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Disease and Evolution

Genetic and Environmental Factors in the Genesis of Spondyloarthritis

An ancient DNA study

Doctoral Thesis

Imanol Martín Laza

Thesis supervisors

Dra. Concepción de la Rúa

Dra. Montserrat Hervella

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Lista de publicaciones

1. **Laza, I.M.**, Hervella, M., de-la-Rúa, C. Genetic markers in a medieval case of ankylosing spondylitis. *The Journal of Rheumatology*. 43(3): 679-681 (2016).
2. Ventades, N.G., **Laza, I.M.**, Hervella, M., de-la-Rúa, C. A recording form for differential diagnosis of arthropathies. *International Journal of Paleopathology*. 20: 45-49 (2018).
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5. **Laza, I.M.**, Hervella, M., Neira Zubieta, M., de-la-Rúa, C. Environmental factors modulated ancient mitocondrial DNA variability and the prevalence of rheumatic diseases in the Basque Country. *Scientific Reports*. 9(1): 20380 (2019).
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1. **Laza, I.M.**, Hervella, H., Izagirre, N., de la Rúa C. Estudio genético de un caso de espondilitis anquilosante de un enterramiento medieval de la catedral de Santa María (Vitoria-Gasteiz). Comunicación oral. XIX Congreso de la Sociedad Española de Antropología Física. Madrid (2015). Publicado: *Libro de Actas de XIX Congreso de la Sociedad Española de Antropología Física*; Poblaciones Humanas, Genética, Ambiente y Alimentación. Montero, P. Prado, C., Acevedo, P., Carmenate, M., del Valle, A., Herrerín, J., Romero, J.F., Keller, K., López, N., Mora, A.I. (Eds.). Universidad Autónoma de Madrid. pp. 281-90 (2016).

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LISTADO DE ABREVIATURAS

ACPAs: Anti-citrullinated protein antibodies

aDNA: Ancient DNA

ALDH1A2: Aldehyde dehydrogenase 1 family, member A2

AS: Ankylosing spondylitis

ASAS: Assessment of Spondyloarthritis International Society

ATP: Adenosine triphosphate

axSpA: Axial spondyloarthritis

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index

BASFI: Bath Ankylosing Spondylitis Functional Index

BASMI: Bath Ankylosing Spondylitis Metrology Index

bp: Base pair

BMI: Body mass index

BSA: Bovine serum albumin

CARD9: Caspase recruitment domain-containing protein 9

CD: Crohn´s disease

CRP: C-reactive protein

CTLA4: Cytotoxic T-lymphocyte antigen 4

DNA: Desoxirribonucleid acid

ER: Endoplasmic reticulum

ERAP1: Endoplasmic reticulum aminopeptidase 1

ERAP2: Endoplasmic reticulum aminopeptidase 1

ESR: Erythrocyte sedimentation rate

GDF-5: Growth differentiation factor 5

GPR-25: G-protein coupled receptor 25

GWAS: Genome-wide association study

HLA: Human leukocyte antigen

HLA-B27: Human leukocyte antigen B27

HLA-B40: Human leukocyte antigen B40

HUB: Hospital Universitario de Basurto

HVS: Hypervariable segment

HVS-I: Hypervariable segment I

HVS-II: Hypervariable segment II

IBD: Inflammatory bowel disease

IBD-SpA: Inflammatory bowel disease-associated spondyloarthritis

IFN-γ: Gamma interferon

IL-1: Interleukin 1

IL-6: Interleukin 6

IL-17: Interleukin 17

IL-22: Interleukin 22

IL-23: Interleukin 23

IL-23R: Interleukin 23 receptor

KIR3DL2: Killer cell immunoglobulin-like receptor 3DL2

LIA: Little Ice Age

LILR: Leucocyte immunoglobulin-like receptor

LGM: Last Glacial Maximum

MCA: Medieval Climate Anomaly

MHC: Major histocompatibility complex

MRI: Magnetic resonance imaging

mtDNA: Mitochondrial DNA

NCOA3: Nuclear receptor coactivator 3

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NK cell: Natural killer cell

NOD2: Nucleotide-binding oligomerization domain-containing protein 2

nr-axSpA: non-radiographic axial spondyloarthritis

NSAID: Nonsteroidal anti-inflammatory drug

OA: Osteoarthritis

O_R: Origin of replication

OXPHOS: Oxidative phosphorylation system

PCA: Principal component analysis

PCR: Polymerase chain reaction

Ps: Psoriasis

PsA: Psoriatic arthritis

PTGER4: Prostaglandin E_2 receptor 4

PTPN22: Protein tyrosine phosphatase, non-receptor type 22

RA: Rheumatoid arthritis

rCRS: revised Cambridge Reference Sequence

ReA: Reactive arthritis

RF: Rheumatoid factor

ROS: Reactive oxygen species

RNA: Ribonucleic acid

r-axSpA: Radiographic axial spondyloarthritis

SIJ: Sacroiliac joint

SME: San Miguel de Ereñozar

SNP: Single nucleotide polymorphism

SpA: Spondyloarthritis

STAT4: Signal transducer and activator of transcription 4

T^a: Temperature

Th17 cell: T helper 17 cell

TNF-α: Tumor necrosis factor α

UC: Ulcerative colitis

UPR: Unfolded protein response

uSpA: Undiferentiated spondyloarthritis

ABSTRACT

The present Doctoral Thesis is focused on rheumatic bone pathologies and genetic variants associated to diseases that may present bone manifestations. The analysis was conducted on the human skeletal remains recovered from the medieval site of San Miguel de Ereñozar (SME) (Ereño, Basque Country, 13th-16th centuries). The exceptional nature of this osteological collection lies both in the high frequency of rheumatic bone-signatures detected among the individuals of this site, and in a chronology for the site that coincides with a period of climatic instability known as the Little Ice Age (LIA) (14th-19th centuries), which may have influenced the genesis of rheumatic diseases in the individuals of this medieval population.

The morphological analysis of the bone remains of the 163 individuals recovered from SME allowed the identification of three different types of rheumatic pathologies: spondyloarthritis (SpA), osteoarthritis (OA) and rheumatoid arthritis (RA). The most frequent rheumatic pathologies in the medieval population of SME were SpA, whose prevalence in this population was very high (12.9%), with respect to the results reported in epidemiological studies of current and archaeological populations. The morphological analysis showed the importance of certain joint manifestations for the diagnosis of SpA, such as the affectation of the sacroiliac joints, spine and enthesis, especially of the hip. However, it is necessary to identify the morphology and the location of this type of lesions. In addition, the age of the individuals is also of relevance in order to reach a differential diagnosis, both among the diseases that encompass the group of SpA and with other rheumatic pathologies, such as OA.

The analysis of mitocondrial variability in the individuals of the SME necropolis, showed a high frequency of mitochondrial haplogroup H (73.3%). However, the frequency of haplogroup H is higher in individuals with rheumatic bone manifestations than in those individuals who did not show joint bone lesions. This observation suggests that haplogroup H could increase or could be associated to the risk of developing rheumatic diseases, more specifically SpA, as 81% of the individuals that showed rheumatic characteristics of SpA carried this mitochondrial haplogroup. This could be the result of a higher efficiency of haplogroup H during the process of obtaining energy from food,

which is a process that results in a high oxidative stress, cell damage and cartilage degeneration, which are processes that promote the development of rheumatic diseases. From an adaptive perspective, haplogroup H could be beneficial in adverse environmental conditions, such as the LIA, therefore we suggest that in the population of SME the LIA may have influenced the increase in the frequency of haplogroup H given its energy efficiency. However, this would imply a biological trade-off, by simultaneously increasing the risk of developing rheumatic diseases.

The association between some alleles of the *HLA-B* gene and SpA has been traditionally described. One of the most significant and reasserted associations in the literature is between *HLA-B27* allele and SpA, especially with ankylosing spondylitis (AS). The analysis of the *HLA-B* gene showed seventeen alleles for this gene in the individuals of the medieval population of SME, with *HLA-B40*, *HLA-B27* and *HLA-B35* being the most frequent ones. *HLA-B40* allele was the most frequent allele in individuals of SME with SpA. Although these alleles have been traditionally described as genetic markers associated to the development of SpA, in this study they were also found in individuals with other rheumatic diseases (OA and RA) and even in individuals without pathologies. These data confirm the complexity of the relationship between *HLA-B* gene variants and SpA, since it is not possible to establish a diagnosis of SpA with these genetic variants alone. However, we suggest that alleles *HLA-B40* and *HLA-B27*, in combination with some characteristics (rheumatic bone manifestations and age), would facilitate the diagnosis of SpA in ancient populations.

GWAS studies have revealed a considerable number of genes or gene regions that contribute to the risk to develop SpA. In the present doctoral thesis, we have analyzed in the individuals recovered from the necropolis of SME a set of 43 SNPs located in genes associated with risk to develop this group of rheumatic diseases. This methodology was previously validated in a sample of 62 patients diagnosed with axial spondyloarthritis (axSpA) from the University Hospital of Basurto (HUB) (Bilbao, Basque Country, Spain) in order to evaluate the hypothesis that AS and non-radiographic axial spondyloarthritis (nr-axSpA) are subsets of a single disease. Regarding the frequencies of the set of risk SNPs associated with SpA, no statistically significant differences were found in this study

between the patients with AS and those with nr-axSpA, with *HLA-B27* being the only genetic marker that showed statistically significant differences between two group of patients of HUB. The high frequency of *HLA-B27* allele among the individuals with AS may be correlated with more severe radiographic damage observed and a greater progression of AS. Therefore, from the genetic perspective, it could be hypothesized that the two entities constitute two different expressions of the same disease. Furthermore, this study showed the genetic complexity of SpA, with two SNPs (rs27222427, rs2076756) localized in *NOD2* gene associated with Crohn´s disease being of special interest, which were found preferentially in individuals diagnosed with AS compared to those with nr-axSpA, suggesting that these two SNPs may be associated with a greater progression of AS, although this relationship has not been described until now. However, the complex genetic nature of the SpA may indicate the involvement of environmental factors such as infections by pathogens, smoking or obesity, in the triggering of the disease. This could be the reason that patients with different genotypes would have the same pathogenic phenotype.

The genotyping of a set of 43 risk SNPs associated to SpA in the individuals recovered from the necropolis of SME provided only partial results, which is possibly due to the low hybridization of some genotyping probes. However, very interesting perspectives for the evolutionary study of diseases and the influence of environmental factors in the genesis of rheumatic pathologies are opened, particularly to evaluate the relevance of the change in the lifestyle of human populations (from hunter-gatherers to farmers) in the development of this type of diseases. Changes in the environmental conditions and lifestyle have played a key role in the evolutionary process of human immunity, which would explain both the appearance of autoimmune and inflammatory diseases, such as SpA, and the adaptive evolution of specific genetic variants.

RESUMEN

La presente Tesis Doctoral se centró en el análisis de patologías óseas reumáticas y las variantes genéticas asociadas a estas enfermedades. El análisis se llevó a cabo sobre los restos óseos humanos recuperados del yacimiento medieval de San Miguel de Ereñozar (SME) (Ereño, País Vasco, España, siglos XIII-XVI). La excepcionalidad de esta colección osteológica radica tanto en la alta frecuencia de signos óseos reumáticos detectados entre los individuos recuperados de este yacimiento, como en su cronología, que coincide con un período de inestabilidad climática conocido como la Pequeña Edad de Hielo (LIA) (siglos XIV-XIX), que pudo haber influido en la génesis de las enfermedades reumáticas que padecieron los individuos de esta población medieval.

El análisis morfológico de los restos óseos de los 163 individuos recuperados de SME permitió identificar tres tipos diferentes de patologías reumáticas: espondiloartritis (SpA), osteoartritis (OA) y artritis reumatoide (RA). Las patologías reumáticas más frecuentes en la población medieval de SME fueron las SpA, cuya prevalencia en esta población fue muy elevada (12,9%), en comparación con los resultados obtenidos en estudios epidemiológicos llevados a cabo en poblaciones actuales y arqueológicas. El análisis morfológico demostró la importancia de ciertas manifestaciones articulares para el diagnóstico de las SpA, como la afectación de las articulaciones sacroilíacas, la columna vertebral y las entesis, especialmente de cadera. Sin embargo, es necesario identificar la morfología y la ubicación de este tipo de lesiones. Además, la edad de los individuos también es relevante para establecer un diagnóstico diferencial, tanto entre las enfermedades que engloban el grupo de las SpA, como con otras patologías reumáticas, como la OA.

El análisis de la variabilidad mitocondrial en los individuos recuperados de SME, reveló una elevada frecuencia del haplogrupo mitocondrial H (73,3%). Además, la frecuencia del haplogrupo H fue mayor entre los individuos que presentaban manifestaciones óseas reumáticas que en aquellos que no mostraban dichas lesiones. Esta observación sugiere que el haplogrupo H podría estar asociados con un mayor riesgo a desarrollar enfermedades reumáticas, y más específicamente SpA, ya que el 81% de los individuos que mostraban manifestaciones reumáticas características de SpA portaban el

haplogrupo H. Esto podría ser el resultado de una mayor eficiencia del haplogrupo H durante el proceso de obtención de energía a partir de la dieta, proceso que da lugar a un elevado estrés oxidativo que conduce al daño celular y a la consecuente degeneración del cartílago, procesos que promueven el desarrollo de enfermedades reumáticas. Desde una perspectiva adaptativa, el haplogrupo H podría ser beneficioso frente a condiciones ambientales adversas, como las que tuvieron lugar durante la LIA, por lo que sugerimos que la LIA podría haber influido en el incremento de la frecuencia del haplogrupo H en la población de SME, dada su eficiencia energética. Sin embargo, esto tendría consecuencias biológicas, al aumentar simultáneamente el riesgo de desarrollar patologías de carácter reumático.

Tradicionalmente se ha descrito la asociación entre algunos alelos del gen *HLA-B* y el desarrollo de las SpA. Una de las asociaciones más significativas y reiteradas en la bibliografía es la que hace referencia al alelo *HLA-B27* con las SpA, y especialmente con la espondilitis anquilosante (AS). El análisis del gen *HLA-B* reveló la presencia de 17 alelos diferentes para este gen entre los individuos de la población de SME, siendo los más frecuentes el *HLA-B40*, el *HLA-B27* y el *HLA-B35*. El alelo *HLA-B40* fue el más frecuente entre los individuos de SME con SpA. Aunque estos alelos han sido tradicionalmente descritos como marcadores genéticos asociados con el desarrollo de SpA, en el presente estudio también fueron identificados en individuos con otras enfermedades reumáticas (OA y RA) e incluso en individuos carentes de estas patologías. Estos resultados confirman la complejidad de la relación entre las variantes del gen *HLA-B* y la patogénesis de las SpA, ya que no es posible establecer un diagnóstico de SpA sólo con el análisis de estas variantes genéticas. Sin embargo, sugerimos que los alelos *HLA-B40* y *HLA-B27*, en combinación con algunas características (manifestaciones óseas reumáticas y edad), facilitarían el diagnóstico de SpA en las poblaciones antiguas.

Los estudios GWAS han identificado un número considerable de genes que contribuyen al riesgo a desarrollar SpA. En la presente Tesis Doctoral se analizó un conjunto de 43 SNPs localizados en genes asociados al riesgo de desarrollar este grupo de enfermedades reumáticas en los individuos recuperados del yacimiento de SME. La metodología para llevar a cabo el genotipado de los SNPs fue previamente validada en

una muestra de 62 pacientes diagnosticados con espondiloartritis axial (axSpA) del Hospital Universitario de Basurto (HUB) (Bilbao, País Vasco, España) con el fin de evaluar la hipótesis que postula que la AS y la espondiloartritis axial no radiográfica (nr-axSpA) son subconjuntos de una misma enfermedad. En relación a las frecuencias del conjunto de SNPs de riesgo asociados a las SpA, no se encontraron diferencias estadísticamente significativas entre los pacientes diagnosticados con AS y los diagnosticados con nr-axSpA. El alelo *HLA-B27* fue el único marcador genético que mostró diferencias estadísticamente significativas entre los dos grupos de pacientes del HUB (AS y nr-axSpA). La elevada frecuencia del alelo *HLA-B27* entre los individuos con AS podría estar relacionada con un daño radiográfico más severo y una mayor progresión de esta entidad patológica. Por lo tanto, desde el punto de vista genético, se podría plantear la hipótesis de que la AS y la nr-axSpA constituirían dos expresiones diferentes de la misma enfermedad. Además, este estudio demostró la complejidad genética de la patogénesis de las SpA, siendo de especial interés dos SNPs (rs27222427 y rs2076756) localizados en el gen *NOD2* que se encuentra asociado a la enfermedad de Crohn. Estos dos SNPs fueron identificados preferentemente entre los individuos con AS en comparación con los individuos con nr-axSpA, lo que sugiere que estos dos SNPs pueden estar asociados con una mayor progresión de la AS, aunque esta relación no ha sido descrita hasta el momento. Sin embargo, la compleja naturaleza genética de las SpA indicaría la participación de otros factores como los ambientales, entre los que destacan las infecciones por patógenos, el tabaquismo o la obesidad, en el desencademiento de estas enfermedades. Esto podría ser la razón que explicaría porque pacientes con diferentes genotipos presentarían el mismo fenotipo patogénico.

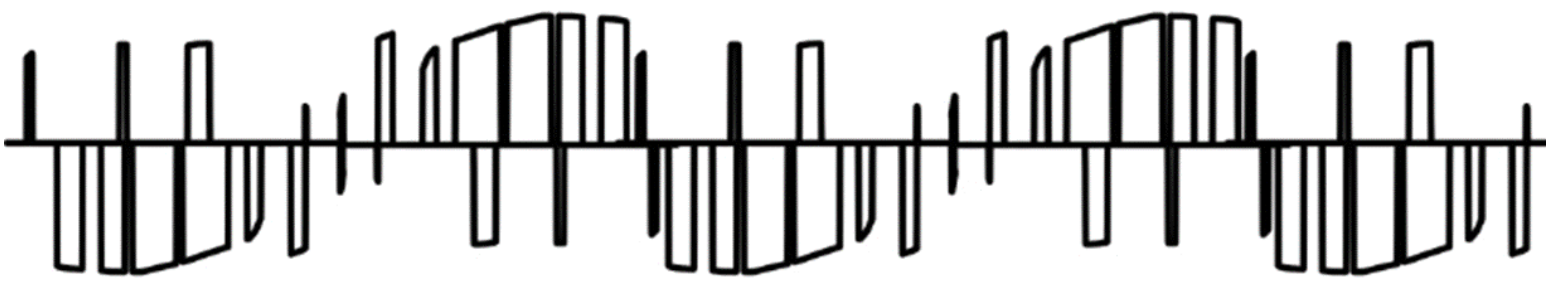
El genotipado de los 43 SNPs de riesgo en los individuos de SME ofreció resultados parciales, lo que posiblemente sea debido a la degradación del DNA de estas muestras, obteniendo bajas tasas de hibridación en algunas de las sondas diseñadas para el genotipado. A pesar de ello, se abren perspectivas muy interesantes para el estudio evolutivo de las enfermedades y la influencia de los factores ambientales en la génesis de las patologías reumáticas, resultando muy sugerente evaluar el cambio del modo de vida de las poblaciones humanas (de cazador-recolector a agricultor) en el desarrollo de este tipo de patologías. Tanto los cambios en las condiciones ambientales como en el

estilo de vida han desempeñado un papel fundamental en el proceso evolutivo de la inmunidad humana, lo que explicaría tanto la aparición de enfermedades autoinmunes e inflamatorias, como las SpA, como la evolución adaptativa de determinadas variantes genéticas.

PRÓLOGO

En la presente Tesis Doctoral se ha llevado a cabo el análisis de los restos óseos recuperados de la necrópolis medieval de San Miguel de Ereñozar (Ereño, País Vasco, España, siglos XIII-XVI), debido por un lado a la elevada frecuencia de manifestaciones óseas de carácter reumático presentes en los individuos de dicha necrópolis y, por otro lado, a los cambios ambientales acontecidos durante el periodo de existencia de la misma. El análisis de los restos esqueléticos fue llevado a cabo desde diversas perspectivas: morfológica, genética, ambiental y evolutiva. El análisis morfológico pretende definir las manifestaciones óseas que contribuyen al diagnóstico de las enfermedades reumáticas que padecieron los individuos recuperados de esta necrópolis. El análisis de marcadores genéticos asociados a enfermedades reumáticas ayudará al diagnóstico establecido mediante el análisis morfológico y permitirá extender los resultados de marcadores genéticos descritos en estudios de poblaciones actuales a las poblaciones antiguas, que a veces presentan manifestaciones óseas avanzadas. La perspectiva ambiental nos permitirá evaluar la influencia de factores exógenos como el clima durante la Pequeña Edad de Hielo en la prevalencia de enfermedades reumáticas en la población medieval de San Miguel de Ereñozar. Por último, el punto de vista evolutivo nos informará de la posible adaptación de individuos a climas fríos y sus posibles consecuencias.

I. Introducción



1. Enfermedades reumáticas

1.1. Espondiloartritis

Las espondiloartritis (SpA, del inglés *spondyloarthritis*), son un grupo de enfermedades reumáticas, inflamatorias y autoinmunes que comparten una serie de características como la afectación del esqueleto axial [columna vertebral y las articulaciones sacroiliacas (SIJs, del inglés *sacroiliac joints*)], artritis periférica, entesitis, dactilitis, otras afectaciones extra-articulares (Sieper *et al*., 2015, Sieper and Poddubnyy, 2017) **(Figura 1)** y una fuerte asociación con el antígeno leucocitario humano B27 (*HLA-B27*, del inglés *human leukocyte antigen B27*) (Bowness, 2015). Las SpA tradicionalmente engloban a la espondilitis anquilosante (AS, del inglés *ankylosing spondylitis*), artritis psoriásica (PsA, del inglés *psoriatic arthritis*), artritis reactivas (ReA, del inglés *reactive arhtritis*), espondiloartritis indiferenciadas (uSpA, del inglés *undifferentiated spondyloarthritis*) y las enfermedades inflamatorias intestinales asociadas a SpA (IBD-SpA, del inglés *inflammatory bowel disease associated spondyloarthritis*), entre las que se encuentran la enfermedad de Crohn (CD, del inglés *Crohn´s disease*) y la colitis ulcerosa (UC, del inglés *ulcerative colitis*) (Moll *et al*., 1974; Wright, 1978; Amor *et al*., 1990; Khan, 2002; Sheehan, 2004; Dougados and Beaten, 2011) **(Figura 1)**.

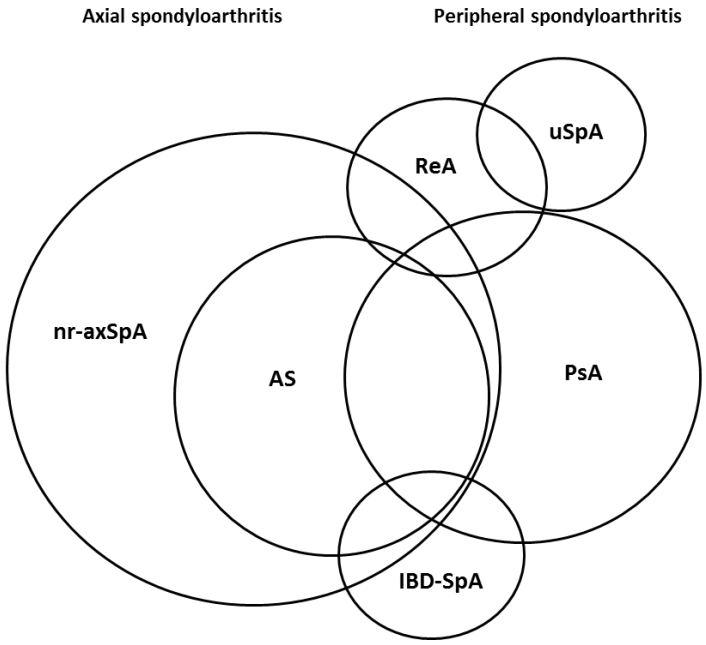


Figura 1. Conjunto de enfermedades que forman el grupo de las SpA y la superposición entre las diferentes formas de SpA. Modificado de Proft and Poddubnyy, 2018.

1.1.1. Características clínicas y radiográficas de las espondiloartritis

Los pacientes diagnosticados con SpA suelen presentar dolor y rigidez en glúteos y en la región lumbar, que se agrava con la inactividad. Con el paso del tiempo, estos síntomas pueden progresar y causar una limitación significativa en la movilidad de la columna vertebral. Sin embargo, el dolor lumbar es un síntoma común en la población general, ya que aproximadamente el 80% de las personas pueden verse afectadas por un episodio de dolor lumbar a lo largo de su vida (Calin *et al*., 1977; Rudwaleit *et al*., 2006; Braun and Inman, 2010), con lo que es necesario identificar y diferenciar el dolor inflamatorio y el dolor mecánico de la región lumbar de la espalda (Jois *et al*., 2008).

La inflamación del esqueleto axial en las SpA afecta a las SIJs (sacroileitis) y a las articulaciones de la columna vertebral y espondilitis, siendo estos cambios observados en cualquiera de los subtipos de SpA **(Figuras 2A y 3)**, aunque de manera diferente, ya que por ejemplo en la AS y en las IBD-SpA la sacroileitis es bilateral y simétrica y los sindesmofitos son simétricos, mientras que en la PsA y la ReA la sacroileitis es unilateral y asimétrica y los sindesmofitos son asimétricos (Resnick, 1979; Amor *et al*., 1994; Gran and Skomsvolly, 1997; Baraliakos *et al*., 2015) **(Figuras 3 y 4)**. La artritis, dactilitis, tenosinovitis y entesitis son manifestaciones periféricas características de las SpA (Olivieri *et al*., 1998). La artritis periférica involucra a articulaciones de fuera de la columna vertebral y generalmente es asimétrica y oligoarticular y puede afectar a grandes y a pequeñas articulaciones (hombro, codo, manos y pies), aunque a menudo afecta a grandes articulaciones de los miembros inferiores (cadera, rodilla y tobillo) (Amor et al., 1994; Moltó *et al*., 2016) **(Figura 2A)**. La dactilitis o "dedo en salchicha", se caracteriza por una tumefacción difusa de un dedo de la mano o del pie y afecta especialmente a los pacientes con PsA (Torre *et al*., 1991; Veale *et al*., 1994; Taurog *et al*., 2016). La tenosinovitis implica la inflamación de la membrana sinovial que reviste la vaina protectora que cubre los diferentes tendones. Las entesis son estructuras esenciales que permiten el anclaje estable de tendones y ligamentos en los huesos y proporcionan una transducción suave de las fuerzas mecánicas de musculo a hueso (tendones) y estabilidad (ligamentos) (Schett *et al*., 2017). La entesitis se caracteriza por la inflamación en los sitios donde los tendones y ligamentos se unen al hueso. En las SpA, el proceso patológico comienza en la médula ósea y en las entesis (Benjamin and

McGonagle, 2001; François *et al*., 2001), donde las reacciones innatas y adaptativas ocurren inicialmente como un proceso de reparación, pero que en fases posteriores pueden generar procesos de remodelación que incluyen edema óseo, osteítis, formación de hueso nuevo y en algunos casos fusión y anquilosis (Bridgewood *et al*., 2018; Watad *et al*., 2018).

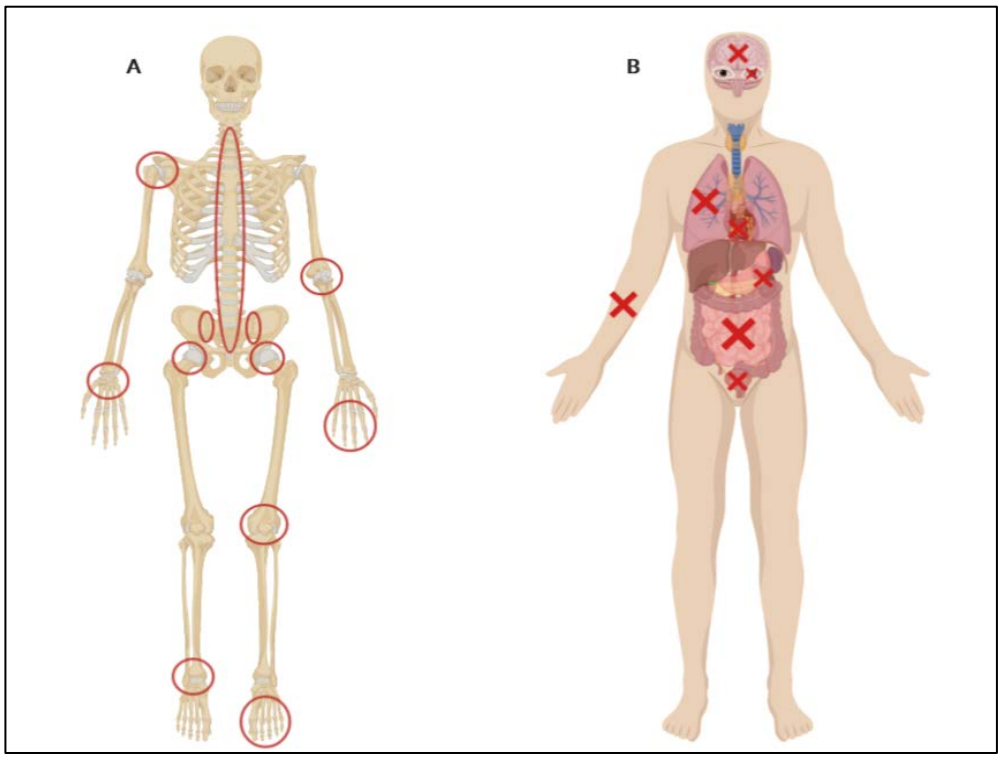


Figura 2. Manifestaciones clínicas asociadas a las SpA. A) Articulaciones clásicas afectadas por las SpA. Las entesis son los lugares primarios de inflamación y patología de las SpA. B) Manifestaciones extra-articulares asociadas a las SpA. Creado en Biorender.com.

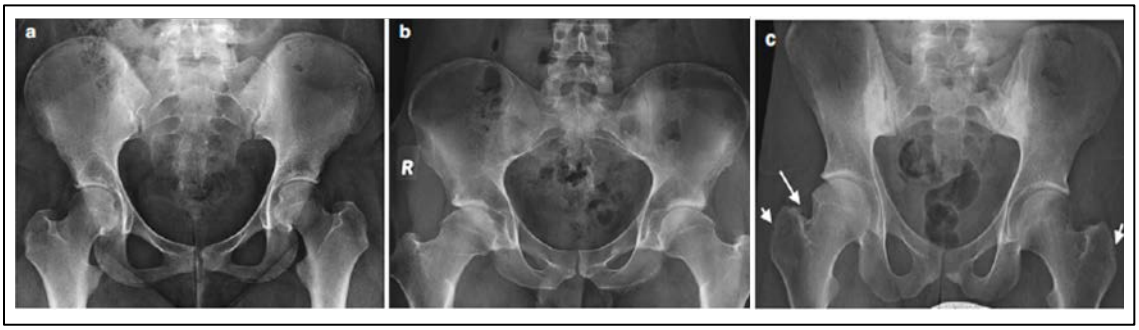


Figura 3. Radiografía convencional de la pelvis. A) Pelvis con SIJs normales. B) Pelvis de hombre de 34 años de edad diagnosticado con AS, con pérdida simétrica de espacio en las SIJs, erosiones, esclerosis subcondral y afectación bilateral de la cadera. C) Pelvis de hombre de 35 años de edad diagnosticado con AS, con pérdida simétrica de espacio en las SIJs, erosiones, esclerosis subcondral, afectación bilateral de la cadera y erosiones en el trocánter mayor (flechas pequeñas) y formación de entesofitos (flecha grande) (O´Neill, 2015).

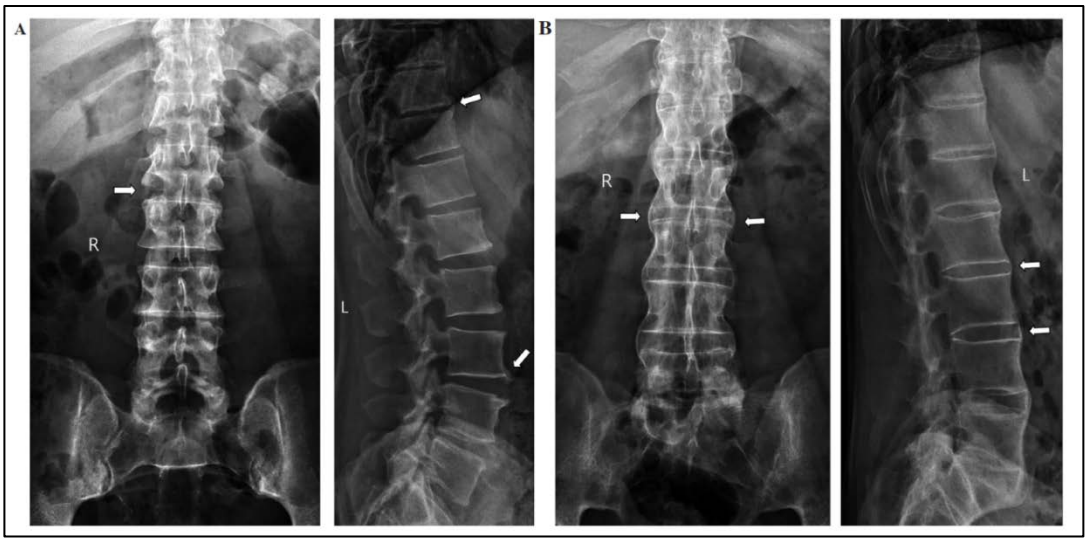


Figura 4. Radiografía convencional de la región lumbar de la columna vertebral en un paciente diagnosticado con PsA con afectación axial (A) y en un paciente diagnosticado con AS (B). La afectación de la columna vertebral en la PsA (A) es más frecuentemente unilateral y los sindesmofitos no siguen el curso del ligamento longitudinal anterior y no aparecen en vértebras consecutivas, mientras que la AS (B) es bilateral y los sindesmofitos siguen el curso del ligamento longitudinal anterior y aparecen en vertebras consecutivas (caña de bambú) (Baraliakos *et al*., 2015).

Actualmente se desconoce la relación entre la inflamación y la formación de hueso nuevo en las SpA (Maksymowych *et al*., 2012). La homeostasis ósea es un equilibrio estrechamente regulado entre los osteoclastos responsables de la reabsorción ósea y los osteoblastos que forman el hueso. Diversos factores pueden interrumpir este equilibrio en las SpA. La inflamación ósea puede conducir a la perdida de hueso e incrementa el riesgo de fracturas, pero la principal causa de daño estructural en las SpA es la formación de hueso nuevo aberrante, que resulta de la fusión de las SIJs y articulaciones zigoapofisarias y sindesmofitos que crean puentes entre los cuerpos vertebrales dando lugar a la anquilosis (Lories and Haroon, 2014) **(Figuras 5 y 6)**. La principal paradoja en las SpA es que la perdida y formación de hueso, a menudo se regula por señales opuestas que ocurren simultáneamente y frecuentemente en yuxtaposición.

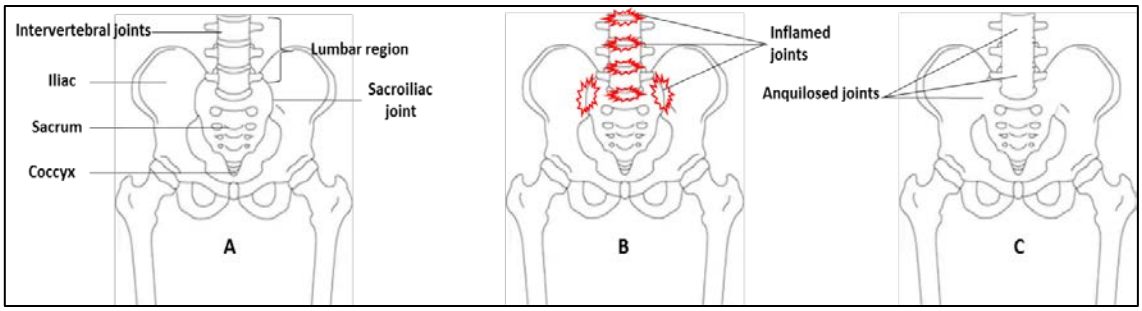


Figura 5. Etapas en el desarrollo de la espondilitis anquilosante. A) Estadio normal. B) Durante el proceso inflamatorio. C) Anquilosis.

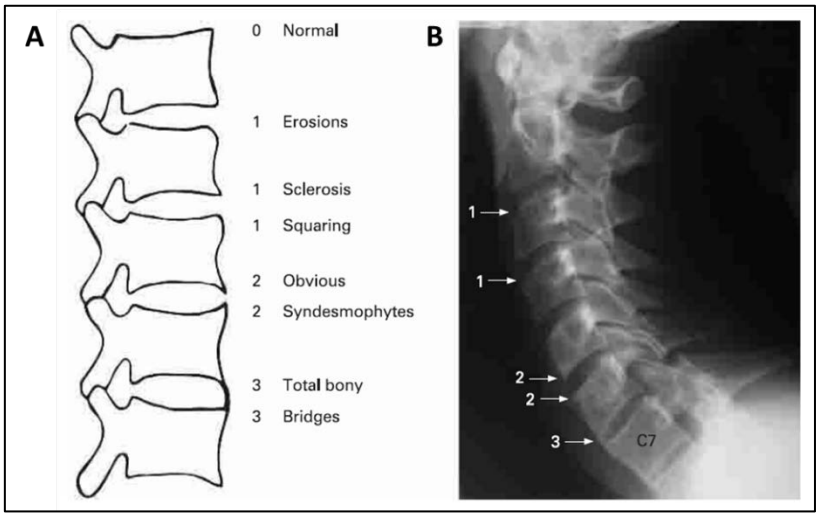


Figura 6. A) Cambios estructurales en la columna vertebral y valores de puntuación según modified Stoke AS Spine Score (mSASSS). B) Ejemplo de puntuación según mSASSS. Modificado de Sieper *et al.*, 2009.

Estos rasgos radiográficos son característicos de las SpA, pero pueden encontrarse ausentes durante las primeras fases de la enfermedad. A pesar de que la radiografía simple es el método inicial, en ausencia de estas manifestaciones radiográficas es necesario recurrir a la imagen por resonancia magnética (MRI, del inglés *magnetic resonance imaging*) o tomografía computarizada, para detectar los estadios iniciales de la enfermedad caracterizados por cambios inflamatorios. Entre las manifestaciones extra-articulares asociadas a las SpA se encuentran afectaciones mucocutáneas (psoriasis, Ps, del inglés *psoriasis*), oculares (uveítis), gastrointestinales, genitourinarias, pulmonares, renales, neurológicas y cardiovasculares **(Figuras 2B and 7)**.

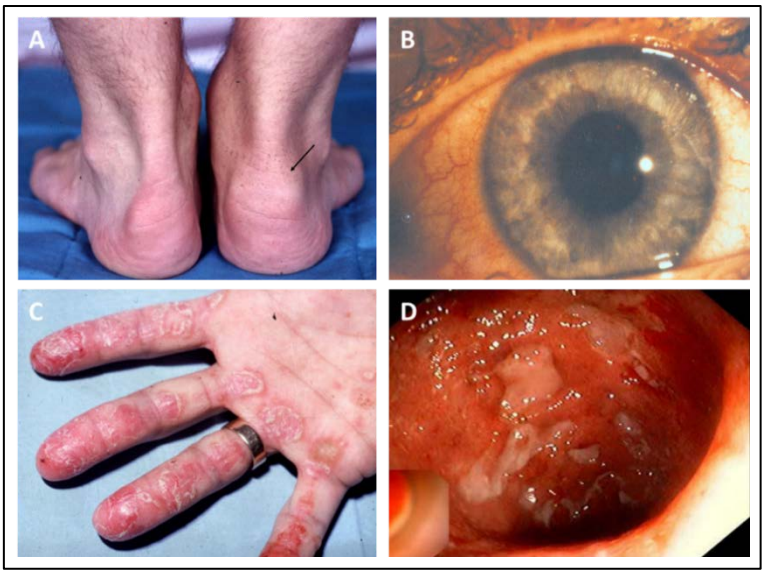


Figura 7. Ejemplos de manifestaciones extra-articulares de las SpA. A) Entesitis del tendón de Aquiles derecho. B) Uveítis anterior aguda. C) Psoriasis. D) Lesiones de la CD en el colon. Modificado de ASAS website.

1.1.2. Diagnóstico y criterios de clasificación de espondiloartritis

Actualmente no existe ningún criterio diagnóstico disponible para las SpA, aunque existen diversos criterios de clasificación. A pesar de la existencia de estos criterios, estas enfermedades a menudo son diagnosticadas tarde con una media en el retraso de diagnóstico de 8-10 años (Sykes *et al*., 2015). En 1930, gracias a la emergencia de la radiografía simple, se confirmó que el daño estructural en la AS comienza en las SIJs, considerándose el daño estructural en esta región (sacroileitis), una característica esencial para su diagnóstico (Moll *et al*., 1974).

En 1961, se propuso el primer conjunto de criterios para la clasificación de pacientes con AS, aunque para cumplir estos criterios no era obligatoria la evidencia de sacroileitis radiográfica (Kellgren, 1962; Wilkens, 1984). En 1963, la interpretación de la radiografía de las SIJs fue estandarizada y se estableció una escala gradual de puntuación que permite la clasificación de las SpA axiales radiográficas (r-axSpA, del inglés *radiographic axial spondyloarthritis*) (Kellgren and Jeffrey, 1963; Bennet and Bruch, 1968) **(Tabla 1)**.

Tabla 1. Clasificación de la sacroileitis radiográficas. Modificado de Bennett and Bruch, 1968.

Grading of radiographic sacroiliitis	
Grade 0	Normal
Grade 1	Suspicious changes
Grade 2	Minimal abnormality – small localized areas with erosions or sclerosis, without alteration of the joint width
Grade 3	Unequivocal abnormality – moderate or advanced sacroiliitis with one or more erosions, evidence sclerosis, widening, narrowing or partial ankylosis
Grade 4	Severe abnormality – total ankylosis

Tabla 2. Criterios modificados de Nueva York. Modificado de van der Linden *et al*., 1984a.

The modified New York criteria for ankylosing spondylitis
Clinical criteria
1. Low back pain of at least 3 months´ duration improved by exercise and not relieved by rest
2. Limitation of lumbar spine in sagittal and frontal planes
3. Chest expansion decreased relative to normal values for age and sex
4. Bilateral sacroiliitis grade 2 to 4
5. Unilateral sacroiliitis grade 3 or 4
Definite ankylosing spondylitis
Unilateral grade 3 or 4, or bilateral grade 2 to 4 sacroiliitis and any clinical criterion
Grading of radiographs
Normal 0; suspicious, 1; minimal sacroiliitis, 2; moderate sacroiliitis, 3; ankyloses, 4

En 1966, basándose en los criterios de Roma y utilizando los grados de sacroileitis, se establecieron los criterios de Nueva York, con una subsecuente actualización en 1984 (criterios modificados de Nueva York) (van der Linden *et al*., 1984a) **(Tabla 2)**. El cumplimiento de estos criterios exige la presencia de daño estructural en las SIJs **(Figura 3)**, que a menudo aparece varios años después del inicio de la enfermedad, lo que provoca un retraso en el diagnóstico (Feldtkeller *et al*., 2000), además de solo incluir síntomas de tipo axial. Para prevenir el retraso en el diagnóstico, en 1990 se propusieron los criterios de Amor, que incorporan características periféricas, buena respuesta a fármacos antiinflamatorios no esteroideos (NSAIDs, de inglés nonsteroidal anti-inflammatory drugs) y excluyen la sacroileitis radiográfica, aunque lo mantienen con un peso importante (Amor *et al*., 1990).

