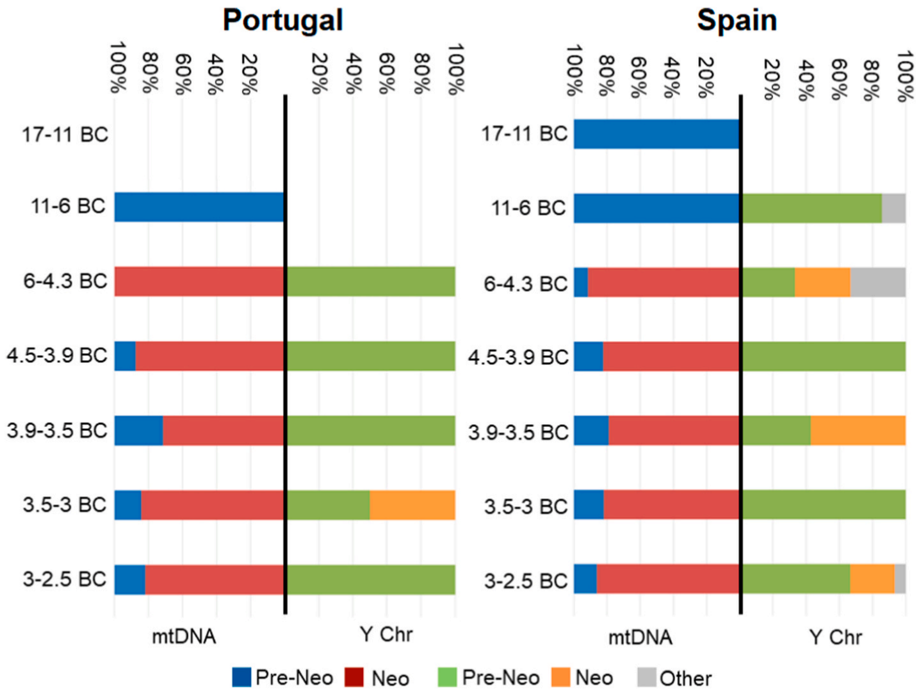

Fig. 2. Percentage of mtDNA and Y chromosome haplogroups of pre-Neolithic (local), Neolithic (imported from the Near East) and unknown/other origin in the time transects studied in Portugal and Spain. Pre-Neolithic mtDNA haplogroups: U, U4, U5; Neolithic mtDNA haglogroups: H, HV, J, K, N, T, U3, W, X; Pre-Neolithic Y chromosome haplogroups: C1, CT, I, I2, IJ; Neolithic Y chromosome haplogroups: G2a, H2, J2; other Y chromosome haplogroups: F, G, H, I, J, K, R1b. Dates in Kyrs BC.

new subsistence practices but also through social reorganisation and acquisition of new symbolic behaviours and worldviews—while maintaining a distinctive ancestry profile through enduring endogamic practices. If a demographic imbalance in favour of local hunter-gatherers and a population "boom" event after the introduction of farming—as simulation models predict for the Early Neolithic (Balsera et al., 2015; Drake et al., 2016; Pardo-Gordó and Carvalho, 2020)—are taken in consideration, these facts would explain the dominance of WHG ancestry thereafter. Consequently, patrilocality rules (which are presumed to have been the norm during the Neolithic) might also explain the above-mentioned prevalence of Mesolithic Y DNA in later Neolithic phases. It is important to note, though, that additional hunter-gatherer genetic contribution of non-local origin across the Neolithic cannot be discarded. Unfortunately, a distinction between local and non-local sources of hunter-gatherer ancestry in this region cannot be achieved with current available data.

If confirmed by future research, the persistence of endogamic practices could have been possible only under some form of political autonomy or autarchy within larger social units. A number of studies have suggested that such a type of organisation was common in Neolithic societies (e.g., Renfrew, 1973). In central-southern Portugal this explanation is supported by the observation that two main levels of social structuration can be observed among megalith builders. At the socio-economic level, the existence of several politically and economically independent small-scale human groups (or "segments") settled in distinct ecological-geographical environments has been inferred from isotopic studies of subsistence and mobility (Carvalho et al., 2019); at a broader scale, the recurrence of homogeneous material cultures and a shared system of values visible in common funerary practices and rituals (of megalithic type) shows a strong cultural, supra-regional unity (e.g., Cardoso, 2007; Carvalho, 2016). In short, a segment-based social organisation combined with shared political and ideological traits bringing together communities dispersed across a wider geography is, in our view, the general scenario within which the observed diachronic genetic patterns must be understood.

