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High frequency of mitochondrial haplogroups H and H2 in medieval individuals from the Cathedral of Santa María (Basque Country). Their contribution to the study of inflammatory arthropathies

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ABSTRACT

The haplogroup H has been associated with high mitochondrial energy efficiency, which may have favoured survival and reproduction under adverse environmental conditions. On the other hand, it enhances the production of Reactive Oxygen Species (ROS), which are involved in the pathogenesis of several arthropathies, especially those of inflammatory origin (IAs). In the present study, we analysed mtDNA haplogroups H and H2 in a sample composed of 55 individuals recovered from the Cathedral of Santa María (Basque Country, 12th-18th centuries) including both individuals with arthropathies (N = 31) and a control group (N = 24). The obtained results showed a particularly high frequency of haplogroup H (64.5%) compared with other present and ancient populations from Northern Spain. This frequency was higher (83.3%) in individuals with Inflammatory Arthropathies (IAs). A relevant relationship was found between sub-haplogroup H2 and Spondyloarthropathies (SpAs), which is a type of Inflammatory Arthropathy (IA) that affects the axial skeleton. This relationship indicates that the H2 individuals present a greater tendency toward developing SpAs, which allows suggesting the influence of this sub-haplogroup on the etiopathogenesis of this type of diseases. The present study demonstrates that the application of a simple and straightforward methodology of aDNA analysis can be used to improve the knowledge on ancient populations, in this case the dwellers of Vitoria-Gasteiz during the Middle Ages. This approach requires taking into account the historical context, due to its important influence on the health of the population, especially when considering pathologies with a strong environmental background, such as IAs.

1. Introduction

Throughout evolution, many Single Nucleotide Polymorphisms (SNPs) have accumulated sequentially in the mtDNA genome. Specific combinations of these polymorphisms in the mitogenome define human mitochondrial haplogroups (Torroni et al., 1996), which have been widely used to elucidate past human migrations and reconstruct human history, due to their geographical specificity at the continental level (García et al., 2011; Hervella et al., 2015; Richards et al., 2000). However, many studies have clearly shown that some polymorphisms defining mtDNA haplogroups are not phenotypically neutral and have been epidemiologically associated with different diseases (Chinnery and Gómez-Durán, 2018; Herrnstadt and Howell, 2004), including arthropathies (Blanco et al., 2011; Rego-Pérez et al., 2008). Therefore, some mtDNA haplogroups have been proposed as useful biomarkers for the diagnosis and prognosis of Osteoarthritis (OA) (Rego-Pérez et al., 2020, 2008; Soto-Hermida et al., 2014).

According to published data, depending on the mitochondrial haplogroup, a different effect on susceptibility to certain diseases has been described; this may be explained by biochemical differences between haplogroups, which present dissimilar bioenergetic capacities and coupling efficiencies (Gómez-Durán et al., 2010). It has been suggested that these differences are probably the result of a process of selective adaptation to colder climates when *Homo sapiens* migrated out of Africa (Mishmar et al., 2003; Ruiz-Pesini et al., 2004). In this respect, it has been proposed that the geographic distribution of mtDNA haplogroups in current Europe is the result of a selection process driven by adaptation not only to climate, but also to nutritional changes based on food or caloric supply (Tranah et al., 2011; Wallace, 2005; Wallace et al., 2003). Specifically, haplogroup H is highly efficient at converting dietary

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calories into ATP with minimal heat release, which may have favoured survival and reproduction under climate change and food scarcity (Wallace, 2013). However, while haplogroup H could have been advantageous in adverse environmental conditions (Wallace, 2005), the increased Reactive Oxygen Species (ROS) production could lead to a higher prevalence of several arthropathies (Filippin et al., 2008; Rego-Pérez et al., 2008; Solmaz et al., 2016).