A finales de los años 90, la MRI permitió por primera vez detectar la presencia de inflamación del esqueleto axial antes de la presencia de sacroileitis radiográfica (Zeidler *et al*., 2004) **(Figura 8)**. Los criterios de clasificación para el diagnóstico de las SpA desarrollados por el grupo Assessment of Spondyloarthritis International Society (ASAS) **(Tabla 3)**, fueron elaborados y publicados en 2009 (Rudwaleit *et al*., 2009a, 2009b, 2011), incluyendo las manifestaciones detectadas por MRI y niveles elevados de proteína C reactiva (CRP, del inglés *C-reactive protein*), ya que los descritos anteriormente presentaban algunas limitaciones para el diagnóstico (Sieper and van der Heijde, 2013). Basándose en los criterios de ASAS, las SpA pueden ser clasificadas como axial (axSpA) o periférica (Sieper *et al*., 2009; Rudwaleit *et al*., 2009a, 2009b, 2011). Dentro de la axSpA se encuentran pacientes con cambios radiográficos en las SIJs (AS) y otros sin manifestaciones radiográficas (nr-axSpA, del inglés *non-radiographic axial spondyloarthritis*) **(Figuras 1, 3, 8 y 9)**.

Tabla 3. Los criterios de clasificación ASAS para las axSpA en pacientes con dolor lumbar durante 3 o más meses y con una edad de inicio inferior a 45 años. Modificado de Rudwaleit *et al*., 2011.

Sacroiliitis on Imaging + ≥1 SpA Feature	**OR *HLA-B27* + ≥2 Other SpA Features**
SpA Features	**Sacroiliitis on Imaging**
Inflammatory back pain	
Arthritis	Active (acute) inflammation on MRI highly suggestive of sacroiliitis associated with SpA
Enthesitis (heel)	
Uveitis	
Dactylitis	OR
Psoriasis	
Crohn´s disease/ulcerative colitis	Definitive radiographic sacroiliitis according to modified New York criteria
Good response to NSAIDs	
Family history for SpA	
HLA-B27^{+}	
Elevated CRP	

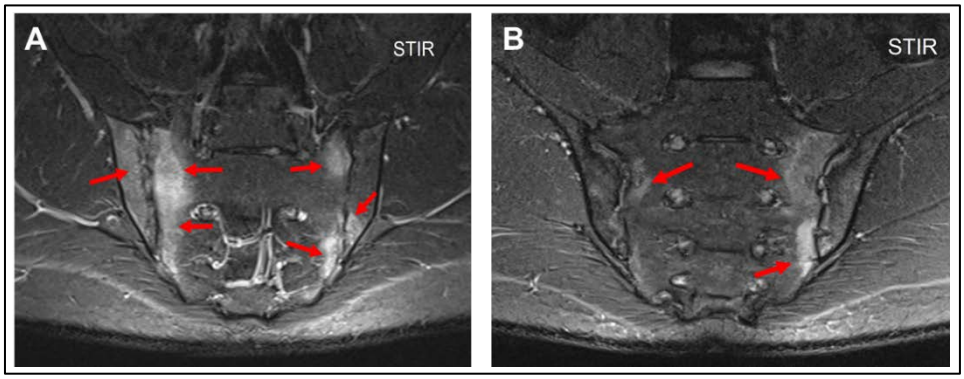


Figura 8. Lesiones inflamatorias activas de las SIJs detectadas por MRI de dos pacientes (STIR, del inglés *short tau inversion recovery*). Las flechas rojas indican edema de médula ósea subcondral. Modificado de ASAS website.

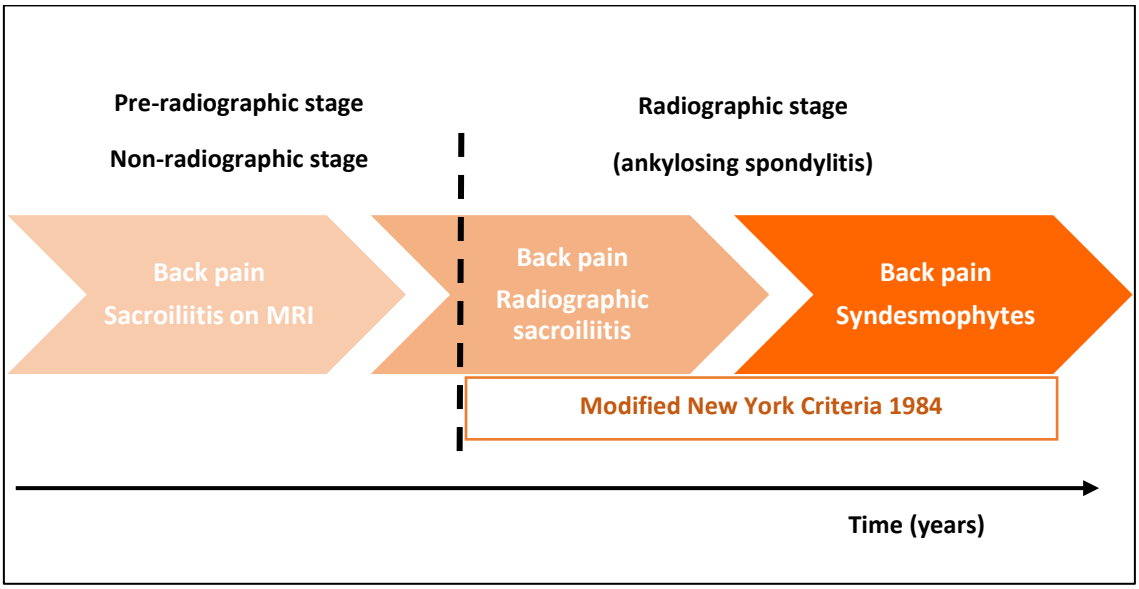


Figura 9. Concepto de axSpA. Modificado de Rudwaleit *et al*., 2005.

En la actualidad, existe un debate sobre si la nr-axSpA es una forma diferente de la AS (Robinson *et al*., 2013; Wallis *et al*., 2013), una forma temprana de la AS (Huerta-Sil *et al*., 2006; Kiltz *et al*., 2012; Ciurea *et al*., 2013) o bien ambas son dos expresiones de una misma enfermedad (Rudwaleit *et al*., 2005, 2009c; Baeten *et al*., 2013a), ya que algunos estudios han encontrado características demográficas, clínicas y de laboratorio similares entre la AS y la nr-axSpA, mientras que otros estudios, por el contrario, han mostrado diferencias estadísticamente significativas referentes a estas características entre ambas entidades patológicas. Algunos pacientes con nr-axSpA progresarán a AS después de años de enfermedad (Sampaio-Barros *et al*., 2001, 2010; Poddubnyy *et al*., 2011,

2012a). Sin embargo, otros pacientes con nr-axSpA sufrirán la enfermedad durante décadas y probablemente de por vida, sin ninguna evidencia de daño radiográfico (Said-Nahal *et al*., 2000), e incluso algunos de ellos pueden experimentar una remisión de forma espontánea o mediante el uso de fármacos.

1.1.3. Etiología de las espondiloartritis

Actualmente se desconoce cuál es la etiopatogenia de estas enfermedades, aunque se sabe que las SpA son consecuencia de la compleja interacción entre factores genéticos y ambientales (Brown *et al*., 1997; Sieper *et al*., 2002). La contribución relativa de genes y ambiente puede variar entre las diferentes formas de SpA (Brown *et al*., 1997).

1.1.3.1. Factores genéticos

Se ha sugerido que algunos genes del sistema HLA (HLA, del inglés *human leucocyte antigen*) del complejo mayor de histocompatibilidad (MHC, del inglés *major histocompatibility complex*), en concreto los genes de clase I (HLA-A, B y C) y de clase II (DP, DQ y DR), poseen un papel crítico en la respuesta autoinmune de estas enfermedades (Kochi, 2016) **(Figura 10)**. El MHC se caracteriza por presentar un alto grado de polimorfismo en algunos de sus genes, más notablemente en genes de clase I y II del sistema HLA que presentan un elevado nivel de desequilibrio de ligamiento (Breban *et al*., 2015).

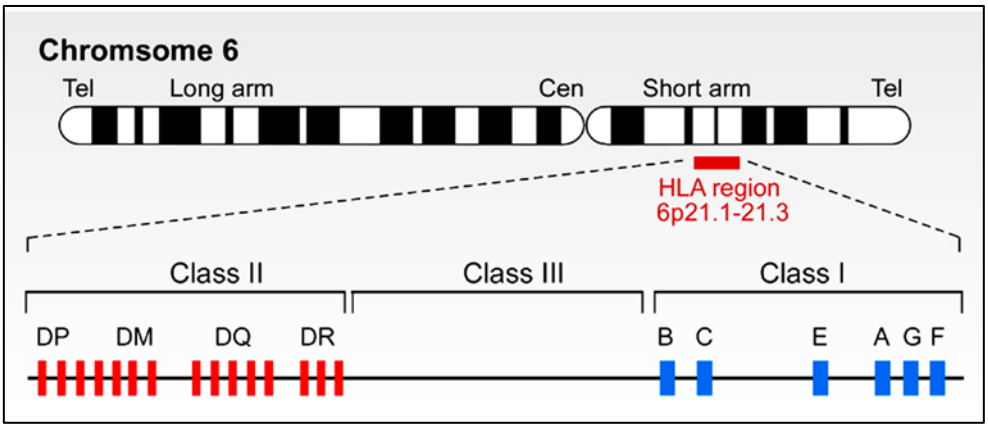


Figura 10. Mapa genético del sistema HLA localizado en el brazo corto del cromosoma 6 en humanos. Modificado de Zhang *et al*., 2014.

El *HLA-B27* es el factor de riesgo genético dominante para las SpA (Brewerton *et al*., 1973; Caffrey and James, 1973; Schlosstein *et al*., 1973), cuya asociación fue descrita en 1973 (Brewerton *et al*., 1973) y desde entonces se han identificado más de 150 alelos conocidos, aunque para la mayoría de estos alelos se desconoce si están asociados con alguna enfermedad (Khan, 2010, 2013, 2017; Dashti *et al*., 2018). La presencia de polimorfismos generalmente localizados en los exones 2 y 3 de los 8 exones que presente el gen *HLA-B*, da lugar a los más de 150 alelos descritos hasta el momento (Little and Parham, 1999; Khan and Ball, 2002; Khan, 2010). EL *HLA-B27* es un antígeno de superficie de clase I codificado por el locus *HLA-B* del MHC en el cromosoma 6, cuya función es presentar antígenos microbianos a linfocitos T **(Figura 10)**. El complejo HLA es fundamental para que el sistema inmune sea capaz de diferenciar lo propio de lo extraño y para permitir la comunicación de células del mismo individuo. Dentro de las SpA, la asociación más fuerte del *HLA-B27* es con la AS, puesto que el 90% de los pacientes con esta enfermedad son *HLA-B27*$^{+}$ (Chatzikyriakidou *et al*., 2011), en comparación con menos del 10% que aparece en la población general. Sin embargo, solo el 5% de individuos *HLA-B27*$^{+}$ desarrollarán finalmente la enfermedad (van der Linden *et al*., 1984b) e incluso la AS se desarrolla en individuos *HLA-B27*$^{-}$. Este porcentaje se incrementa hasta el 15-20% para aquellos individuos que presentan un familiar de primer grado afectado (Brown, 2010; Reveille, 2011). La tendencia familiar de la AS es muy importante con riesgos relativos de 94X, 25X y 4X para parientes de primer, segundo y tercer grado respectivamente (Taurog, 2007). También se ha descrito que los homocigotos para el *HLA-B27* tienen aproximadamente el doble de riesgo de desarrollar AS en comparación con los heterocigotos (Jaakkola *et al*., 2006). Además, se ha observado que los individuos diagnosticados con AS y *HLA-B27*$^{+}$ presentan un inicio más temprano de la enfermedad, un diagnóstico más temprano y un mayor daño radiográfico que los individuos diagnosticados con AS y *HLA-B27*$^{-}$ (Feldtkeller *et al*., 2003; Coates *et al*., 2020). Este alelo muestra, también una asociación con otros tipos de SpA, como la ReA [50-70% de pacientes *HLA-B27*$^{+}$] (Sieper, 2001), PsA [20-50% de pacientes *HLA-B27*$^{+}$] (Gladman *et al*., 1986; Chandran *et al*., 2010; Feld *et al*., 2018) y IBD-SpA [50-70% de pacientes *HLA-B27*$^{+}$] (Wollheim, 2005).

De todos los alelos del gen *HLA-B27* descritos hasta la fecha, el más común es el *HLA-B27:05*, con una distribución mundial. Se ha propuesto a este alelo como el alelo *HLA-B27* ancestral, que evoluciono tras la salida de *Homo sapiens* de África y a partir del cual han surgido el resto de los subtipos a partir de polimorfismos puntuales, aunque no todos los subtipos de *HLA-B27* predisponen la enfermedad (Khan, 2017; Dashti *et al*., 2018) **(Figura 11)**. Se ha descrito que la AS se desarrolla con los siguientes subtipos de *HLA-B27*, aunque la fuerza de esta asociación difiere entre estos subtipos: *B27:01*, *B27:02*, *B27:03*, *B27:04*, *B27:05*, *B27:06*, *B27:07*, *B27:08*, *B27:09*, *B27:10*, *B27:14*, *B27:15* y *B27:19*.

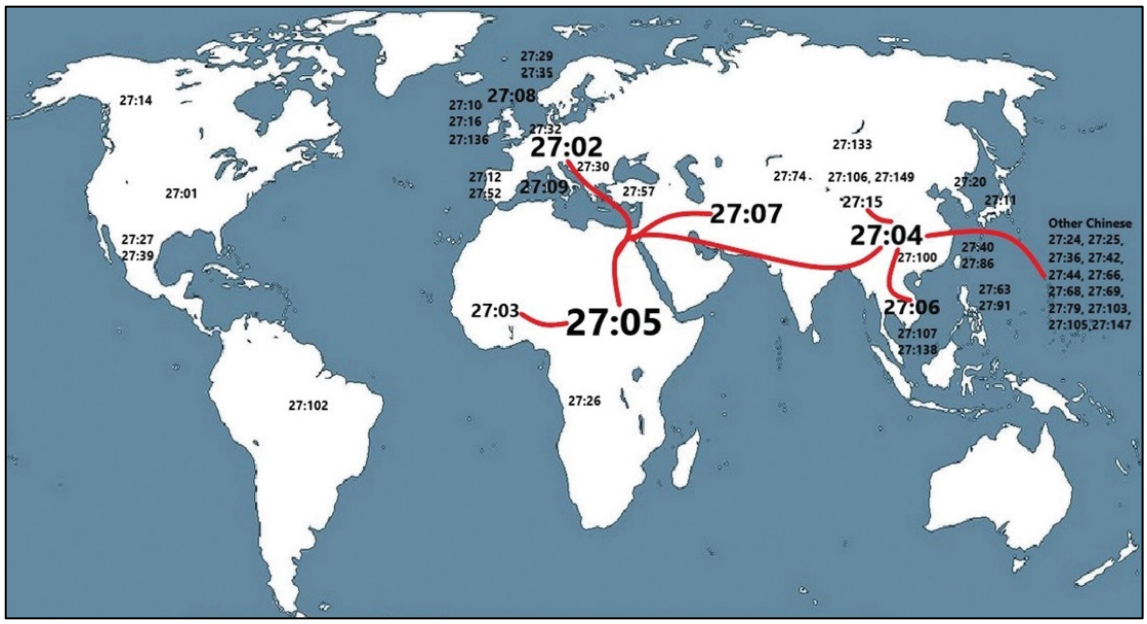


Figura 11. Origen y distribución de los subtipos del alelo *HLA-B27* (Naovarat and Reveille, 2019).

A pesar de la asociación descrita entre el *HLA-B27* y la susceptibilidad a las SpA, actualmente se desconocen los mecanismos moleculares responsables de la asociación entre el alelo *HLA-B27* y las SpA (Bowness, 2015), aunque, sin embargo, diversos estudios han postulado que el *HLA-B27* no presenta un papel directo en la formación de hueso nuevo y cartílago (van Tubergen *et al*., 2012; Lories and Haroon 2014; Neerinkx *et al*., 2017), sino que desempeña un rol indirecto en el proceso inflamatorio (Neerinkx *et al*., 2017). Las siguientes hipótesis han sido postuladas para explicar el posible papel patogénico del *HLA-B27* en las SpA **(Figura 12)**:

a. *Hipótesis del péptido artritogénico* **(Figura 12A)**: Esta hipótesis postula que existe un mimetismo molecular entre las secuencias aminoacídicas de epítopos de algunas bacterias y un ligando endógeno del *HLA-B27*. Esta hipótesis sugiere que el alelo *HLA-B27* desencadena la enfermedad a través de la interacción con células $TCD8^+$ mediante la presentación de péptidos propios que se convierten en el objetivo de la respuesta inmune. Basado en esta hipótesis, las células T citotóxicas responden al *HLA-B27* expresado en varias localizaciones anatómicas (por ejemplo, entesis, SIJs), pudiendo generar inflamación tejido-específica que explicaría el fenotipo de las SpA (Benjamin and Parham, 1992).

b. *Hipótesis del plegamiento anómalo de HLA-B27* **(Figura 12B)**: El *HLA-B27* tiene tendencia natural a plegarse de forma anormal y a acumularse en el retículo endoplásmico (ER, del inglés *endoplasmic reticulum*), lo que genera una respuesta inflamatoria (Mear *et al*., 1999). Esta acumulación puede causar estrés en el ER, lo que activaría un mecanismo de señalización conocido como UPR (del inglés *unfolding protein response*) y la autofagia (Colbert *et al*., 2010). En modelos animales con AS, la sobre-regulación de genes UPR induce e incrementa el número de linfocitos Th17 (del inglés *T helper 17*) y de citocinas pro-inflamatorias como IL-17, IL-23 e IFN-γ (DeLay *et al*., 2009).

c. *Hipótesis de la formación de homodímeros en la superficie celular y reconocimiento inmune* **(Figura 12C)**: EL *HLA-B27* puede formar homodímeros de cadena pesada en la superficie celular (Allen *et al*., 1999), que pueden unirse a receptores específicos como *KIR3DL2* (del inglés *killer cell immunoglobulin-like receptor 3DL2*) (Wong-Baeza *et al*., 2013) y *LILR* (del inglés *leucocyte immunoglobulin-like receptor*) (Giles *et al*., 2012), lo que puede incrementar la expresión de IL-17 e IL-23 a través de la activación de células $TCD8^+$ y las células NK (del inglés *natural killer*) (Bowness *et al*., 2011).

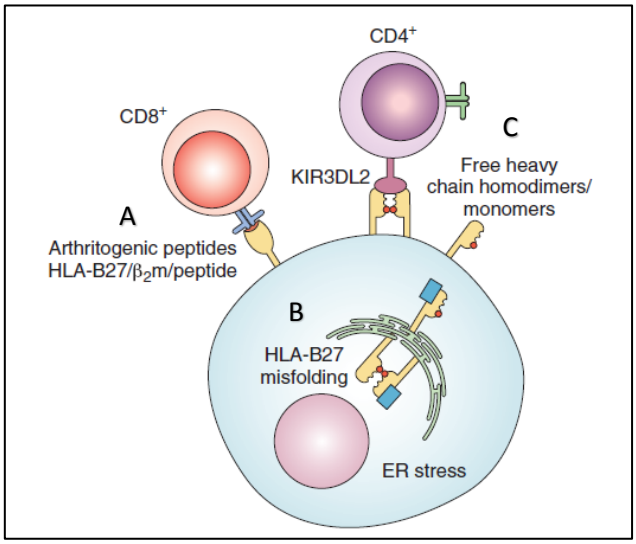


Figura 12. Esquema sobre las hipótesis postuladas acerca del posible rol del alelo *HLA-B27* en la patogénesis de las SpA. A) Hipótesis del péptido artritogénico. B) Hipótesis del plegamiento anómalo de HLA-B27. C) Hipótesis de la formación de homodímeros en la superficie celular y reconocimiento inmune. Modificado de Colbert et al., 2014.

Además del *HLA-B27*, también han sido identificados otras asociaciones entre alelos del gen *HLA-B* y las SpA. Para el caso de la AS se encontraron asociaciones positivas para los alelos *HLA-B13*, *HLA-B14*, *HLA-B38*, *HLA-B40*, *HLA-B47*, *HLA-B49*, *HLA-B51* y *HLA-B52*, y negativas para los alelos *HLA-B07*, *HLA-B08* y *HLA-B57* (Robinson *et al*., 1989; Brown *et al*., 1996; Breban, 1998; López-Larrea *et al*., 2002; van Gaalen *et al*., 2013; Cortes *et al*., 2013, 2015; Díaz-Peña *et al*., 2016; Reveille *et al*., 2019), mientras que para la PsA, los alelos *HLA-B08*, *HLA-B13*, *HLA-B37*, *HLA-B38*, *HLA-B39* y *HLA-B57* incrementan el riesgo a desarrollar dicha enfermedad (Gladman *et al*., 1986; Chandran and Rahman, 2010; Eder *et al*., 2012; Chandran, 2013; Okada *et al*., 2014a). También se han encontrado asociaciones entre las SpA con variantes de las regiones *HLA-A*, *HLA-C*, *HLA-DPB1* y *HLA-DRB1*.

Aunque el *HLA-B27* muestra una presencia genéticamente dominante en este grupo de patologías, se ha sugerido que por ejemplo en la AS contribuye en un 20-25% de la herencia total de la enfermedad (Reveille, 2012). Los estudios de asociación de genoma completo (GWAS, del inglés *genome-wide association studies*) han identificado numerosas variantes genéticas adicionales que contribuyen a la susceptibilidad a desarrollar AS, aunque estos parecen contribuir con un 3-7% a la herencia de la

enfermedad (Ellinghaus *et al*., 2016). Fuera del MHC, se han identificado numerosos genes involucrados en la patogénesis de las SpA, entre los que destacan genes que codifican aminopeptidasas expresadas en el ER, como *ERAP1* y *ERAP2*, o el gen que codifica el receptor de la IL-23 (*IL-23R,* del inglés *interleukin-23 receptor*).

Las aminopeptidasas del ER están involucradas en el recorte de péptidos hasta una longitud de 8-9 aminoácidos para que puedan ser presentados por los receptores de moléculas de MHC de clase I, como el *HLA-B27* (Saveanu *et al*., 2005; Reveille *et al*., 2010) y han sido asociadas con la AS (Burton *et al*., 2007a; Evans *et al*., 2011; Cortes *et al*., 2013) **(Figura 13)**. Esta función junto con la fuerte interacción génica entre variantes del gen que codifica la aminopeptidasa del retículo endoplasmático 1 (*ERAP1*, del inglés *endoplasmic reticulum aminopeptidase 1*) y el alelo *HLA-B27*, señalaron a la presentación anómala de péptidos como el mecanismo clave involucrado en las SpA. La asociación de *ERAP1* con la AS está limitada a los pacientes *HLA-B27*$^{+}$ o *HLA-B40*$^{+}$ (Robinson *et al*., 1989; Cortes *et al*., 2015), mientras que la asociación entre el gen que codifica la aminopeptidasa del retículo endoplasmático 2 (*ERAP2*, del inglés *endoplasmic reticulum aminopeptidase 2*) y la AS ha sido descrita en pacientes *HLA-B27*$^{+}$ y *HLA-B27*$^{-}$ (Cortes *et al*., 2013).

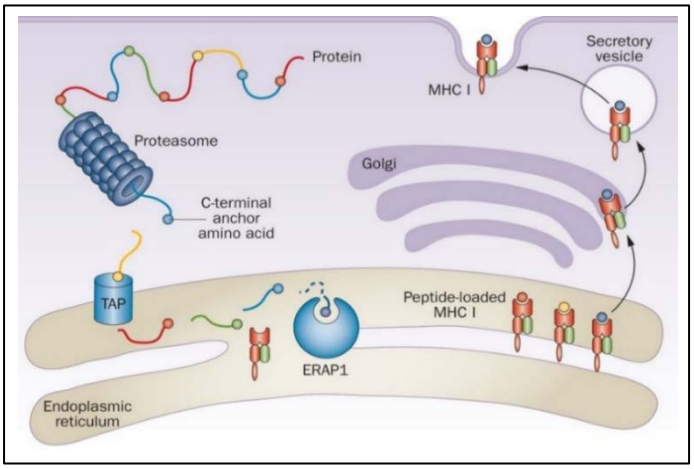


Figura 13. Mecanismo molecular de procesamiento de péptidos mediado por *ERAP1*. Las proteínas intracelulares propias y ajenas son degradadas por el proteosoma y los polipéptidos resultantes entran en el ER por medio de un transportador asociado al procesamiento de péptidos (TAP). Los péptidos son recortados hasta una longitud óptima de 8-9 aminoácidos para ser luego cargados por *ERAP1* en moléculas de clase I del MHC, como el *HLA-B27*. Los complejos péptido-proteína de clase I son transferidos a la superficie de la célula para su presentación a las células T (Kirino and Remmers, 2015). TAP, del inglés *transporter associated with antigen processing*.

Los estudios GWAS han identificado variantes en *IL-23R* implicadas en la ruta de señalización de la IL-23 en distintas enfermedades como la AS (Burton *et al*., 2007a), la IBD (Duerr *et al*., 2006), PsA (Hüffmeier *et al*., 2009) y la Ps (Cargill *et al*., 2007), lo que implica que *IL-23R* es un factor de susceptibilidad común en todo el espectro de las SpA. La IL-23 es una citoquina clave involucrada en la diferenciación de los linfocitos $TCD4^+$ naive en linfocitos Th17 que producen IL-17, IL-6, IL-22, factor de necrosis tumoral α (TNF-α, del inglés *tumor necrosis factor α*) y otras citocinas pro-inflamatorias (Cua and Tao, 2010; Sherlock *et al*., 2012; Razawy *et al*., 2018). La IL-23 es sintetizada sobre todo por macrófagos y células dendríticas en respuesta a infecciones bacterianas y su actividad está mediada por la unión con el complejo del *IL-23R*, que se expresa en los linfocitos T (Tan *et al*., 2009) **(Figura 14)**.

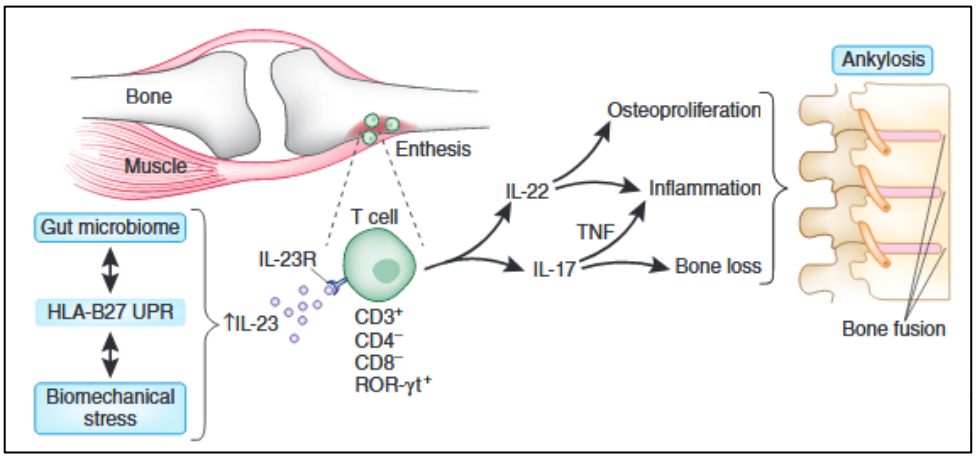


Figura 14. Mecanismo mediante el cual la IL-23 y las células T residentes en las entesis participan en la patogénesis de las SpA. La IL-23 puede activar células T residentes en las entesis, que a su vez pueden promover la inflamación local y la remodelación del hueso a través de una variedad de mediadores efectores, entre los que se incluyen la IL-17 e IL-22, que llevan al típico aspecto anquilosado de la columna vertebral (Lories and McInnes, 2012).

En el contexto de la AS, las células Th17 producen IL-17 desencadenando la activación de los osteoclastos suprimiendo la regeneración del hueso y generando una pérdida ósea, pero también pueden secretar IL-22 tras la exposición a IL-23 que puede estimular la osteoproliferación (Babaie *et al*., 2018) **(Figura 14)**. Este proceso contradictorio podría explicar la coexistencia de la pérdida ósea y la formación de hueso nuevo que tiene lugar en la AS. La asociación de *IL-23R* se ha encontrado tanto en individuos diagnosticados con AS *HLA-B27*$^+$ como *HLA-B27*$^-$ (Reveille *et al*., 2010).

Se ha observado la desregulación del eje de citocinas IL-23/IL-17 en pacientes con SpA, detectándose niveles más elevados de IL-23 e IL-17 en el suero de pacientes diagnosticados con AS y PsA (Mei *et al*., 2011) y en el líquido sinovial de pacientes con PsA, ReA y uSpA (Singh *et al*., 2011; Celis *et al*., 2012). Además, también se observó la presencia de células productoras de IL-17 en las articulaciones facetarias de pacientes con AS (Appel *et al*., 2011) y diversos estudios han demostrado que la inflamación de las entesis depende de IL-23 (Ruutu et al., 2012; Sherlock *et al*., 2012; Benham *et al*., 2014) **(Figura 10 y 11)**. Por esta razón, se ha planteado la hipótesis de que la entesis es el sitio primario de inflamación y el objetivo principal de las SpA **(Figura 15)**. Esta zona contiene una población única de células T residentes, que cuando son activadas por IL-23, pueden provocar la patogénesis característica de estas enfermedades (Lories and McInnes, 2012) **(Figura 14 y 15)**.

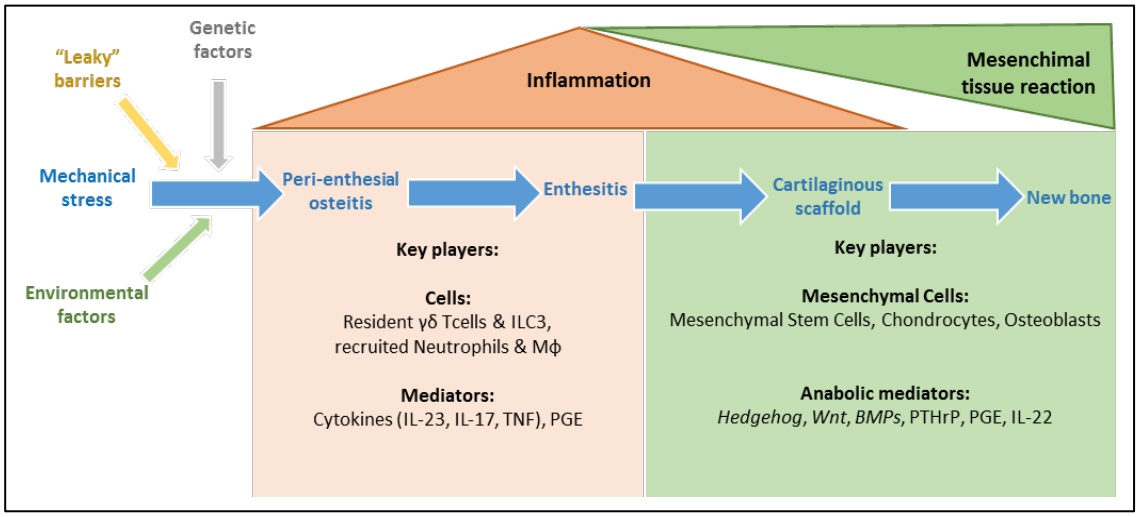


Figura 15. Secuencia de eventos que resultan en la inflamación de las entesis y en la formación de hueso nuevo. Según la hipótesis del estrés mecánico, los microtraumas repetidos en individuos genéticamente predispuestos junto con otros desencadenantes adicionales, inducen la inflamación de la médula ósea y las entesis y la reacción del tejido mesenquimal que resulta en la formación anormal de hueso. Los mediadores moleculares clave para el proceso inflamatorio temprano son *PGE2* y citocinas (IL-23, IL-17, TNF-α). En los pacientes con SpA, la fase inflamatoria es seguida de una formación cartilaginosa mediada por condroblastos continuada de una osificación mediada por osteoblastos. Los mediadores anabólicos clave que promueven la activación de los condroblastos y osteoblastos son las *Hedgehog proteins*, *Wnt agonists*, *bone morphogenic proteins* (BMPs), *parathytoid hormone-related peptide* (PTHrP) e IL-22. Modificado de Kalliolias and Skokos, 2019.

El TNF-α ha sido encontrado sobreexpresado en la circulación, en el fluido sinovial y en las SIJs y articulaciones facetarias de pacientes con AS (Braun *et al*., 1995; Chen *et al*., 2009). Además, la eficacia de inhibidores de TNF-α en pacientes con AS y axSpA apoya la participación de TNF-α en la patofisiología de las SpA. El TNF-α es una citocina producida por diversas células T en respuesta a IL-23, con lo que su función es compatible con la ruta IL-23/IL-17 e induce la actividad de los osteoclastos y puede conducir a erosiones y pérdida ósea.

También se han identificado otras asociaciones genéticas con la AS con efectos sobre la ruta IL-23 (*CARD9*, *EOMES*, *ICOSLG*, *IL1R1*, *IL1R2*, *IL6R*, *IL7R*, *IL12B*, *IL27*, *PTGER4*, *STAT3*, *RUNX3*, *TBX21*, *TYK2* y *ZMIZ1*) (Cortes *et al*., 2013; Ellighaus *et al*., 2016), que pueden influir en la producción de IL-23, en el número o frecuencia de células sensibles a IL-23 y en la señalización de IL-23, lo que ha fortalecido la evidencia de que el eje de citocinas IL-23/IL-17 estaría involucrado en la susceptibilidad a las SpA (Burton *et al*., 2007a; Evans *et al*., 2011; Cortes *et al*., 2013; Parkes *et al*., 2013; Gaffen *et al*., 2014). Dentro de estos genes, son de particular interés, el gen que codifica el receptor 4 de la prostaglandina E_2 (*PTGER4, del inglés prostaglandin E_2 receptor 4*) que vincula el estrés físico en las entesis con la inducción de la producción de IL-23 y las señales anabólicas de formación ósea, identificando así un mecanismo por el cual la inflamación podría conducir a la formación de hueso (Cortes *et al*., 2015), y el gen que codifica la proteína 9 que contiene el dominio de reclutamiento de caspasa (*CARD9*, del inglés *caspase recruitment domain-containing protein 9*), que codifica una proteína involucrada en la señalización entre el receptor dectin-1 de la inmunidad innata y el núcleo, conduciendo a la producción de IL-23 (Cortes *et al*., 2013).

Si bien todos estos genes tienen influencias en la vía de IL-23, también afectan a su vez a otras vías, por lo que no es posible estar seguro si su influencia principal es directamente a través de IL-23 o aguas abajo. Sin embargo, la gran cantidad de genes que influyen en esta vía respalda firmemente la hipótesis de que el eje de citocinas IL-23/IL-17 es fundamental en la patogénesis de las SpA y, en consecuencia, un objetivo importante para el desarrollo de terapias para este grupo de enfermedades (Evans *et al*., 2011; Cortes *et al*., 2013; Parkes *et al*., 2013; Gaffen *et al*., 2014). Además, los datos

de ensayos clínicos bloqueando IL-17A e IL-12/IL-23, proporcionaron un apoyo importante de la ruta IL-23/IL-17 en la patogénesis de las SpA (Baeten *et al*., 2013b; van den Berg and McInnes, 2013; Poddubnyy *et al*., 2014).

Además, existen otros genes involucrados en la diferenciación de células T (*EOMES*, *IL7R*, *RUNX3*, *ZMIZ1*, *BACH2* y *SH2B3*) y los receptores acoplados a proteínas G (*GPR25*, *GPR35*, *GPR37* y *GPR65*) (Reveille *et al*., 2010; Cortes *et al*., 2013) que han sido relacionados con la susceptibilidad a desarrollar estas patologías.

1.1.3.2. Factores ambientales

El microbioma intestinal juega un rol muy importante en el desarrollo del sistema inmune y en el mantenimiento de la homeostasis celular (Fung *et al*., 2014). La disbiosis del microbioma intestinal y las infecciones microbianas están implicadas en varias enfermedades mediadas por el sistema inmune, incluida la esclerosis múltiple, la IBD y la diabetes tipo 1 (Costello *et al*., 2015a, 2015b). La disbiosis en la IBD está asociada con la perdida en diversidad microbiana y una relativa reducción en Firmicutes y Bacteroidetes y un incremento en Enterobacteriaceae (Frank *et al*., 2007; Packey and Sartor, 2009). Existen evidencias de que la inflamación inmune innata puede conducir a la disbiosis intestinal (Craven *et al*., 2012). Diversos investigadores en el campo de las SpA creen que la disbiosis intestinal puede ser la responsable de la génesis de este grupo de enfermedades (Costello *et al*., 2015a, 2015b; Rizzo *et al*., 2017) **(Figura 16)**. En las SpA, la inflamación intestinal es común, donde un 60% de los pacientes con AS muestran inflamación subclínica y un 6,5% desarrollan IBD durante el curso de la enfermedad (Jacques *et al*., 2012), lo que demuestra una superposición en la susceptibilidad genética. Un 30% de los pacientes con IBD pueden cumplir con los criterios de las SpA (de Vlam *et al*., 2000), con un 11-52% mostrando sacroileitis asintomática y un 10% que cumple los criterios de la AS (Jacques *et al*., 2012). Existe la hipótesis de que un efecto del *HLA-B27* puede alterar el microbioma intestinal de una forma que promueve la enfermedad inflamatoria (Rosenbaum and Davey, 2011; Rizzo *et al*., 2017), ya que los pacientes *HLA-B27*$^{+}$ exhiben diferencias en las comunidades de microorganismos en la zona terminal del íleon comparado con controles sanos y una mayor abundancia de Prevotellaceae y Bacteroidaceae (Costello *et al*., 2015b), además de conducir al

desarrollo de la enfermedad induciendo procesos inmunológicos como la producción de IL-23 (Kenna and Brown, 2013). Actualmente se desconoce si estas diferencias pueden ser causa o consecuencia de la inflamación.

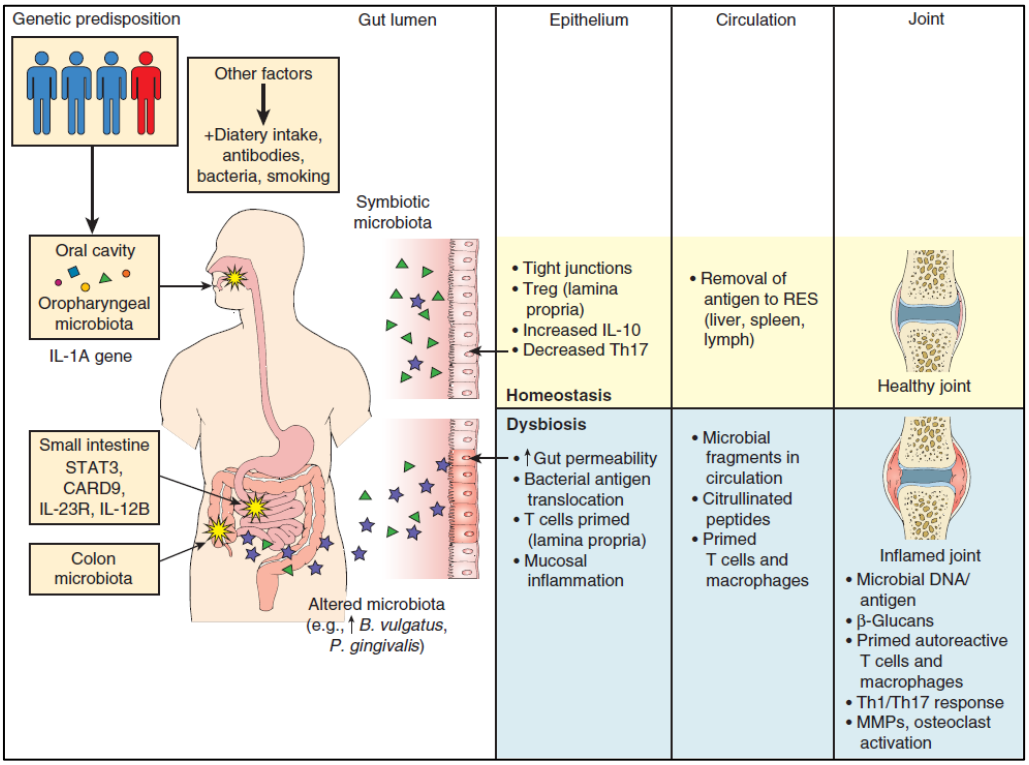


Figura 16. Interacción microbiota-huésped. Homeostasis *versus* disbiosis y la supuesta generación de artritis enteropática (Yeoh *et al*., 2013).

La ReA es otro ejemplo en donde la exposición a un factor ambiental desencadena la enfermedad en el huésped. La ReA suele ser desencadenada por infecciones gastrointestinales por *Campylobacter*, *Salmonella*, *Shigella* o *Yersinia* (Ajene *et al*., 2013; Costello *et al*., 2015b), urogenitales por *Chlamydia trachomatis* (Taylor-Robinson *et al*., 1992) o respiratorias por *Chlamidia pneumoniae* (Carter and Hudson, 2017) o *Mycoplasma pneumoniae* (Natarajan *et al*., 2001). En la mayoría de los pacientes con ReA, la recuperación es completa sin daño articular o enfermedad crónica, aunque algunos pacientes progresarán a una SpA crónica que incluye afectación axial con cambios radiográficos (Kaarela *et al*., 2009).

Otro factor ambiental relacionado con el desarrollo de las SpA hace referencia a la vitamina D. La vitamina D es una hormona esteroidea que juega un papel crucial en el metabolismo del calcio, la homeostasis ósea (Reynolds and Bruce, 2017) y en la

regulación del sistema inmune, modulando las reacciones inflamatorias (Bikle, 2007; Hewison, 2012; Wöbke *et al*., 2014). Las personas principalmente obtienen la vitamina D a partir de la acción de la luz solar (~90%) y también de los alimentos de la dieta o suplementos alimenticios (~10%) (Holick, 2007). En la actualidad, cada vez es más reconocido el papel de la vitamina D en la etiología de las enfermedades autoinmunes (Bikle, 2007; Holick, 2009; Hewison, 2012; Reynolds and Bruce, 2017). Estudios epidemiológicos han mostrado evidencias de una mayor prevalencia de enfermedades autoinmunes y reumáticas en poblaciones que viven en latitudes más altas, donde la deficiencia de vitamina D, probablemente causada por una menor exposición solar, puede desempeñar un papel importante en su patogénesis (Podolsky, 1991; Sonnenberg and Wasserman, 1991; Cantorna, 2006; Cutolo *et al*., 2009; Hamzaoui *et al*., 2010; Arnson *et al*., 2011; Wen and Baker, 2011).