4. Conclusions

In articulation with palaeogenetic datasets available in the literature for the whole of Iberia, some only recently obtained, the mitochondrial aDNA we retrieved from Portuguese Mesolithic and Neolithic populations (Table 3) suggests a three-stage process of population change starting with the last hunter-gatherers. Though most genetic data have been obtained from cemetery sites located in central-southern Portugal, data from contiguous regions (Fig. 1) and elsewhere in Iberia suggest that the events we detected reflect processes developing across the entire western façade of the peninsula. This exercise provides therefore a population/demographic background model for the historical-cultural shifts that took place in the region during the *ca.* 5500–3000 BC time interval (Table 1).

Although still scarce, this evidence can be summarized as follows:

i) Local Mesolithic hunter-gatherers show a remarkable mtDNA homogeneity, in which U5-derived mitochondrial haplogroups are exclusive. This is found in the two main core areas of Mesolithic settlement in the country—Muge and Sado (Fig. 1A)—and further confirms similar conclusions previously reached elsewhere in Iberia. This is in good accord with the long-acknowledged cultural homogeneity that globally characterizes the peninsula's last hunter-gatherer communities.

ii) The arrival of farming lifeways to western Iberia is due to a migration process that eventually triggers the neolithization of the entire region. The earliest well-documented entry point of new human populations is the middle and upper sectors of Portuguese Estremadura (Fig. 1A), resulting in the presence of HV, J2, T2, N and H mitochondrial haplogroups at Caldeirão, Picoto, and Galeria da Cisterna (this study; Olalde et al., 2015; Allentoft et al., 2022). Two of the Caldeirão individuals show 27−43% of local Iberian WHG ancestry. The limited number of analysed Cardial samples does not allow a finer characterisation of the exact location and time of admixture, though local admixture of the immediate ancestors of this group seems highly likely given that the Caldeirão individuals (the earliest of which is dated to 5477–5364 BC, 2σ calibrated interval; see Zilhão, 2021) are among the country's first farmers.

iii) Unlike seen among Early Neolithic individuals, no evidence for exogenous genetic inputs is observed around 3500–4000 BC, among the earliest megalith builders. A significant percentage of typically WHG haplogroups is visible in the number of analysed individuals from Barrão and Bom Santo belonging to U5-derived mitochondrial haplogroups (known in Early Neolithic Spain but so far undetected in coeval individuals from Portugal) (Table 3). Though these data were obtained from cave sites in the limestone massifs of Estremadura, not from dolmens (due to taphonomic limitations derived from the latter's location in acidic soils), current archaeological evidence supports that we are dealing with the same populational pool. Explanations for this pattern—which, in western Iberia, would seem to be better described as one of persistence rather than resurgence—can tentatively be sought on these communities' social organisation, of segmentary type, along with population growth (see discussion above).

Regardless of which factors are responsible for the Middle Neolithic pattern, the fact is that views proposing a direct derivation of megalithic builders from hunter-gatherer groups lack empirical support, not only chronometric but also genetic—even in the case of proto-megalithic contexts (Fig. 1B), as recently demonstrated by the evidence from the Campo de Hockey necropolis, Cádiz (Vijande et al., 2022). Population admixture processes clearly took place in western Iberia during the Early Neolithic, prior to the advent of megalith-building traditions.

Author contributions

António F. Carvalho: Project direction, funding acquisition, sample supply, conceptualization, investigation, writing. **Eva Fernández-Domínguez**: Conceptualization, funding acquisition. investigation, formal analysis, writing. **Eduardo Arroyo-Pardo**: Project direction, funding acquisition, draft revision. **Catherine Robinson**: Formal analysis. **João L. Cardoso**, **João Zilhão**, and **Mário V. Gomes**: Sample supply, draft revision.

Data availability

Detailed datasets, supplementary methods, and short descriptions (with main references) of the analysed archaeological sites may be found in the Supplementary Material and Methods.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.quaint.2023.03.015.

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