Some studies suggest that ROS and other free radicals that are produced during mitochondrial oxidative metabolism (mROS) are involved in the pathogenesis of inflammatory chronic arthropathies (Escames et al., 2012), enhancing inflammatory response through the alteration of several cellular processes and metabolic routes (Cillero-Pastor et al., 2008; Vaamonde-García et al., 2012). This is due to their role as signalling molecules that participate in important homeostatic pathways, including innate immunity (Shadel and Horvath, 2015). On the contrary, some haplogroups, such as T and J, have been associated with a protective effect, due to their higher capacity to cope with oxidative stress (Mueller et al., 2012), which results in a decrease in ATP generation, but also in a lower production of ROS and oxidative damage (Castro et al., 2007; Coto-Segura et al., 2012).

Although many epidemiological studies in current populations have established a relation between some mitochondrial haplogroups and joint diseases, research in ancient populations is scarce, with most studies focusing on associations with certain nuclear markers, such as Ankylosing Spondylitis (AS) and *HLA-B27* (Haak et al., 2005; Laza et al., 2016; Leden et al., 2009). Therefore, the study performed in a medieval necropolis in the Basque Country (San Miguel de Ereñozar, Spain, 13th-16th centuries) was particularly relevant (Laza et al., 2019), since it established a relation between some arthropathies and mitochondrial haplogroups and, particularly, between Spondyloarthropathies (SpAs) and haplogroup H.

According to Laza et al., (2019), the climate changes that occurred during the Little Ice Age (LIA; 14th-19th centuries) gave some advantage to haplogroup H carriers, due to its greater energy efficiency, which allowed them to endure lower temperatures and other adverse events that affected food availability. These factors were considered in the present study about the population of Vitoria-Gasteiz recovered from the Cathedral of Santa María (Basque Country, 12th-18th centuries), where a high prevalence of skeletal pathologies, such as rickets, was found (Ventades et al., 2020). That high prevalence of rickets was related to several adverse events, some of which involved crop failures and food shortages (e.g., famines, conflicts, LIA, epidemics).

In this context, in the present study of the Cathedral of Santa María, we evaluated the frequency of mitochondrial haplogroups H and H2 in a