Varios autores han señalado una posible asociación entre el déficit de vitamina D y diferentes enfermedades como las SpA (Lange *et al*., 2005; Mermerci *et al*., 2010; Arends et al., 2011; Cantorna, 2012). Se ha observado que las SpA son más prevalentes en áreas con exposición a la luz solar disminuida, como América del Norte y el norte de Europa (Podolsky, 1991; Sonnenberg and Wasserman, 1991; Cantorna, 2006). Distintos meta-análisis han sugerido que la deficiencia de vitamina D podría estar relacionado con el desarrollo de AS (Fischer *et al*., 2012), ya que se han observado niveles inferiores en suero del metabolito de la vitamina D [25(OH)D_3] en pacientes con AS en comparación con controles sanos (Lange *et al*., 2005; Mermerci *et al*., 2010; Muntean *et al*., 2011; Erten *et al*., 2013; Cai *et al*., 2015). Además, también se ha observado una correlación negativa entre los niveles de séricos de vitamina D y la actividad de la AS indicada mediante el *Bath Ankylosing Disease Activity Index* (índice BASDAI) y los niveles de velocidad de sedimentación globular (ESR, del inglés *erythrocyte sedimentation rate*) y CRP (Lange *et al*., 2001, 2005; Durmus *et al*., 2012; Hmamouchi *et al*., 2013, 2016; Erten *et al*., 2013; Zhao *et al*., 2017). Estos resultados podrían sugerir que niveles más elevados de vitamina D disminuirían el riesgo de desarrollar AS.

También existen evidencias de la influencia nociva del tabaquismo en las SpA y en particular en la AS (Wendling and Prati, 2013). Estudios previos asociaron el tabaquismo

con una mayor actividad y gravedad de la enfermedad, un mayor daño radiográfico, un aumento de los niveles de CRP, una peor respuesta al tratamiento con inhibidores de TNF-α y una mayor incidencia en la AS (Chung *et al*., 2012; Poddubnyy *et al*., 2012b, 2013; Wendling and Prati, 2013; Videm *et al*., 2014; Azizi *et al*., 2015; Sakellariou *et al*., 2015; Ciurea *et al*., 2016). El tabaquismo presenta efectos inflamatorios a través del aumento de la producción de citocinas pro-inflamatorias como IL-6, IL-17 y TNF-α y de la activación y diferenciación de linfocitos Th17, además de alterar la microbiota intestinal y aumentar el riesgo a desarrollar enfermedad cardiovascular (Torii *et al*., 2011; Kazantseva, *et al*., 2012; Rom *et al*., 2013; Biedermann *et al*., 2014; Wendling and Prati, 2015).

Por otro lado, debido a las propiedades inmunomoduladoras del tejido adiposo y sus vínculos con la inflamación y la autoinmunidad se ha sugerido que la obesidad es una afección inflamatoria crónica, implicada como un factor de riesgo para el desarrollo de enfermedades inmunomediadas (Harpsøe *et al*., 2014; Nikiphorou and Fragoulis, 2018). En la obesidad, se ha observado que existe una mayor producción de citocinas pro-inflamatorias (IL-6 y TNF-α) y adipoquinas que pueden conducir al estado inflamatorio, lo que sugiere que el tejido adiposo presenta un rol importante en los procesos inflamatorios (Hauner *et al*., 2005; Versini *et al*., 2014; Vargas *et al*., 2016). Distintos estudios han observado que la obesidad es más prevalente en pacientes diagnosticados con PsA y AS en comparación con la población sana (Bhole *et al*., 2012; Maas *et al*., 2016; López-Medina *et al*., 2017). Además, la obesidad incrementa la actividad de la enfermedad e influye en la eficiencia al tratamiento de la PsA y AS basadas en inhibidores de TNF-α, lo que sugiere que un índice de masa corporal (BMI, del inglés *body mass index*) elevado incrementaría el riesgo a desarrollar PsA y AS (Durcan *et al*., 2012; Ottaviani *et al*., 2012; Di Minno *et al*., 2013; Gremese *et al*., 2014; Hojgaard *et al*., 2016; Micheroli *et al*., 2017). Además, la obesidad incrementa el riesgo a desarrollar enfermedad cardiovascular (Jamnitski *et al*., 2013; Bengtsson *et al*., 2017a, 2018), ateroesclerosis (Eder *et al*., 2013, 2015) y conduce a un incremento del estrés mecánico especialmente en entesis y tendones, localizaciones anatómicas caracterizadas por su inflamación en las SpA (Scott *et al*., 2015).

1.1.4. Epidemiología de las espondiloartritis

La prevalencia de las SpA es de 0,1-1,4% a nivel global y de 0,5-2% en población caucásica (Zeidler *et al*., 2006), y muestra diferencias geográficas que pueden ser explicadas por la prevalencia del alelo *HLA-B27* (Stolwijk *et al*., 2012), muy fuertemente asociado con el desarrollo de este grupo de patologías. Se ha observado la existencia de una correlación entre la prevalencia de la AS y la prevalencia del *HLA-B27*, es decir, cuanto mayor es la frecuencia de dicho alelo en la población, mayor es la prevalencia de la AS (Khan, 1996). Por un lado, las prevalencias más elevadas del *HLA-B27* se encuentran en la tribu Pawaia en Papúa Nueva Guinea (53%) (Bhatia *et al*., 1988), seguido de los indios Haida en el oeste de Canadá (50%) (Gofton *et al*., 1984) y en la tribu de los esquimales Chukotka en Rusia Oriental (40%) (Alexeeva *et al*., 1994; Krylov *et al*., 1995) **(Figura 17)**, correlacionándose con la gran prevalencia de SpA en los indios Haida (6-10%) (Gofton *et al*., 1984) **(Figura 18)**. Por otro lado, las frecuencias más bajas han sido descritas en Japón (1%) (Hukuda *et al*., 2001) y en población árabe (3%) (Mustafa *et al*., 2012), siendo en la población de Japón donde menor prevalencia presentan las SpA (Hukuda *et al*., 2001) **(Figura 17)**. Además, las poblaciones genéticamente no mezcladas como los nativos de Australia y América del Sur, los africanos del Sahara y los de la Polinesia Oriental exhiben frecuencias muy bajas o incluso nulas de este alelo (Khan, 1995; López-Larrea *et al*., 1995) **(Figura 17)**.

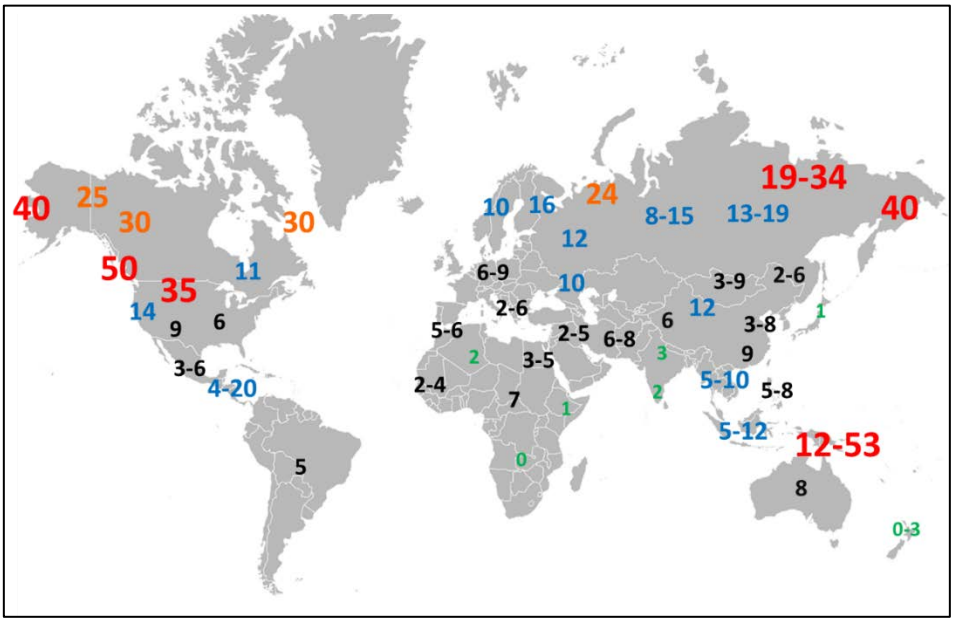


Figura 17. Prevalencia del alelo *HLA-B27* en poblaciones indígenas (%). Datos adaptados de Khan, 1995.

En Europa, las frecuencias más elevadas de *HLA-B27* han sido encontradas en las poblaciones del norte de Europa, como las detectadas en poblaciones escandinavas (10-16%) (Sieper *et al*., 2006) **(Figura 17)**. En estas poblaciones, a su vez presentan valores elevados de prevalencia de SpA, siendo las poblaciones del norte de Noruega las que presentan los valores más elevados de prevalencia en Europa (1,8%) (Johnsen *et al*., 1992), seguido de Alemania (0,86%) (Braun *et al*., 1998) **(Figura 18)**. Sin embargo, en el sur de Europa se detectan valores de frecuencia del *HLA-B27* y de prevalencia de SpA menores, siendo la población griega la que exhibe los valores más bajos de prevalencia de las SpA (0,24%) y de *HLA-B27* (Andrianakos *et al*., 2003) **(Figuras 17 y 18)**.

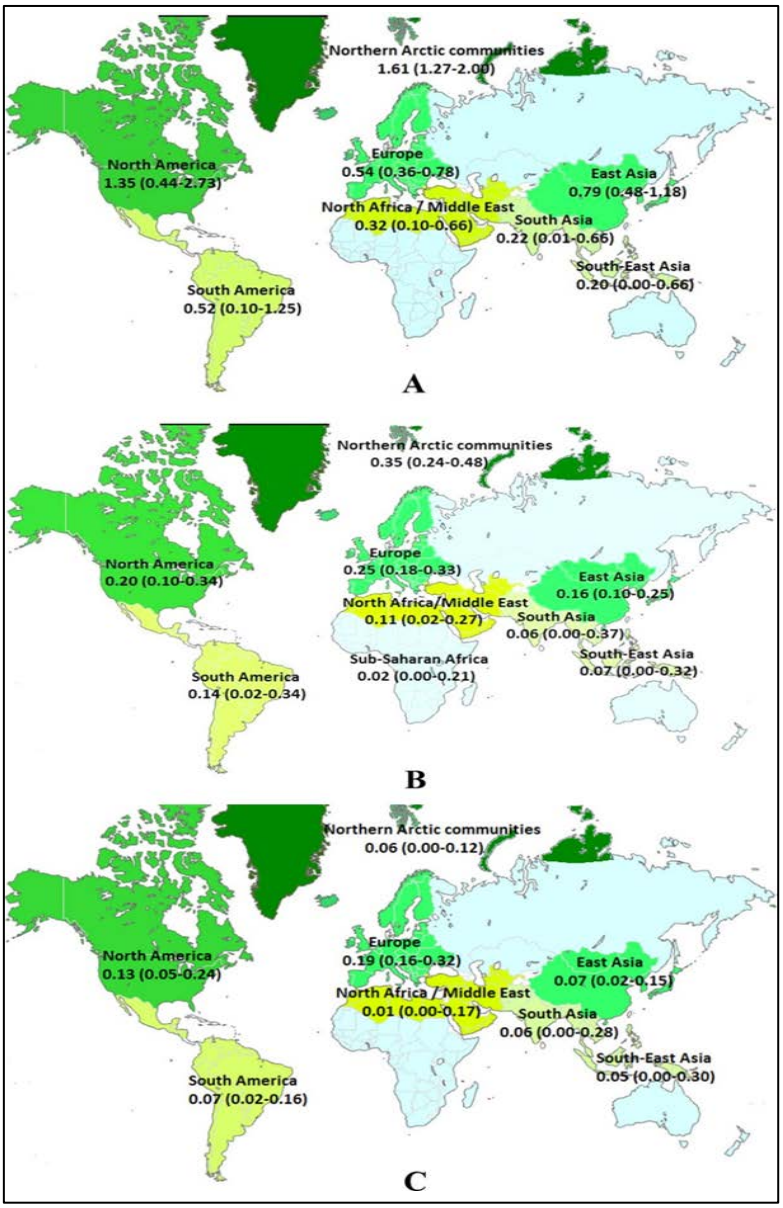


Figura 18. Prevalencia global de: A) SpA, B) AS, C) PsA (Stolwijk *et al*., 2016).

Por otro lado, el género y la edad de inicio de los síntomas se relaciona de forma diferencial con las distintas entidades que conforman las SpA. La AS es una enfermedad que afecta principalmente a jóvenes, ya que ~80% de los pacientes desarrollan los primeros síntomas antes de los 30 años, y menos del 5% de los pacientes los presenta después de los 45 años de edad, siendo más prevalente en hombres que en mujeres (2-3:1) (Feldtkeller *et al*., 2003). Al igual que la AS, la ReA es más prevalente en hombres sí se origina por una infección urogenital e igualmente prevalente entre los dos sexos si la infección es de origen gastrointestinal (Barth and Segal, 1999; Flores *et al*., 2003; Carter and Hudson, 2009) y afecta a individuos generalmente entre los 20-40 años (Carter and Hudson, 2009). La PsA afecta a hombres y mujeres por igual y la edad de inicio de los síntomas es entre los 30-50 años (Gladman *et al*., 2005). En relación a la IBD, tanto la CD como la UC presentan una edad de inicio de los síntomas de 20-30 años y no presentan diferencias en relación al sexo, aunque en algunas cohortes se ha observado una mayor frecuencia de mujeres para la CD (Vegh *et al*., 2014; Rönnblom *et al*., 2016; Su *et al*., 2016; Shivashankar *et al*., 2017). La edad de inicio de la uSpA suele ser anterior a los 40 años para pacientes con predominio axial y posterior a los 40 años para pacientes con predominio periférico (Skare *et al*., 2012) y afecta a más hombres que a mujeres (3:2) (Cruzat *et al*., 2010).

1.2. Otras enfermedades reumáticas

1.2.1. Osteoartritis

La osteoartritis (OA) es una enfermedad degenerativa articular que afecta principalmente a personas mayores de 50 años. La OA es la forma más común de artritis y está caracterizada por la erosión del cartílago articular, hipertrofia, esclerosis subcondral, formación de hueso nuevo (osteofitos) en los márgenes de la articulación, en un intento reparador del tejido óseo, y por alteraciones bioquímicas y morfológicas en la membrana sinovial y en la capsula articular de cualquier articulación (Kraus *et al*., 2015; Di Cesare *et al*., 2017). Las articulaciones afectadas más comunes son las manos, cadera, rodilla, columna vertebral (región cervical y lumbar), pies y hombros **(Figura 19)**. En la mano se ven afectadas las articulaciones interfalángica distal (Bijsterbosch *et al*., 2010, 2011), interfalángica proximal (Bijsterbosch *et al*., 2010, 2011),

metacarpofalángica (Kraus *et al*., 2007) y carpometacarpiana (Dominick *et al*., 2005) **(Figura 20)**.

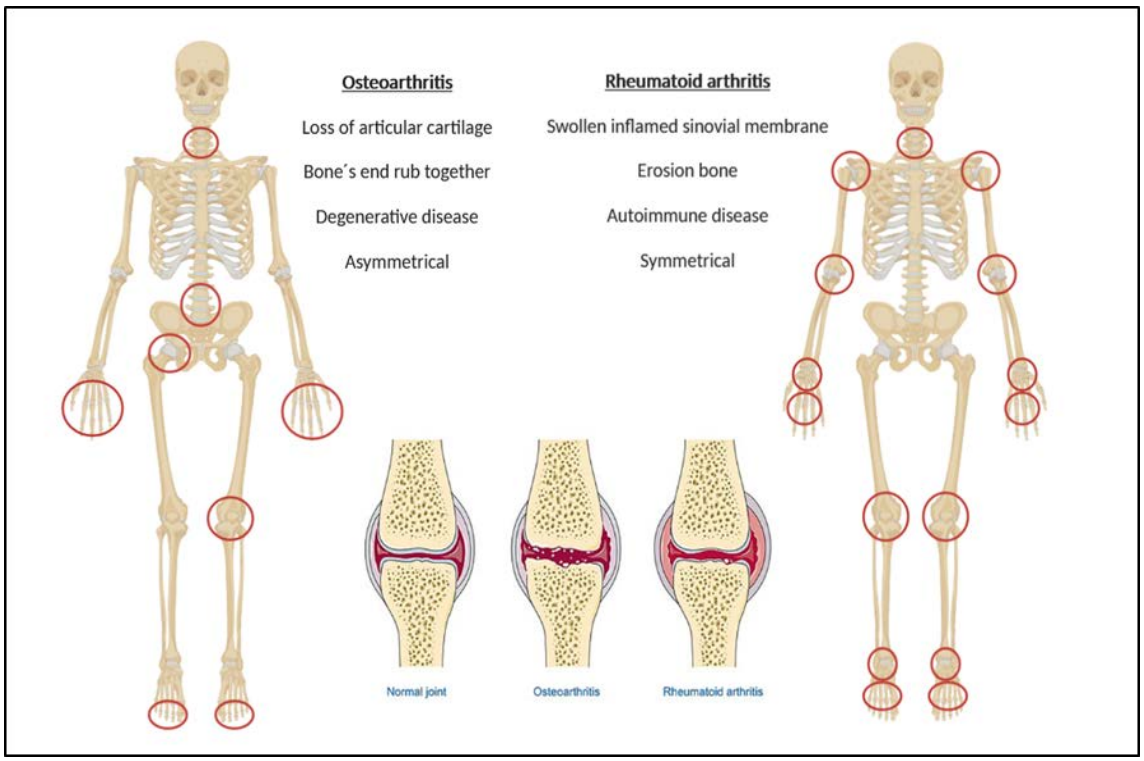


Figura 19. Diferencias clínicas articulares entre la OA y la RA. Creado en Biorender.com.

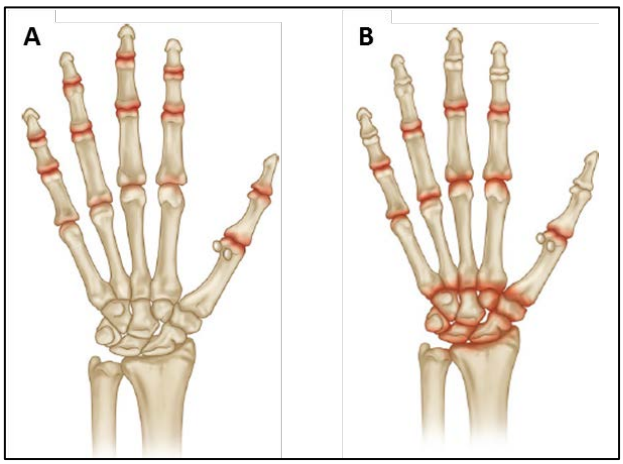


Figura 20. Regiones anatómicas comunes de afectación de la mano A) OA y B) RA. Modificado de Khalidi and O´Neill, 2015.

En estas articulaciones, los condrocitos responden a la agresión mediante la producción de enzimas degradantes y con respuestas reparadoras inadecuadas. Además, una gran cantidad de mediadores y moléculas pro-catabólicas presentes en el proceso, como proteasas y citocinas pro-inflamatorias (IL-1, IL-6, IL-8, TNF-α), comprometen la síntesis de las macromoléculas de la matriz, esenciales para la homeostasis del cartílago (Di

Cesare *et al*., 2017) **(Figuras 21 y 22)**. Estas alteraciones generan dolor y en los estadios más avanzados, hinchazón, deformidad articular y finalmente, incapacidad funcional (Dieppe and Lohmander, 2005) **(Figura 22)**.

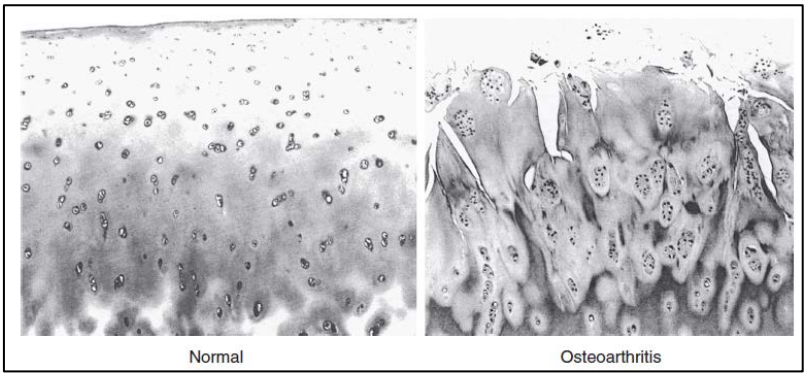


Figura 21. Secciones histológicas de cartílago articular normal y cartílago con OA obtenidas de la cabeza del fémur. El cartílago articular con OA presenta irregularidades en la superficie, con hendiduras o surcos en la zona radial y clonación de condrocitos (Di Cesare *et al*., 2017).

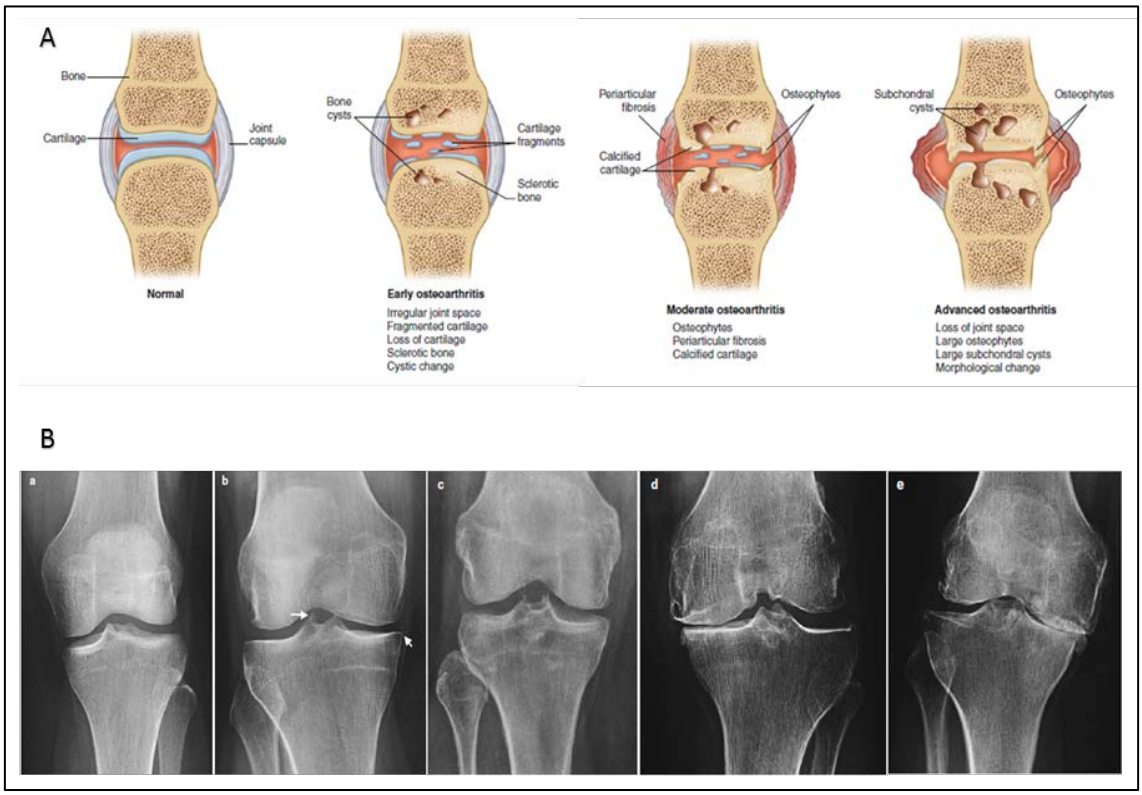


Figura 22. A) Desarrollo y progresión de la OA. B) Radiografía convencional de una rodilla con OA. a) Grado 0: Rodilla normal. b) Grado 1: dudoso estrechamiento del espacio articular y osteofitos tempranos (flechas blancas). c) Grado 2: osteofitos definidos, posible estrechamiento del espacio articular. d) Grado 3: osteofitos múltiples moderados, estrechamiento definido del espacio articular, ligera esclerosis y posible deformidad del contorno óseo. e) Grado 4: osteofitos grandes, marcado estrechamiento del espacio articular, esclerosis severa y deformidad definida del hueso (Cividino and O´Neill, 2015).

1.2.2. Etiología de la osteoartritis

Actualmente, la patogénesis de la OA es desconocida, aunque se sabe que es una enfermedad multifactorial resultante de la interacción de diferentes factores genéticos y ambientales (Fernández-Moreno *et al*., 2008; Valdes *et al*., 2010; Rego-Pérez *et al*., 2013) **(Figura 19)**. La edad es el factor de riesgo más fuertemente asociado a la OA, incrementándose su riesgo progresivamente en todas las articulaciones con la edad (Peyron, 1984; Martin and Buckwalter, 2002). La OA rara vez ocurre antes de los 40 años y afecta a un 10-20% de la población mayor de 50 años y a un 80% de los mayores de 75 años (Blanco *et al*., 2011; Di Cesare *et al*., 2017). Otros factores ambientales que se asocian al desarrollo OA son la obesidad (Anderson and Felson, 1988; Felson *et al*., 1995; Hunter *et al*., 2002), el sexo femenino (Wluka *et al*., 2000), la localización de las articulaciones (Cole and Kuettner, 2002), el padecimiento continuado de traumatismos (Lane *et al*., 1977; Bullough, 1981) y la actividad mecánica repetitiva asociada a actividades deportivas y/o profesionales (Radin and Paul, 1971; Conaghan, 2002; Thelin *et al*., 2006).

La susceptibilidad a desarrollar OA no depende de un factor genético aislado, sino de la acumulación del efecto de múltiples factores genéticos interactuando con los factores ambientales comentados anteriormente **(Figura 23)**. Dentro de los factores genéticos, la susceptibilidad está determinada por múltiples polimorfismos autosómicos, cuya contribución en cada caso concreto supone un pequeño efecto. Una de las características de los polimorfismos asociados al desarrollo de la OA es que son comunes en la población general, incluso en sujetos que no desarrollarán la enfermedad. Diversos estudios han estimado la heredabilidad de la OA en un 50-65% (Cicuttini and Spector, 1997; Loughlin, 2001; MacGregor *et al*., 2000a), aunque existen diferencias sobre la influencia genética ejercida que determina la articulación afectada en la OA (Spector *et al*., 1996; Hirsch *et al*., 1998; Bijkerk *et al*., 1999). A pesar de que la patogénesis de la OA no está clara, está ampliamente aceptado que la genética juega un papel fundamental en la prevalencia y progresión de la OA (McDonnell *et al*., 2007; Minafra *et al*., 2014). Se ha postulado que la OA puede ser causada por mutaciones en varios genes que se expresan en el cartílago, entre los que se incluyen aquellos que codifican los tipos II, IV, V y VI de colágeno, así como la proteína de la matriz oligomérica del cartílago (Ala-Kokko

et al., 1990; Jimenez *et al*., 1998). Por otro lado, existen otros genes candidatos entre los que se incluyen el receptor de la vitamina D (Sokoloff, 1969; Radin and Paul, 1970; Glowacki *et al*., 2003), *NCOA3* (Evangelou *et al*., 2014), *ALDH1A2* (Styrkarsdottir *et al*., 2014), *GDF5* (Zhang *et al*., 2015), además de diferentes citocinas por-inflamatorias (IL-1, IL-6, TNF-α) asociadas con el daño al cartílago en la OA (Loughlin, 2002).

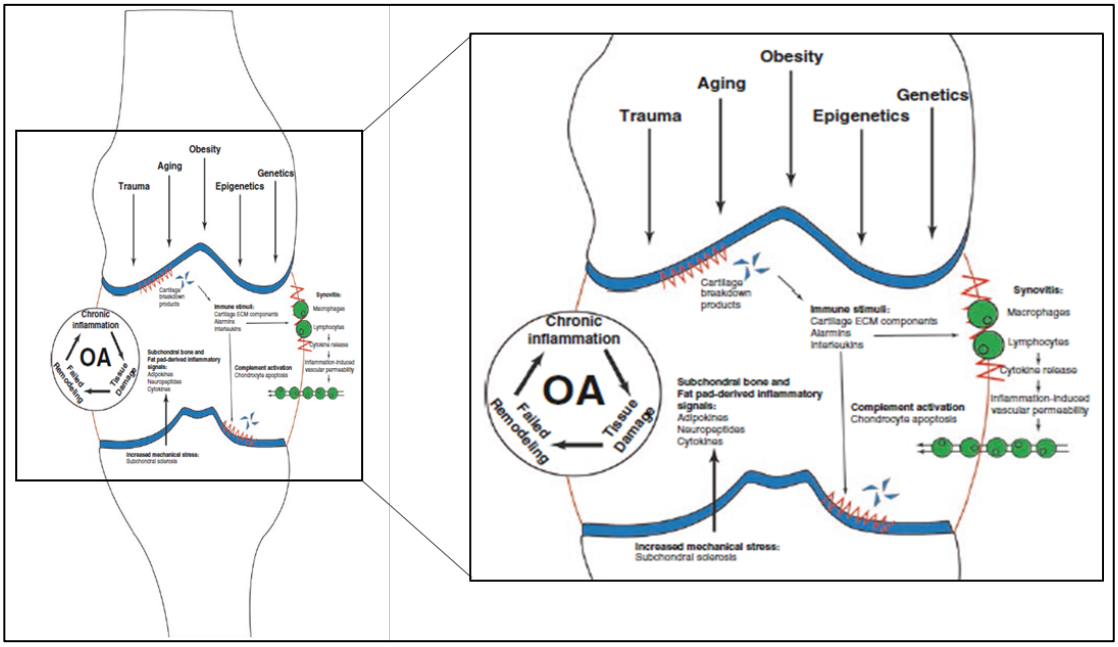


Figura 23. La fisiopatología de la OA implica varios factores, incluyendo factores genéticos, epigenéticos y ambientales como el envejecimiento, la obesidad o los traumatismos. La inflamación crónica juega un papel importante en la patogénesis de la OA, resultando en un daño tisular crónico. Modificado de Jeffries, 2020.

1.2.2. Artritis reumatoide

La RA es una enfermedad inflamatoria, autoinmune y sistémica que afecta al 0.5%-1% de la población general (Silman and Pearson, 2002; Smolen *et al*., 2016). La RA está dividida típicamente en dos subtipos: RA seronegativa o seropositiva. La RA seropositiva está definida por la presencia de niveles séricos elevados del factor reumatoide (RF, del inglés *rheumatoid factor*) y de antiproteínas citrulinadas (ACPAs, del inglés *anti-citrullinated protein antibodies*). La inflamación puede afectar a cualquier articulación sinovial (Imboden, 2009), siendo las pequeñas articulaciones de manos y pies las más comúnmente afectadas, con una distribución simétrica y bilateral **(Figura 19)**. El daño radiográfico y las erosiones en la superficie de las articulaciones provoca deformación y destrucción articular acompañada de una pérdida de función asociada con discapacidad

y reducción de la calidad de vida del paciente (Silman and Pearson, 2002) **(Figura 24)**. Además, la inflamación en la RA no solo afecta a las estructuras articulares, sino que también afecta a tejidos extra-articulares, produciendo complicaciones mucocutáneas, cardiopulmonares, neurológicas, oculares y hematológicas (Mielants and van den Bosch, 2009).

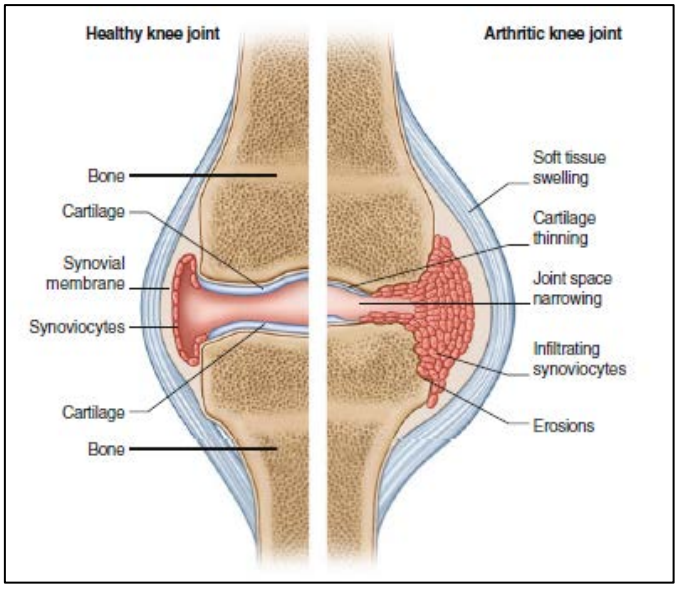


Figura 24. Desarrollo de las erosiones y la sinovitis (inflamación del sinovio) en la RA. La RA está caracterizada por la proliferación sinovial y la inflamación. Esto resulta en la hinchazón del tejido blando debido a la sinovitis, la formación de nuevos vasos en el sinovio, erosiones óseas, edema de médula ósea, destrucción del cartílago y osteopenia periarticular (Larche, 2015).

Las articulaciones de manos y pies se ven afectadas en fases más tempranas de la enfermedad **(Figura 25A)**. Las articulaciones metacarpofalángica, metatarsofalángicas e interfalángicas proximales están casi siempre afectadas, especialmente en los tres primeros dedos, además de las articulaciones de muñecas y tobillos (Llopis *et al*., 2017) y raramente afecta a la articulación interfalageal distal y a la primera articulación metacarpofalángica y metatarsofalángica (Ichikawa *et al*., 2012; Longo *et al*., 2015) **(Figuras 20 y 25A-F)**. Las articulaciones medianas y grandes (codo, hombro, rodilla, cadera) suelen verse afectadas en fases más avanzadas de la enfermedad (Llopis *et al*., 2017) **(Figura 19)**. La afectación de la columna vertebral se limita a la región cervical ya que aparece en aproximadamente el 80% de los casos, siendo normalmente afectada la zona superior, especialmente la articulación atlantoaxoidea (C1-C2) (Joaquim and Appenzeller, 2014) **(Figuras 19 y 25G)**. La RA rara vez afecta a la región torácica y

lumbosacra de la columna vertebral y típicamente no causa sacroileitis **(Figura 25)**. En fases más avanzadas puede observarse anquilosis, especialmente en las zonas del carpo y del tarso **(Figura 25F)**.

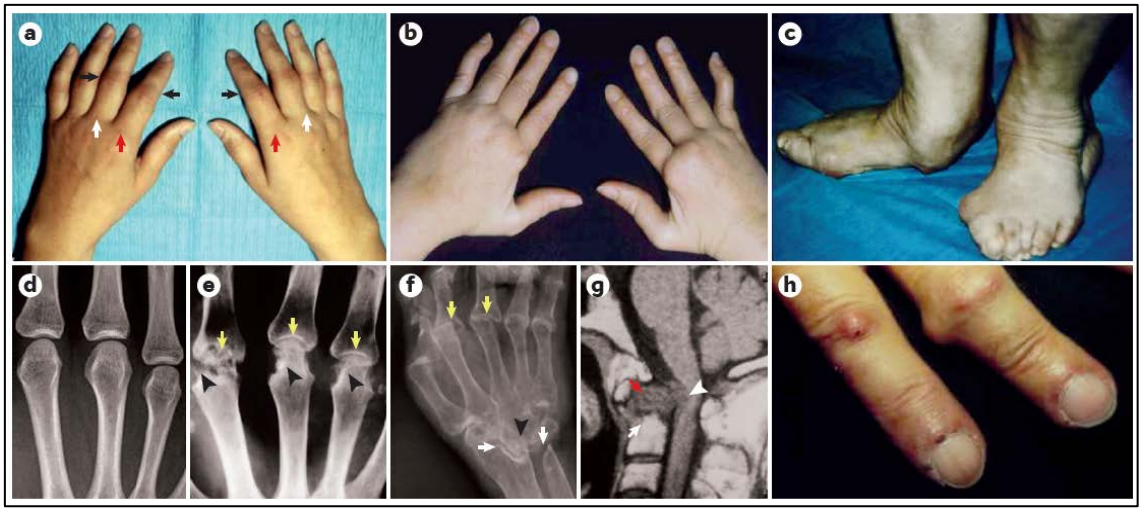


Figura 24. Manifestaciones clínicas de la RA. A) RA temprana caracterizada por una leve y apenas perceptible hinchazón de la segunda (flecha roja) y tercera (flecha blanca) articulación metacarpofalángica de ambas manos y de varias articulaciones interfalángicas proximales (flechas negras). B) RA establecida con varias deformidades, incluida la subluxación en las articulaciones metacarpofalángica, deformidades de cuello de cisne de varios dedos, que se observan sobre todo en el dedo meñique, y una deformidad en Z del pulgar de la mano derecha. C) RA severa de inicio tardío con afectación mutilante de las articulaciones del pie y del tobillo. D-F) Radiografías convencionales de la mano que van desde una articulación normal (D) a daños graves (E y F) con erosiones óseas (E, puntas de flechas negras) y estrechamiento del espacio articular que corresponde a la pérdida de cartílago (E, flechas amarillas) y cambios mutilantes, donde, por ejemplo, el espacio articular (cartílago) entre los diversos huesos carpianos pequeños se reduce y se fusionan (F, punta de flecha negra). Los cambios mutilantes también implican deformaciones (F, flechas amarillas) y la destrucción del radio distal y el cúbito distal donde interactúan con el carpo (F, flechas blancas). G) MRI de la región cervical de la columna que muestra una severa formación de pannus (proliferación del tejido sinovial) (flecha roja) en la articulación atlantoaxoidea con compresión de la médula (punta de flecha blanca) debido a la hiperplasia sinovial. Las cavidades muestran erosiones severas (flecha blanca). H) Presencia de nódulos reumatoides en las caras dorsal y lateral de varios dedos y vasculitis periungual en los pliegues de las uñas (puntos negros) (Smolen *et al*., 2018).

1.2.2.1. Etiología de la artritis reumatoide

A pesar de que las causas precisas que desencadenan la RA siguen siendo inciertas, está ampliamente aceptado que, al igual que como ocurre con otras enfermedades, son el resultado de una compleja interacción entre factores genéticos y ambientales

(Pedersen, *et al*., 2006; Deane, *et al*., 2017) **(Figura 26)**. Se ha sugerido que los factores genéticos son la mayor influencia en el desarrollo de la RA, ya que contribuyen aproximadamente en el 50-60% del riesgo de desarrollar RA (MacGregor *et al*., 2000b) **(Figura 26)**.

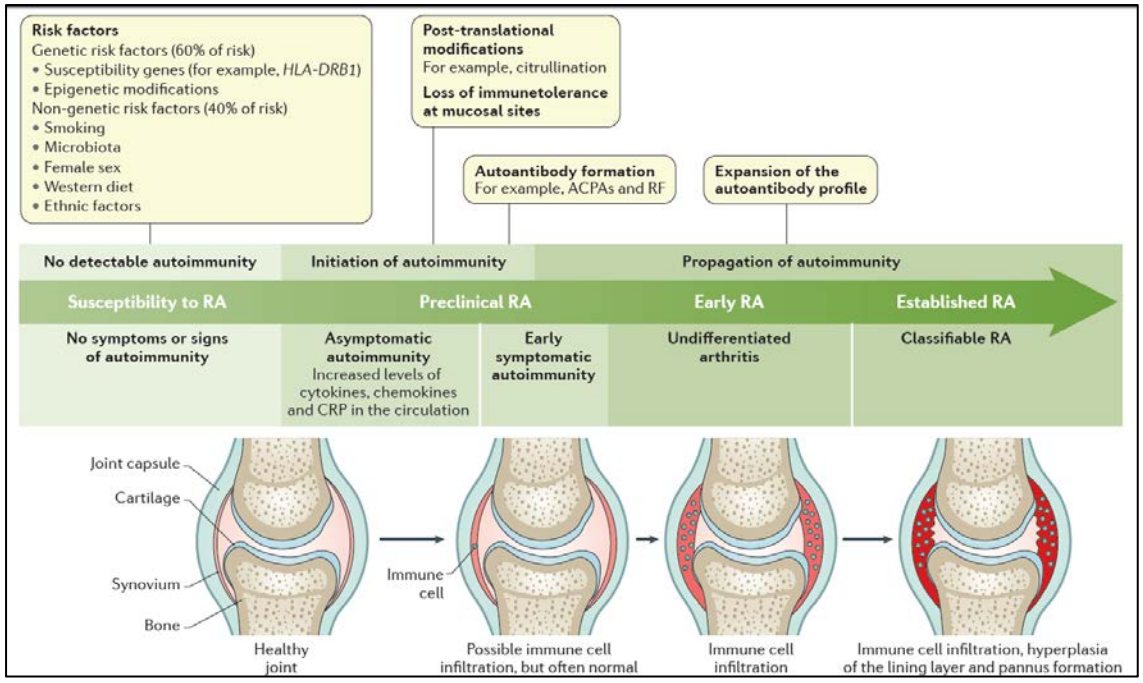


Figura 26. Desarrollo y progresión de la RA. Tanto los factores de riesgo genéticos como los no genéticos contribuyen al desarrollo de la RA, y es posible que se requieran múltiples factores de riego antes de que se alcance un umbral por encima del cual se desencadena la RA. En un individuo genéticamente predispuesto con genes de susceptibilidad, los estímulos ambientales, las modificaciones epigéneticas y las modificaciones postraduccionales pueden conducir a la pérdida de tolerancia en las mucosas con la subsiguiente sinovitis asintomática, que en última instancia conduce a la RA clínica. La progresión de la enfermedad implica la iniciación y propagación de la autoinmunidad frente a proteínas propias, lo que puede ocurrir años antes de la sinovitis subclínica y de los síntomas clínicos de la RA (Smolen *et al*., 2018).

Los factores genéticos identificados incluyen múltiples genes implicados en la respuesta inmune, siendo los más conocidos un conjunto de alelos del gen *HLA-DR* dentro del MHC (Weyand *et al*., 1992). Estas variantes alélicas comparten la secuencia de aminoácidos glutamina-leucina-arginina-alanina-alanina (QKRAA) en el dominio funcionalmente implicado en su función de unión y presentación de antígenos al receptor del linfocito T (Gregersen *et al*., 1987). Esta secuencia común a los diferentes alelos de *HLA-DR* asociados a la RA ha sido denominada como epítopo compartido y son considerados como la influencia genética más importante en la susceptibilidad a la RA (Gregersen *et*

al., 1987; Raychaudhuri *et al*., 2012). En la actualidad se considera que estos alelos contribuyen hasta aproximadamente un 40% del riesgo genético para la RA, aunque en otros estudios se sugiere una contribución menor (Gregersen *et al*., 1987; Plenge, 2009a; van der Woude *et al*., 2009; Raychaudhuri *et al*., 2012; Frisell *et al*., 2013; Kurkó *et al*., 2013). Muchos de estos alelos que contienen el epítopo compartido se encuentran en la región *HLA-DRB1*.

Los estudios GWAS han permitido identificar más de 100 loci asociados con la susceptibilidad a desarrollar RA, aunque únicamente se les ha otorgado aproximadamente un 5% de la asociación genética con la RA (Plenge, 2009b; Stahl *et al*., 2010; Eyre *et al*., 2012; Frisell *et al*., 2013, 2016; Okada *et al*., 2014b; Messemaker *et al*., 2015). Dentro de este grupo, destacan los genes *PTPN22* (Rieck *et al*., 2007; Stanford and Botini, 2014; Messemaker *et al*., 2015), *CTLA4* (Plenge *et al*., 2005), *STAT4* (Remmers *et al*., 2007), IL-6 (Ferreira *et al*., 2013) o *NF-κB* (Spurlock *et al*., 2015) y genes relacionados con enzimas que posiblemente podrían participar en respuestas autoinmunes (Plenge *et al*., 2005; Messemaker *et al*., 2015).

Se ha estudiado la relación de diversos factores ambientales en la patogénesis de la RA **(Figura 26)**. El tabaquismo es el factor ambiental de riesgo más asociado en la patogénesis de la RA y las estimaciones indican que el tabaquismo representa aproximadamente el 20-30% del riesgo ambiental a la RA (Vessey *et al*., 1987; Klareskog *et al*., 2010; Arnson *et al*., 2010; Deane *et al*., 2017). Diversos estudios ponen de manifiesto que el consumo de tabaco puede influir en la expresión clínica, produciendo un curso evolutivo de la enfermedad más grave y una mayor destrucción articular, además de una peor respuesta al tratamiento, aunque no todos los estudios apoyan estas conclusiones (Ruiz-Esquide and Sanmartí, 2012).

Otros factores ambientales que aumentan la susceptibilidad a la RA se encuentran la exposición al polvo de sílice (Sluis-Cremer *et al*., 1986; Turner and Cherry, 2000; Stolt *et al*., 2010) y a una mayor contaminación del aire (Gan *et al*., 2013; Hart *et al*., 2013; Chang *et al*., 2016; Sun *et al*., 2016), un estatus socioeconómico más bajo (Bengtsson *et al*., 2005; Bergstrom *et al*., 2011), una dieta rica en azúcar, sodio, carne roja, proteínas y

hierro (Pattison *et al*., 2004; Benito-Garcia *et al*., 2007; Hu *et al*., 2014; Sundstrom *et al*., 2015), bajos niveles séricos de vitamina D (Patel *et al*., 2007; Haque and Bartlett, 2010; Kerr *et al*., 2011), la obesidad (Crowson *et al*., 2013; de Hair *et al*., 2013; Lu *et al*., 2014; Nikiphorou *et al*., 2017) y la presencia de varios agentes infecciosos. Entre los agentes infecciosos relacionados con la RA, cabe destacar *Porphyromonas gingivalis,* principal agente de la periodontitis, enfermedad aproximadamente el doble de frecuente en pacientes con RA en comparación con la población sana (de Pablo *et al*., 2008; Lundberg *et al*., 2010; Wegner *et al*., 2010; Laugisch *et al*., 2016). En general, se ha sugerido que la microbiota y la inflamación de la mucosa pueden influir en los cambios inmunológicos que conducen a la RA (Yeoh *et al*., 2013).

Debido a que la RA afecta más frecuentemente a mujeres que a hombres (2-3:1) y que existen estudios epidemiológicos que señalan diversos factores relacionados con el sexo en el riesgo de la RA, se ha considerado el posible papel diferencial de las hormonas sexuales en la susceptibilidad a la enfermedad. Los factores que se han asociado con un mayor riesgo a desarrollar RA son la menopausia precoz (Pikwer *et al*., 2012; Beydoun *et al*., 2013; Bengtsson *et al*., 2017b), el síndrome del ovario poliquístico (Merlino *et al*., 2003) y la preeclampsia (Jørgensen *et al*., 2010).

2. DNA mitocondrial

La mitocondria es un orgánulo celular encargado de suministrar la mayor parte de la energía necesaria para la actividad celular, convirtiendo moléculas nutricionales en ATP mediante fosforilación oxidativa (Henze and Martin, 2003). La mitocondria contiene su propio material genético, el DNA mitocondrial (mtDNA, del inglés *mitochondrial DNA*), una pequeña molecular circular de 16.569 pares de bases (bp, del inglés *base pairs*) de longitud y supone el 0,0005% del genoma humano **(Figura 27)**. Aproximadamente el 90% del genoma mitocondrial es codificante y contiene la información de 37 genes: 2 RNA ribosómicos, 22 RNA de transferencia y 13 proteínas. El 10% restante consiste en una pequeña región no codificante de unas 1.100 bp de longitud, denominada región control o *D-loop*, donde se encuentra el origen de replicación del genoma mitocondrial (O_R). Dentro de la región control se encuentran dos segmentos denominados segmento hipervariable I (HVS-I, del inglés *hypervariable segment I*) y segmento hipervariable II

(HVS-II, del inglés *hypervariable segment II*), siendo el HVS-I el más utilizado en distintos tipos de análisis, ya que presenta una mayor variabilidad.

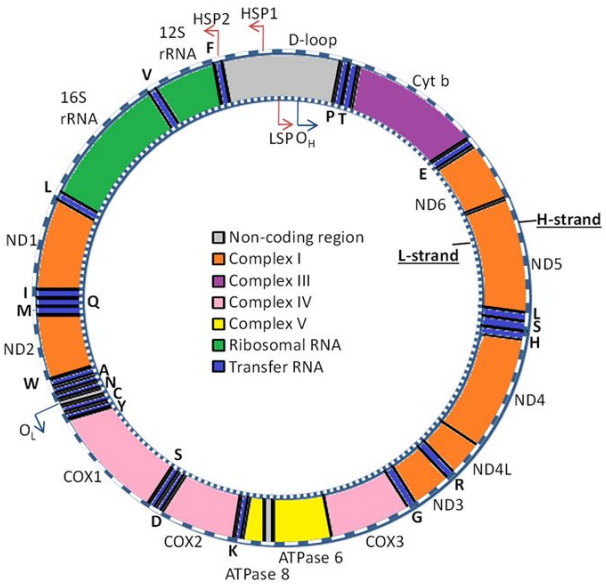


Figura 27. Genoma mitocondrial humano (van der Wijst *et al*., 2017).

2.1. **Características del DNA mitocondrial**

Las características que presenta el mtDNA hacen que sea un marcador especialmente útil en estudios de evolución humana. A continuación, se describen algunas de las características más importantes:

1. El mtDNA presenta un elevado número de copias por célula eucariota. Una mitocondria puede contener entre 2-10 moléculas de mtDNA, por lo tanto, el número de copias por célula eucariota, oscila entre 1.000-10.000 (Robin and Wong, 1988; Malyarchuk *et al*., 2002), facilitando así, su supervivencia y recuperación para su análisis en estudios de DNA antiguo (aDNA, del inglés *ancient DNA*).
2. El mtDNA no presenta recombinación, siendo los cambios de una generación a la siguiente el resultado de mutaciones puntuales, lo que permite interpretar de forma directa su variabilidad (Stoneking, 1993; Stoneking and Soodyall, 1996; Wallace *et al*., 1999).
3. El mtDNA presenta una elevada tasa de mutación, 5-10 veces mayor que el DNA nuclear (Brown, 1980; Ingman *et al*., 2000; Pakendorf and Stoneking, 2005), debido a que el genoma mitocondrial no presenta un sistema eficaz de

reparación de daño del DNA. Esta característica hace que el mtDNA sea adecuado para el estudio de la reconstrucción de la historia evolutiva reciente de las poblaciones humanas (Sigurğardóttir *et al*., 2000).

4. El mtDNA se transmite únicamente por vía materna, lo que significa que solo las mujeres lo transmiten a su descendencia (Stoneking, 1993; Stoneking and Soodyall, 1996; Wallace *et al*., 1999). Esto es debido a que, durante la fecundación del óvulo, las mitocondrias presentes en la cola de los espermatozoides no penetran en el óvulo, por lo tanto, las mitocondrias presentes en el citoplasma del cigoto serán las portadas por el óvulo.

A pesar de la gran utilidad del mtDNA, los resultados derivados de su estudio deben ser interpretados con cautela ya que se trata de un único locus que nos informa únicamente de la historia evolutiva de las mujeres.

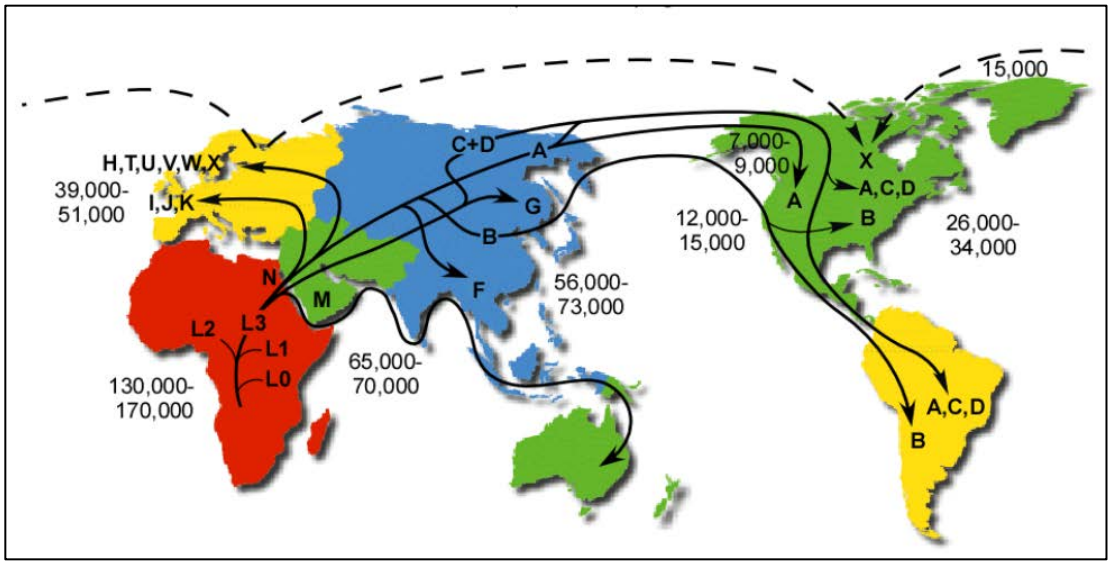


Figura 28. Historia migratoria de los linajes mitocondriales humanos. Modificado de http://www.mitomap.org.

La variabilidad de las secuencias mitocondriales se puede clasificar en distintos grupos denominados haplogrupos, que son una agrupación de haplotipos que se diferencian por presentar una serie de mutaciones estables y específicas de las distintas regiones geográficas a nivel continental (Torroni *et al*., 1996) **(Figura 28)**.

Dentro de la variabilidad de los haplogrupos mitocondriales de la población euroasiática, por un lado, destaca el haplogrupo H que actualmente es el linaje mitocondrial más común y diverso en Europa (55-40%) (Richards *et al*., 2000; Achilli *et al*., 2004; Loogväli *et al*., 2004; Brotherton *et al*., 2013) **(Figura 29)**, planteándose su origen hace aproximadamente 25.000-30.000 años en el suroeste de Asia, llegando a Europa desde Oriente Próximo antes del Último Máximo Glaciar (LGM, del inglés *Last Glacial Maximum*) hace 25.000-19.500 años (Torroni *et al*., 1996, 2001; Richards *et al*., 2000; Achilli *et al*., 2004; Soares *et al*., 2010) **(Figura 28)**. Por otro lado, el haplogrupo U es el linaje materno más antiguo en Europa, sugiriéndose su origen en el suroeste de Asia hace aproximadamente 55.000 años, desde donde se expandió hacía Europa y actualmente presenta una frecuencia del 11% en la población europea (Torroni *et al*., 1996; Achilli *et al*., 2005; Soares *et al*., 2010; Secher *et al*., 2014) **(Figura 28)**. Ambos haplogrupos subsistieron en los refugios glaciares del suroeste de Europa durante el LGM, para su posterior re-expansión. En relación al haplogrupo K, linaje descendiente del U8, presenta una edad de coalescencia de 17.800-13.500 años y se expandió por Europa después del LGM (Soares *et al*., 2010) **(Figura 28)**. Los haplogrupos J y T surgieron en algún lugar de Asia Occidental y llegaron y se dispersaron por Europa a través de Oriente Próximo tras el LGM, hace aproximadamente 12.000 años, junto con la difusión de la cultura neolítica (Soares *et al*., 2010) **(Figura 28)**.

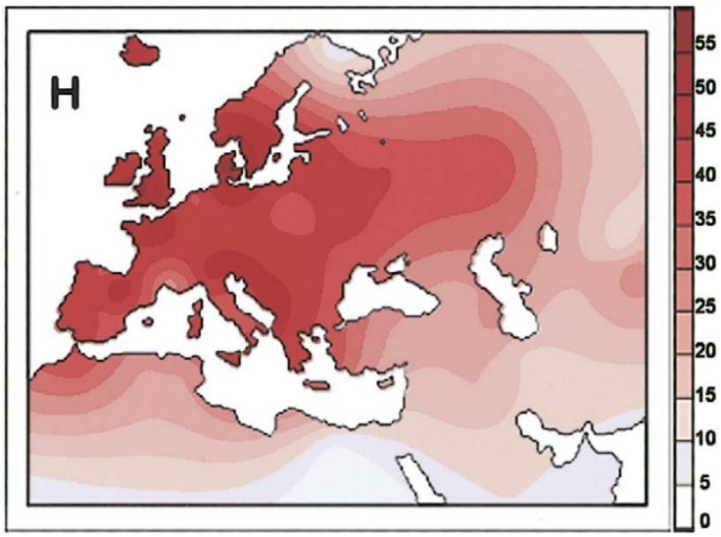


Figura 29. Distribución espacial de la frecuencia (%) del haplogrupo mitocondrial H en Europa, Oriente Próximo y Norte de África (Achilli *et al*., 2004).

Algunas variantes mitocondriales podrían haber sido críticas para la adaptación humana en diferentes ambientes, siendo favorables para la supervivencia y la reproducción de las poblaciones que habitan en zonas climáticas particulares y no resultar adaptativas en otros ambientes (Wallace, 2005). Los efectos de episodios climáticos extremos son perjudiciales para la supervivencia de nuestra especie, como por ejemplo las glaciaciones del Pleistoceno y del Holoceno, entre las que se encuentra la Pequeña Edad de Hielo (LIA, del inglés *Little Ice Age*, siglos XIV-XIX) y que quizás moldearon o seleccionaron mutaciones de nuestro genoma para poder sobrevivir a estas condiciones.

2.2. Relación entre el DNA mitocondrial y las enfermedades reumáticas

Se ha sugerido que la asociación diferencial de algunos haplogrupos mitocondriales con patologías reumáticas, podría estar relacionada con un proceso de adaptación de *Homo sapiens* a climas fríos (Mishmar *et al*., 2003; Wallace *et al*., 2003; Ruiz-Pesini *et al*., 2004). Además de cómo mecanismo adaptativo, distintas evidencias sugieren que el mal funcionamiento mitocondrial podría influir en la patogenia de algunas enfermedades humanas, entre las que se incluyen desordenes neurodegenerativos (Hudson *et al*., 2013), enfermedades metabólicas (Kwak and Park, 2016), patologías reumáticas (Roach 2008; Blanco *et al*., 2011; Wang *et al*., 2015), procesos asociados con la edad (de Benedictis *et al*., 1999; Niemi *et al*., 2003; Courtenay, 2012) y cáncer (Wallace, 2015). Una posible explicación radica en el funcionamiento de la mitocondria, cuyo metabolismo produce especies reactivas de oxígeno (ROS, del inglés *reactive oxygen species*) e intermediarios metabólicos, que son señales que transmiten la información entre la mitocondria y el núcleo, y que son capaces de variar la expresión de genes nucleares, alterando numerosos procesos celulares y rutas metabólicas que pueden influir en el desarrollo de diferentes enfermedades (Wallace and Fan, 2010; Shadel and Horvath, 2015; Picard *et al*., 2016).

Existen evidencias de la relación existente entre distintos haplogrupos mitocondriales y la prevalencia, incidencia y progresión de la OA en grupos étnicos de distinta procedencia geográfica (Rego-Pérez *et al*., 2008, 2010, 2013; Fang *et al*., 2014; Hudson *et al*., 2014; Soto-Hermida *et al*., 2014), lo que podría ser debido a interacciones con el

medio ambiente, como por ejemplo la adaptación a climas fríos (Mishmar *et al*., 2003; Wallace *et al*., 2003; Ruiz-Pesini *et al*., 2004).

Distintos estudios apoyan el papel de la mitocondria en la patogénesis de la OA, ya que se ha observado que la función de la mitocondria está alterada en los condrocitos de individuos con OA (Roach, 2008; Blanco *et al*., 2011; Wang *et al*., 2015), produciendo estrés oxidativo, aumentando la apoptosis de los condrocitos y la degradación del cartílago (Li *et al*., 2003; Kim *et al*., 2010; Blanco *et al*., 2011) **(Figura 30)**.

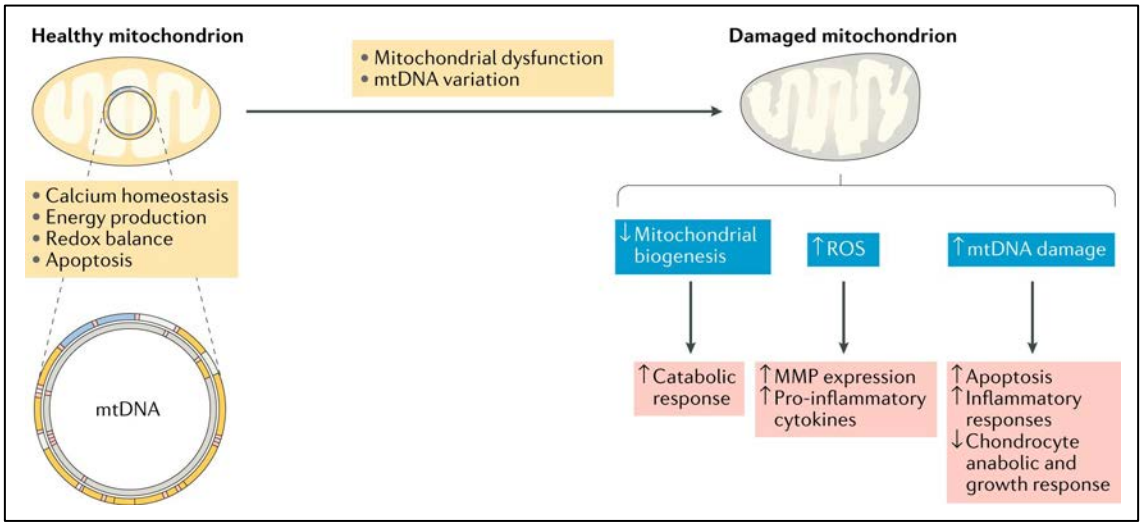


Figura 30. Relación entre el mtDNA y la patogénesis de la OA. La mitocondria regula diferentes procesos como la homeostasis del calcio, la producción de energía, el equilibrio redox o la apoptosis, todos ellos modulados por las variaciones del mtDNA. El daño mitocondrial puede conducir a diferentes procesos que posteriormente contribuyen al desarrollo de OA. MMP, del inglés *matrix metalloproteinase*; MRC, del inglés *mitochondrial respiratory complex* (Blanco *et al*., 2018).

Por un lado, se ha postulado que los haplogrupos H y U están significativamente relacionados con un mayor riesgo y severidad a desarrollar enfermedades reumáticas (Rego-Pérez *et al*., 2008; Duhn *et al*., 2017). El rol del haplogrupo H en la patogénesis de las enfermedades de tipo reumático se basa en que debido a que presenta una elevada eficiencia energética, genera un mayor estrés oxidativo, incrementando la producción de ROS, que produce la degradación del cartílago y el riesgo a desarrollar enfermedades óseas degenerativas (Duhn *et al*., 2017) **(Figura 31)**. Por otro lado, se ha descrito que los haplogrupos J y T están relacionados significativamente con una disminución de la

incidencia y progresión de la OA (Rego-Pérez *et al*., 2010, 2011, 2013; Shen *et al*., 2014; Soto-Hermida *et al*., 2014, 2015; Fernández-Moreno *et al*., 2017a, 2017b) y, además, de la PsA para el caso del J (Coto-Segura *et al*., 2012), sugiriendo un posible rol protector de estos haplogrupo frente a estas enfermedades. Este posible efecto protector descrito para los haplogrupos T y J reside en una menor eficiencia en el proceso de conversión de calorías en ATP, lo que genera una menor producción de ROS y un menor daño oxidativo. Este hecho protege a la célula frente a la apoptosis y disminuye la degradación del cartílago involucrado en la patogénesis de la OA (Mueller *et al*., 2012; Wallace and Chalkia, 2013) **(Figura 31)**.

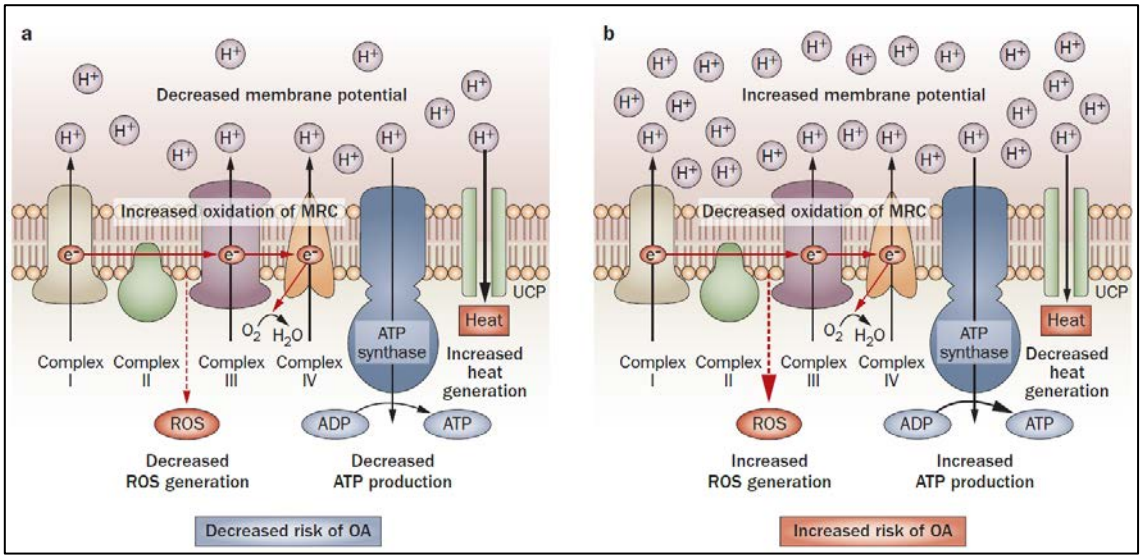


Figura 31. Influencia de los haplogrupos mitocondriales J, T y H en la OA. a) Los individuos que portan los haplogrupos J y T presentan mutaciones en el mtDNA que desacoplan parcialmente la fosforilación oxidativa mitocondrial. Estas mutaciones conducen a una disminución de la producción de ATP y de la producción de ROS, lo que reduce el daño oxidativo causado a la célula, protege frente a la apoptosis celular y disminuye la degradación del cartílago, lo que da lugar a una disminución del riesgo de desarrollar OA. b) En los individuos que portan el haplogrupo H, la fosforilación oxidativa mitocondrial está estrechamente acoplada, lo que conduce a un aumento de la producción de ATP y la generación de ROS, lo que incrementa la degradación del cartílago y el riesgo de desarrollar OA. MRS, del inglés *mitochondrial respiratory chain*; UCP, del inglés *uncoupling protein* (Blanco *et al*., 2011)

Para el caso de la RA, no se encontró hasta el momento ninguna asociación significativa entre los haplogrupos mitocondriales europeos y esta patología (Duhn *et al*., 2017), al igual que para las SpA.

Estudios donde se analizaron los haplogrupos mitocondriales asiáticos (B y G), han permitido sugerir que el haplogrupo B parece tener un papel protector frente al desarrollo de la OA de rodilla, mientras que el haplogrupo G incrementaría su riesgo (Fang *et al*., 2014). Estos estudios sugieren que distintos haplogrupos mitocondriales están asociados al desarrollo de la OA, sin embargo, las diferencias étnicas y factores ambientales modulan esta asociación.

3. DNA antiguo

El aDNA, es el DNA recuperado a partir de restos biológicos de organismos ya extintos o de cierta antigüedad. La información obtenida a partir del aDNA, permite abordar cuestiones científicas de diversa naturaleza, como por ejemplo la reconstrucción de la historia evolutiva y demográfica de las poblaciones del pasado o la reconstrucción de las filogenias entre especies extintas y sus parientes actuales. En humanos el aDNA se recupera principalmente de los huesos y dientes, ya que son los restos mejor conservados en contextos arqueológicos y su estudio se realiza mediante técnicas paleogenéticas.

3.1. Características del DNA antiguo

Entre las principales características del aDNA destacan las siguientes:

1. *Recuperación de una escasa cantidad de aDNA.* En muestras antiguas, el DNA representa únicamente entre el 0,1-1% del DNA que se esperaría encontrar en una muestra moderna (Tuross, 1994), e incluso, en algunas ocasiones no se encuentra ninguna traza de DNA **(Figura 32)**. Debido a ello, en la mayoría de los casos la única parte del genoma que se puede amplificar de forma reproducible es el mtDNA, ya que representa entre 1.000-10.000 copias por célula (Handt *et al*., 1994).

2. *Degradación del aDNA.* En las células vivas de los organismos, existen sistemas de reparación del DNA que evitan o atenúan el daño que se produce en el DNA. Cuando el organismo muere, estos sistemas cesan su funcionamiento, por lo que el ataque físico-químico endógeno y exógeno, no encuentra ningún

impedimento (Höss *et al*., 1994, 1996; Handt *et al*., 1994). El resultado es que el DNA recuperado de tejidos antiguos se encuentra severamente dañado **(Figura 33)**. Además, el aDNA se degrada en función del tiempo y de los factores tafonómicos acontecidos, principalmente en los primeros momentos del enterramiento (pH del suelo, humedad, temperatura, …) (Lindahl, 1993; Höss *et al*., 1996; Burger *et al*., 1999; Hofreiter *et al*., 2001a). Como resultado de esta degradación, el DNA se encuentra fragmentado y los nucleótidos de la secuencia del DNA pueden presentar modificaciones. La degradación del DNA nos limita a trabajar con fragmentos de una longitud en torno a 100-200 bp. Por otro lado, la modificación post-mortem de los nucleótidos de la secuencia de DNA puede ser la causa de resultados fallidos o erróneos en el análisis del aDNA, principalmente durante la amplificación *in vitro* de la cadena de DNA (Handt *et al*., 1994).

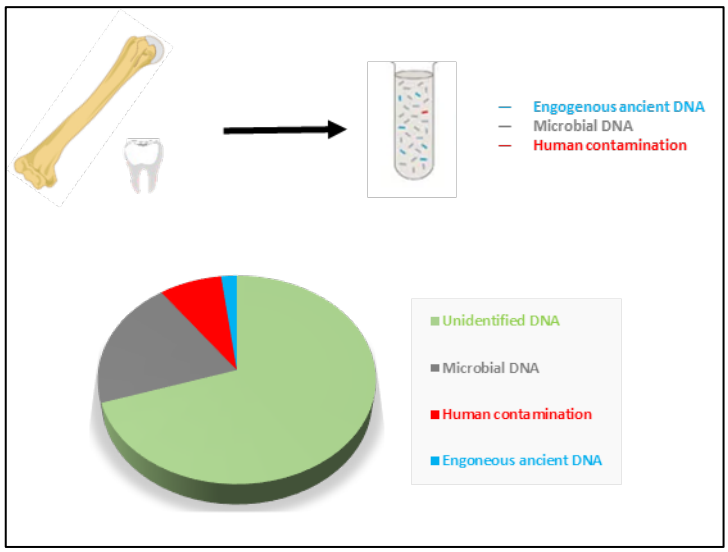


Figura 32. Representación esquemática de las proporciones de los diferentes tipos de DNA que pueden encontrarte en un extracto de aDNA. Modificado de Stoneking and Krause, 2011.

3. *Contaminación de aDNA.* La principal fuente de contaminación es el DNA exógeno humano procedente de arqueólogos, personal del museo o de los propios investigadores que manipulan las muestras, que pueden contaminar los restos esqueléticos antes, durante y después de su análisis, bien a partir de las células de la piel, el pelo, la saliva o de los aerosoles producidos durante la respiración **(Figura 32)**. Por ello, los restos esqueléticos deben de ser manipulados siempre con guantes, gorro y mascarilla, tanto durante la excavación como posteriormente. Con el fin de identificar esta posible

contaminación, se analiza el DNA del personal que ha manipulado las muestras y se compara con el DNA obtenido de las muestras esqueléticas (Willerslev *et al*., 2004; Gilbert and Willerslev, 2006). Otro vehículo de contaminación puede ser el material y los reactivos empleados en el análisis molecular en el laboratorio. La posible contaminación ocurrida durante alguno de estos procesos es fácil de detectar mediante la utilización de los controles correspondientes. Sin embargo, los productos de DNA generados de experimentos previos realizados en el laboratorio, constituyen la fuente de contaminación más pertinaz. La apertura de los tubos utilizados en la fase de análisis del DNA provoca la contaminación del ambiente del laboratorio. Este tipo de contaminación se evita utilizando laboratorios independientes, en nuestro caso, contamos con un laboratorio exclusivo para la extracción del DNA y la preparación de las muestras para su posterior análisis y otro físicamente aislado del anterior, para analizar los productos de DNA amplificados y secuenciados. Además, se debe utilizar material y equipamiento exclusivo para trabajar con aDNA y mantener la esterilidad de los mismos y de las superficies de trabajo, mediante lavado con lejía e irradiación con luz UV, de forma rutinaria. Por último, todo el personal debe utilizar de forma obligatoria y rutinaria una vestimenta adecuada: buzo, bata, gorro, guantes y mascarilla desechables, de un único uso.

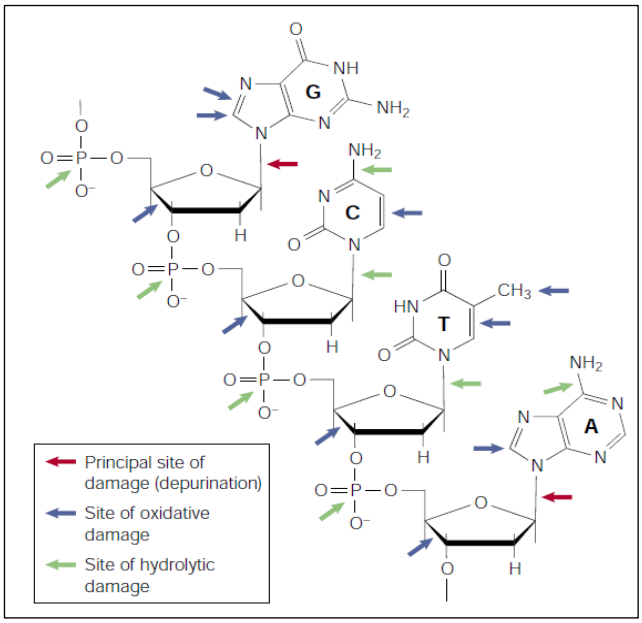


Figura 33. Daño oxidativo e hidrolítico del DNA. Lugares de acción de los procesos de hidrólisis y oxidación. Modificado de Hofreiter *et al*., 2001b.

4. La Pequeña Edad de Hielo (siglos XIV-XIX)

La LIA está enmarcada en la Edad Media, donde Europa se caracterizaba por ser un continente de naturaleza rural con un sistema político basado en el feudalismo y con numerosos conflictos políticos, económicos o religiosos. Entre los años 900 y 1300 d.C. la Tierra presenció un incremento de la temperatura conocido como período cálido medieval (MCA, del inglés *Medieval Climate Anomaly*) (Lamb, 1965). Durante este periodo la sociedad experimentó un crecimiento y un incremento de la creatividad y vitalidad, siendo una época benigna ya que permitió la cosecha de productos como trigo, grano y cereal, con varios siglos de buenas cosechas interrumpidas en ocasiones por cortos inviernos. En esta época, la población dependía principalmente de la agricultura (Kershaw, 1973) y durante el MCA las cosechas fueron más productivas incrementando la producción de alimentos y disminuyendo en general las enfermedades, lo que produjo un aumento de la población europea, especialmente de la población rural (Lopez, 1971; Campbell, 2016). Sin embargo, a finales del siglo XIII, esta prosperidad que caracterizó el MCA se vio interrumpida por factores climáticos desfavorables que dieron lugar al inicio de la LIA (Campbell, 2016) **(Figura 34)**.

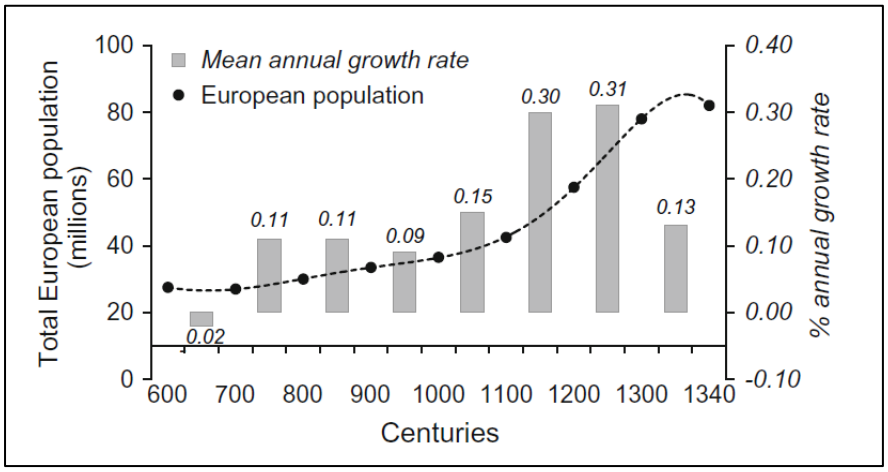
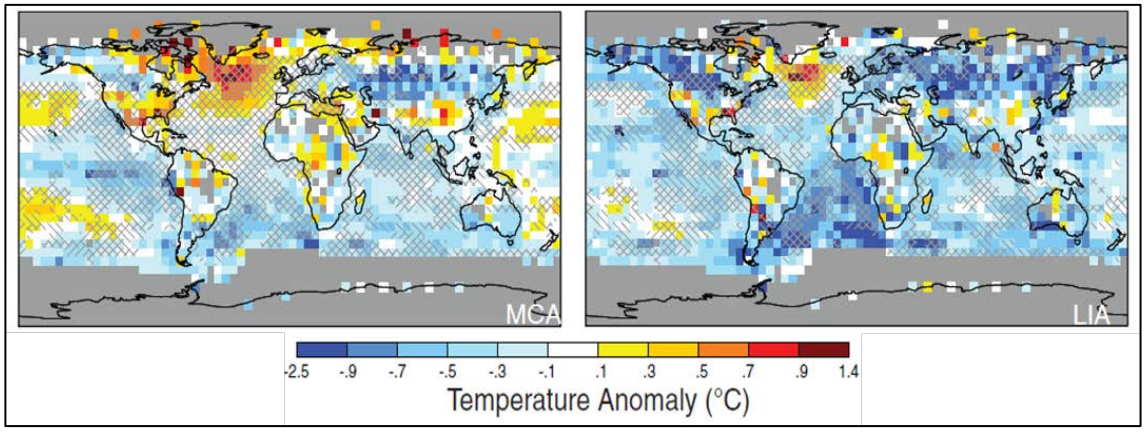


Figura 34. Estimación de la población total de Europa y de las tasas medias de crecimiento anual entre los años 600 y 1340. Durante la primera mitad del siglo XIV, el crecimiento de la población europea cesó y disminuyo debido a los fracasos de las cosechas (Biraben, 1979).

La LIA hace referencia a un período histórico climático frío, que prevaleció entre el siglo XIV, finalizando el MCA, hasta aproximadamente mediados del siglo XIX (Mann *et al*., 2009; Díaz *et al*., 2011). Aunque las temperaturas durante esta época fueron generalmente más bajas que en la actualidad (1-2ºC) (Lamb, 1982) **(Figuras 35 y 36)**, la

LIA se caracterizó por una gran inestabilidad climática (Mann, 2002), con alternancia de períodos cálidos y fríos, y una mayor variabilidad espacial y temporal de la precipitación (Lamb, 1977; Rodrigo *et al*., 1999; Alcoforado *et al*., 2000; Wanner *et al*., 2004, 2011; Pauling *et al*., 2006) **(Figura 36)**. Las condiciones climáticas más frías afectaron particularmente al hemisferio norte, aunque es considerado un evento a escala global, ya que el enfriamiento ha sido documentado en otros lugares del mundo (Jones and Mann, 2004; Mann *et al*., 2009) **(Figura 36)**.

Figura 35. Patrón reconstruido de la temperatura superficial durante el MCA y la LIA. Modificado de Mann *et al*., 2009.



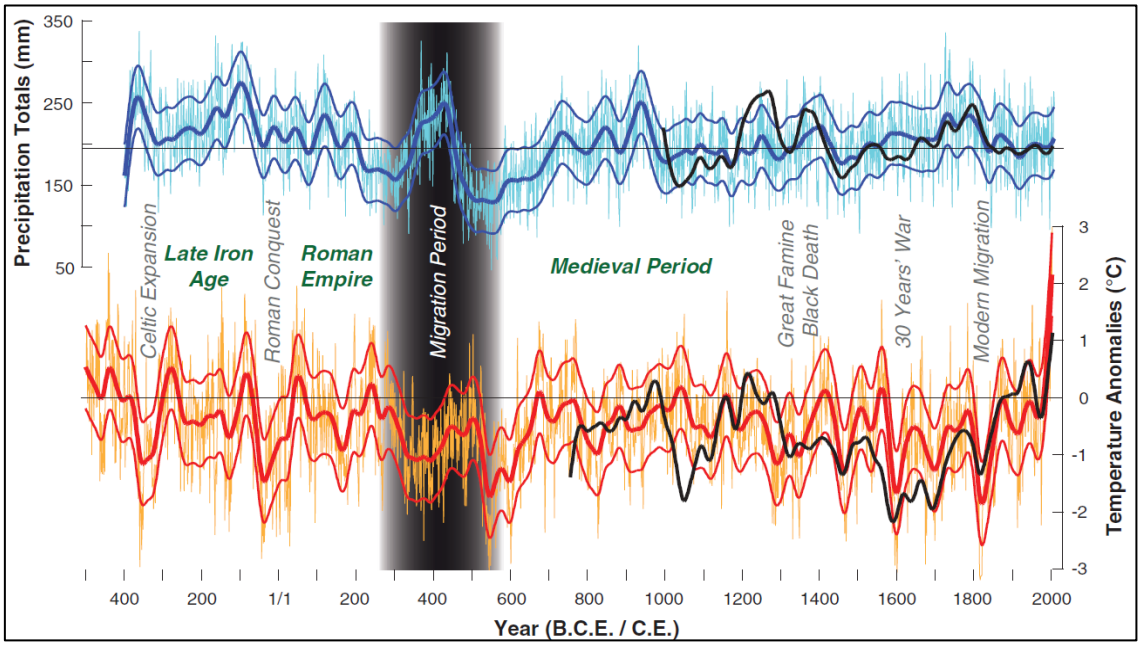


Figura 36. Precipitaciones totales y anomalías en la temperatura en Europa de los últimos 2500 años (Büntgen *et al*., 2011).

Actualmente no se sabe con exactitud que desencadenó la LIA, aunque entre las principales causas se han identificado:

1. *Erupciones volcánicas*: Durante la LIA se produjo un incremento de una potente actividad volcánica, que desencadenó un enfriamiento de la superficie de la Tierra (Lamb, 1970; Porter, 1986; Lean *et al*., 1995; Bradley and Jones, 1992; Mann *et al*., 1998; Prohom *et al*., 2003; Hegerl *et al*., 2011) **(Figura 37)**. Una erupción volcánica lanza gases, principalmente vapor de agua y dióxido de carbono, y partículas a la atmósfera, aunque son los gases de azufre los que influyen principalmente en el clima. Si una erupción es lo bastante fuerte, la fumarola que desprende puede llegar a la estratosfera y al reaccionar con el vapor de agua forma una nube fina y difusa de gotas de ácido sulfúrico que se puede mantener durante años en la estratosfera y que refleja los rayos del sol, reenviándolos al espacio y produciendo un enfriamiento de la superficie de la Tierra. Estos eventos volcánicos parecen haber desencadenado el enfriamiento de los veranos que caracterizaron la LIA, que provocaron un aumento de la extensión del hielo marino del Ártico, produciendo una expansión del frío a latitudes medias (Wanner *et al*., 2011; Miller *et al*., 2012) **(Figura 37)**.

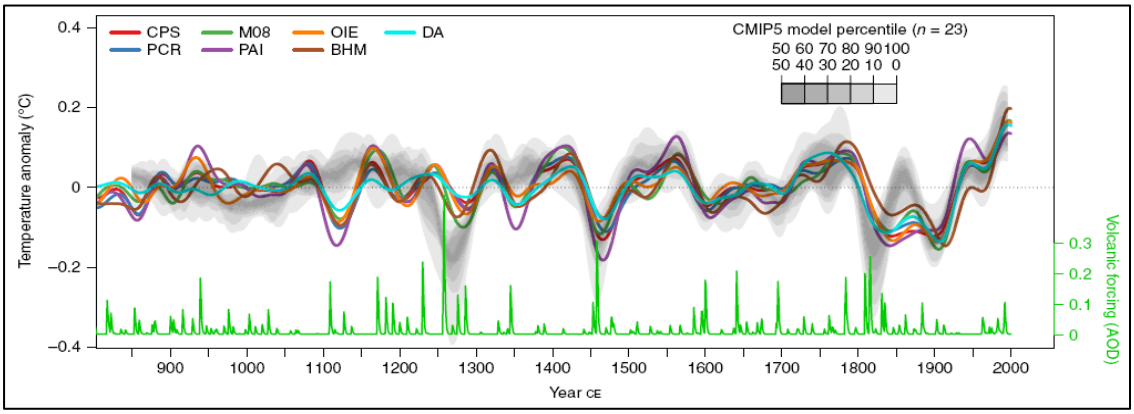


Figura 37. Reconstrucción multidecanal de las anomalías en la temperatura y de las erupciones volcánicas durante los últimos 1100 años. Las líneas de color representan el conjunto de reconstrucciones realizadas mediante los diferentes métodos. El sombreado gris muestra los percentiles de simulación del modelo. La gráfica verde muestra las distintas erupciones volcánicas ocurridas en el último milenio. CPS, del inglés *composite-plus-scaling*; PCR, del inglés *principal component regression*; M08, del inglés *regularized errors in variables*; PAI, del inglés *pairwise comparison*; OIE, *optimal information extraction*; BHM, del inglés *bayesian hierarchical model*; DA, del inglés *offline data assimilation*; CMIP5, del inglés *Coupled Model Intercomparison Project Phase 5*; AOD, del inglés *aerosol optical depth* (Neukom *et al*., 2019).

2. *Radiación solar*: Durante la LIA, se produjo una pequeña disminución de la radiación solar (Benedict and Maisch *et al*., 1989; Lean *et al*., 1995; Beer *et al*., 2000; Shindell *et al*., 2001; Bradley, 2003; Solanski *et al*., 2004; Usoskin *et al*., 2004), teniendo lugar cuatro mínimos solares (Wolf: 1280-1350, Spörer: 1450-1540, Maunder: 1645-1715 y Dalton: 1790-1830) correlacionados con períodos fríos (Lamb, 1979) **(Figura 38)**. Estos mínimos hacen referencia al número mínimo de manchas solares en la superficie del sol. Cuando hay menos manchas solares en su superficie, el sol emite menos radiación y por lo tanto la Tierra recibe menos radiación produciéndose un enfriamiento global. En Europa Occidental varios eventos claros de frío mínimo tuvieron lugar dentro de estos cuatro mínimos solares (Mörner, 1996).