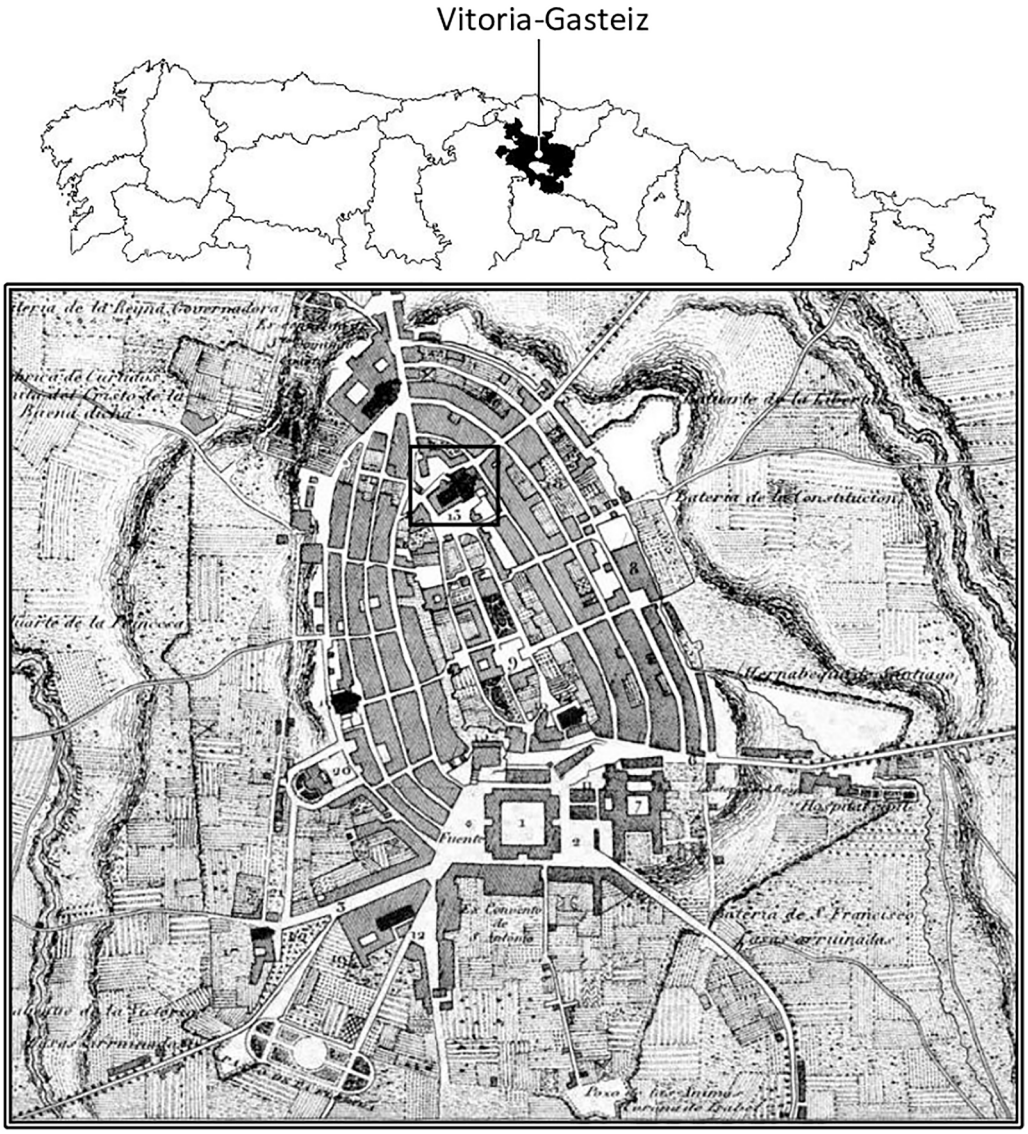

Fig. 1. Location of Vitoria-Gasteiz and the temple of Santa María on a map of the city from 1846 modified from Galarraga (1996).

sample of individuals suffering from arthropathies, especially those of inflammatory origin, given the relation proposed between these haplogroups and joint diseases (Laza et al., 2019; Rego-Pérez et al., 2020, 2008; Soto-Hermida et al., 2014).

2. Materials and methods

The human bone remains analysed in this study were recovered from the Cathedral of Santa María (12th-18th centuries), a catholic temple located in Vitoria-Gasteiz, a city in Northern Spain (Basque Country) (Fig. 1). Archaeological works recovered around 2,200 individuals in different states of conservation. Skeletons with an acceptable state of conservation, at least those that preserved important skeletal parts to allow diagnosing joint diseases, such as the axial skeleton and/or the limbs (27% approx.; 590 individuals), were selected for palaeopathological analysis. The diagnosis of arthropathies was established through macroscopic examination based on previously described features commonly associated with arthropathies (Rogers et al., 1987; Ventades et al., 2018).

Arthropathies constitute a complex group of pathologies with different origin (e.g., inflammatory, degenerative, neurological, endocrine). Our study was focused on Inflammatory Arthropathies (IAs), which include, among others, Spondyloarthropathies (SpAs) and Rheumatoid Arthritis (RA). The palaeopathological analysis allowed identifying 31 individuals with joint diseases, 12 of whom had an Inflammatory Arthropathy (IA), which means a prevalence of 2.03% (12/590) in the sample. In addition, degenerative arthropathies (e.g., Osteoarthritis and DISH) were also analysed for comparative purposes, since they do not have an inflammatory origin, thus another sample of equal size was gathered (non-IA; N = 12). Some individuals with arthropathies that were difficult to classify within the IA/non-IA subgroups (N = 7) were also included. In total, 31 individuals with joint diseases were analysed in this study, giving a prevalence of 5.25% (31/590). A "control group" (N = 24) was also selected according to the following criteria: (1) absence of pathological manifestations, (2) adults (>25–30 years), and (3) well-preserved skeletons. These inclusion criteria for the control group ensured the absence of pathological traits related to joint diseases, as well as their unlikely development in the future. Therefore, in the present study, the sample was composed of 55 individuals, including both individuals with arthropathies (N = 31) and a control group (N = 24).