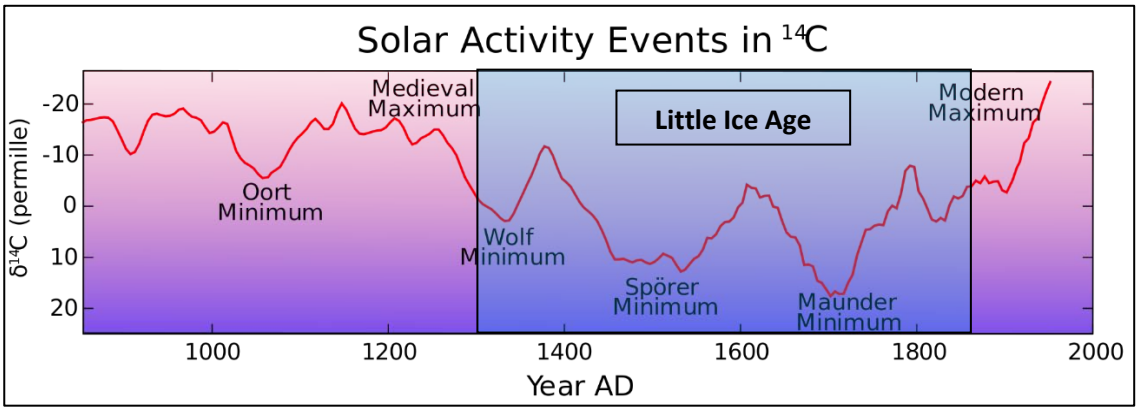


Figura 38. Patrón de actividad solar reconstruido a partir de datos de anillos de crecimiento de los árboles y de carbono-14 (^{14}C) durante los últimos 1100 años, en el que se observan los mínimos solares de Oort, Wolf, Spörer y Maunder, además del MCA. Modificado de McInnes, 2007 tomando los registros de ^{14}C de Reimer *et al*., 2004.

3. *Circulación termohalina o cinta transportadora oceánica*: Se trata de un potente flujo de agua templada que traslada el calor a las latitudes del norte. En el trópico la superficie del mar se calienta por la gran cantidad de radiación solar, y esta agua fluye y se traslada a latitudes más altas donde pierde calor que se fuga a la atmósfera. A medida que el agua se enfría, se vuelve más densa, se hunde y fluye hacia el sur para mantener el equilibrio con el agua templada que fluye hacia el norte por la superficie **(Figura 39)**. Se ha sugerido que la LIA se produjo cuando las fuerzas naturales interrumpieron ese flujo, sugiriendo que el catalizador fue el flujo de agua dulce del hielo ártico que se había derretido durante el MCA, ya que la adición de agua dulce desequilibra la salinidad interrumpiendo las corrientes

(Broecker, 2000). Si se añade mucha agua dulce, la superficie del agua es menos densa y no se hunde, lo que produce que la corriente se detenga. Si la corriente se detiene, el efecto sobre el clima de la Tierra, y en especial el de Europa sería enorme, ya que el calor no se transportaría a la atmósfera del Atlántico Norte y los vientos dominantes del Este soplarían aire frío del mar sobre el continente en lugar de aire cálido.

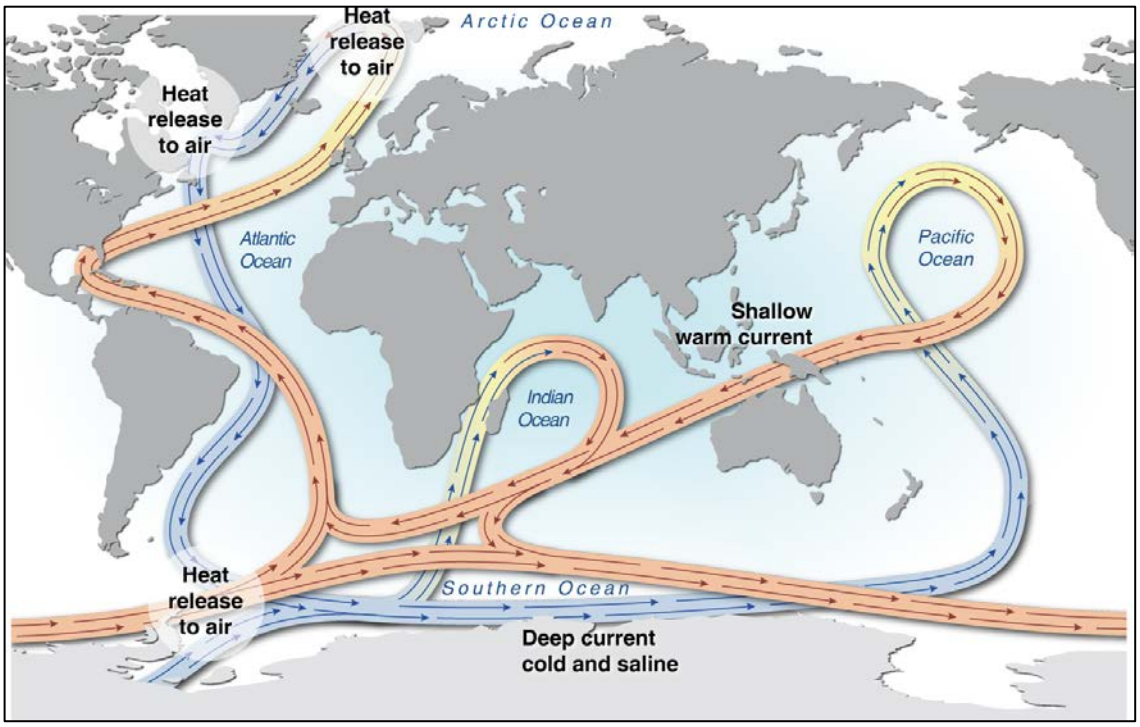


Figura 39. Patrón de circulación termohalina. Este proceso natural transporta y mezcla el agua de los diferentes océanos. Cuando se produce un aporte en exceso de agua dulce procedente de precipitaciones, escorrentías o derretimiento del hielo la cinta transportadora se debilita o incluso puede verse interrumpida. La variabilidad en la fuerza de la cinta transportadora puede conducir a un cambio climático en Europa y también puede influir en otras zonas del océano mundial (Hugo Ahlenius, UNEP/GRID-Arendal, 2007).

Las consecuencias más significativas de este recrudecimiento del clima durante la LIA fueron un avance de los glaciares (especialmente en zonas de alta montaña), sequías, inundaciones, olas de frío y de calor y un aumento de la actividad tormentosa. El avance de los glaciares de montaña tuvo importantes consecuencias para las poblaciones humanas, ya que produjo la pérdida de numerosas granjas y pueblos (Mann, 2002). En la Península Ibérica, únicamente hubo glaciares durante la LIA en las zonas más elevadas de los Pirineos, la cordillera Cantábrica y Sierra Nevada (González Trueba *et al.*, 2007;

Oliva *et al*., 2018) **(Figura 40)**. También durante este periodo fueron muy frecuentes y particularmente severas las olas de frío seguidas de fuertes nevadas (Rodrigo *et al*., 1998), mientras que las olas de calor estuvieron asociadas a menudo a incendios y sequías. Durante la LIA se produjo un incremento de las inundaciones en los ríos Atlánticos y Mediterráneos, y de un incremento de la actividad tormentosa en el Atlántico Norte (Jelgersma *et al*., 1995; Aagaard *et al*., 2007; Sorrel *et al*., 2009).

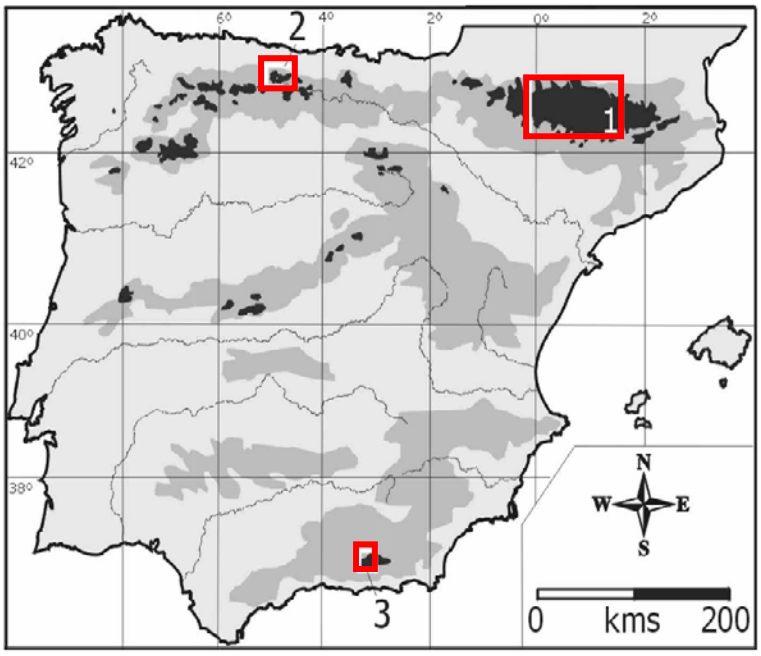


Figura 40. Ubicación de los glaciares de montaña durante la LIA en la Península Ibérica (recuadros rojos). 1. Pirineos. 2. Picos de Europa en la cordillera Cantábrica. 3. Sierra Nevada. El color negro señala la zona glaciar durante el Pleistoceno en la Península Ibérica. Modificado de González Trueba *et al*., 2007.

Los efectos socioeconómicas de la variabilidad climática extrema que tuvo lugar durante la LIA fueron muy perjudiciales (Fagan, 2002), ya que las sociedades de la época eran más susceptibles al impacto destructivo de los episodios climáticos intensos (Dezileau *et al*., 2011) **(Figura 41)**, principalmente debido a los efectos en el suministro de agua y la productividad agrícola y ganadera (Weiss and Bradley, 2001; deMenocal *et al*., 2001**;** Haug *et al*., 2003; Patterson *et al*., 2010), la salud humana (McMichael *et al*., 2006) y el desencadenamiento de conflictos civiles (Burke *et al*., 2009). Los fenómenos climáticos más extremos que tuvieron un mayor impacto social y natural en la Península Ibérica fueron las sequías, inundaciones y olas de frío y de calor (Barriendos *et al*., 2003; Domínguez-Castro *et al*., 2008).

Figura 41. Cuadros representando los efectos de la LIA. A) *The Frozen Thames* por Abraham Hondius, 1677 (Museum of London, Londres, Reino Unido). B) *Ice-skating in a Village* por Hendrick Avercamp, 1610 (Rijksmuseum, Amsterdam, Holanda).

Las fluctuaciones climáticas de la LIA tuvieron efectos devastadores, especialmente en la producción de alimentos ya que la economía estaba basada en la agricultura (Domínguez-Castro *et al*., 2012). La pérdida de cosechas, produjo una escasez de alimentos que junto con el encarecimiento de estos derivaron en grandes hambrunas por todo Europa dando lugar a un incremento de la mortalidad (Jordan, 1996; Clark, 2007, 2009; Campbell, 2009, 2010; Schofield *et al*., 2013) **(Figura 42)**. La actividad pesquera también se vio afectada, ya que el clima provocó cambios en los circuitos tradicionales de pesca obligando a los pescadores a buscar nuevos caladeros, y dificultó la propia actividad, sobre todo en invierno (Aragón Ruano, 2011).

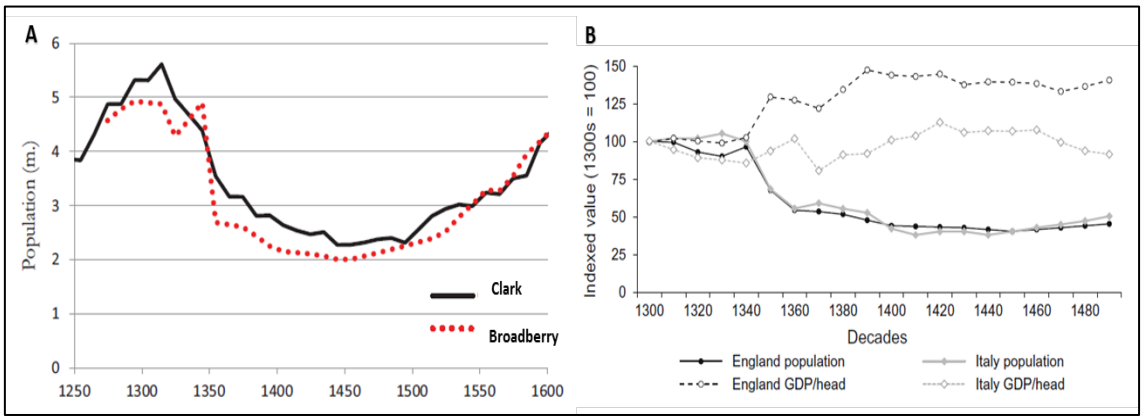


Figura 42. A) Población de Inglaterra durante la Edad Media (Clark, 2007; Broadberry *et al*., 2015). B) Datos demográficos y económicos de Inglaterra e Italia entre los años 1300 y 1480 (Malanima, 2011; Broadberry *et al*., 2015). GDP, del inglés *gross domestic product*.

Asimismo, el clima también causó la muerte del ganado y afectó a los cultivos madereros y al transporte terrestre y marítimo, perjudicando a su vez las rutas comerciales. Los episodios de malnutrición y otras dolencias debilitaron a la población y la hicieron más vulnerable a diversas enfermedades. Además del deterioro climático, el incremento de los movimientos poblacionales y los conflictos bélicos fueron responsables de cambios en la dieta y la dispersión de enfermedades (Campbell, 2016), que tuvieron un fuerte impacto en la sociedad de la época debido al debilitamiento físico de la población producido como consecuencia del hambre (Coltrain, 2009). Durante este periodo numerosas pandemias afectaron a Europa, como por ejemplo la de la Peste Negra que llegó a Europa en 1347 procedente de Asia y diezmo la población europea **(Figura 43)**, acabando con un tercio de su población (Benedictow, 2004, 2010; Kaplan *et al*., 2009; Büntgen *et al*., 2010; Kausrud *et al*., 2010). Las sociedades pre-industriales eran sensibles a la hambruna, la enfermedad y la guerra, que a menudo eran impulsadas por eventos climáticos (Brázdil *et al*., 2005) **(Figura 44)**.

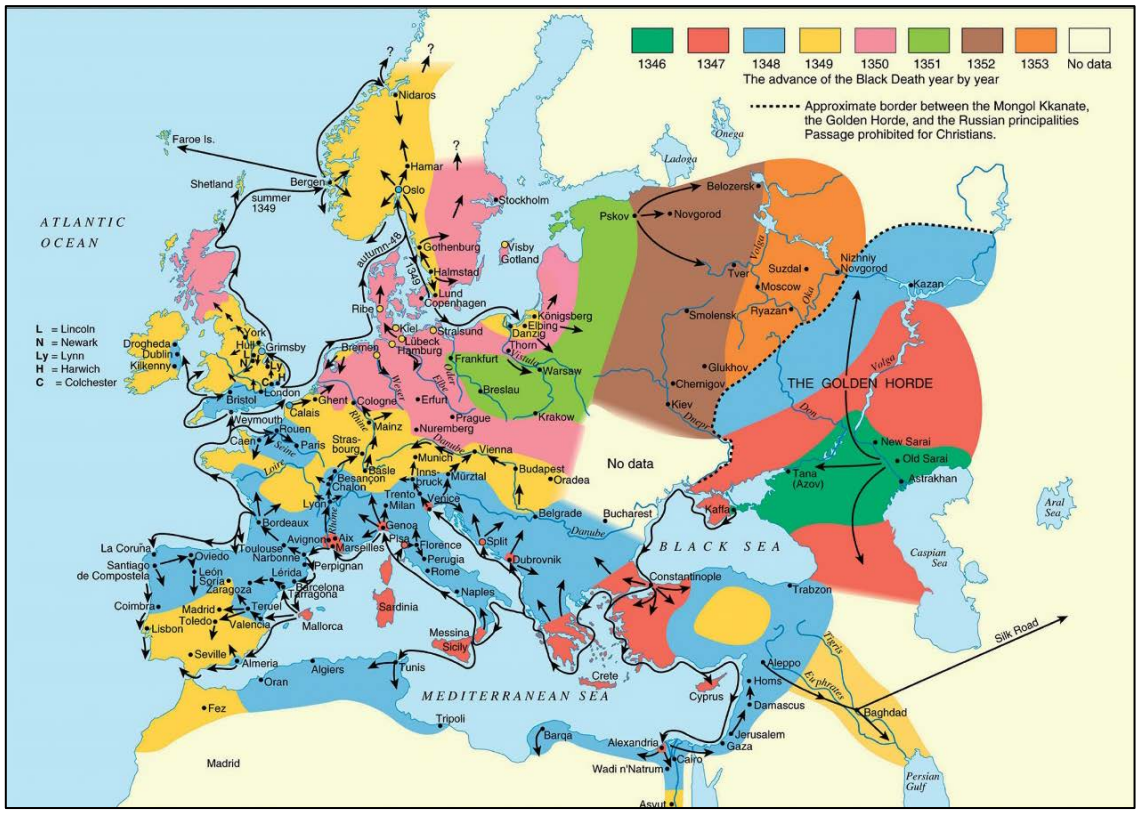


Figura 43. Propagación de la peste negra en Europa y Oriente Próximo (1346-1353) (Cesana *et al*., 2017).

Figura 44. The Triumph of Death (De Triomf van de Dood) por Pieter Bruegel the Elder, 1562 (Museo del Prado, Madrid, España).

Alrededor de 1850 la LIA finalizó abruptamente, y este cambio tuvo lugar a través de solo una década, existiendo diversos factores que pudieron influir en este proceso como: el incremento de la radicación solar a mediados del siglo XIX, la disminución del número de erupciones volcánicas, la industrialización, la polución antropogénica, los gases de efecto invernadero y la fluctuación natural del océano (Lean and Rind, 1999) **(Figuras 37, 45 y 46)**.

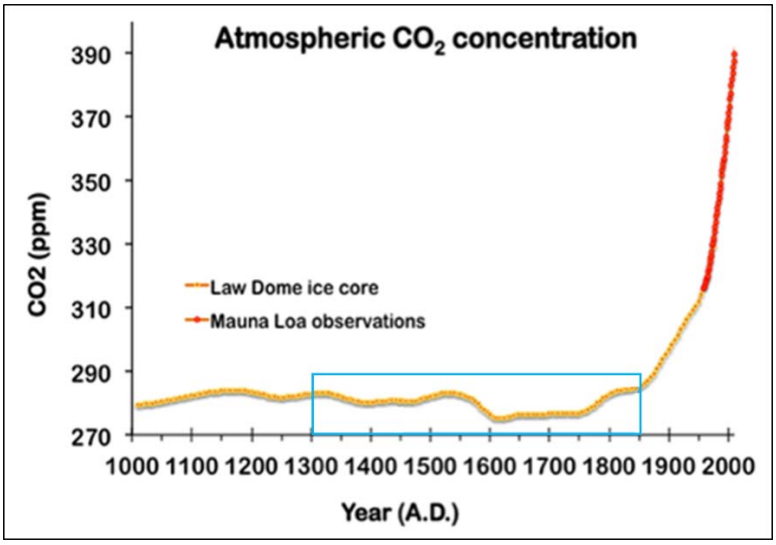


Figura 45. Registro de la concentración de dióxido de carbono (CO_2) en la atmósfera, a partir de las mediciones directas en el volcán Mauna Loa (Hawái, Estados Unidos) y las mediciones de burbujas de aire en el hielo de Law Dome (Antártida). El recuadro azul representa el período cronológico en el que tuvo lugar la LIA. Modificado de Etheridge *et al*., 1998.

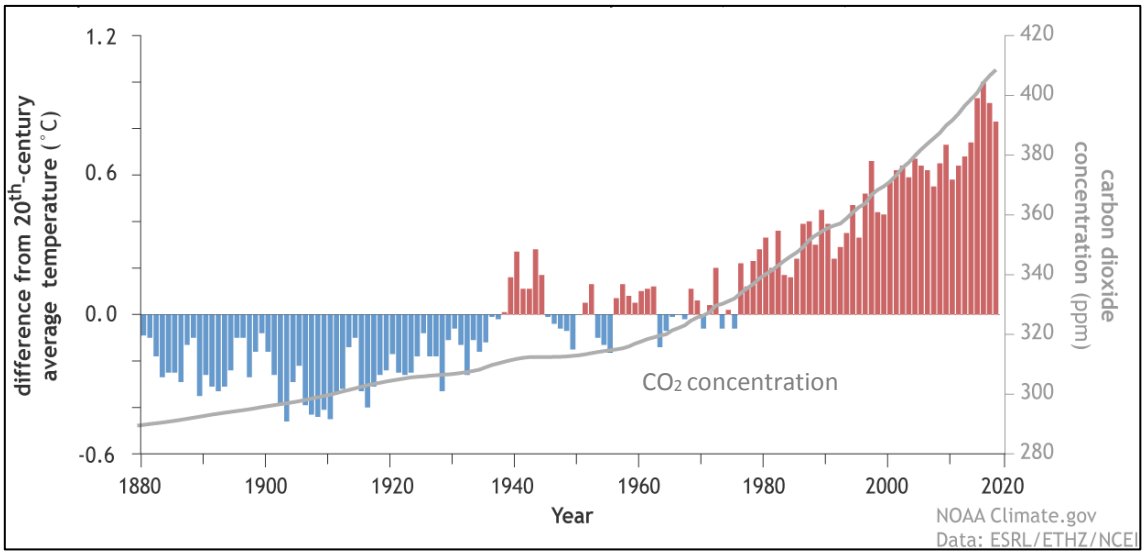


Figura 46. Datos de la concentración atmosférica de CO_2 y de la temperatura de la superficie terrestre (1880-2018). La temperatura anual global ha aumentado más de 0,8ºC desde 1880. Las barras rojas muestran las temperaturas por encima de la media y las barras azules indican las temperaturas por debajo de la media. La línea gris indica la concentración atmosférica de CO_2 en partes por millón (ppm, del inglés *parts per million*). Datos de *NOAA Climate.gov*.

II. Objetivos del estudio

El objetivo general de la presente tesis doctoral ha sido el análisis antropogenético de los restos óseos humanos recuperados de la necrópolis medieval de San Miguel de Ereñozar (siglos XIII-XVI, Ereño, País Vasco, España), centrado en evaluar la influencia de factores genéticos y ambientales en la génesis de enfermedades reumáticas. Este objetivo general se llevó a cabo mediante los siguientes objetivos específicos:

1. Análisis de los cambios morfológicos a nivel óseo de los individuos recuperados de la necrópolis de San Miguel de Ereñozar con el fin de identificar las manifestaciones óseas características de las enfermedades reumáticas presentes en esta población.
2. Análisis de marcadores genéticos (nucleares y mitocondriales), asociados con el desarrollo de enfermedades reumáticas, mediante el diseño de un protocolo metodológico que permita este análisis a partir del DNA obtenido de los restos óseos recuperados de la necrópolis de San Miguel de Ereñozar.
3. Valorar la posible relación existente entre enfermedades reumáticas y ciertos linajes mitocondriales identificados en la necrópolis estudiada, teniendo en cuenta la influencia de los factores ambientales que afectaron a los individuos que habitaron la región de Ereño durante los siglos XIII-XVI, en concreto, la influencia de la Pequeña Edad de Hielo (siglos XIV – XIX).
4. Identificar la variabilidad de alelos del gen *HLA-B* en los individuos recuperados de la necrópolis de San Miguel de Ereñozar con el objetivo de analizar e interpretar su prevalencia en los individuos que presentan manifestaciones óseas reumáticas y en aquellos individuos carentes de ellas.
5. Desarrollo de una herramienta metodológica que combine las manifestaciones óseas patológicas y genéticas, con el objetivo de contribuir al diagnóstico de enfermedades reumáticas. Esta metodología podría ayudar tanto a identificar estas patologías articulares en fases iniciales, como al diagnóstico paleopatológico, que habitualmente se enfrenta con limitaciones relacionadas con la escasa preservación de los restos esqueléticos.

6. Analizar un conjunto de alelos de riesgo presentes en genes involucrados en la patogénesis de la espondilitis anquilosante, psoriasis, artritis psoriásica y de la enfermedad inflamatoria intestinal en los individuos recuperados la necrópolis de San Miguel de Ereñozar, con el fin de analizar genes involucrados en rutas patogénicas compartidas por estas enfermedades y que puede contribuir a comprender la complejidad multifactorial de las espondiloartritis. Además, investigar la posible influencia de factores exógenos, como el clima, que podría haber afectado en la génesis de estas enfermedades en los individuos de dicha necrópolis.
7. Evaluar si las variaciones fenotípicas observadas en pacientes hospitalarios del Hospital Universitario de Basurto diagnosticados con espondiloartritis axial corresponden a entidades diferenciadas desde el punto de vista genético, mediante el análisis de un conjunto de SNPs de riesgo localizados en genes involucrados en la patogénesis de la espondilitis anquilosante, psoriasis, artritis psoriásica y de la enfermedad inflamatoria intestinal. Además, indagar la influencia de factores exógenos, como obesidad y tabaquismo, en las manifestaciones clínicas de las espondiloartritis axiales.

III. Material y Métodos

1. Material

1.1. Restos óseos humanos recuperados del yacimiento de San Miguel de Ereñozar

En la presente Tesis Doctoral se analizaron los restos esqueléticos humanos correspondientes a 163 individuos recuperados del yacimiento medieval de San Miguel de Ereñozar (SME). Este yacimiento se encuentra situado en el monte Ereñozar, a 447 metros de altura, y emplazado a 1.31 km al suroeste del municipio de Ereño. El municipio de Ereño se localiza al noreste de la provincia de Bizkaia (País Vasco, España), en la región de Busturialdea-Urdaibai **(Figura 47)**.

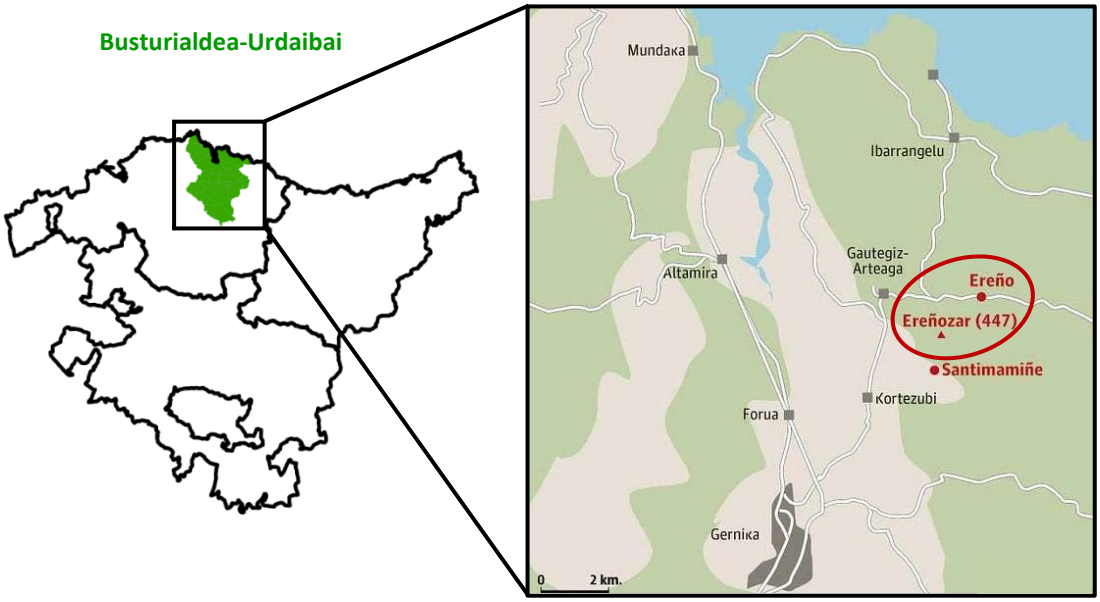


Figura 47. Localización geográfica del yacimiento medieval de San Miguel de Ereñozar (Ereño, Bizkaia, siglos XIII-XVI).

El yacimiento medieval se localiza en la cumbre del monte Ereñozar, donde en el siglo XI se construyó un castillo con fines militares, políticos o defensivos dada su estratégica situación geográfica ocupando una posición preferente sobre la ría de Gernika, su desembocadura y un extenso tramo costero (García Camino, 2008; Sarasola and Moraza, 2012) **(Figura 47)**. En la segunda mitad del siglo XIII se produjo el abandono del castillo, para su uso como lugar cementerial, aunque el castillo fue usado de nuevo con fines militares en el siglo XIV (Neira Zubieta, 2013). La excavación arqueológica, dirigida por el arqueólogo Mikel Neira Zubieta, puso de manifiesto la existencia de una necrópolis medieval, probablemente asociada a una antigua iglesia, de la que se

recuperaron un total de 163 individuos **(Figura 48)**. La necrópolis medieval comprende desde mediados/finales del siglo XIII hasta la mitad del siglo XVI y está dividida en 3 fases con distinta cronología: 1) XIII-XIV, 2) XIV-XV y 3) XV-1560 **(Figura 49)**. La inhumación en fosa simple es el método más usado, aunque también aparecen individuos recuperados de fosas antropomorfas y de estructuras sepulcrales, aunque hay que destacar que muchas de estas fosas fueron reutilizadas (Neira Zubieta, 2013) **(Figura 48)**.

Figura 48. Imágenes del yacimiento medieval de San Miguel de Ereñozar durante la excavación arqueológica que tuvo lugar entre los añós 2008 y 2012, junto con la actual ermita de San Miguel de Ereñozar. Imágenes cedidas por Mikel Neira Zubieta.

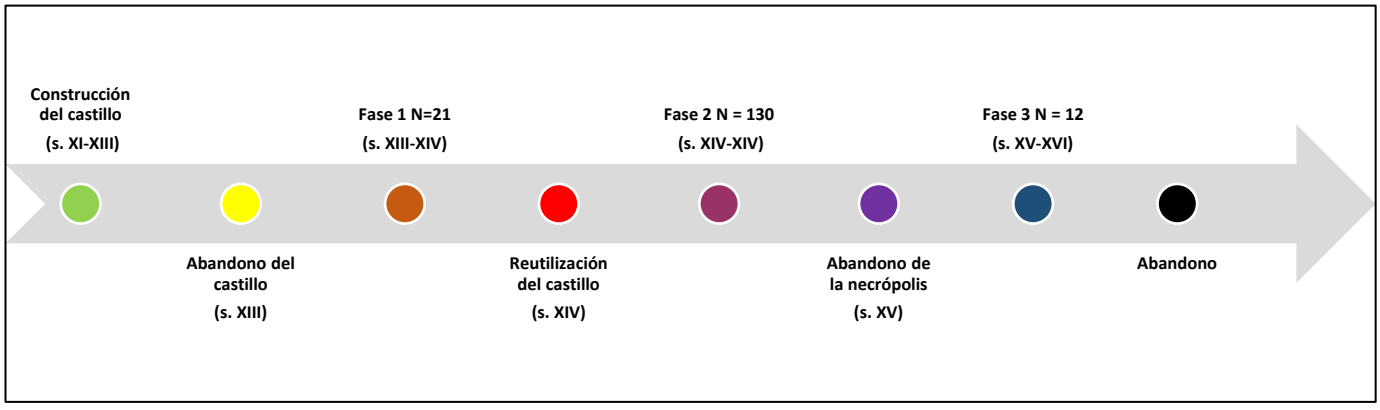


Figura 49. Cronología de la utilización de la cumbre del monte Ereñozar entre los siglos XI y XVI.

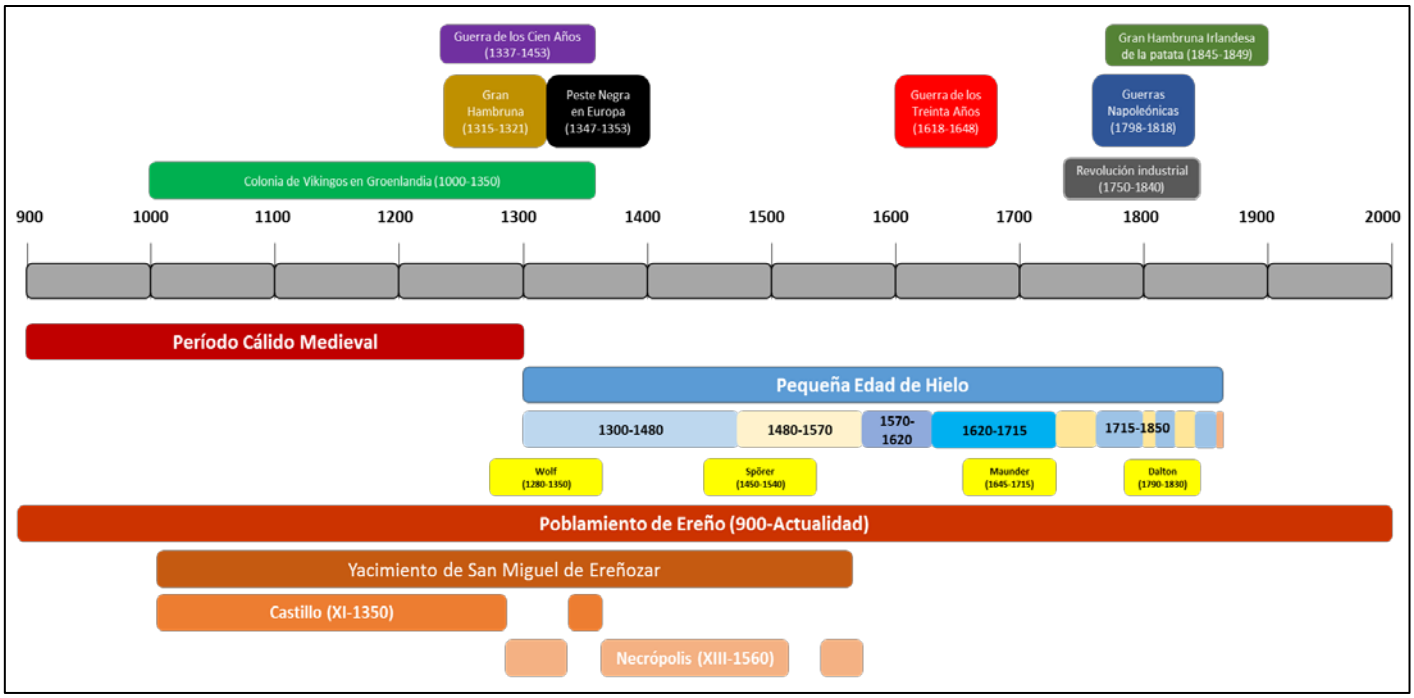


Figura 50. Línea de tiempo que muestra la cronología de los sucesos más importantes relacionados con la presente Tesis Doctoral y que tuvieron lugar durante los último 1.100 años.

1.2. Muestras de pacientes con espondiloartritis axial del Hospital Universitario de Basurto

El DNA fue obtenido de 62 pacientes reclutados en el Hospital Universitario de Basurto (HUB) (Bilbao, País Vasco) a partir de la extracción de muestras de sangre. Estas muestras fueron suministradas por el Biobanco Vasco para la Investigación (Biobanko, Bioef). Los pacientes incluidos en el estudio eran mayores de 18 años, sufrían dolor inflamatorio lumbar y cumplían los criterios modificados de Nueva York para la AS (van der Linden *et al*., 1984a) o los criterios de clasificación ASAS para la axSpA (Rudwaleit *et al*., 2009a, 2009b, 2011). Además, se registraron los datos clínicos, demográficos y de laboratorio de los pacientes (CRP, ESR, *HLA-B27*). El estado funcional y la actividad de la enfermedad fueron evaluados mediante los índices *Bath Ankylosing Spondylitis Functional Index* (BASFI) y BASDAI, respectivamente. También se evaluó la movilidad espinal y de cadera mediante el índice *Bath Ankylosing Spondylitis Metrology Index* (BASMI).

La radiografía pélvica fue evaluada por tres reumatólogos y la existencia de sacroileitis, definida según los criterios modificados de Nueva York (van der Linden *et al*., 1984a)

(Tabla 2), fue decidida en consenso, por al menos dos de los tres reumátologos. Los pacientes con sacroileitis radiográfica fueron diagnosticados con AS (N = 49), mientras que los pacientes que no presentaban sacroileitis radiográfica fueron diagnosticados por MRI de sacroiliacas (nr-axSpA) (N = 13), siguiendo los criterios de clasificación propuestos por ASAS (Rudwaleit *et al*., 2009A, 2009b, 2011) **(Tabla 3)**. Toda esta información fue registrada en una base de datos junto con un consentimiento informado firmado por los pacientes siguiendo los protocolos del Comité de Ética de la Investigación con medicamentos de Euskadi (CEImE, PI2017141).

2. Métodos

2.1. Análisis morfológico de los esqueletos recuperados del yacimiento de San Miguel de Ereñozar

2.1.1. Estimación de sexo y edad

La estimación del sexo y la edad de los restos óseos de los 163 individuos recuperados del yacimiento de SME fue llevada a cabo a nivel morfológico. La estimación del sexo se llevó a cabo siguiendo los criterios propuestos por White *et al*., (2011), estableciéndose cuatro grupos: masculino, femenino, alofiso e indeterminado. El carácter alofiso se aplica a aquellos individuos que, a pesar de presentar todos los elementos básicos para efectuar el diagnóstico sexual, éste resulta dudoso debido a que el dimorfismo sexual no está definido. En cambio, el carácter indeterminado se debe a la ausencia de elementos óseos suficientes para abordar la estimación sexual (Campillo, 2001). La estimación de la edad en individuos adultos se centró en los cambios morfológicos ocurridos en la carilla auricular de la pelvis (Byers, 2001), en la región acetabular (Calce, 2012), la sínfisis del pubis (Brooks and Suchey, 1990) y las suturas ectocraneales (Eguía *et al*., 1983).

Respecto al sexo, se identificaron 55 individuos del sexo masculino (33,7%), 44 del sexo femenino (27%), 60 individuos de carácter indeterminado (36,8%) y 4 individuos alofisos (2,5%). En relación a la edad, se identificaron 21 individuos infantiles de 0-12 años, 19 juveniles de 13-19 años, 26 adultos jóvenes de 20-34 años, 35 adultos de 35-44 años, 22 adultos maduros de 45-59 años y 10 adultos seniles de más de 60 años. La ausencia de restos óseos importantes del esqueleto para la estimación de la edad imposibilitó la

inclusión de 30 individuos dentro de los rangos de edad establecidos. No obstante, el nivel de maduración de los huesos de estos individuos permitió estimar que todos ellos eran adultos de más de 20 años de edad.

2.1.2. Diagnóstico de enfermedades reumáticas: análisis de las manifestaciones óseas

En los restos óseos recuperados de la necrópolis de SME se aplicó el protocolo descrito por Ventades *et al*. (2018), para llevar a cabo el diagnóstico diferencial de las enfermedades incluidas dentro de las artropatías inflamatorias. Este análisis se realizó de forma jerárquica, centrándose principalmente en la pelvis y en la columna vertebral.

La afectación de la articulación sacroiliaca es diferente entre cada una de las entidades patológicas. Cabe destacar que, en el caso de la AS, la afectación de la articulación sacroiliaca es simétrica **(Figura 3)**, mientras que, en la PsA, ReA y en las uSpA es unilateral y asimétrica, y en las IBD-SpA sacroileitis puede ser simétrica o asimétrica, aunque normalmente estas lesiones suelen ser similares a las de la AS (Resnick, 1979; Amor *et al*., 1994; Gran and Skomsvolly, 1997; Baraliakos *et al*., 2015).

La afectación a nivel raquídeo también presenta diferencias entre las patologías estudiadas. La AS se caracteriza por la aparición de sindesmofitos simétricos y verticales, normalmente localizados en las regiones torácica y lumbar, aunque pueden extenderse a toda la columna vertebral (formando la denominada *caña de bambú*), y también por la anquilosis de las articulaciones interapofisarias (Resnick, 1979; Gran and Skomsvolly, 1997) **(Figura 4)**. En la PsA, la afectación se produce en las regiones cervical y lumbar, y los sindesmofitos son asimétricos y verticales en forma de lesiones salteadas (Gladman *et al*., 1993, Baraliakos *et al*., 2015) **(Figura 4)**. La ReA se caracteriza por presentar lesiones vertebrales similares a las de la PsA, localizadas en la región cervical y en menor medida en las regiones torácica y lumbar (Resnick, 1979). En la uSpA, principalmente se ve afectada la región lumbar, mientras que en la IBD se desarrollan lesiones parecidas a la AS (Resnick, 1979).

2.2. Selección y procesamiento de las muestras óseas para el análisis de DNA antiguo

Siempre que fue posible, se seleccionaron piezas dentarias frente a hueso compacto, ya que es el material que ofrece mayores garantías a la hora de recuperar el DNA (Zierdt *et al*., 1996; Barrio-Caballero, 2013). Únicamente en los casos en los que no se conservaban ninguna pieza dentaria o estas presentaban un deficiente estado de conservación (caries profundas o fisuras), se seleccionaron huesos, preferentemente costillas, por ser una de las regiones anatómicas que presenta menor interés antropológico. Para este análisis, se incluyeron 47 individuos que presentaban manifestaciones óseas reumáticas y 43 individuos que no presentaban ningún signo artropático a modo de grupo control. Para este último grupo se seleccionaron individuos adultos maduros (> 45 años de edad) garantizándose la posibilidad de no desarrollar ninguna SpA, por esta razón no se analizaron los 163 individuos estudiados a nivel morfológico. En total se seleccionaron 2 muestras de cada uno de los 90 individuos para poder llevar a cabo el análisis molecular por duplicado.

Tras la selección de las muestras, se llevó a cabo, un registro fotográfico de cada una de las caras de los dientes y del hueso seleccionados, además de un registro métrico, consistente en la medición del diámetro buco-lingual y mesio-distal, así como de la longitud de la corona y de la raíz en el caso de tratarse de diente, mientras que en el caso de hueso se llevó a cabo la medición de los diámetros de las partes distal, central y proximal, así como de la longitud total.

Posteriormente, las muestras se sometieron a un proceso de limpieza superficial para eliminar el posible DNA contaminante de origen humano procedente del personal que ha manipulado la muestra y/o microbiano, así como posibles inhibidores y suciedad propia del enterramiento.

El proceso de limpieza se realizó de forma diferente según el material de partida seleccionado (diente o hueso). En el caso de tratarse de hueso, la superficie externa se limpió mediante abrasión con una lima odontológica (*Sofu*) adaptada a un micromotor odontológico (*Novfrom*). Posteriormente se cortó un fragmento de hueso de 5 cm de longitud y se pulverizó de forma manual. En el caso de tratarse de diente, este fue

sometido a un proceso depurinización mediante ácidos durante 10 minutos (20% ácido acético y 15% ácido clorhídrico). A continuación, el diente es lavado, primero con etanol 70% durante 15 minutos para esterilizar su superficie de cualquier agente microbiano y posteriormente con H_2O destilada durante 30 minutos para eliminar los posibles restos de las soluciones anteriores a las que ha sido sometido. Finalmente, el diente es irradiado con luz ultravioleta durante 30 minutos por cada una de sus caras. Tras este proceso, el diente se sierra entre la raíz y la corona para facilitar el acceso a la cavidad pulpar, la cual es limada mediante una lima odontológica para la recuperación de una mayor cantidad de restos celulares (Hervella *et al*., 2014).

2.3. Extracción de DNA antiguo de muestras esqueléticas

La extracción de DNA se realizó siguiendo dos metodologías diferentes: *fenol:cloroformo* si se partía de diente (Hervella *et al*., 2012), y *DNAzol* si se partía de hueso pulverizado (Maca-Meyer *et al*., 2005; Laza *et al*., 2016).

El método de extracción de *fenol:cloroformo* consiste en una incubación inicial del diente en 5 ml de una solución de lisis (0,5 M EDTA; 0,5 mM Tris HCl; SDS 0,5%; 200 µl proteinasa K) durante dos horas a 56ºC con rotación. Tras esta incubación, se realiza la extracción de DNA de los restos celulares mediante *fenol:cloroformo*. El componente proteico del extracto se desnaturaliza y queda disuelto en la fase fenólica, mientras que los ácidos nucleicos permanecen disueltos en la fase acuosa. La fase acuosa obtenida se purifica y se concentra mediante filtración utilizando *Centricon-30* siguiendo las instrucciones del fabricante (*C-30 Amicon)* (Hervella *et al*., 2012).

El método de extracción de *DNAzol* consiste en una variante del método de extracción tradicional de la sílica. Siguiendo este método, el hueso pulverizado se incuba durante 3 días con agitación y en oscuridad con *DNAzol* (*DNAzol™ Reagent*, *Invitrogen*) que contiene tiocianato de guanidinio (GuSCN) y el sobrenadante obtenido se concentra y purifica con columnas con base de sílica (*PCR Purification Kit*, *QIAgen*). En esta solución, los ácidos nucleicos se separan debido a su alta afinidad para enlazarse con las matrices de sílica mediante el GuSCN. Finalmente, se eluye el DNA retenido en la sílica con un tampón de elución (Maca-Meyer *et al*., 2005; Laza *et al*., 2016).

En cada tanda de extracción se incluyeron al menos dos blancos de extracción, que consisten en muestras que se someten a todo el proceso de extracción, pero a las que no se les añade tejido óseo. Estos controles sirven para detectar posibles contaminaciones que pudieran tener lugar durante la fase de extracción.

Tras obtener el extracto de DNA de cada una de las muestras esqueléticas, este fue cuantificado mediante fluorimetría (QUBIT, *Life Technologies*), para estimar la concentración y la calidad del DNA obtenido.

2.4. Análisis de la variabilidad del DNA mitocondrial

2.4.1. Secuenciación del Segmento Hipervariable I de la región control del DNA mitocondrial

La secuenciación del HVS-I del mtDNA a partir de los extractos de aDNA obtenido fue llevada a cabo mediante la utilización de 6 parejas de primers, que permiten la amplificación de 6 fragmentos solapantes de aproximadamente 100 bp de longitud cada uno, de modo que finalmente se obtiene una secuencia consenso de DNA comprendida entre los nucleótidos 15.995 y 16.399 (404 bp) (Alonso *et al*., 2003) **(Tabla 4)**.

Tabla 4. Secuencia de los primers utilizados para llevar a cabo la amplificación del HVS-I, junto con la temperatura de anillamiento (T^a) y el tamaño del fragmento obtenido en pares de bases (bp) para cada pareja de primers.

SIGLA	Secuencia nucleotídica (5 → 3´)	T^a (ºC)	Tamaño (bp)
A1	CAC CAT TAG CAC CCA AAG CT (20)	60	112
A1R	ACA TAG CGG TTG TTG ATG GG (20)		
1F	GAA GCA GAT TTG GGT ACC AC (20)	57	123
1R	GTA CTA CAG GTG GTC AAG TAT (21)		
2F	CAC CAT GAA TAT TGT ACG GT (20)	58	125
2R	ATG TGT GAT AGT TGA GGG TTG (21)		
3F	CCC CAT GCT TAC AAG CAA GT (20)	55	132
3R	TGG CTT TAT TGT ACT ATG TAC (21)		
4F	CAC TAG GAT ACC AAC AAA CCT A (22)	58	130
4R	CAA GGG ACC CCT ATC TGA GG (20)		
5F	CGT ACA TAG CAC ATT ACA GT (20)	57	92
5R	TGA TTT CAC GGA GGA TGG TG (20)		

Cada fragmento del HVS-I fue amplificado en PCRs independientes con una mezcla de reacción en un volumen final de 25 µl: 10 mM Tris-HCl pH 8.3; 50 mM KCl; 2 M $MgCl_2$; 25 µM de cada dNTP; 20 µg BSA; 0.4 µM de primer **(Tabla 4)**; 1 U de HotStart AmpliTaq Gold polimerase (*Applied Biosystems*) y 10 µl de DNA diluido con BSA (1:10) (*Sigma-Aldrich*) y siguiendo las condiciones de la PCR: 95 ^{o}C x 10 min + 45 cycles (95 ^{o}C x 30 sec + T^{a} x 30 sec + 72 ^{o}C x 30 sec) + 72 oC x 10 min.

En todas las PCRs, además de los blancos de extracción se incluyeron dos controles negativos de la PCR, consistentes en muestras que se someten a todo el proceso de amplificación, pero a las que no se les añade DNA, y su análisis tiene la finalidad de detectar una posible contaminación que pudiera tener lugar durante el proceso de amplificación.

El tamaño de los amplificados y la ausencia de contaminación se comprobaron mediante migración electroforética en un gel horizontal de agarosa 2% y 0,01% de *RedSafe™* (*iNtRON Biotechnology*) en tampón *TBE 1X* (Tris-Borate-EDTA, *Invitrogen*). El tamaño de los productos obtenidos en la PCR se calcula por comparación con un marcador de peso molecular (*Marcador XIII 50 bp*, *Roche Diagnostic*)

Los productos de PCR a secuenciar deben estar previamente purificados para eliminar los restos de primers, nucleótidos y sales que provienen de la reacción de la PCR. La purificación se realiza mediante acción enzimática de *ExoSap-IT* siguiendo las instrucciones del fabricante (*ExoSap-IT*, *USB Corporation*). A continuación, se llevó a cabo la reacción de secuenciación (*BigDye 1.1 Terminator Cycle Ready Reaction Kit*, *Applied Biosystems*) siguiendo la metodología propuesta por Hervella *et al*., (2012). Posteriormente, el producto de la reacción de secuenciación fue purificado mediante el uso de columnas *AutoSeq™ G-50* diseñadas para la eliminación de los terminadores fluorescentes de la reacción de secuenciación, siguiendo las instrucciones del fabricante (*GE Healthcare Life Sciences*). La acumulación de terminadores puede impedir la correcta lectura de la secuencia resultante. Las muestras purificadas fueron desnaturalizadas añadiendo *HI-DI Formamida* (*Applied Biosystem*) y migradas en un secuenciador automático *ABI PRISM 310* (*Applied Biosystem*).

Las secuencias obtenidas del HVS-I fueron editadas mediante el programa informático *BioEdit* (Hall, 1999), comparándolas con la secuencia de referencia publicada por Anderson *et al*., (1981) y que fue revisada por Andrews *et al*., (1999), denominándose rCRS (del inglés *revised Cambridge Reference Sequence*), obteniendo una secuencia consenso de una longitud de 404 bp. Con el fin de determinar el haplogrupo, sub-haplogrupo y haplotipo mitocondrial de las secuencias obtenidas, estas fueron analizadas y filtradas usando las bases de datos *Haplogrep* (*https://haplogrep.uibk.ac.at*) (van Oven and Kayser, 2009) y *Phylotree* (*https://www.phylotree.org*) (Kloss-Brandstätter *et al*., 2011).

2.4.2. Secuenciación del Segmento Hipervariable II (HVS-II) de la región control del DNA mitocondrial

En los casos en los que los individuos presentaban el haplotipo rCRS para el HVS-I, se secuenció un fragmento (7F/7R) del HVS-II del mtDNA que contiene la posición 73 (Hervella *et al*., 2012) **(Tabla 5)**. Se siguió el mismo procedimiento de análisis que en el apartado de secuenciación del HVS-I (Apartado 2.4.1.). En aquellos individuos que portaban el polimorfismo A073G, se realizó PCR-RFLPs con la enzima *AluI* para determinar el nucleótido en la posición 7025 con el fin de verificar si esa secuencia correspondía al haplogrupo H (Alzualde *et al*., 2005).

Tabla 5. Secuencia de los primers utilizados para llevar a cabo la amplificación de un fragmento de la región HVS-II, junto con la temperatura de anillamiento (T^a) y el tamaño del fragmento obtenido en pares de bases (bp) (Alzualde *et al*., 2005).

SIGLA	Secuencia nucleotídica (5 → 3´)	T^a (ºC)	Tamaño (bp)
7F	TTC CTA CTT CAG GGT CAT AAA GCC (24)	61	115
7R	ACC AAA TGC ATG GAG AGC TC (20)		

2.5. Secuenciación de los exones 2 y 3 del gen *HLA-B*

El gen *HLA-B*, que posee 8 exones, presenta un alto grado de polimorfismo genético localizado principalmente en los exones 2 y 3 (Little and Parham, 1999; Khan and Ball, 2002; Khan, 2010). Existen más de 150 de alelos del gen *HLA-B* descritos hasta el momento, definidos en base a las diferencias en la secuencia nucleotídica (Khan, 2010,

2013, 2017; Dashti *et al*., 2018). En el presente estudio se realizó la secuenciación de los exones 2 y 3 del gen *HLA-B* en los individuos recuperados de la necrópolis SME. Para ello, se utilizaron tres parejas de primers descritas en Laza *et al*., 2016 y una pareja de primers descrita en Domínguez *et al*., (1992) **(Tabla 6)**, con el fin de obtener fragmentos solapantes de entre 61 y 117 bp de longitud y generar la secuencia consenso de estos dos exones, siguiendo el protocolo descrito por Laza *et al*., (2016), y siendo similar al descrito para la secuenciación del HVS-I (Apartado 2.4.1).

Tabla 6. Secuencia de los primers utilizados para la amplificación del exón 2 y 3 del gen *HLA-B*, junto con la temperatura de anillamiento (T^a) y el tamaño del fragmento obtenido en pares de bases (bp) para cada pareja de primers. *Primers descritos en Domínguez *et al*., 1992. $^+$Primers descritos en Laza *et al*., 2016.

SIGLA	Secuencia nucleotídica (5 → 3´)	T^a (ºC)	Tamaño (bp)
HLAB2-1F$^+$	GCC GCG AGT CCG AGA GA (17)	65	117
HLAB2-1R$^+$	GGC CTC GCT CTG GTT GTA (18)		
HLAB2-2F*	CCG GAG TAT TGG GAC CG (17)	65	67
HLAB2-2R*	GGC CTC GCT CTG GTT GT (17)		
HLAB3-2F$^+$	GGG CAG GGT CTC ACA CCC TCC (21)	65	61
HLAB3-2R$^+$	GAT GTA ATC CTT GCC GTC GTA (21)		
HLAB3-3F$^+$	GGA TTA CAT CGC CCT GAA CG (20)	60	92
HLAB3-3R$^+$	TCC ACG CAC TCG CCC TCC AGG T (22)		

Las secuencias resultantes del análisis fueron editadas mediante el programa bioinformático *BioEdit* (Hall, 1999), y para ello se utilizó la secuencia de referencia del gen *HLA-B* publicada en la base de datos IPD-IMGT/HLA (*http://www.ebi.ac.uk/ipd/imgt/hla/*) (Robinson *et al*., 2015a). La identificación de los diferentes alelos del gen *HLA-B* fue llevada a cabo mediante la comparación de las diferentes secuencias nucleotídicas obtenidas para el gen *HLA-B* con la base de datos IPD-IMGT/HLA *(http://www.ebi.ac.uk/ipd/imgt/hla/*) (Robinson *et al*., 2015a). Este análisis se llevó a cabo en las muestras esqueléticas recuperadas del yacimiento medieval de SME y en las muestras de pacientes del HUB.

2.6. Genotipado de SNPs asociados a la susceptibilidad a desarrollar espondiloartritis

La selección de los SNPs asociados a las SpA se llevó a cabo a partir de la búsqueda en distintas bases de datos bibliográficas: 1) PubMed (*http://www.ncbi.nlm.nih.gov/pubmed/*), 2) *dbSNP* (*http://www.ncbi.nlm.nih.gov/snp/*) del *NCBI* (*National Center for Biotechnology Information*, EEUU), y 3) bases de datos que recopilan los resultados obtenidos de diferentes análisis *GWAS* (*Immunobase*, *GWAS Catalog*, *GWAS Central*, *Emsembl Genome Browser*), en los que se identifican aquellas variantes alélicas de SNPs asociadas a la susceptibilidad a desarrollar una enfermedad.

En este estudio se seleccionaron todos aquellos SNPs que previamente habían sido asociados al diagnóstico, pronóstico y tratamiento de AS, Ps, PsA, e IBD **(Tabla 7)**. Estos SNPs se localizan en genes que se encuentran involucrados en distintas rutas y mecanismos que podrían explicar en parte la patogénesis de la enfermedad.

Tabla 7. SNPs asociados con el desarrollo de AS, Ps, PsA e IBD (CD y UC). Se indica el locus, el gen, código rs y la enfermedad asociada. AS: ankylosing spondylitis; Ps: psoriasis; PsA: psoriatic arthritis; IBD: inflammatory bowel disease; CD: Crohn´s disease; UC: ulcerative colitis. [1] Jostins *et al*., 2012; [2] Cortes *et al*., 2013; [3] Evans *et al*., 2011; [4] Strange *et al*., 2010; [5] Kenny *et al*., 2012; [6] Anderson *et al*., 2011; [7] Hüffmeier *et al*., 2009; [8] Burton *et al*., 2007b; [9] Reveille *et al*., 2010; [10] Tsoi *et al*., 2012; [11] Ellinghaus *et al*., 2012; [12] Liu *et al*., 2015; [13] Hüffmeier *et al*., 2010; [14] Stuart *et al*., 2015; [15] Franke *et al*., 2010.

Locus	Gen	SNP	Enfermedad asociada
1p13	*PTPN22*	rs6679677	IBD, CD [1]
1q21	*IL6R*	rs4129267	AS [2]
1q23	*FCGR2A*	rs1801274	AS [2]
1p31	*IL23R*	rs11209026	AS, Ps, PsA, IBD [1-7]
1p31	*IL23R*	rs12141575	AS [2]
1q32	*GPR25-KIF21B*	rs41299637	AS[2]
1p36	*RUNX3*	rs6600247	AS[2]
2q11	*IL1R2*	rs2310173	IBD, UC [8]
2p15	Intergenic	rs10865331	AS, Ps, IBD [1,3,9, 10]
2p16	*REL*	rs702873	Ps, PsA [4,11]
2p23	*UCN*	rs1728918	IBD, CD [2]
2q37	*ATG16L1*	rs12994997	IBD, CD [2,13]
5q15	*ERAP1*	rs27434	AS [9]

5q15	*ERAP1*	rs30187	AS [2,3]
5q15	*ERAP2*	rs2910686	AS [2]
5q15	*ERAP2*	rs10045403	AS [2]
5q33	*IL12B*	rs6556416	AS [2,3]
5q33	*IL12B*	rs6871626	AS [2]
5q33	*IL12B*	rs12188300	Ps, PsA [9,13]
5q33	*TNIP1*	rs2233278	Ps [9]
6p21	*HLA-B27*	rs116488202	AS [2]
6p21	*HLA-C*	rs4406273	Ps [9,14]
6p21	*HLA-C*	rs13191343	PsA [7]
6q21	*TRAF3IP2*	rs33980500	Ps, PsA [9,11,13,14]
6q23	*TNAFAIP3*	rs6920220	IBD, UC [1,12]
9q34	*CARD9*	rs1128905	AS [2]
10q22	*ZMIZ1*	rs1250550	AS, IBD [2,15]
10q24	*NKX2-3*	rs11190133	AS [2]
12p13	*LTBR-TNFRSF1A*	rs1860545	AS [2]
12p13	*LTBR-TNFRSF1A*	rs11616188	AS [3]
12q24	*SH2B3*	rs11065898	AS [2]
14q31	*GPR65*	rs11624293	AS [2]
16p11	*IL27*	rs75301646	AS [2]
16q12	*NOD2*	rs2076756	IBD, CD [5,15]
16q12	*NOD2*	rs17221417	IBD, CD [8]
17q11	*NOS2*	rs2531875	AS [2]
17q21	*NPEPPS-TBKBP1-TBX21*	rs9901869	AS [2]
19p13	*TYK2*	rs35164067	AS [2]
19p13	*SBNO2*	rs2024092	IBD, CD [1,12]
21q22	Intergenic	rs2242944	AS [9]
21q22	Intergenic	rs2836883	AS [2]
21q22	*ICOSLG*	rs7282490	AS [2]
21q22	*IFNGR2*	rs2284553	IBD, CD [1,12]

Posteriormente se redujo la selección de SNPs realizada mediante la utilización de los siguientes criterios: 1) GWAS descritos en población caucásica, ya que algunas variantes alélicas presentan diferencias entre poblaciones de distinto origen étnico, 2) p_{value} de asociación del SNP con la enfermedad fuera menor de $5*10^{-6}$, y 3) los SNPs que se encontraron en desequilibrio de ligamiento ($r^2 > 0.8$), fueron excluidos del estudio.

Los SNPs seleccionados en este estudio fueron genotipados mediante la plataforma BioMark HD de *Fluidigm* (*96.96 Dynamic Array™ IFC, Fluidigm, South San Francisco, CA, USA*). El genotipado fue llevado a cabo en la Unidad de Secuenciación y Genotipado del SGIker de la Universidad del País Vasco (UPV/EHU, Leioa, Bizkaia, España), mediante el uso de sondas *SNP Type*. Los análisis *SNP Type* son ensayos PCR alelo-específicos realizados por *Fluidigm* que utilizan tres primers y dos sondas universales (SNPtype-FAM y SNPtype-HEX) para distinguir entre dos alelos **(Figura 51)**. El Fluidigm Chip (*96.96 Dynamic Array™ IFC*) realizado consta de 96 pocillos, correspondiendo cada pocillo a una única muestra de DNA, con la excepción de dos pocillos en los que no se les añade muestra y son utilizados a modo de controles del análisis (94 muestras + 2 controles).

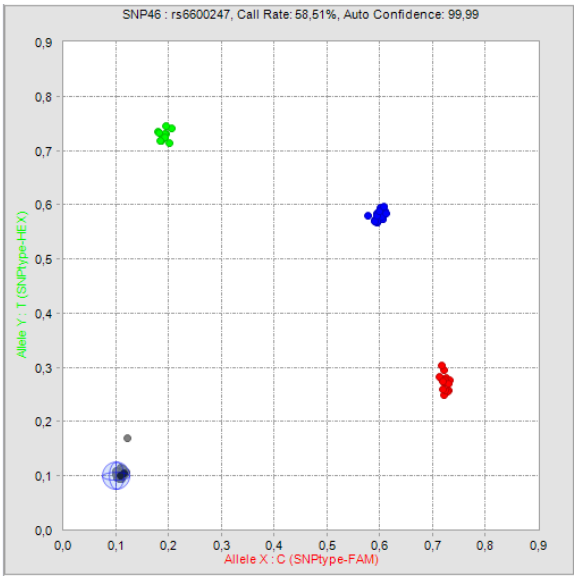


Figura 51. Gráfico resultante del genotipado a través del programa *Fluidigm SNP Genotyping Analysis*. Cada punto en la gráfica corresponde a un genotipo que proviene de una muestra determinada. Los genotipos marcados en rojo y verde corresponden a las muestras homocigotas (XX y YY) mientras que los genotipos marcados en azul a las muestras heterocigotas (XY).

Las sondas diseñadas superaron la fase de calidad/éxito teórico por parte de *Fluidigm* para poder llevar a cabo el correcto genotipado de los SNPs. Todos los SNPs que no superaron una tasa de éxito <0.90 fueron descartados del análisis. El genotipado del *HLA-B27* también se realizó mediante el tagSNP rs11688202 en la plataforma BioMark HD de *Fluidigm* comentada anteriormente corroborando los resultados obtenidos mediante secuenciación automática (Cortes *et al*., 2013, 2015; Robinson *et al*., 2015b).

Este SNP posee una sensibilidad y especificidad de >98% para el tipaje del *HLA-B27* (Cortes *et al*., 2013). Este análisis se realizó tanto en el DNA procedentes de muestras actuales (HUB) como de muestras antiguas (SME).

2.7. Criterios de autentificación en los resultados de DNA antiguo

La comunidad científica exige unos estrictos criterios de autentificación de los resultados de aDNA obtenidos por PCR y secuenciación automática (Cooper and Poinar, 2000; Hofreiter *et al*., 2001a, 2001b; Pääbo *et al*., 2004; Gilbert and Willerslev, 2006). Los criterios de autentificación que se han seguido en el presente estudio son los siguientes:

- Cuantificación del número de moléculas amplificables del DNA en los extractos mediante fluorimetría (*QUBIT*), con el fin de valorar el estado de preservación del DNA y la reproducibilidad de los resultados. Todas las muestras indicaban un valor de concentración de DNA >5 ng/μl.
- En todos los procesos de extracción y PCRs se incluyeron al menos dos blancos de extracción y varios controles negativos de la PCR, con el fin de determinar la existencia de contaminación tanto en la fase de extracción como en la de amplificación.
- Todas las muestras analizadas fueron duplicadas en nuestro laboratorio, en diferentes momentos y por diferentes investigadores, siendo todos los resultados coincidentes.
- Los productos de PCR fueron clonados con el fin de diferenciar las mutaciones endógenas de la propia muestra a las originadas por el daño del DNA. La clonación de los productos amplificados y su posterior secuenciación, permite diferenciar las mutaciones endógenas de la muestra, que deben aparecer en todos los clones, de aquellas mutaciones debidas a la incorporación errónea de un nucleótido en una posición dañada del aDNA (Krings *et al*., 1997). La clonación fue llevada a cabo mediante la utilización de *TOPO TA CloningR Kit* siguiendo las instrucciones del fabricante (*Invitrogen*). La ventaja que ofrece este kit para el análisis de muestras aDNA frente a otros reside en que presenta una elevada eficiencia de clonación de los productos de PCR de una longitud menor a 200 bp.

- Tipaje del personal que manipuló las muestras óseas con el fin de identificar la posible contaminación de DNA procedente de dicho personal.

2.8. Análisis estadístico

La distancia F_{ST} y la diversidad genética (Nei, 1987) de los diferentes grupos de individuos fueron calculadas con el programa informático *Arlequin 3.11* (Schneider and Excoffier, 1999), teniendo en cuenta las frecuencias de los haplogrupos y sub-haplogrupos mitocondriales y los alelos del gen *HLA-B*.

Para la realización del análisis de componentes principales (PCA, del inglés *principal component analysis*) y Heatmap se utilizaron los paquetes *FactoMineR* (Lê *et al*., 2008) y *Pheatmap* (Kolde, 2015) del programa *RStudio* (RStudio Team, 2018), respectivamente.

El análisis de las variables continúas obtenidas del estudio de pacientes del HUB, expresadas como la media y la desviación estándar, fueron comparadas utilizando la prueba t de Student. Del mismo modo, las variables categóricas (variables clínicas y de laboratorio), fueron comparadas considerándose estadísticamente significativo un $p_{value} < 0.05$. Por otro lado, las frecuencias alélicas y genotípicas fueron comparadas utilizando el test exacto de Fisher, considerándose estadísticamente significativo un $p_{value} < 0.05$ (aplicando la corrección de Bonferroni para múltiples test). Estos análisis fueron llevados a cabo mediante el programa estadístico *SPSS Statistic v25* (IBM Corp, 2017). El test de Equilibrio de Hardy-Weinberg para las frecuencias genotípicas de los SNPs seleccionados fue realizado mediante el paquete *HardyWeinberg* del programa *RStudio* (Graffelman and Morales-Camarena, 2008; Graffelman, 2015; RStudio Team, 2018), considerando que no se encontraban en equilibrio de Hardy-Weinberg aquellos SNPs con un $p_{value} < 0.05$ (aplicando la corrección de Bonferroni para múltiples test).

IV. Resultados

The results of the present Doctoral Thesis are published in four different articles.

- **Article 1:** Laza, I.M., Hervella, M., de-la-Rúa, C. Genetic markers in a medieval case of ankylosing spondylitis. *The Journal of Rheumatology*. 43(3): 679-681 (2016).
- **Article 2:** Laza, I.M., Ventades, N.G., Hervella, M., de-la-Rúa, C. Contribution of ancient human remains analysis to the understanding of the variability in HLA-B gene variants in relation to the diagnosis of spondyloarthropathies. *Journal of Autoimmunity*. 94: 70-82 (2018).
- **Article 3:** Laza, I.M., Hervella, M., Neira Zubieta, M., de-la-Rúa, C. Environmental factors modulated ancient mitochondrial DNA variability and the prevalence of rheumatic diseases in the Basque Country. *Scientific Reports*. 9(1): 20380 (2019).
- **Article 4:** Laza, I.M., Hervella, M., Izagirre, N., Blanco Madrigal, J.M., Galíndez, E., Rivera, N.A., Garcia Vivar, M.L., de-la-Rúa, C. Clinical and genetic study of axial spondyloarthritis. *Research Square*. DOI: 10.21203/rs.2.22687/v1 (2020).

V. Discusión General

In the present Doctoral Thesis, we analyzed the human bone remains recovered from the medieval site of San Miguel de Ereñozar (SME) (Basque Country, Spain, 13th-16th centuries). The analyzed sample has unprecedented features, due to the high frequency of joint pathologies found among its individuals and to the high efficiency obtained in the DNA extraction from human bone remains. The study has involved the analysis of spondyloarthritis (SpA), a group of inflammatory and autoimmune rheumatic diseases that traditionally encompass ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), undifferentiated spondyloarthritis (uSpA) and inflammatory bowel disease-associated spondyloarthritis (IBD-SpA) such as Crohn´s disease (CD) and ulcerative colitis (UC), from several perspectives: i) to analyze of key bone manifestations to achieve a differential diagnosis of the different diseases that encompass the group of SpA, ii) to evaluate the potential relationship between mitochondrial DNA (mtDNA) and rheumatic diseases, iii) to analyze the association of the *HLA-B* gene alleles with rheumatic disease and vi) to study the putative role of a set of risk alleles located in genes associated with different types of rheumatic disease. Furthermore, the chronology of this site coincides with a period of climatic instability known as the Little Ice Age (LIA) (14th–19th centuries), which could be one of the environmental factors involved in the development of SpA in this population.

Ancient populations, such as the one of this study, offer an excellent opportunity to analyze rheumatic pathologies, since joint manifestations are usually found in advanced states due to the absence of treatment. Furthermore, the development of analytical techniques in the field of ancient DNA (aDNA) facilitates the analysis of different nuclear markers associated with the development of SpA beyond the allele *HLA-B27*, which up to the present has been the only marker tested in ancient populations, limited only to confirm its presence or absence (Haak *et al*., 2005; Leden *et al*., 2009).

1. Analysis of bone manifestations characteristics of spondyloarthritis: contribution to diagnosis at the skeletal level

The morphological analysis of the bone remains of the 163 individuals recovered from the SME site has shown that the key manifestations for SpA diagnosis are sacroiliitis and hip enthesitis **(Article 2 - Figure 1)**. The affectation of the sacroiliac joint (SIJ) is a

characteristic feature of SpA, although not exclusively for this group of pathologies, since this feature was also found in the individuals diagnosed with osteoarthritis (OA) **(Article 2 – Figure 1)**. The age of the individuals may help to discriminate between both diseases (SpA and OA), since in OA the SIJ are affected in more advanced stages of life (>45 years). On the other hand, sacroiliitis and hip enthesitis may contribute to differentiate various types of SpA, sacroiliitis being symmetric in the case of AS and SpA IBD-SpA, and asymmetric in psoriatic arthritis PsA and reactive arthritis ReA. In SpA, the pathological process begins in the bone marrow and in the enthesis (Benjamin *et al*., 2001; François *et al*., 2001), where innate and adaptive immune responses are initially activated as a repair process. However, in subsequent stages these responses may generate remodeling processes that include bone marrow edema, osteitis, new bone formation and fusion and ankylosys in some cases (Bridgewood *et al*., 2018; Watad *et al*., 2018). For this reason, it has been postulated that enthesis is the primary site of inflammation and the main target of SpA. These areas contain unique populations of resident T cells that, upon activation by IL-23, they can cause the characteristic pathogenesis of this group of rheumatic disease (Lories and McInnes, 2012).

Despite the great importance of the SIJ and hip enthesis affectation in the diagnosis of SpA, it is necessary to identify other joint lesions to differentiate the various types of SpA. Interestingly, the location, symmetry and direction of spine lesions are crucial for this purpose. In AS, syndesmophytes are symmetric and vertical, and they usually affect the thoracic and lumbar regions, there are severe cases in which the entire spine is affected (bamboo cane) (Amor *et al*., 1994; Gran and Skomsvolly, 1997; Baraliakos *et al*., 2015). In PsA and ReA, syndesmophytes are asymmetric, vertical and skip, and they mainly affect the cervical region (Gladman *et al*., 1993; Baraliakos *et al*., 2015). The IBD-SpA usually causes spine lesions similar to those in AS, whereas in uSpA, lesions mainly affect the lumbar region.

In conclusion, at the morphological level, SIJ, spine joints and enthesis, mainly from the hip, are the target locations in SpA that help to establish their diagnosis. Thus, we can discriminate the pathologies that encompass the group of SpA from other rheumatic

diseases by the analysis of the location and morphology of bone lesions, and by the measurement of the age of those individuals.

From the morphological analysis carried out in the individuals recovered from the SME site (N = 163), we estimated that 30% of the individuals showed rheumatic bone features. The most frequent diseases identified in this population were SpAs (45%), being four cases also positive for AS **(Article 2- Figure 1B)**. Furthermore, individuals with OA (36%) and rheumatoid arthritis (RA) (4%) were also found. We were unable to stablish an accurate diagnosis for the 15% of the individuals studied, due to their poor state of conservation. The prevalence of SpA in the population studied is very high (12.9%), in comparison to that reported in epidemiological studies of current populations, where the prevalence of SpA ranges between 0.1% and 1.4% globally and between 0.5 and 2% in the Caucasian population (Zeidler *et al*., 2006). This high prevalence of SpA from the necropolis of SME is also observed when compared to the current populations with the highest prevalence of SpA, such as Haida (6-10%) and Bella Coola (2%) in Canada (Gofton *et al*., 1984), Eskimos in Alaska (2.5%) (Gofton *et al*., 1966) and populations in Northern Norway (1.8%) (Johnsen *et al*., 1992), which are also subject to adverse environmental factors just as it might occur in the medieval population of SME. The prevalence of SpA in the necropolis of SME is also higher when compared to other archaeological populations in North America (0.7-5.1%) (McKelvey, 1945; Neumann, 1966; Lamphear, 1988) and in South America (7% in Chile) (Arriaza *et al*., 1993). Most European reports of SpA appear to represent isolated cases, with accurate prevalence information unavailable to date in ancient human populations (Ruffer, 1919; Zorab, 1961; Bourke, 1967; Thould and Thould, 1983; Tamás *et al*., 2006).

2. Rheumatic diseases and mitochondrial lineages

Currently, it has been postulated the existence of a relationship between some rheumatic diseases and certain mitochondrial lineages, based on the critical role that the mitochondrion plays in cellular functioning and in survival against oxidative stress (Rego-Pérez *et al*., 2013; Hwang and Kim, 2015). During the energy generation process, via oxidative phosphorylation, the mitochondrion produces Reactive Oxygen Species (ROS), whose production causes damage to lipids, proteins and DNA, promoting

mitochondrial dysfunction and inflammation in different pathologies (Cillero-Pastor *et al*., 2008; Grishko *et al*., 2009; Kim *et al*., 2010; Rego-Pérez *et al*., 2013; Lepetsos and Papavassiliou, 2016).

Different studies have shown that some mitochondrial haplogroups can increase the risk of developing rheumatic diseases, such as haplogroup H, whereas other haplogroups, such as J and T, can constitute a protection factor and may be associated to a lower risk of developing OA and PsA (Rego-Pérez *et al*., 2010, 2011, 2013; Coto-Segura *et al*., 2012; Shen *et al*., 2014; Soto-Hermida *et al*., 2014, 2015; Fernandez-Moreno *et al*., 2017a, 2017b).

Considering this context, in the present Doctoral Thesis we analyzed the mitochondrial variability of 90 adult individuals of the medieval necropolis of SME (47 individuals with rheumatic bone manifestations and 43 without rheumatic bone manifestations) and we identified 7 haplogroups (H, U, T, K, R, J and HV) and 18 different sub-haplogroups (H2, H1, H3, T2, U5, H14, R8, H5, J1, K1, U*, H7, H11, H24, HV*, K2, U1, U3) **(Article 3 - Tables 1 y 2)**. The most frequent mitochondrial haplogroup was H (73.3%), whose frequency is higher than that reported in the current population of Busturialdea-Urdaibai, where the SME necropolis is located (Palencia-Madrid *et al*., 2017) **(Article 3 – Table 1)**. We found statistically significant differences between these two populations for the different mitochondrial haplogroups. These differences can be attributed to the high frequency of haplogroup H found in this necropolis.

The analysis of the mitochondrial haplogroups revealed the existence of 55 different haplotypes among the 90 individuals. Haplotype rCRS showed a very high frequency in the necropolis (≃24%) **(Article 3 – Table S1)**, although similar to the values found in the current populations of the Basque Country (27-21%). Therefore, we can discard the existence of endogamy among the individuals recovered from the necropolis of SME.

Among the causes that could explain the distribution of haplogroup H in the current European population, the climate change that took place during the Last Glacial Maximum (LGM) seems to have played an important role. Moreover, the demographic

changes that occurred during the Neolithic were a key factor for the diversification of haplogroup H and for the increase of its frequency, becoming the main haplogroup at present in Western Europe (55-40%) (Richards *et al*., 2000; Achilli *et al*., 2004; Loogväli *et al*., 2004; Brotherton *et al*., 2013).

We further studied the relationship between haplogroup H and rheumatic diseases in the necropolis of SME and, interestingly, we observed that 56% of the individuals that carried this haplogroup had rheumatic lesions, which suggests that carrying haplogroup H can increase the risk of developing rheumatic diseases **(Article 3 – Figure 1)**. This relationship is supported by the hypothesis that haplogroup H may be related to greater oxidative stress, greater cartilage degradation and higher risk of developing rheumatic pathologies (Rego-Pérez *et al*., 2010, 2011; Hudson *et al*., 2014; Soto-Hermida *et al*., 2015). In the present Doctoral Thesis, we observed that haplogroup H was more frequent among the individuals who had bone manifestations characteristic of SpA (81%) than in the rest of the rheumatic pathologies that had been identified in the necropolis of SME **(Article 3 – Figures 3 and 4B)**. This haplogroup is also present with lower frequencies in individuals diagnosed with OA and RA **(Article 3 – Figure 3)**.

Furthermore, in regard to the H sub-haplogroups identified in the SME necropolis (H1, H2, H3, H5, H11, H14, H24), we observed that H2 was the most frequent (36.7%), followed by H1 (21.11%) and H3 (6.67%) **(Article 3 – Table 2)**. The frequency of sub-haplogroup H2 showed marked differences in the individuals with joint manifestations compared to those who did not have these manifestations **(Article 3 – Figure 5)**, which suggests that this haplogroup is strongly related to a higher risk of developing rheumatic diseases. Of note, H2 sub-haplogroup showed a high frequency in the individuals diagnosed with SpA (57%) **(Article 3 – Figure 6)**, which corroborates that sub-haplogroup H2 may increase the risk of developing a SpA.

On the other hand, haplogroup U, the oldest European lineage, (Achilli *et al*., 2005; Soares *et al*., 2010; Secher *et al*., 2014) and the second most frequent in this necropolis (10%) **(Article 3 - Table 1)**, showed a higher frequency in the individuals with joint lesions (66.7%) than in those without joint lesions (33.3%) **(Article 3 – Figure 1)**. This marked

difference in the frequency of haplogroup U between the two groups and the reported relationship of this haplogroup with a higher risk of developing a knee OA (Rego-Pérez *et al*., 2008), allows extending this relationship to other rheumatic pathologies, although it is not possible to establish further explanation, since in the present study we found similar frequencies of U in individuals with SpA, OA and other rheumatic diseases without a specific diagnosis **(Article 3 – Figure 3)**.

Haplogroup T, with a frequency of 6.7% in the necropolis, was greater represented in individuals without bone manifestations (66.7%) **(Article 3 - Table 1)**. This observation is in agreement with a study that describes the protective role of haplogroup T against knee OA (Soto-Hermida *et al*., 2014), and suggests that haplogroup T diminishes the risk of developing rheumatic diseases.

The other mitochondrial haplogroups (K, R, J, HV) were poorly represented in this study, with frequencies below 5%, which does not allow determining their role in the susceptibility of developing rheumatic diseases **(Article 3 - Table 1)**.

The proposed relationships between these mitochondrial haplogroups and their protective or non-protective role in different pathologies are based on the nature of the mitochondrion, in which, by means of the Oxidative Phosphorylation System (OXPHOS), energy is generated to produce heat and thus maintain the body temperature, as well as ROS (Mishmar *et al*., 2003; Ruiz-Pesini *et al*., 2004; Sahin *et al*., 2011). Different studies have suggested that European haplogroups were shaped by selective mechanisms related to low temperatures during the last glacial period, when *Homo sapiens* dispersed over Europe (Ambrose, 1998; Mishmar *et al*., 2003; Wallace *et al*., 2003; Ruiz-Pesini *et al*., 2004). Furthermore, we suggest that the LIA (14^{th}–19^{th} centuries), a climate instability period that coincided chronologically with medieval necropolis of SME (13^{th} – 16^{th} centuries), could be an environmental factor that influenced the selection of some mitochondrial haplogroups, favoring those haplogroups that were more efficient at obtaining energy and heat from the diet to endure lower temperatures and food shortage.

In brief, we could say that haplogroup H is one of the most energy-efficient mitochondrial lineages. Consequently, it produces high oxidative stress, cell damage and cartilage degeneration, promoting the development of rheumatic diseases (Wallace, 1999; Ruiz-Pesini *et al*., 2004; Martínez-Redondo *et al*., 2010; Wallace and Chalkia, 2013). In the medieval population of SME, whose inhabitants lived through the LIA, a period in which energy demands had risen, we observed that the individuals who carried haplogroup H had a significantly higher frequency of rheumatic pathologies, more specifically SpA, a relationship that was also verified for sub-haplogroup H2. Therefore, we suggest that in the medieval population of SME, the LIA may have been a factor that it had an influence in the increase of haplogroup H frequency given its energy advantage. However, this could imply a biological trade-off, the increase of the risk to develop rheumatic diseases.

3. Rheumatic disease and *HLA-B* gene variability

In addition to the relationship between different mitochondrial lineages and SpA, several authors have suggested a link between different nuclear markers and the pathogenesis of this group of rheumatic disease (Brown *et al*., 1996, 1997; Hamersma *et al*., 2001; Burton *et al*., 2007a; Reveille *et al*., 2010; Evans *et al*., 2011; Lin *et al*., 2011; Cortes *et al*., 2013; Ellinghaus *et al*., 2016). The genetic component plays a key role in the immunopathogenesis of SpA, although the primary trigger is still unknown. One of the most significant and reiterated genetic association described in the literature is the one of *HLA-B27* allele and SpA (Brewerton *et al*., 1973; Cafrey and James, 1973; Schlosstein *et al*., 1973; Chatzikyriakidou *et al*., 2011), especially AS, since this allele has been found in almost 90% of patients with AS (Chatzikyriakidou *et al*., 2011). However, in the general population, only 5% of *HLA-B27*$^{+}$ individuals will eventually develop this disease (van der Linden *et al*., 1984b) and even AS can develop in *HLA-B27*$^{-}$ individuals. Furthermore, it has been proposed that other *HLA-B* alleles are associated with the susceptibility to develop SpA, such as alleles *HLA-B07*, *HLA-B13*, *HLA-B40*, *HLA-B47*, *HLA-B51* o *HLA-B57*, among others (Reveille *et al*., 2019).

In regard to the *HLA-B* gene variants discovered in the medieval population of SME, allele *HLA-B40* is the most frequent one (18%). The 90% of the individuals with this allele

had pathological manifestations, although 10% did not have any arthropathy **(Article 2 - Figure 3)**. This allele has been traditionally associated with the development of AS (Robinson *et al*., 1989), however, it was only found in one out of 4 diagnosed cases of AS in the necropolis of SME, finding *HLA-B44* in 2 individuals and *HLA-B27* in one individual **(Article 2 - Table 1)**. Allele *HLA-B40* was also detected in five individuals suffering from some SpA, without an accurate diagnosis based on the morphological criteria **(Article 2 - Table 1)**. These data indicate that the *HLA-B40* allele is the most common allele in individuals diagnosed with SpA, although *HLA-B40* could not be considered as a unique allele of AS, since this allele also appeared in one individual diagnosed with OA **(Article 2 - Table 2)**. On the other hand, allele *HLA-B27* which is associated with SpA and is a genetic marker of AS par excellence (Brewerton *et al*., 1973; Cafrey and Jones, 1973; Schlosstein *et al*., 1973; Chatzikyriakidou *et al*., 2011), showed a much higher frequency among the individuals with pathological manifestations in the necropolis of SME (85.7% *vs.* 14.3%) **(Article 2 - Tables 1 and 2, Figure 3)**. Therefore, we indicate that this allele, traditionally associated with the development of SpA and especially AS, cannot be considered as a unique marker of AS, since in this study was also identified in individuals diagnosed with OA **(Article 2 - Table 1)**. The third most frequent allele in this study was *HLA-B35* which, despite being involved in susceptibility to SpA in *HLA-B27*$^-$ patients (Said-Nahal *et al*., 2000), no differences were observed in the frequencies of *HLA-B35* between the individuals with pathologies and those without pathologies (57% *vs*. 43%) recovered from the necropolis of SME **(Article 2 - Figure 3)**. Furthermore, HLA-B35 was found in individuals with different joint pathologies: two diagnosed with OA, one diagnosed with RA and one diagnosed with SpA, thus it cannot be considered a specific allele of SpA as had traditionally been suggested (Said-Nahal *et al*., 2000) **(Article 2 – Table 1)**. Likewise, among the individuals with alleles *HLA-B44* and *HLA-B07* the same frequency of cases with and without pathologies was found (50% vs 50%) **(Article 2 - Tables 1 and 2, Figure 3)**. Allele *HLA-B44*, which is associated with the development of AS (Purrmann *et al*., 1988) and CD (Orchard *et al*., 2000; Peeters *et al*., 2004; Mahdi, 2015), was detected in 3 individuals with SpA (2 of them with AS), but also in individuals without pathologies. This result indicates that *HLA-B44* is not a specific allele of AS, although it may be associated with the development of CD, which is an inflammatory disease of the intestine and in some cases it causes joint manifestations

like AS. Allele *HLA-B07*, which is believed to reduce the risk of developing AS (Cortes *et al*., 2015), was found in two individuals with OA and in 3 individuals without pathological bone manifestations in the necropolis of SME, whereas it was not identified in any individual with AS, which supports the protecting role of this allele against the risk of developing AS.