To determine the mtDNA haplogroup and sub-haplogroup in this sample, DNA extraction was performed following the procedure described in Hervella et al., (2015, 2012) and Laza et al., (2016). Dental pieces were preferably selected, since these provide more reliable results (Barrio-Caballero, 2013; Zierdt et al., 1996). In those cases in which the teeth were absent, ribs were employed, since they are an anatomic region with low anthropological interest.

Distinction between haplogroup H and other mtDNA haplogroups (hereinafter "non-H") was assessed by performing PCR-RFLPs for diagnostic marker −7025 *Alu*I (Alzualde et al., 2005). Haplogroup H is defined by the absence of the *Alu*I restriction endonuclease site at position 7025, thus, after amplification and digestion, only a 120 bp fragment is detected by electrophoresis on agarose gel. However, non-H haplogroups do present this restriction site, generating two fragments (78 and 42 bp). This diagnostic RFLP marker prevents unnecessary sequencing of the entire mtDNA D-loop hypervariable segment I (HVS-I) to discern between haplogroup H and non-H haplogroups, resulting in a faster and cost-efficient screening (Santos et al., 2004).

To define sub-haplogroup H2, the mtDNA HVS-I fragments were sequenced by automated Sanger (15,995 to 16,399 pb) following established protocols (Alonso et al., 2003). Then, the mtDNA sequences were aligned using BioEdit software and compared to the revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999), with the aim of identifying individual polymorphisms and determining sub-haplogroups using Phylotree database (van Oven and Kayser, 2009). To guarantee reliable results, the following authentication criteria were applied in this study: (1) controls of DNA extraction and amplification, (2) fluorimetric quantification (QUBIT) of the number of template DNA molecules recovered from the extracts obtained, validating the reproducibility of the results, and (3) replication of the results by independent researchers at different times (Cooper and Poinar, 2000; Gilbert and Willerslev, 2006; Pääbo et al., 2004).

The statistical analyses were performed with SPSS software (*v.26*). In the case of chi-square contingency tables, Fisher's exact test was applied when the expected frequencies were less than 5. P-values < 0.05 were considered statistically significant. Genetic diversity (Nei, 1987) was calculated based on haplotype frequencies from individuals using Arlequin 3.11 (Excoffier et al., 2005).

3. Results and discussion

3.1. Haplogroup H in the sample from the Cathedral of Santa María

In the present study, the frequency of haplogroup H in a sample of 55 individuals from the Cathedral of Santa María was analysed considering both individuals with arthropathies (N = 31) and those without pathological manifestations, called "control group" (N = 24) (Table 1). The frequency of haplogroup H in this population was 63.6%, which can be considered high with respect the frequency described in other current populations from Northern Spain (Table 2) and Europe (55–40%) (Achilli et al., 2004; Pereira et al., 2005; Soares et al., 2010).

To discard the idea that this high frequency could be due to possible family matrilineal relationships, the haplotype variability within haplogroup H (N = 35) was analysed, revealing 13 different haplotypes (gene diversity: 0.8271 +/- 0.0451): 10 unique haplotypes (each of them appearing in a single individual), another 2 haplotypes (one being

Table 1
Frequency of the mtDNA haplogroup H and the other haplogroups (called "non-H") in individuals with arthropathy and without joint manifestations ("control") in the sample from the Cathedral of Santa María (Basque Country, Spain, 12th-18th centuries).

	Arthropathy		Control		Total	
	N	%	N	%	N	%
H	20	64.5	15	62.5	35	63.6
non-H	11	35.5	9	37.5	20	36.4
TOTAL	31	100	24	100	55	100

Table 2
Frequency of the mtDNA haplogroup H (%) in several present and ancient populations from Northern Spain (CN: Central-Northern; N: Northern).

Population	N	n (H)	Freq. (%)	Reference
Basque Country and CN Navarre	427	217	50.8	Behar et al., 2012
Bizkaia	91	37	40.7	García et al., 2011
Gipuzkoa	113	63	55.8	García et al., 2011
Asturias	429	225	52.5	Pardiñas et al., 2012
Cantabria, N Burgos, La Rioja, N Aragón	124	60	48.4	Behar et al., 2012
Aldaieta (6th-7th c., Álava)	34	18	52.9	Alzualde et al., 2006
S. M. Ereñozar (13th-16th c., Bizkaia)	90	66	73.3	Laza et al., 2019
Santa María (12th-18th c., Álava)	55	35	63.6	Present study

Table 3
Frequency of the mtDNA haplogroup H and the other haplogroups (called "non-H") in individuals with Inflammatory Arthropathy (IA) and other types of arthropathy (non-IA) in the sample from the Cathedral of Santa María (Basque Country, Spain, 12th-18th centuries).

	IA		non-IA	
	N	%	N	%
H	10	83.3	8	66.7
non-H	2	16.7	4	33.3
TOTAL	12	100	12	100

Table 4
Frequency of the mtDNA sub-haplogroup H2 and the other sub-haplogroups of haplogroup H (called "non-H2") in individuals with arthropathy and without joint manifestations ("control") in the sample from the Cathedral of Santa María (Basque Country, Spain, 12th-18th centuries).

	Arthropathy		Control		Total	
	N	%	N	%	N	%
H2	8	42.1	3	20.0	11	32.4
non-H2	11	57.9	12	80.0	23	67.6
TOTAL	19	100	15	100	34	100

present in 9 individuals and the other in 4 individuals), and finally, CRS haplotype (H2a2), which was detected in 11 individuals, as expected, given the high frequency of this haplotype in the current populations. One individual was discarded, as the haplotype could not be correctly determined. According to these data, the diversity obtained within haplogroup H in the sample of Santa María can be considered representative of the original population.

Comparing the frequency of haplogroup H in the sample of Santa María with the frequencies of other present and ancient populations from Northern Spain (Table 2), higher values were observed in those populations corresponding to the Middle Ages, especially that from San Miguel de Ereñozar (13th-16th centuries, Bizkaia). In the study of that population, a relation between haplogroup H and the prevalence of arthropathies was proposed, considering an adverse climatic period with possibly higher energy demands.

In view of the association between joint diseases and haplogroup H (Laza et al., 2019; Rego-Pérez et al., 2020, 2008; Soto-Hermida et al., 2014), the present study analysed the frequency of this haplogroup in the individuals with arthropathies and in a control group, obtaining values of 64.5% and 62.5%, respectively (Table 1). According to these data, it was observed that haplogroup H was the most frequent in the population from the Cathedral of Santa María, with very similar frequencies in both groups, i.e., those who present joint lesions and those without pathological manifestations.

From the results obtained, no relationship was detected between haplogroup H and joint pathologies in the sample from the Cathedral of Santa María (Table 1), although it has to be considered that arthropathies constitute a complex group of pathologies with different origin (inflammatory, degenerative, neurological, endocrine, etc.). Particularly, mitochondrial ROS (mROS) and oxidative damage have been shown to be involved in the pathogenesis of Inflammatory Arthropathies (IAs) (Li et al., 2020; Mateen et al., 2016; Phillips et al., 2010). Therefore, we analysed this relationship in this particular type of joint disease. To this end, the individuals from Santa María were classified based on the inflammatory nature of the lesions (IA and non-IA) after an exhaustive palaeopathological analysis (Table 3). Given that 7 of the 31 individuals with joint lesions presented an arthropathy that was difficult to classify within the IA/non-IA subgroups, we only considered the 24 individuals with a clear diagnosis (12 IA and 12 non-IA individuals).