The combination of pathological data with that corresponding to the different alleles of the *HLA-B* gene in the medieval population of SME could help establishing a more accurate diagnosis in some individuals with absence of obvious bone manifestations or in individuals with insufficient skeletal remains. In the present study, we can suggest that the presence of *HLA-B* gene alleles is associated with the development of SpA, especially in the case of *HLA-B40* and *HLA-B27* alleles. Thus, the study of these alleles, together with the analysis of other characteristics (rheumatic bone manifestations and age), could facilitate the diagnosis of SpA. The results also demonstrate that, although these alleles appear mainly in individuals with SpA, they cannot be considered exclusive genetic markers of these diseases, since they are also shared by other pathological entities such as OA and RA. However, it is worth highlighting that *HLA-B40* is the most frequent allele in the medieval necropolis among the individuals diagnosed with SpA. Therefore, we can attribute a greater relevance in the pathogenesis of SpA and a certain diagnostic relevance to *HLA-B40* allele, at expense of *HLA-B27*, a classic and dominant genetic marker of this group of rheumatic pathologies (Brewerton *et al*., 1973; Caffrey and James, 1973; Schlosstein *et al*., 1973).

Therefore, it is not possible to establish an arthropathy diagnosis based only on *HLA-B* gene alleles, but it is necessary to reconcile the genetic data with the pathological skeletal manifestations. If the latter are not clear, either due to very mild manifestations or poor preservation remains, it is more difficult to establish a diagnosis. The data obtained about the relationship of the *HLA-B* gene variants with SpA confirm the complexity of the pathogenesis in these immune-mediated pathologies.

4. Genes associated with the susceptibility to develop rheumatic diseases: risk SNPs associated with spondyloarthritis

In addition to the *HLA-B* gene, GWAS studies have revealed a considerable number of genes or gene regions that contribute to the susceptibility to develop SpA and in particular AS (Brown *et al*., 1996, 1997; Hamersma *et al*., 2001; Burton *et al*., 2007a; Reveille *et al*., 2010; Evans *et al*., 2011; Cortes *et al*., 2013; Ellinghaus *et al*., 2016). In the present Doctoral Thesis we analyzed different SNPs associated with the susceptibility to develop SpA (AS: 28 SNPs, PsA: 6 SNPs, IBD: 9 SNPs) using the *BioMark HD* platform of *Fluidigm* (*96.96 Dynamic ArrayTM IFC, Fluidigm*). Given the limitations of aDNA, the genotyping of nuclear SNP in this type of samples may lead to obtain inconclusive partial results. For this reason, the viability of the genotyping technique was evaluated in 62 patients of the University Hospital of Basurto (HUB) (Bilbao, Basque Country, Spain) diagnosed with axial spondyloarthritis (axSpA). Within axSpA, there are patients with classical radiographic changes (AS), whereas other patients do not show radiographic manifestations (non-radiographic axial spondyloarthritis, nr-axSpA), characterized by the presence or absence of structural changes in the SIJs, respectively.

There is an extensive debate in the field to determine whether nr-axSpA is a different form of AS (Wallis *et al*., 2013; Robinson *et al*., 2013), an early form of AS (Huerta-Sil *et al*., 2006; Kiltz *et al*., 2012; Ciurea *et al*., 2013), or whether both are two expressions of the same disease (Rudwaleit *et al*., 2005, 2009c; Baeten *et al*., 2013a). To clarify, we carried out the analysis of a set of risk SNPs localized in genes involved in the pathogenesis of AS, Ps, PsA and IBD in a sample of patients of HUB diagnosed with AS and nr-axSpA, with the aim of determining whether these two entities had a common genetic background. We also we analyzed the clinical and demographic characteristics and several molecular laboratory markers associated with these two groups of patients.

The analysis of a set of 28 risk SNPs located in genes involved in the pathogenesis to develop AS showed that over 75% of the patients of this study shared 60% of the risk alleles **(Article 4 - Figure 1)**. However, no statistically significant differences were found in the frequencies of the risk alleles of these 28 SNPs between the individuals with AS and those with nr-axSpA. These results indicate that both pathological entities have a

common genetic background, at least at the level of risk SNPs. At the genotype level, no statistically significant differences were found between the two groups of patients for risk SNPs located in genes involved in the pathogenesis of AS. Some SNPs showed similar genotypes in most of the individuals of the two groups, e.g., *ERAP1* (rs30187), *ERAP2* (rs10045403), *IL-23R* (rs11209026), *GPR25* (rs41299637), and in intergenic region 2p15 (rs10865331), all of them located outside of the MHC **(Article 4 - Figure 2)**.

The *ERAP1* gene encodes an aminopeptidase that shows a strong association with the susceptibility to develop AS (Saveanu *et al*., 2005; Reveille *et al*., 2010). The SNPs located in the *ERAP1* gene only appear to be associated with AS in *HLA-B27*$^{+}$ and *HLA-B40*$^{+}$ patients (Robinson *et al*., 1989; Cortes *et al*., 2015). On the other hand, *ERAP2* gene encodes another aminopeptidase, whose association with AS has been described in *HLA-B27*$^{+}$ and *HLA-B27*$^{-}$ patients (Cortes *et al*., 2013). The high frequency of risk SNPs of the *ERAP-1* (rs30187) and *ERAP-2* (rs10045403) genes found in the analyzed patients suggest the alteration of the correct functioning of both genes (Reeves *et al*., 2013, 2014). This alteration can affect to the supply of optimum peptides for MHC class I molecules such as *HLA-B27*, which could explain their relationship with the development of SpA, both radiographic (AS) and non-radiographic form (nr-axSpA) (Dangoria *et al*., 2002; Evans *et al*., 2011; Haroon *et al*., 2012; Chen *et al*., 2014; Martín-Esteban *et al*., 2014).

IL-23 is an essential cytokine involved in a key pathway in the pathogenesis of SpA (Burton *et al*., 2007a; Evans *et al*., 2011; Cortes *et al*., 2013; Parkes *et al*., 2013; Gaffen *et al*., 2014). The *IL-23R* gene encodes for IL-23 receptor. There are polymorphisms of *IL-23R*, such as rs11209026, associated with IBD, AS, Ps and PsA (Duerr *et al*., 2006; Burton *et al*., 2007a; Cargill *et al*., 2007; Hüffmeier *et al*., 2009). The A variant of this SNP leads to a nonsynonymous substitution of amino acid residue R381Q, which considerably weakens *IL-23R* function and the production of several proinflammatory cytokines. Besides, R381Q can exert a protective effect against AS inflammation and development (Di Meglio *et al*., 2011). In the present study, the G variant of rs11209026 showed a very high frequency among the patients of two groups (AS and nr-axSpA), thus increasing *IL-23R* function, the risk of inflammation and the risk of developing AS (Di

Meglio *et al*., 2011) **(Article 4 - Figure 2)**. The results confirm the evidence that IL-23 and its entire pathogenic route are involved in the susceptibility to develop axSpA, granting inflammation a main role in the triggering of the disease.

We also identified the association between the SNPs of intergenic region 2p15 (rs10865331) and G-protein coupled receptor 25 (*GPR25*) (rs41299637) with AS (Reveille *et al*., 2010; Cortes *et al*., 2013), although their role in the pathogenesis of AS is unknown. It has been hypothesized that 2p15 region has non-coding RNA species or protein-coding genes unknown to date, which could be involved in the susceptibility to develop AS (Reveille *et al*., 2010). In the case of *GPR25*, this gene is strongly expressed in memory T-cells and NK-cells and involved in the positive regulation of B-cell proliferation (Ricaño-Ponce *et al*., 2016), suggesting a potential mechanism in different autoimmune diseases However, the high prevalence of these SNPs in both groups of patients could suggest a key role in the onset and development of axSpA **(Article 4 - Figure 2)**.

On the other hand, no statistically significant differences for any of the SNPs associated with Ps, PsA and IBD were found between AS and nr-axSpA patients. However, the combination of the risk genotypes of 2 SNPs located in the *NOD2* gene associated with CD (rs27222427, rs2076756), was found mainly in individuals with AS (AS: 30.7% vs nr-axSpA: 7.7%), suggesting that these two SNPs are associated with a greater progression of the disease **(Article 4 - Figures 4 y 5)**. The *NOD2* gene was identified as the most relevant risk factor of CD (Risch, 2000; Hugot *et al*., 2001), although its association with AS had not been described to date. The *NOD2* gene is an intracellular pathogen recognition receptor (Strober *et al*., 2006) and plays a role in the immune response to bacterial lipopolysaccharides. This gene is involved in the regulation of the response of Th17 cells for the elimination of bacteria by inducing the secretion of cytokines IL-23 and IL-1B, which appear in individuals with CD who have a mutation in *NOD2* gene (Philpott *et al*., 2014). The results obtained could suggest that individuals with these two SNPs of the *NOD2* gene will be at greater risk of developing intestinal lesions characteristic of IBD throughout their lives **(Article 4 - Figure 4)**. Furthermore, the presence of these and other SNPs located in genes associated with AS and IBD may confirm the relevance of

intestinal dysbiosis in the genesis of SpA and especially in AS, given the high frequency of these two SNPs in patients diagnosed with this pathology (Costello *et al*., 2015a, 2015b).

After confirming the viability of genotyping of a set of risk SNPs associated with the susceptibility to develop SpA in patients of HUB, we analyzed a sample of 21 individuals recovered from the medieval necropolis of SME and diagnosed with SpA, in order to assess whether the same genetic complexity pattern existed as that observed in patients of HUB. A low hybridization efficiency was obtained from the probes that were designed for the genotyping analysis, which can be explained by the high degree of degradation and fragmentation of aDNA, which prevents the correct hybridization of the probes. Despite these difficulties, some samples showed a correct hybridization rate, which showed the genetic complexity of these rheumatic pathologies. In relation to the 5 most frequent SNPs presented in the HUB patients, SNPs localized in *ERAP1* (rs30187), *IL-23R* (rs11209026) genes and in intergenic region 2p15 (rs10865331), appeared at high frequencies in the individuals diagnosed with SpA of medieval necropolis, whereas the SNPs located in *ERAP2* (rs10045403) and *GPR25* (rs41299637) genes showed partial results. In a similar manner to the study of patients from the HUB diagnosed with axSpA, the results obtained in the genetic analysis of the medieval population of SME confirmed the genetic complexity of SpA and the importance of the *ERAP1* and *IL-23R* genes and the intergenic region 2p15 in the pathogenesis of this group of rheumatic diseases.

In addition to the SNPs described above, we also carried out the analysis of *HLA-B27* allele in 62 patients of the HUB diagnosed with axSpA. This analysis revealed that HLA-B27 allele showed a significantly greater frequency among the patients with AS (87.8%) compared to those with nr-axSpA (38.5%), suggesting a correlation between severe radiographic damage and the presence of the *HLA-B27* allele (Coates *et al*., 2020). Interestingly, the aforementioned data is in line with the results obtained in other studies (Haibel *et al*., 2008; Rudwaleit *et al*., 2009c; Althoff *et al*., 2013; Sieper and van der Heijde, 2013; Kilic *et al*., 2015) **(Article 4 – Table 2)**.

The results obtained for SNPs located in genes (*ERAP1*, *ERAP2*, *IL-23R*, *GPR25*) and gene regions (2p15), along with the different *HLA-B* gene subtypes (*HLA-B27* y *HLA-B40*) demonstrate their importance in the pathogenesis of axSpA, probably with an essential role in the onset of both AS and nr-axSpA. Despite the fact that the individuals who suffer from different axSpA have common genotypes for some SNPs of genes that are important in the pathogenesis of AS, we observed great genetic heterogeneity within each group and between the two pathological entities, which demonstrates the complex nature of this type of diseases **(Article 4 - Figure 3)**. In the SNPs analyzed in the present study, we observed that AS and nr-axSpA have a common genetic background associated with the pathogenic development of these diseases; therefore, from the genetic perspective, it could be hypothesized that AS shares its main pathogenic pathways with nr-axSpA.

Between 20-25% of the known inheritance of AS is attributed to allele *HLA-B27*, and 3-7% to SNPs identified in GWAS studies (Reveille, 2012; Ellinghaus *et al*., 2016). Thus, approximately 70% of the inheritance of this pathology could be related to genetic variants that have not been described to date. Missing heritability is a common issue in complex genetic diseases, and it may be caused by multiple factors. It has also been suggested that general heritability is overestimated, with the probability that epigenetic factors, especially environmental factors, may be more relevant in the susceptibility to develop AS.

Smoking is an important environmental factor in the inflammatory process of rheumatic diseases, including SpA. In the sample of patients of HUB we detected a higher proportion of smokers in the individuals diagnosed with AS (69.4%) with respect to those diagnosed with nr-axSpA (53.9%) **(Article 4 - Table 2)**. This suggests that smoking could contribute to increasing the progression to the radiographic form of SpA, promoting spine damage and an earlier onset of the inflammatory back pain, which characterizes AS (Poddubnyy *et al*., 2013; Sakelariou *et al*., 2015). This, in turn, is correlated with the presence of syndesmophytes, the BASMI index and an earlier diagnosis of the disease in the group of patients of HUB diagnosed with AS **(Article 4 - Table 2)**.

Furthermore, we observed that 61.3% of the patients were obese (BMI>29.99 kg/m^2) or overweight (BMI>24.99 kg/m^2) **(Article 4 - Table 2)**. Excess adipose tissue in overweight and obese individuals may have immunomodulating properties that affect the course of the disease (Gremese *et al*., 2014; Harpsøe *et al*., 2014; Nikiphorou and Fragoulis, 2018) which could be associated with an increase in the production of proinflammatory cytokines (Hauner *et al*., 2005; Versini *et al*., 2014; Vargas *et al*., 2016). Previous studies have reported a greater prevalence of obesity and overweight in AS and PsA patients compared to the healthy population (Bhole *et al*., 2012; Maas *et al*., 2016; López-Medina *et al*., 2017). Besides, AS and PsA patients also show greater functional limitations, greater activity of the disease and a lower response to anti-TNF therapies (Durcan *et al*., 2012; Ottaviani *et al*., 2012; Di Minno *et al*., 2013; Gremese *et al*., 2014; Hojgaard *et al*., 2016; Micheroli *et al*., 2017), which in turn contributes to increasing physical inactivity and, thus, gaining weight (Durcan *et al*., 2012; Maas *et al*., 2016).

The high prevalence of smoking and obesity or overweight among the individuals analyzed in the present study suggest a link between these life habits and the development of these diseases, with inflammation playing a key role in their triggering. In regard to the individuals recovered from the necropolis of SME, we cannot relate these environmental factors to their high prevalence of SpA since we are unaware of their life habits. However, we envision that the unfavorable climate of the LIA and a lifestyle based on physical work would increase the risk of inflammation, joint involvement and the development of rheumatic disease.

Regarding the demographic and clinical characteristics and laboratory markers of patients of HUB, we only found statistically significant differences between the patients with AS and nr-axSpA in the age at diagnosis, the disease duration, the presence of syndesmophytes and the BASMI index **(Article 4 - Table 2)**. These results could be explained because AS can be more aggressive than nr-axSpA, with a faster radiographic progression, which would imply an earlier symptom onset and diagnosis compared to nr-axSpA. Furthermore, the greater frequency of *HLA-B27* in the AS group confirms the link between the presence of *HLA-B27* and a lower onset age typical of AS, together with

an earlier diagnosis and more severe radiographic damage (Feldtkeller *et al*., 2003; Coates *et al*., 2020) **(Article 4 - Table 2)**.

Regarding the gender of the patients, our results confirmed the predominance of males among the individuals diagnosed with AS (77.55%) **(Article 4 - Table 2)**, whereas in nr-axSpA patients the proportion of women (46.15%) was very similar to that of men (53.85%) **(Article 4 - Table 2)**.This observation is in agreement with other studies conducted in European and North American cohorts (Rudwaleit *et al*., 2009c; Ciurea *et al*., 2013; Wallis *et al*., 2013). An interesting explanation for the differential prevalence between the two entities in terms of gender is that women have a lower radiographic damage and a slower progression to a radiographic state, remaining in the non-radiographic form for longer periods of time (Baraliakos *et al*., 2011; Sieper and van der Heijde, 2013; Poddubnyy and Sieper, 2014), due to a possible differential role of hormones in the formation of new bone material in patients with SpA.

VI. Bibliografía

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VII. Conclusiones

1. When performing a differential diagnosis of the different rheumatic diseases in skeletal samples, the most helpful bone manifestations are sacroiliitis and hip enthesitis along with spine lesions. Moreover, it is important to know the symmetry and topography of the lesions to stablish a differential diagnosis between the diseases that encompass the group of spondyloarthritis (ankylosing spondylitis, psoriatic arthritis, reactive arthritis, inflammatory bowel disease associated spondyloarthritis, undifferentiated spondyloarthritis). To achieve a differential diagnosis with osteoarthritis, the age of individuals is an important feature.
2. The development of an effective methodological protocol to analyze nuclear variants in ancient DNA (aDNA) samples allowed to genotype the HLA-B gen allele and a set of risk SNPs associated with rheumatic diseases (multiplexing) in ancient skeletal samples. Furthermore, the aDNA extraction method was optimized according to the provided skeletal sample selected, in order to recover the highest quantity and quality of aDNA. In the case of tooth, *phenol:chloroform* method was the most effective, whereas the silica/DNAzol method was in the case of crushed bone.
3. Regarding the variability of *HLA-B* gene in rheumatic diseases, the alleles *HLA-B40*, *HLA-B27* and *HLA-B35* are the most frequent alleles in the medieval population analyzed, being *HLA-B40* and *HLA-B27* the most prevalent ones in the individuals with pathologies. Although these alleles have been traditionally described as genetic markers associated to SpA, in this study we also detected them in individuals with other rheumatic diseases (osteoarthritis and rheumatoid arthritis), as well as in individuals without pathologies. Consequently, there is not a single *HLA-B* gene allele that is uniquely associated with a specific rheumatic disease.
4. We suggest that genotyping *HLA-B40* and additionally *HLA-B27* alleles will greatly improve the diagnostic of SpA in ancient populations, together with the analysis of several characteristics such as rheumatic bone manifestations and the age.
5. In the San Miguel de Ereñozar population, we suggest that the mitochondrial haplogroup H increased the risk to develop rheumatic pathologies, and more

specifically the risk to develop SpA. This link is supported by the hypothesis that haplogroup H is related to a greater oxidative stress, greater cartilage degradation and a higher risk of developing rheumatic pathologies. Moreover, this connection was also verified for sub-haplogroup H2 due to their higher frequency among individuals diagnosed with SpA in comparison to other H sub-haplogroups.

6. From the evolutionary perspective, the high frequency of haplogroup H among the population of San Miguel de Ereñozar could be explained by a biological adaptation to adverse environmental conditions, such as the ones that took place during the Little Ice Age, since this mitochondrial haplogroup is more energy-efficient and favors survival under these conditions. However, this would imply a biological trade-off, producing greater oxidative stress, which in turn, would increase the risk of developing rheumatic pathologies.
7. The clinical characteristics of the patients of the University Hospital of Basurto (Bilbao, Spain) diagnosed with axial spondyloarthritis (axSpA) analyzed in the present study, suggest that ankylosing spondylitis (AS) is a more aggressive form of axSpA than the variant that does not show radiographic signs (nr-axSpA), because it is diagnosed at an earlier age, the disease lasts longer and shows a lesser degree of mobility of the spine and sacroiliac joints measured by the BASMI index.
8. The genotyping of a set of risk SNPs associated with rheumatic pathologies in patients of the University Hospital of Basurto diagnosed with axSpA, suggested that, from the genetic perspective, AS and nr-axSpA share the same pathogenic pathways. The majority of the patients have common genotypes for some SNPs of genes that are important in the pathogenesis of AS (*ERAP1*, *ERAP2*, *IL-23R*, *GPR25*, 2p15), whose role may influence the onset, development and severity of the disease. However, we found differences between AS patients and those with nr-axSpA in a higher frequency of *HLA-B27* allele among AS patients, which can be related to more severe radiographic damage, and in a higher frequency of two SNPs in *NOD2* gene in patients diagnosed with AS, which can be associated with a higher risk to develop inflammatory bowel disease.

9. The complex genetic nature of SpA may indicate the involvement of exogenous factors that can trigger the disease. In present day populations, the most relevant factors are: infections by pathogens that alter the intestinal microbiota, endocrine alterations such as overweight and obesity, and unhealthy life habits (e.g., smoking). These factors could explain that patients with different genotypes may present the same pathogenic phenotype. From evolutionary perspective, the interactions between microorganisms and humans are defined by the environmental changes and lifestyle and, thus have played a key role in the evolutionary process of human immunity, which would explain both the appearance of autoimmune and inflammatory diseases, such as SpA, and the adaptive evolution of specific genetic variants.

VIII. ANEXO

1. Publicaciones del autor relativas a la presente Tesis Doctoral

Trabajos publicados o en revisión relativos a la presente Tesis Doctoral:

- **Article 1:** Laza, I.M., Hervella, M., de-la-Rúa, C. Genetic markers in a medieval case of ankylosing spondylitis. *The Journal of Rheumatology*. 43(3): 679-681 (2016). Category: Rheumatology; Rank: 13/30 (Q2); Impact Factor: 3.150.

- **Article 2:** Laza, I.M., Ventades, N.G., Hervella, M., de-la-Rúa, C. Contribution of ancient human remains analysis to the understanding of the variability in HLA-B gene variants in relation to the diagnosis of spondyloarthropathies. *Journal of Autoimmunity*. 94: 70-82 (2018). Category: Immunology; Rank: 18/158 (Q1); Impact Factor: 7.543.

- **Article 3:** Laza, I.M., Hervella, M., Neira Zubieta, M., de-la-Rúa, C. Environmental factors modulated ancient mitochondrial DNA variability and the prevalence of rheumatic diseases in the Basque Country. *Scientific Reports*. 9(1): 20380 (2019). Category: Multidisciplinary Sciences; Rank: 15/69 (Q1); Impact Factor: 4.011.

- **Article 4:** Laza, I.M., Hervella, M., Izagirre, N., Blanco Madrigal, J.M., Galíndez, E., Rivera, N.A., Garcia Vivar, M.L., de-la-Rúa, C. Clinical and genetic study of axial spondyloarthritis. *Research Square*. DOI: 10.21203/rs.2.22687/v1 (2020).

Article 1

Genetic markers in a medieval case of ankylosing spondylitis

Genetic Markers in a Medieval Case of Ankylosing Spondylitis

IMANOL MARTÍN LAZA, MONTSERRAT HERVELLA and CONCEPCIÓN DE-LA-RÚA

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Genetic Markers in a Medieval Case of Ankylosing Spondylitis

To the Editor:

Ankylosing spondylitis (AS) is a chronic rheumatic autoimmune disease that affects mainly the sacroiliac joints and spine, where it causes ankylosis through processes of inflammation and ossification. Although the exact accurate etiology of AS is unknown, the disease's development has been shown to be influenced by genetic factors. The HLA-B27 allele is the strongest genetic marker associated with AS[1], but there are other genes both within and outside the MHC that are involved in the development of AS. The most significant laboratory test in the diagnosis of AS involves detecting the presence of the allele HLA-B27, because 90–95% of patients with AS have this allele[2]. Nevertheless, this allele is found in about 10% of the world's population; of them, only 5% ultimately develop this disease[2]. This means that when there are several radiological and clinical symptoms, detecting the allele HLA-B27 is not enough to diagnose the disorder.

The aim of our study was to identify several genetic markers associated

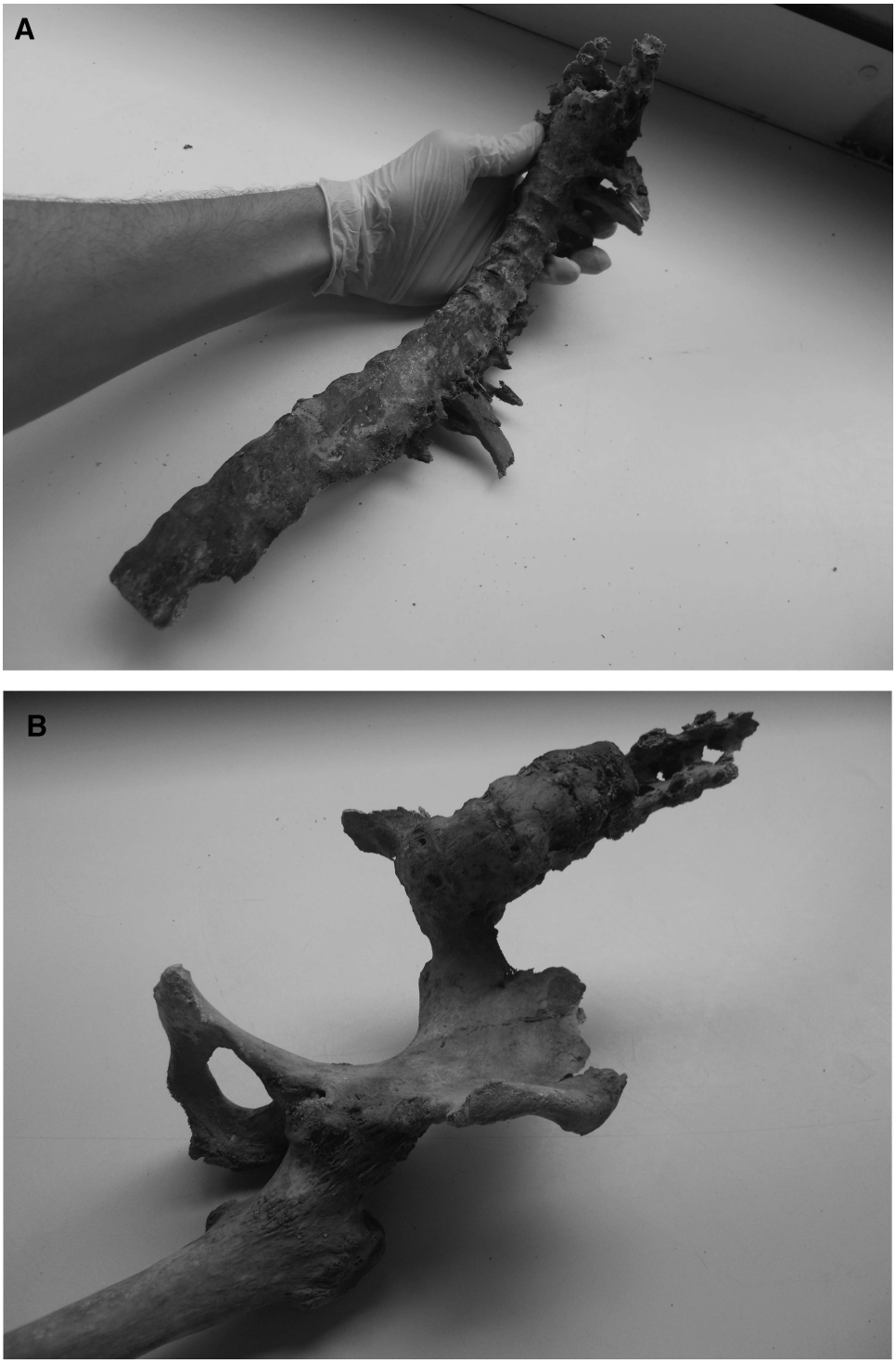

Figure 1. Human bone remains belonging to a medieval individual (16th century) from Santa María in Vitoria-Gasteiz (Basque Country, Spain). The diagnostic bone manifestations of AS are shown: A. Fused bamboo-shaped spinal column. B. Bilateral fusion of the sacroiliac joints and of the lumbosacral region.

with the development of AS [HLA-B27 allele and single–nucleotide polymorphisms (SNP) in interleukin 23R (*IL-23R*) and *ERAP1* genes] in a medieval burial site (16th century Basque Country, Spain) that shows human remains with morphological and radiological manifestations of AS, i.e., a bamboo-shaped spinal column and bilateral fusion of the sacroiliac joints and of the lumbosacral region (Figure 1). It was identified as a female (based on the morphology and the presence of the amelogenine gene). This is the first ancient DNA (aDNA) study, to our knowledge, to address the nuclear markers associated with the development of AS, instead of focusing only on the presence/absence of the HLA-B27 allele[3,4]. The identification of nuclear mutations in an ancient individual with unequivocal signs of having had AS can help to define a set of mutations that contribute to the early diagnosis of this disease.

We extracted DNA from 3 ribs to obtain the sequence of exons 2 and 3 of *HLA-B* gene and determine the SNP in *IL-23R* and *ERAP1* genes by using 10 pairs of primers that we designed (Table 1). The *HLA-B27* gene records a high degree of genetic polymorphism, mainly in exons 2 and 3 of the gene's 8 exons[5]. There are 105 known subtypes encoded by 132 alleles, defined on the basis of the differences in the nucleotide sequence[5]. The present study involved the extraction and analysis of aDNA, while taking the usual precautions in aDNA studies to avoid any possible contamination as negative controls for extraction and polymerase chain reaction amplification. Further authentification criteria are cloning of amplified products (at least 10 clones for each amplified product), analysis in triplicate, and genetic typing of archaeologists who handled these bones, to identify any possible contamination in the sample[6]. On the other hand, the mitochondrial DNA of this individual (revised Cambridge Reference Sequence) was not found in any of the researchers involved in the present study, therefore contamination was avoided[6].

We determined that this individual was homozygous for HLA-B27 allele. This implies a risk of developing AS 3-fold higher than in heterozygous counterparts[7]. The consensus sequence obtained has shown that the individual has the *HLA-B*27:90:01* subtype, for which there are no prior studies; but we may propose a possible link to AS.

The analysis of the SNP within the *IL-23R* gene associated with AS has determined that this individual presented the derived variant (C) rs2201841 and the ancestral variants (G) rs11209026 and rs11209032, which are associated with AS. Further, cloning has been used to confirm that the individual is a homozygote for the 3 SNP. Although the biological effect of these variants in the expression and function of *IL-23R* is currently unknown, it seems clear that the SNP within this gene are major factors in the development of AS[8,9].

The SNP studied within the *ERAP1* gene in this medieval individual has the derived variant (C) rs27044 and the ancestral variants (T) rs30187 and rs2287987. All 3 SNP are homozygous. In the case of the individual studied, who is positive for the HLA-B27 allele, the SNP analyzed could lead to the malfunction of the *ERAP1* gene, which would affect the presentation of antigens by the class I molecules of the MHC such as HLA-B27, thus entailing a greater risk of developing AS[8].

Although there are diverse studies associating different genes with AS, an analysis of several nuclear genetic markers (*HLA-B27* gene and SNP in the *IL-23R* and *ERAP1* genes) in an individual with unmistakable signs of AS has allowed us to confirm the implication of *IL-23R* and *ERAP1* genes in the development of AS. We also propose that the subtype *HLA-B*27:90:01* as a possible genetic marker associated with the disease. Given the lack of clear differential diagnostic criteria for early stages of AS, the determination of a haplotype associated with the disease will contribute to the early diagnosis of AS and other related disorders within the spondyloarthropathies.

IMANOL MARTÍN LAZA, MS; MONTSERRAT HERVELLA, PhD; CONCEPCIÓN DE-LA-RÚA, PhD, Department of Genetics, Physical Anthropology, and Animal Physiology, Faculty of Science and Technology, University of the Basque Country (UPV-EHU), Biscay, Spain. Address correspondence to I. Martín Laza, Department of Genetics, Physical Anthropology, and Animal Physiology, UPV/EHU, Barrio Sarriena s/n 48940 Leioa, Bizkaia, Spain.
E-mail: imanol.martinl@ehu.es

Table 1. Sequence of the primers used for amplifying exons 2 and 3 of the *HLA-B27* gene and SNP in *IL-23R* and *ERAP1* genes, together with the annealing temperature (T) corresponding to each pair of primers and the size of the amplification product obtained.

Primer	Primer Sequence (5′ to 3′)	T (ºC)	Length of Amplification (pb)
HLAB2-1F*	GCC GCG AGT CCG AGA GA (17)	65	117
HLAB2-1R*	GGC CTC GCT CTG GTT GTA (18)		
HLAB2-2F	CCG GAG TAT TGG GAC CG (17)	65	67
HLAB2-2R	GGC CTC GCT CTG GTT GTA G (19)		
HLAB3-2F	GGG CAG GGT CTC ACA CCC TCC (21)	65	61
HLAB3-2R	GAT GTA ATC CTT GCC GTC GTA (21)		
HLAB3-3F	GGA TTA CAT CGC CCT GAA CG (20)	60	92
HLAB3-3R	TCC ACG CAC TCG CCC TCC AGG T (22)		
IL23R-rs2201841-F	GTG ATG ATT TGT GAC AGT AGT A (22)	60	69
IL23R-rs2201841-R	AAG TGC TGG GCT TAC AGG CA (20)		
IL23R-rs11209026-F	CTT TGA TTG GGA TAT TTA AC (20)	55	86
IL23R-rs11209026-R	CAT ATA CAT GTA GTC TAA ATC AG (23)		
IL23R-rs11209032-F	GCT ATC CTG ACA ATT CCT C (19)	53	83
IL23R-rs11209032-R	CTT CAA GCT GAA TTG CA (17)		
ERAP1-rs2287987-F	ATG AGC TTA TAC CTG GTG AGC (21)	60	32
ERAP1-rs2287987-R	AGT CTT CTG CAT CAA GTA AGC (21)		
ERAP1-rs30187-F	GCT CTT GCT TCA TGT GTA CA (20)	62	87
ERAP1-rs30187-R	CCA TGA TGA ACA CTT GGA CAC (21)		
ERAP1-rs27044-F	AGC CTT CTG CCC TCT GTA (18)	58	110
ERAP1-rs27044-R	GCA GAC ATG GAC AGA CGA G (19)		

* Primer from Dominguez, *et al*, Immunogenetics, 1992. SNP: single-nucleotide polymorphism; IL: interleukin.

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Article 2

Contribution of ancient human remains analysis to the understanding of the variability in HLA-B gene variants in relation to the diagnosis of spondyloarthropathies

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Contribution of ancient human remains analysis to the understanding of the variability in *HLA-B* gene variants in relation to the diagnosis of spondyloarthropathies

Imanol M. Laza*, Nerea G. Ventades, Montserrat Hervella, Concepción de-la-Rúa**

Department of Genetics, Physical Anthropology and Animal Physiology, Faculty of Science and Technology, University of the Basque Country (UPV-EHU), Barrio Sarriena s/n, 48940, Leioa, Bizkaia, Spain

A B S T R A C T

Genetic investigations on ancient human remains affected by rheumatological pathologies are a research field of particular interest for identifying the pathogenesis of diseases, especially those having an autoimmune background such as spondyloarthopaties (SpA). Reliable studies concerning this topic require collaboration between multiple disciplines, usually starting from paleopathologic observations up to molecular genetic screening. Here, we focused our investigation in a medieval necropolis in the Basque Country (13th-15th century, N = 163), which presents a high frequency of joint pathologies through two approaches: on the one hand, the analysis of joint manifestations for the differential diagnosis of the SpA and, on the other hand, the determination of the alleles of the *HLA-B* gene. The morphological analysis allowed determining that 30% of the individuals had rheumatic bone manifestations, with SpA being the most frequent (45%). The genetic analysis of individuals with and without pathologies, based on the study of the *HLA-B* gene, allowed finding 17 alleles for this gene, with *HLA-B40*, *HLA-B27* and *HLA-B35* being the most frequent. Although these alleles have been traditionally described as genetic markers associated to the development of SpA, in this study they were also found in individuals with other rheumatic diseases (osteoarthritis and rheumatoid arthritis) and even in individuals without pathologies. These data confirm the complexity of the relationship of the *HLA-B* gene variants with SpA, since it is not possible to establish a diagnosis of SpA with these variants alone. However, we suggest that allele *HLA-B40*, in combination with some specific rheumatic bone manifestations, facilitates the diagnosis of SpA.

1. Introduction

Spondyloarthropathies (SpA) are a group of inflammatory and autoimmune rheumatic diseases that traditionally encompass ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), undifferentiated spondyloarthropathies (uSpA), juvenile spondyloarthropathies (JSpA) and spondyloarthropathies associated with inflammatory bowel disease (IBD) (such as Crohn's disease (CD) and ulcerative colitis (UC)) [1]. This group of diseases share a series of bone manifestations, such as the affectation of the spine and of the sacroiliac joint (sacroiliitis), peripheral arthritis, enthesitis, dactylitis and extra-articular affectations (eyes, skin, intestine and heart), although they also show differential characteristics [2].

Currently, the etiopathogeny of these diseases is still unknown, although it is known that SpA are the consequence of a complex interaction between genetic and environmental factors, which may vary depending on the different forms of SpA [3]. It has been suggested that some genes of the HLA (Human Leukocyte Antigen) system of the MHC (Major Histocompatibility Complex), specifically class I genes (*HLA-A/B/C*) and class II genes (*HLA-DP/DQ/DR*), play a critical role in the autoimmune response of these diseases [4,5].

Ancient populations offer an excellent opportunity to analyse this type of pathologies, since joint manifestations are usually found in advanced states due to the absence of treatment. Furthermore, the development of analytical techniques in the field of ancient DNA (aDNA) makes it possible to analyse different nuclear markers associated with the development of SpA, in addition to allele *HLA-B27*, which up to the present time has been the only one analysed in ancient populations, although only its presence/absence has been described [6,7].

Understanding the pathogenesis of a complex disease as SpA requires a multidisciplinary approach, including pathological examinations of human remains and genetic analysis. Morphological observations in ancient human remains can not always yield a definite diagnosis, because it may happen that some skeletal parts are missing or they are not always intact. A reliable and complete study often needs information from molecular genetic analysis, fundamentally based on genotyping assays, when autoimmune rheumatic diseases associated to the HLA system are involved.

* Corresponding author.
** Corresponding author.
E-mail addresses: Imanol.martinl@ehu.eus (I.M. Laza), nereagv@hotmail.com (N.G. Ventades), montse.hervella@ehu.eus (M. Hervella), conchi.delarua@ehu.eus (C. de-la-Rúa).

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In the present study, an analysis of SpA was carried out at the bone and genetic level with the medieval population of the San Miguel de Ereñozar site (Ereño, Basque Country, Spain; 13th-15th century), in which a high frequency of bone manifestations characteristic of SpA was detected (30%). The bone analysis was focused on the observation of key joint manifestations for the differential diagnosis of the different SpA, which mainly appear in the sacroiliac joint and in the spine.

The genetic study consisted in the analysis of the different alleles of the HLA system which have been suggested to be associated with the development of SpA. One of the most significant and reasserted associations in the literature is that of allele *HLA-B27* [8,9]. Some studies indicate that this allele is present in 75% of Caucasian patients with SpA and increases 39 times the risk of acquiring these diseases compared to patients who do not have this allele [10]. Within the SpA, *HLA-B27* is most strongly associated with AS, although it is also present in other SpA. Is has been reported that almost 90% of patients with AS have the *HLA-B27* allele [9], although only 5% of *HLA-B27*-positive individuals will eventually develop the disease. Moreover, it has been suggested that other alleles of the *HLA-B* system increase the susceptibility to develop SpA, such as alleles *HLA-B07*, *HLA-B13*, *HLA-B40*, *HLA-B47*, *HLA-B51* or *HLA-B57*, among others.

Other risk factors involved in the pathogenesis of SpA are: sex, age, bacterial infections, microbiota imbalance and vitamin D deficiency. Different authors have reported that patients diagnosed with SpA show lower plasma levels of vitamin D than healthy patients [11], which explains why SpA are more prevalent in populations that are closer to the poles, since the incidence of solar irradiance is lower in these regions [12]. These factors, combined with the genetic factors, seem to be the ones responsible for the beginning and development of these diseases.

The aim of this study was to develop a methodological tool that combines joint manifestations and genetic variants to contribute to the diagnosis of these pathologies. This knowledge could help identifying these diseases at early stages, and also facilitate paleopathological diagnosis, which is usually challenged by limitations related to the poor preservation of skeletons.

2. Materials and methods

The present study was based on the analysis of the human bone remains of 163 individuals recovered from the medieval site of San Miguel de Ereñozar, located in the northeast of the province of Bizkaia (Ereño, Basque Country, Spain; 13th-15th century). This site, which coincided chronologically with a cold period known as the Little Ice Age (14th-19th century), offers the possibility to analyse the influence of the environmental pressure posed by the cooling of the planet due to a decrease in solar irradiance [13], which is one of the environmental factors associated with the development of spondyloarthropathies (SpA).

After the analysis of the joint pathologies in this population, two groups were established: 1) individuals with pathological joint manifestations characteristic of SpA (N = 47) and 2) individuals without pathological bone manifestations of SpA (N = 18). Skeletal samples of the two groups were selected for DNA extraction. Whenever possible, dental pieces were preferred over compact bone, since the former have better results in DNA recovery [14]. Only when the individual did not have any dental pieces left, compact bones were selected, preferably ribs as these are the anatomical region with lesser anthropological interest.

2.1. Analysis of spondyloarthropathies: joint manifestations

The morphological analysis of the bone remains of the 163 individuals recovered from the San Miguel de Ereñozar site consisted in the application of a protocol described by Ventades et al. (2018), in order to perform the differential diagnosis of the set of pathologies included in the group of SpA [15]. This analysis was conducted in a hierarchical manner, focusing mainly on the pelvis and the spine.

The affectation of the sacroiliac joint is symmetric in AS, whereas in PsA, ReA and uSpA this affectation is unilateral and asymmetric, and in IBD sacroiliitis can be either symmetric or asymmetric. At the spinal level, AS is characterised by major features: the emergence of symmetric and vertical syndesmophytes, usually located in the torathic and lumbar regions, although they can expand to the whole spine (*bamboo cane*), and ankylosis of the interapophyseal joints [16]. In PsA, the affectation occurs in the cervical and lumbar areas, and the syndesmophytes are asymmetric and vertical in the shape of “skip lesions” [17]. ReA is characterised by spinal injuries that are similar to those of PsA, located mainly in the cervical region and, to a lesser extent, in the torathic and lumbar regions. In uSpA, the lumbar region is the most affected, whereas the lesions developed in IBD are similar to those of AS [16].

Likewise, sex and age were estimated at the morphological level, given the differential prevalence of some SpA according to these two factors. The estimation of age was based on the morphological changes that took place in the auricular surface of the pelvis [18], the acetabular region [19], the pubic symphysis [20] and the ectocranial sutures [21]. The estimation of sex was conducted following the criteria described by White et al. (2011) [22].

2.2. Molecular analysis

2.2.1. Sample treatment prior to DNA extraction

The achievement of reliable genetic results from anthropological remains requires particular strategies to assure material integrity and authenticity of aDNA, to avoid false-positive results due to traces of contaminating modern DNA. The cleaning process before DNA extraction was carried out in a different way depending on the initial bone piece selected (tooth or compact bone). The compact bones were cleaned by abrasion of the external surface, and the dental pieces by irradiation with ultraviolet light and depurination with acids. After the cleaning, the bones were pulverised and the teeth were serrated between the root and the crown to facilitate access to the pulp cavity and the recovery of cellular remains [23].

2.2.2. Ancient DNA extraction

DNA extraction was performed by means of the *phenol:cloroform* method for teeth [24] and the *DNAzol* method for bones [25,26]. The *phenol:cloroform* method begins with the incubation of the tooth in 5 ml of a lysis buffer (0.5 M EDTA; 0.5 mM Tris HCl; SDS 0.5%; 200 µl proteinase K) rotating for two hours at 56 °C. After this incubation, DNA is extracted from the cellular remains using *phenol:cloroform*. The aqueous phase obtained is purified and concentrated by filtration using *Centricon-30* (*C-30 Amicon)* [24]. In the *DNAzol* method, the sample is incubated and stirred for 3 days in the dark with *DNAzol*, which contains guanidine thiocyanate (GuSCN) and the supernatant obtained is concentrated and purified using purification columns with silica base (*PCR Purification Kit* by *QIAgen*) [25,26].