In those individuals with IAs such as Rheumatoid Arthritis (RA) and Spondyloarthropathies (SpAs), a very high frequency of haplogroup H (83.3%) was found, which was much higher than that found in individuals with other types of arthropathies (non-IA; 66.7%; Table 3). The absence of a statistically significant difference between the frequency of haplogroup H in IA and non-IA individuals (χ^2, $p = 0.640$) may be presumably attributed to the sample size, which is very difficult to increase, since IAs are not easily diagnosed in ancient populations. For their diagnosis at the skeletal level, it is fundamental to recover certain bone elements of the spine and pelvis, and even small bones from the hands and feet, whose absence in archaeological bone remains is quite common due to their small size and extreme fragility (Rogers and Waldron, 1994; Waldron, 2009). Moreover, skeletons with pathologies are usually the most incomplete, since the disease makes them more susceptible to post-mortem damage (Pinhasi and Bourbou, 2008; Stojanowski et al., 2002). This limitation is very common in palaeopathological studies in which the certainty of the diagnosis strongly depends on the representativeness of the bone remains recovered (Thillaud, 1994).

In our case, the exhumed population from the Cathedral of Santa María constitutes an exceptional sample composed by a very large number of individuals, although not all of them present an excellent state of conservation. An exhaustive analysis was carried out, selecting those skeletons with an acceptable state of conservation for palaeopathological analysis (N = 590). The prevalence value (2.03%) provided by the cases of IA identified in the analysed sample (N = 12) is in line with the current prevalence values of this type of diseases (0.5–2%) (Silman and Hochberg, 2001; Stolwijk et al., 2012). Although some analyses did not show statistically significant results, the obtained data showed a considerably higher frequency of H in the group of IAs compared to the group of non-IAs (Table 3), which could indicate the existence of a genetic component in the etiopathogenesis of inflammatory arthropathies; however, the gene-environment interaction is not well-known in the development of these diseases (Zeboulon-Ktorza et al., 2013).

With regard to the environmental factors, we have evidence of the influence of adverse conditions on the health of the population of Vitoria-Gasteiz during the Middle Ages, such as the increased prevalence of rickets (Ventades et al., 2020). The population of Vitoria-Gasteiz, as well as other populations in the Basque Country (Laza et al., 2019), were seriously affected by famines, epidemics and conflicts, mainly during the 14th and 15th centuries. The fall in agricultural production was especially critical, which reached the limit of its possibilities and was unable to increase productivity to provide food for an increasing population (Díaz de Durana, 1986, 1984). Therefore, we suggest that the influence of environmental factors can partly explain the relative contribution of specific mtDNA haplogroups to the pathogenesis of joint diseases, as has been proposed for haplogroup H in relation to certain arthropathies (Laza et al., 2019; Rego-Pérez et al., 2020, 2008; Soto-Hermida et al., 2014).

3.2. Sub-haplogroup H2 in the sample from the Cathedral of Santa María

The association between mtDNA haplogroup H and multifactorial diseases has been broadly investigated (Maruszak et al., 2009; Serrano-Teruel et al., 2019). However, research at the sub-haplogroup level is still scarce, even in epidemiological studies. For example, sub-haplogroup H5 has been reported to be a risk factor for Alzheimer's disease, due to a more coupled OXPHOS system and increased mROS production (Santoro et al., 2010). Regarding arthropathies, sub-haplogroup H1 seems to increase the risk of rapidly progressive Osteoarthritis (OA) of the knee (Durán-Sotuela et al., 2019). In ancient populations, to our knowledge, the mention of a possible relationship between sub-haplogroup H2 and IAs was proposed in an aDNA study concerning a medieval population (Laza et al., 2019).

To further understand this relation in the sample of Santa María, those individuals with sub-haplogroup H2 were analysed in comparison with those carrying other mitochondrial sub-haplogroups, which we called "non-H2". There was only one individual in whom it was not

Table 5
Individuals with Inflammatory Arthropathy (IA): Burial number, type of Inflammatory Arthropathy (RA/SpA), mtDNA haplogroup (H/non-H) and sub-haplogroup (H2/non-H2). (SpA: Spondyloarthropathy; RA: Rheumatoid Arthritis).

Burial number	SpA/RA type	Haplogroup	Sub-haplogroup
SMC.07-512	SpA	H	H2
SMC.00-2	SpA	non-H	non-H2
SMC.07-333	SpA	H	non-H2
SMC.07-218	SpA	H	H2
SMC.02-SpA	SpA	H	H2
SMC.07-123	SpA	H	H2
SMC.97-30	RA	H	non-H2
SMC.97-18	RA	H	non-H2
SMC.00-121	RA	H	non-H2
SMC.02-585	RA	non-H	non-H2
SMC.97-1	RA	H	non-H2
SMC.00-93	RA	H	non-H2

possible to determine the sub-haplogroup, due to unreliable results in its mtDNA sequence (N = 34). The obtained results indicate that 42.1% of the individuals with joint lesions presented sub-haplogroup H2, compared to 20% of the control group (Table 4). It was remarkable that most individuals who do not present joint lesions had other mitochondrial sub-haplogroups ("non-H2"; 80%).

As can be observed, the frequency of sub-haplogroup H2 in individuals with arthropathies was over twice as much as in the control group (Table 4). The absence of a statistical difference could be possibly attributed to the small sample size caused by the subdivision of the sample to analyse those individuals with sub-haplogroup H2 versus the other H mtDNA sub-haplogroups (Fisher exact test, p = 0.2714). Nevertheless, these data suggest a possible relation between arthropathies and sub-haplogroup H2, which has also been suggested in the study of another medieval population of the Basque Country (Laza et al., 2019).

Moreover, in the present study we were able to diagnose two types of IA, i.e., Spondyloarthropathy (SpA) and Rheumatoid Arthritis (RA), comparing the frequency of mitochondrial sub-haplogroup H2 between these two pathologies (Table 5; Fig. 2). It was observed that all the individuals with sub-haplogroup H2 presented a SpA-type arthropathy, whereas none of the H2 individuals were among the RA-type cases (Table 5). These results indicate a relationship between sub-haplogruop H2 and SpAs, although the difference is marginally not significant according to Fisher's exact test (p = 0.0606). When the analysis was performed considering only the individuals with haplogroup H (N = 10), the differences were statistically significant (Fisher exact test, p = 0.0476), thereby supporting the mentioned association.

With the current knowledge it is difficult to explain the differential frequency of sub-haplogroup H2 found in the present study in one IA or the other (Table 5). IAs, particularly SpAs and RA, constitute different and complex pathologies whose development is influenced by genetic, environmental and immunological factors. According to the results obtained in the present study, it seems that the individuals with sub-haplogroup H2 present greater tendency toward developing a SpA and not a RA, which indicates that the weight of the genetic component, in this case the mitochondrial lineage, would be different in the etiopathogenesis of both diseases. We suggest that the behaviour of this sub-haplogroup at the physiological level may influence the pathogeny of SpAs. However, while the higher energy efficiency of haplogroup H has been comprehensively described in relation to the increase of mROS production and oxidative stress (Martínez-Redondo et al., 2010; Wallace, 2013), there is no literature regarding the performance of the H2 mtDNA sub-haplogroup from a physiological perspective.

4. Conclusion

The present analysis in a sample from the Cathedral of Santa María showed a high frequency of haplogroup H (63.6%), which was much higher in those individuals with Inflammatory Arthropathy (IA; 83.3%) compared to those with an arthropathy of a non-inflammatory nature (non-IA; 66.7%). A particularly relevant relationship was described between sub-haplogroup H2 and Spondyloarthropathies (an IA that mainly affects the axial skeleton), which suggests that this sub-haplogroup could be influencing the etiopathogenesis of these diseases.

In addition to a genetic substrate, environmental factors contribute largely to the etiopathogenesis of these diseases, thus the understanding of the demographic, historical and paleoclimatic context of the population buried in Santa María in the Middle Ages must be taken into account. We are aware of the existence of several adverse events

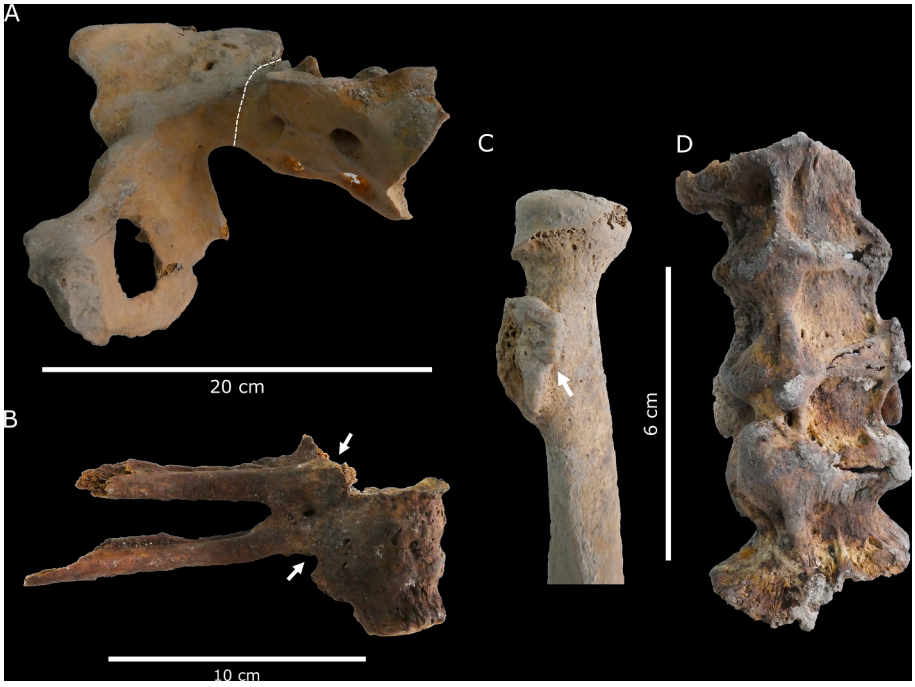

Fig. 2. Skeletal lesions found in several individuals with Spondyloarthropathy (SpA) from the Cathedral of Santa María (Basque Country, Spain, 12th-18th centuries). (A) Ankylosis of the right sacroiliac joint (SMC.07–333); (B) Fusion of the costovertebral joint in two left ribs and fusion of two thoracic vertebrae (SMC.07–218); (C) Enthesial ossification involving the radial tuberosity at the insertion of the biceps brachii muscle (SMC.00–2); (D) Fused thoracic and lumbar vertebrae (T10-L1) (SMC.07–123).

throughout this period (e.g., famines, conflicts, LIA, epidemics), which strongly influenced the health of the population of Vitoria-Gasteiz, especially when considering pathologies with a strong environmental background, such as IAs.

The present study showed that the use of a simple and straightforward methodology of aDNA analysis can help to improve the knowledge of ancient population, in this case the dwellers of Vitoria-Gasteiz during the Middle Ages. This approach requires considering the historical context of the population, in order to provide an integrated and well-supported view of the lifestyle of the population, and its influence on their health and evolution.

CRediT authorship contribution statement

Nerea G. Ventades: Methodology, Formal analysis, Investigation, Writing – original draft. **Concepción de-la-Rua:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Montserrat Hervella:** Conceptualization, Methodology, Resources, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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