2.2.3. Analysis of the HLA-B gene

The *HLA-B* gene shows a high degree of genetic polymorphism, mainly located in exons 2 and 3 of its eight exons [27–29]. There are hundreds of *HLA-B* alleles, defined according to the differences in the nucleotide sequence [29,30]. In the present study, exons 2 and 3 of the *HLA-B* gene from the analysed individuals were sequenced. To this end, four pairs of primers described by Dominguez et al. (1991) and Laza et al. (2016) were used, in order to obtain overlapping fragments of approximately 100 bp in length and generate the consensus sequence of these two exons, following the protocol described by Laza et al. (2016) [26,31].

The sequences obtained in the analysis were edited using the bioinformatics software *BioEdit* (http://www.mbio.ncsu.edu/BioEdit/

bioedit.html); the reference sequence of the *HLA-B* gene published in the database IPD-IMGT/HLA (http://www.ebi.ac.uk/ipd/imgt/hla/) was used for this purpose [32].

2.3. Authentication criteria

The authentication criteria employed in this work were the usual in aDNA studies [33–36]. To avoid contamination: 1) DNA extraction and the amplification reaction were carried out in a sterile chamber with positive pressure, where samples of modern human DNA have never been handled, 2) the study material and the working surfaces assigned to the analysis of the aDNA were kept sterile, through routine treatment with sodium hypochlorite and ultraviolet irradiation and, 3) appropriate clothing and protective equipment was regularly and compulsory used during the process of aDNA analysis to prevent sample contamination (one-use hat, gloves, mask and coverall).

In addition, controls of the DNA extraction and amplification were performed (extraction blank and negative control of PCR). The number of DNA template molecules recovered in the extracts obtained was quantified by fluorometry (QUBIT), in order to assess the reproducibility of the data.

2.4. Statistical analysis

Several principal components analyses (PCA) were carried out using the statistics software SPSS v24 to interpret the variability of the pathological bone manifestations and the variability of the alleles of the *HLA-B* gene in the analysed individuals. The Fst distance and genetic diversity of the two groups (with pathologies and without pathologies) were calculated from the allele frequencies of the *HLA-B* gene using Arlequin 3.11 [37].

3. Results

A study of spondyloarthropathies (SpA) was conducted in a sample of 163 individuals of the medieval population of San Miguel de Ereñozar (13th-15th century, Basque Country) with two approaches: the analysis of joint bone manifestations and the analysis of the *HLA-B* gene sequence.

3.1. Analysis of the pathological bone manifestations in the medieval population of San Miguel de Ereñozar (13th-15th century, Basque Country) for the diagnosis of spondyloarthropathies

This analysis was carried out in all the individuals recovered from the necropolis of San Miguel de Ereñozar (N = 163), following the diagnosis protocol described by Ventades et al. (2018), which allowed identifying almost 30% individuals (N = 47) with some rheumatic bone manifestation, whereas the rest of the individuals did not show such manifestations [15]. Most of the individuals with rheumatic pathologies had bone manifestations characteristic of SpA (N = 31) and the rest (N = 16) showed rheumatic pathologies different from those of SpA, such as osteoarthritis (OA) and rheumatoid arthritis (RA). It was possible to establish an accurate diagnosis of SpA in those individuals who presented a good state of conservation (N = 21), whereas in the rest of the individuals (N = 10) the poor state of conservation did not allow establishing an initial specific diagnosis within the group of SpA based on the bone manifestations.

The 21 individuals with an accurate diagnosis of SpA (E27, E46, E3009, E3014, E4019, E6015, E5007, E6018, E6017, E3007, E4042, E4052, E86, E6022, E6023, E21, E65, E92, E1003, E4030 and E6004), showed affectation of the torathic-lumbar region of the spine (vertical syndesmophytes and affectation of the vertebral apophyses), sacroiliitis and enthesitis. In 4 of them (E27, E5007, E4042 and E4052), it was possible to specify the diagnosis of ankylosing spondylitis (AS), since they had symmetric and vertical syndesmophytes in the torathic and lumbar region of the spine and symmetric sacroiliitis (with the exception of E4052, whose pelvis could not be recovered). Furthermore, one of them (E5007) had a completely fused spine (*bamboo cane*) and another individual (E4052) had several vertebrae fused, which would be the initial state of the complete fusion of the spine, characteristic of AS. In the other 17 individuals (E46, E3009, E3014, E4019, E6015, E6018, E6017, E3007, E86, E6022, E6023, E21, E65, E92, E1003, E4030 and E6004), both the syndesmophytes and the sacroiliitis were symmetric in some cases and asymmetric in others, which made it difficult to specify the type of SpA.

In the group of individuals who presented rheumatic manifestations different from those of SpA (N = 16), it was possible to establish the diagnosis of OA in 14 individuals and RA in 2 (E1019 and E4009). The subjects with OA (E93, E9, E4038, E4047, E78, E1008, E4036, E6021, E6011, E6020, E4013, E6010, E56 and E4024), shared the presence of horizontal syndemorphytes (with the exception of individuals E93, E9 and E4024, in whom their direction could not be determined), located in the cervical and/or lumbar regions of the spine, and affectation of the apophyseal joints. Some of these individuals also showed sacroiliitis and enthesitis (E4038, E4047, E6021 and E56), and lesions in the metacarpophalangeal joint of the hand (E93, E78, E1008, E6010 and E56), in the metatarsophalangeal joint of the foot (E4038, E78, E1008 and E56), in the distal interphalangeal joint of the foot (E6020, E4013 and E4024) and in the proximal interphalangeal joint of the hand (E6010).

The two individuals with RA (E1019 and E4009), showed affectation of the cervical region and of the apophyseal joints of the spine, absence of sacroiliitis and enthesitis, and affectation of the metacarpophalangeal joint in the hands. One of them (E1019) also had the metatarsophalangeal joint of the foot affected.

A principal component analysis (PCA) was carried out considering the joint manifestations in the individuals who showed pathologies (N = 47), obtaining two first components that account for 50.4% of the variance (Fig. 1). The variables associated with the bone manifestations of hands and feet were not included in the PCA since, due to the small size of these bones, it was only possible to recover them in few individuals of this site. The inclusion of these manifestations would cause a decrease in the multivariate analysis sample size.

The variables with stronger correlation in axis 1 of the PCA (32.2% of the variance) are: sacroiliitis and enthesitis (Sacro. Enthesit. −0.711); horizontal syndesmophytes (Syndesm. Hor. 0.647), syndesmophytes in cervical vertebrae (Syndesm. C. 0.646) and affectation of the vertebral apophyses (Apoph. 0.527). In the second component (18.1% of the variance), the variables with stronger correlation are: unspecific syndesmophytes (Syndesm. Unspe. −0.593), vertical syndesmophytes (Syndesm. Ver. 0.541), symmetric syndesmophytes (Syndesm. Sym. 0.533) and syndesmophytes in torathic and lumbar vertebrae (Syndesm. TL. 0.512) (Fig. 1A). The unspecific syndesmophytes are those in which neither the direction nor the symmetry could be estimated due to their low development or the poor conservation of the bone material.

Fig. 1B shows the PCA distribution of the 47 individuals from the necropolis of San Miguel de Ereñozar who had some pathological joint manifestation. In the first axis of the PCA there are 2 groups considering the variable with the strongest correlation for this axis (Sacro. Enthesit. −0.711); on the one hand, the individuals with sacroiliitis and enthesitis and, on the other hand, the individuals without sacroiliitis and enthesitis. In the group with sacroiliitis and enthesitis, the individuals diagnosed at the morphological level with AS (E27, E5007, E4042 and E4052) appear in one end of the first component. It is worth highlighting the slightly isolated position of E4052, which is due to the fact that the pelvis for this individual was not recovered and thus it was not possible to determine whether or not he/she would have developed sacroiliitis (Fig. 1B). However, E4052 had all the other characteristics of AS, i.e., massive vertical syndesmophytes in the lumbar vertebrae, fusion of torathic vertebrae and affectation of the apophyses, which is

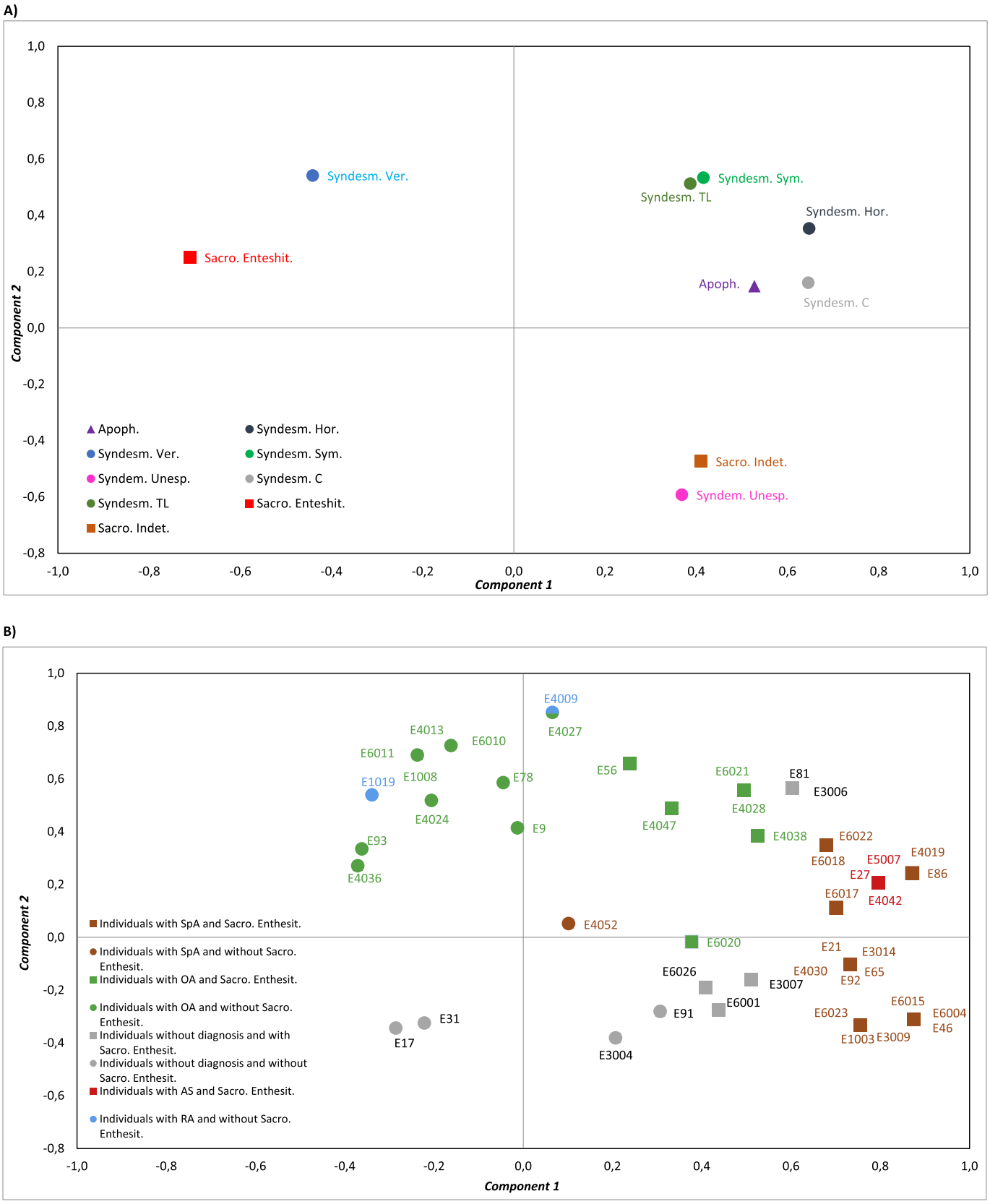


Fig. 1. Principal component analysis of the individuals with pathological bone manifestations (Ereño, Bizkaia, 13th-15th century). A) Distribution of the pathological bone manifestations. B) Distribution of the individuals with pathological features. SpA: spondyloarthropaty; AS: ankylosing spondylitis; OA: osteoarthritis; RA: rheumatoid arthritis; Sacro. Enthesit.: Sacroiliitis and enthesitis; Sacro. Indet.: Indeterminate Sacroiliitis; Syndesm. Hor.: Horizontal Syndesmophytes; Syndesm. Ver.: Vertical Syndesmophytes; Syndesm. Sym.: Symmetric Syndesmophytes; Syndesm. Unspe.: Unspecific Syndesmophytes; Syndesm. C.: Syndesmophytes in Cervical Vertebrae; Syndesm. TL.: Syndesmophytes in Torathic and Lumbar Vertebrae; Apoph: Vertebral apophyses.

Table 1
Alleles of the *HLA-B* gene identified in the individuals who had rheumatic bone manifestations. The table shows the individual code, the *HLA-B* gene allele obtained, the diseases associated with the alleles, and the diseases diagnosed in the present study through the analysis of joint pathologies.

Individual	Allele *HLA-B*	Associated disease	Diagnosed disease Joint pathologies
E27	*B40*	AS [38]	AS
E31	*B40*	AS [38]	–
E46	*B40*	AS [38]	SpA
E93	*B40*	AS [38]	OA
E3009	*B40*	AS [38]	SpA
E3014	*B40*	AS [38]	SpA
E4019	*B40*	AS [38]	SpA
E4027	*B40*	AS [38]	–
E6015	*B40*	AS [38]	SpA
E9	*B27*	SpA, AS [8,9]	OA
E91	*B27*	SpA, AS [8,9]	–
E4038	*B27*	SpA, AS [8,9]	OA
E4047	*B27*	SpA, AS [8,9]	OA
E5007	*B27*	SpA, AS [8,9]	AS
E6018	*B27*	SpA, AS [8,9]	SpA
E78	*B35*	SpA [39]	OA
E1008	*B35*	SpA [39]	OA
E1019	*B35*	SpA [39]	RA
E6017	*B35*	SpA [39]	SpA
E3007	*B44*	CD, AS [40–43]	SpA
E4042	*B44*	CD, AS [40–43]	AS
E4052	*B44*	CD, AS [40–43]	AS
E3006	*B07*	AS [44]	–
E4036	*B07*	AS [44]	OA
E6021	*B07*	AS [44]	OA
E81	*B15*	SpA, uSpA [45–47]	–
E86	*B15*	SpA, uSpA [45–47]	SpA
E6026	*B15*	SpA, uSpA [45–47]	–
E6001	*B58*	IBD [48]	–
E6011	*B58*	IBD [48]	OA
E3004	*B39*	AS, PsA [49,50]	–
E6022	*B55*	–	SpA
E4028	*B14*	AS [50]	–
E6023	*B37*	PsA [51]	SpA
E6020	*B38*	PsA [52]	OA
E4013	*B46*	–	OA
E6010	*B49*	–	OA

AS: ankylosing spondylitis; SpA: spondyloarthropathy; PsA: psoriasic arthritis; ReA: reactive arthritis CD: crohn's disease; IBD: inflammatory bowel disease; uSpA: undifferentiated spondyloarthropathy.

why this individual was included in this group. Next to them, there are 17 individuals who suffered from some type of SpA (E46, E3009, E3014, E4019, E6015, E6018, E6017, E3007, E86, E6022, E6023, E21, E65, E92, E1003, E4030 and E6004), for which it is difficult to specify the type of SpA as they share the characteristics of AS, such as sacroiliitis and enthesitis (Sacro. Enthesit. −0.711), absence of horizontal syndesmophytes (Syndesm. Hor. 0.647), absence of cervical syndesmophytes (Syndesm. C. 0.646) and presence of vertical syndesmophytes (Syndesm. Ver. 0.541), although they had asymmetric syndesmophytes and asymmetric sacroiliitis, which are criteria of differential diagnosis for AS with respect to the rest of the SpA. It was more difficult to specify the type of SpA in individuals E21, E65, E92, E3014 and E4030, from whom the vertebrae could not be recovered and, thus, it was not possible to determine neither the presence/absence of cervical syndesmophytes (Syndesm. C. 0.646) nor the presence/absence of horizontal syndesmophytes (Syndesm. Hor. 0.647), which are variables with a strong correlation for this component (Fig. 1).

In the negative end of axis 1, there are the individuals with neither sacroiliitis nor enthesitis (Sacro. Enthesit. −0.711) (Fig. 1B), which is the variable that defines SpA; therefore, it can be inferred that they had another type of rheumatic disease, such as OA or RA. In addition to the absence of sacroiliitis and enthesitis, these individuals showed cervical syndesmophytes (Syndesm. C. 0.646) and horizontal syndesmophytes (Syndesm. Hor. 0.647), which are two characteristic variables of OA (with the exception of individuals E93, E9, E4047 and E4024, who had very damaged vertebrae, which made it impossible to verify the presence or absence of syndesmophytes).

The rest of the individuals are spread along axis 1 due to the fact that they presented a combination of variables with different correlation values for this axis. It is worth mentioning the two individuals located in the negative side of axes 1 and 2 (E31 and E17); due to the fact that their pelvises could not be recovered, it was not possible to verify the presence of sacroiliitis or enthesitis, thus a diagnosis could not be established.

In the second component of the PCA, which accounts for 18.1% of the variance, no variable was found to have a significant correlation value; the variables with the highest correlation values for this second component of the axis were: unspecific syndesmophytes (Syndesm. Unspe. −0.593), vertical syndesmophytes (Syndesm. Ver. 0.541), symmetric syndesmophytes (Syndesm. Sym. 0.533) and syndesmophytes in the torathic and lumbar regions (Syndesm. TL. 0.512) (Fig. 1A).

The individuals located in the positive end of the second component (E4027, E78, E1008, E1019, E3006, E6021, E81, E6011, E4028, E4013, E6010, E56, E4009 and E4024) share the characteristic of lacking unspecific syndesmophytes (Syndesm. Unspe. −0.593) and vertical syndesmophytes (Syndesm. Ver. 0.541). Moreover, 6 individuals (E78, E1008, E6011, E4013, E56 and E4024) had symmetric syndesmophytes in the torathic and lumbar region of the spine. As an exception, individuals E4027, E3006, E81, E4028 and E4009 lacked syndesmophytes in the entire spine. Except for individuals E81 and E3006, the rest of the individuals had morphological variables that allowed establishing the diagnosis for OA (E4027, E78, E1008, E3006, E6021, E6011, E4028, E4013, E6010, E56 y E4024) and RA (E1019 and E4009).

The rest of the individuals are spread along axis 2 of the PCA, since they did not show a clear pattern regarding the manifestation of the variables with the highest correlation values for this component.

3.2. Analysis of the sequence of HLA-B gene

Given the commonly accepted association of some alleles of the *HLA-B* gene with SpA, exons 2 and 3 of this gene have been analysed in 65 adult individuals recovered from the necropolis of San Miguel de Ereñozar, of which 47 had some type of joint pathology and 18 had no bone lesions. However, in 10 individuals with pathological bone manifestations (E17, E21, E56, E65, E92, E1003, E4009, E4024, E4030 and E6004) no reproducible results were obtained for the *HLA-B* gene allele, due to the poor state of their DNA.

The amplified segments of DNA for the *HLA-B* gene and the comparison of the different nucleotide sequences obtained with the HLA database (http://www.ebi.ac.uk/ipd/imgt/hla/) [32], allowed identifying the different alleles of the *HLA-B* gene for the different individuals analysed in this study (Tables 1 and 2).

Results were obtained from a total of 55 individuals (37 with pathologies and 18 without pathologies), and 17 different alleles of the *HLA-B* gene were detected. In the individuals with bone pathologies, 14 alleles of this gene were identified (Table 1), whereas in those without joint pathologies, 11 alleles were identified (Table 2). The two groups (with and without pathologies) have 8 alleles in common (*HLA-B40*, *HLA-B27*, *HLA-B35*, *HLA-B44*, *HLA-B07*, *HLA-B15*, *HLA-B39* and *HLA-B55*) and 9 alleles are unique to each group, of which 6 alleles were only found in the individuals with pathologies (*HLA-B58*, *HLA-B14*, *HLA-B37*, *HLA-B38*, *HLA-B46* and *HLA-B49*) and 3 in those without pathologies (*HLA-B13*, *HLA-B41* and *HLA-B50*) (Fig. 3).

The genetic diversity of the *HLA-B* alleles is 0.8994 for the individuals with pathologies and 0.8718 for the individuals without pathologies; no statistically significant differences were found between the two groups, despite the fact that the number of alleles, in proportion

Table 2
Alleles of the *HLA-B* gene identified in the individuals who did not have bone pathologies. The table shows the diseases associated with these alleles.

Individual	Allele *HLA-B*	Associated disease
E16	*B40*	AS [38]
E4005	*B27*	SpA, AS [8,9]
E38, E74, E1002	*B35*	SpA [39]
E24, E4001, E4032	*B44*	CD, AS [40–43]
E36, E4016, E6013	*B07*	AS [44]
E1007	*B15*	SpA, uSpA [45–47]
E13, E3005	*B39*	AS, PsA [49,50]
E75	*B55*	–
E3010	*B13*	PsA [44,53]
E6024	*B41*	–
E53	*B50*	–

AS: ankylosing spondylitis; SpA: spondyloarthropathy; PsA: psoriasic arthritis; ReA: reactive arthritis; CD: crohn's disease; IBD: inflammatory bowel disease; uSpA: undifferentiated spondyloarthropathy.

to the sample size, is larger in the group without pathologies (N = 18) than in the group with pathologies (N = 37).

The most frequent allele in this site is *HLA-B40* (18.2%) (Fig. 2), which was found in 10 individuals, 9 of which (90%) had pathological bone manifestations (E27, E31, E46, E93, E3014, E3009, E4019, E4027 and E6015) and only one individual showed no signs of arthropathy (E16) (10%) (Tables 1 and 2, Fig. 3). This allele has been associated with AS [38] (Table 1).

The second most frequent allele, *HLA-B27* (12.73%) (Fig. 2), was detected in 7 individuals, 6 of which (85.7%) had pathological bone lesions (E9, E91, E4038, E4047, E5007 and E6018), and one of them had no signs of bone pathology (E4005) (14.3%) (Tables 1 and 2, Fig. 3). This allele appears in the set of diseases that make up the group of SpA and has been especially associated with the development of AS [8,9] (Table 1).

Allele *HLA-B35* was detected in 7 individuals (12.73%) (Fig. 2), 4 of which (57.14%) had rheumatic bone manifestations (E78, E1008, E1019 and E6017) and 3 individuals (42.86%) showed no manifestations (E38, E74 and E1002) (Tables 1 and 2, Fig. 3). This allele has been associated with the development of SpA [39] (Table 1).

Allele *HLA-B44* was found in 6 individuals (10.91%) (Fig. 2), of which 50% had rheumatic lesions (E3007, E4042 and E4052) and the other 50% had no lesions (E24, E4001 and E4032) (Tables 1 and 2, Fig. 3). Allele *HLA-B44* has been associated with the development of Crohn's disease [40–42] and AS [43] (Table 1).

Allele *HLA-B07* was detected in 6 individuals (10.91%) (Fig. 2), 3 of which had some type of arthropathy (E3006, E4036 and E6021) (50%), and the other 3 had no arthropathies (E36, E4016 and E6013) (50%) (Tables 1 and 2, Fig. 3). This allele been associated with a lower risk of developing AS [44] (Table 1).

Allele *HLA-B15* was detected in 4 individuals (7.27%) (Fig. 2), 3 of which (75%) had some type of bone lesion (E81, E86 and E6026) and one of them (25%) had no pathologies (E1007) (Tables 1 and 2, Fig. 3). This allele has been associated with the development of SpA involving the peripheral skeleton [45,46] and undifferentiated spondyloarthropathies (uSpA) [47] (Table 1).

Allele *HLA-B39* was detected in 3 individuals (5.45%) (Fig. 2), one of which had characteristic manifestations of rheumatic lesions (E3004) (33.3%) and the other two had no manifestations (E13 and E3005) (66.7%) (Tables 1 and 2, Fig. 3). Allele *HLA-B39* has been associated with the development of AS [49] and psoriasic arthritis (PsA) [50] (Table 1).

Alleles *HLA-B55* and *HLA-B58* were found each in two individuals (3.64%) (Fig. 2). *HLA-B55* appeared in an individual with rheumatic bone manifestations (E6022) (50%), and in another without manifestations (E75) (50%) (Tables 1 and 2, Fig. 3). No association has been described between allele *HLA-B55* and the group of diseases analysed in this study. Allele *HLA-B58* was detected in two individuals with bone lesions (E6001 and E6011) (100%) (Table 1). This allele has been associated with the development of inflammatory bowel disease [48]

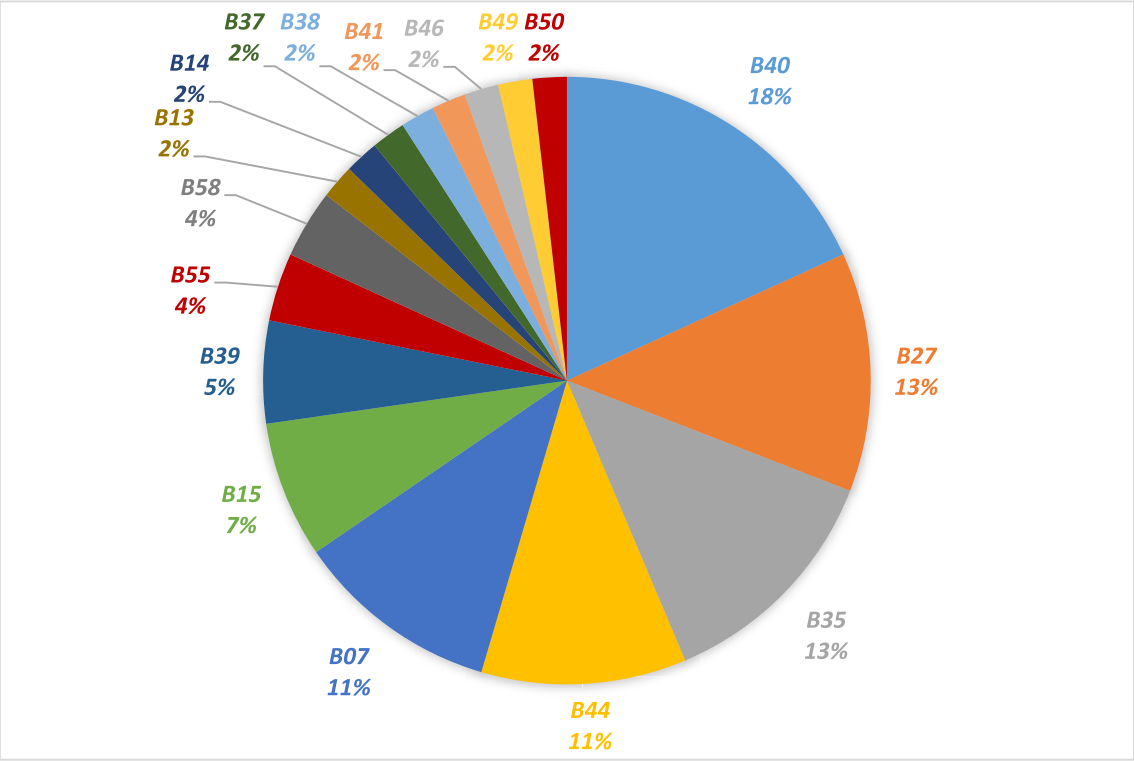


Fig. 2. Frequency of the 17 alleles of the *HLA-B* gene. These alleles were identified in the individuals recovered from the medieval necropolis of San Miguel de Ereñozar.

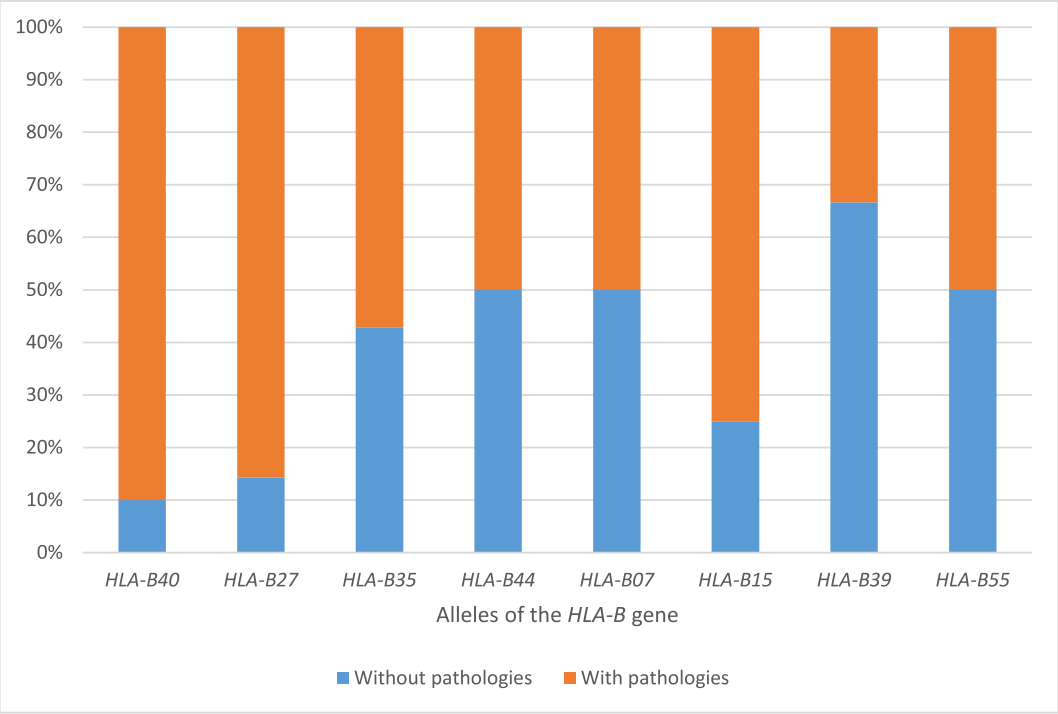


Fig. 3. Frequency of the 8 alleles of the *HLA-B* gene. These alleles were identified in individuals both with and without pathologies recovered from the medieval necropolis of San Miguel de Ereñozar.

(Table 1).

Alleles *HLA-B13*, *HLA-B14*, *HLA-B37*, *HLA-B38*, *HLA-B41*, *HLA-B46*, *HLA-B49* and *HLA-B50* were each found in one individual (1.82%) (Fig. 2). Alleles *HLA-B14*, *HLA-B37*, *HLA-B38*, *HLA-B46* and *HLA-B49* only appeared in individuals with rheumatic joint manifestations, whereas alleles *HLA-B13*, *HLA-B41* and *HLA-B50* were only found in individuals without such manifestations (Table 2, Fig. 2).

Alleles *HLA-B37* and *HLA-B38* have been associated with PsA [51] and allele *HLA-B14* with AS [52] (Table 1). Allele *HLA-B13* has been associated with PsA [53] and AS [44], although in the present study this allele was found in one individual without joint manifestations (E3010) (Table 2). However, despite the fact that no association has been described between alleles *HLA-B41*, *HLA-B46*, *HLA-B49* and *HLA-B50* and the diseases analysed in this study, alleles *HLA-B46* and *HLA-B49* were found in individuals with rheumatic lesions in the necropolis of San Miguel de Ereñozar.

A PCA was conducted considering the different alleles of the *HLA-B* gene identified in 55 individuals recovered from the necropolis of San Miguel de Ereñozar (Bizkaia, Basque Country). The first two components of the PCA account for 32.2% of the variance (Fig. 4). In the positive end of the first component there is a group of 10 individuals (E16, E27, E31, E46, E93, E3009, E3014, E4019, E4027 and E6015), all of them located in the same point since they all have the *HLA-B40* allele, which is the one with the highest frequency in this study (18.2%). In the positive end of the second component there are 7 individuals (E9, E91, E4005, E4038, E4047, E5007 and E6018) who share allele *HLA-B27*, whereas in the negative end there are 7 individuals (E38, E74, E78, E1002, E1008, E1019 and E6017), who share allele *HLA-B35* (Fig. 4). These two alleles have a frequency of 12.73% each.

The rest of the individuals did not show high correlation values that could explain the variance of the PCA. This might be due to the fact that the alleles have low frequency values, or because they are found in individuals with and without pathologies.

3.3. Analysis of the relationship between pathologies and the alleles of the HLA-B gene

In order to relate the pathologies to the alleles of the *HLA-B* gene, a third PCA was performed including all the individuals analysed (Fig. 5). The PCA obtained has a total variance of 36.9% (25.4% for the first component and 11.5% for the second one). This analysis shows that the distribution of the individuals in the PCA varies slightly compared to the PCA conducted with the pathological bone manifestations alone (Fig. 1B), probably due to the low influence of the variations of the *HLA-B* gene alleles.

The variables with the highest correlation values in the first component are: syndesmophytes in the torathic and lumbar regions (Syndesm. TL. 0.830), affectation of the vertebral apophyses (Apoph. 0.685), syndesmophytes in the cervical region (Syndesm. C. 0.650), symmetric syndesmophytes (Syndesm. Sym. 0.641) and horizontal syndesmophytes (Syndesm. Hor. 0.609). In the second component, the variables with the highest correlation values are: vertical syndesmophytes (Syndesm. Ver. 0.762) and sacroiliitis and enthesitis (Sacro. Enthesit. 0.660).

In axis 1 of the PCA there are different groups: on the one hand, the individuals without pathologies and, on the other hand, the individuals with pathologies (Fig. 5B). The individuals without bone pathologies are located around the origin of coordinates in the PCA, due to the fact that they did not show bone manifestations and their variation lies solely in the alleles of the *HLA-B* gene (E13, E16, E24, E36, E38, E53, E74, E75, E1002, E1007, E3005, E3010, E4001, E4005, E4016, E4032, E6013 and E6024) (Table 2). These individuals share 47% of the alleles (*HLA-B07*, *HLA-B15*, *HLA-B27*, *HLA-B35*, *HLA-B39*, *HLA-B40*, *HLA-B44* and *HLA-B55*) with the individuals that had pathological bone manifestations (Table 2, Fig. 3).

With respect to the individuals with pathologies, those diagnosed with SpA are located in the positive end of axis 1 (E27, E46, E3009, E3014, E4019, E6015, E5007, E6018, E6017, E3007, E4042, E4052, E86, E6022, E6023, E21, E65, E92, E1003, E4030 and E6004), since they presented the typical spine manifestations of this group of diseases

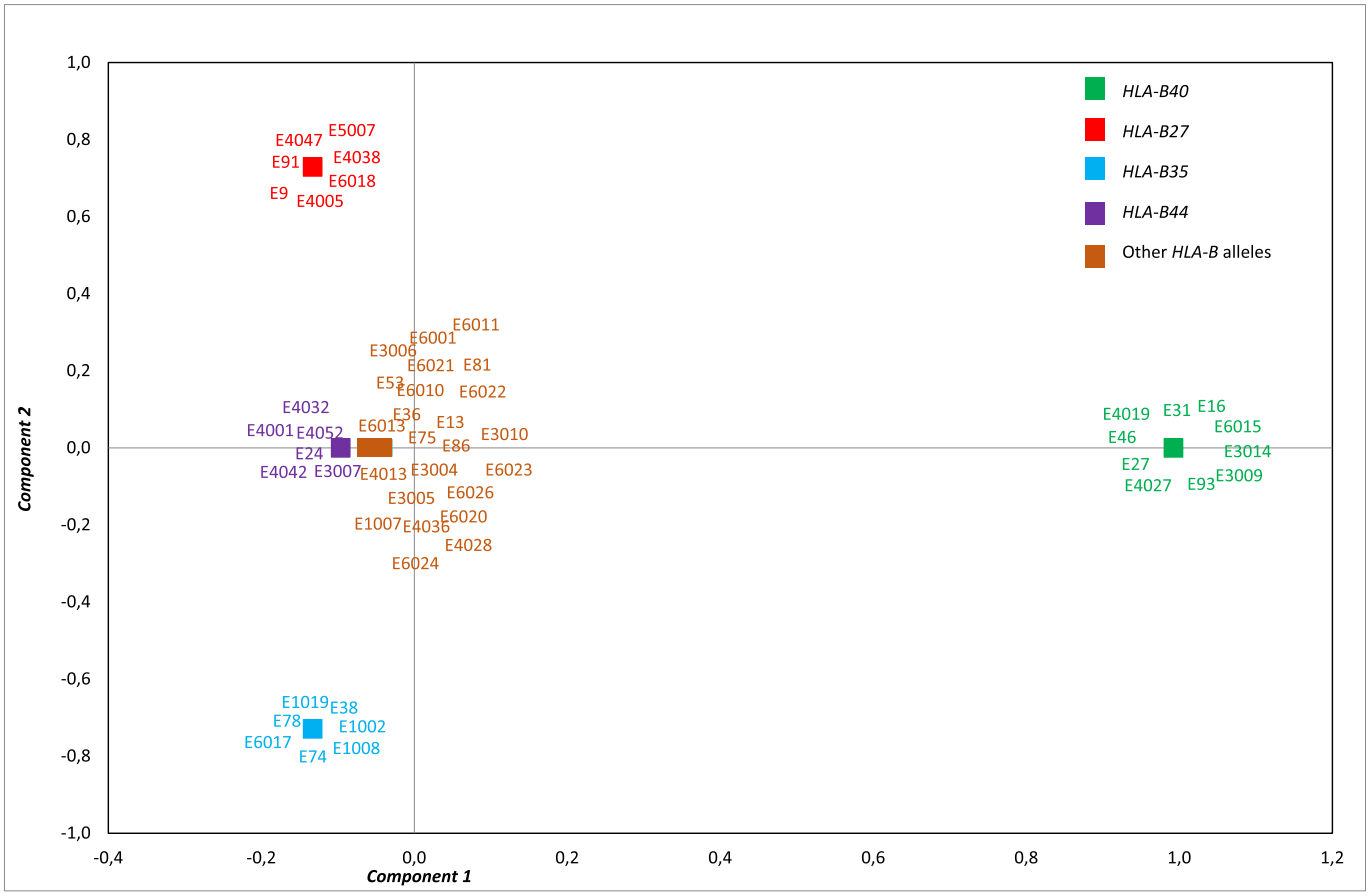


Fig. 4. Principal component analysis of the alleles of the *HLA-B* gene. This principal component analysis shows the distribution of the 55 individuals analysed for the alleles of the *HLA-B* gene. This analysis includes individuals with and without rheumatic bone manifestations (Ereño, Bizkaia, Basque Country, 13th-15th century; N = 55).

(Syndesm. TL. 0.830, Apoph. 0.685); these are also the individuals with the highest correlation value for this axis, with the exception of 5 individuals (E21, E65, E92, E3014 and E4030) who, despite having been diagnosed with SpA, they appear separated since, due to the lack of vertebrae, they did not have such variables. Within this group, there are 4 individuals diagnosed with AS (E27, E5007, E4042 and E4052), as they had symmetric syndesmophytes (Syndesm. Sym. 0.641). These individuals also have the alleles associated with SpA, although with different alleles in each case (*HLA-B40*, *HLA-B27* and *HLA-B44*). In this analysis, subject 4052 is again separated from the group of individuals with SpA as it had no pelvis, although it has allele *HLA-B44*. (Fig. 5B).

The high allele variability of the *HLA-B* gene found in the individuals with SpA is remarkable (E46, E3009, E3014, E4019, E6015, E6018, E6017, E3007, E86, E6022, E6023, E21, E65, E92, E1003, E4030 and E6004). Within these individuals with SpA, 7 different alleles were found in 11 individuals (*HLA-B40*, *HLA-B27*, *HLA-B35*, *HLA-B44*, *HLA-B15*, *HLA-B55* and *HLA-B37*). In the rest of the individuals with SpA (E21, E65, E92, E1003, E4030 and E6004) it was not possible to determine the allele of the *HLA-B* gene. It is worth mentioning that *HLA-B40* is the most frequent allele among these individuals (E46, E3009, E3014, E4019 and E6015), which is an important contribution to the diagnosis of SpA (Fig. 5B).

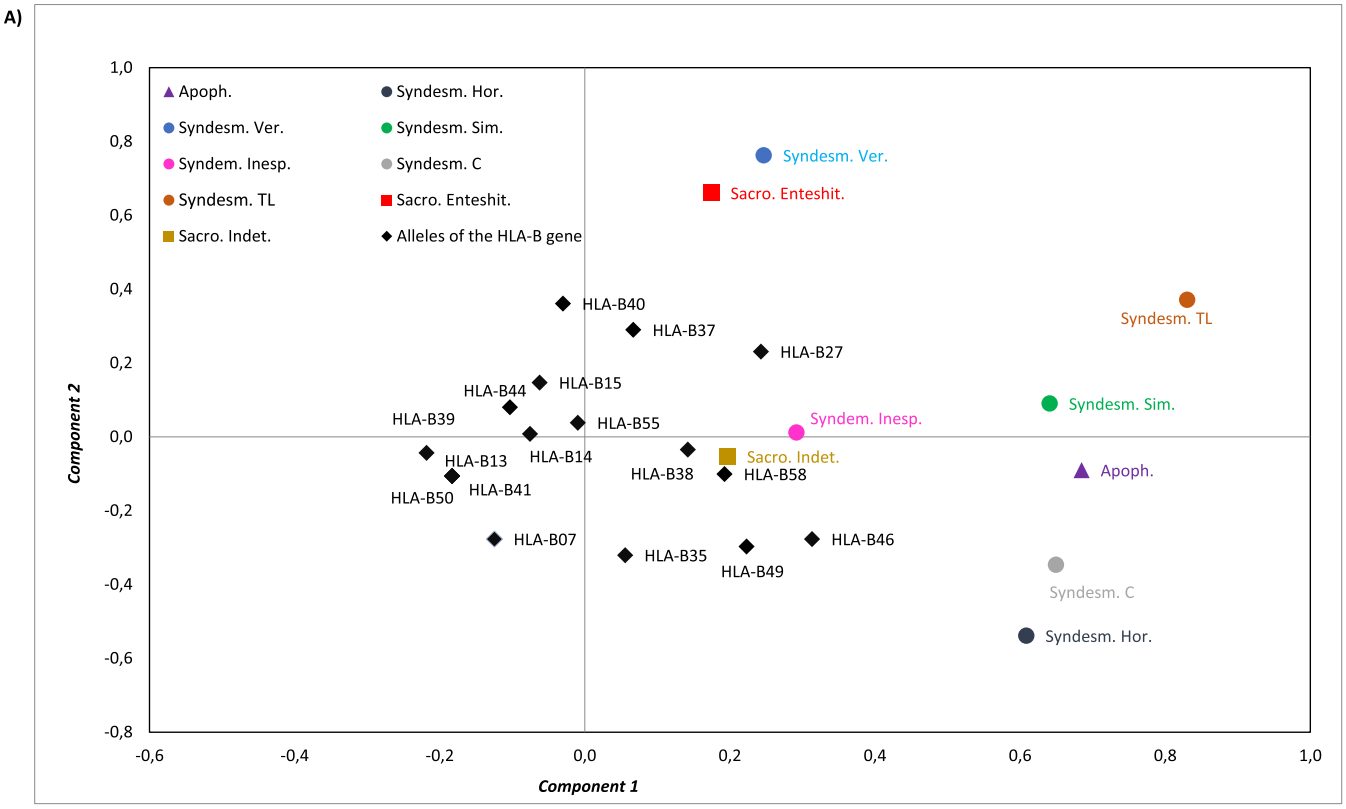
In the second axis of the PCA (11.5% of the variance) the variables with the highest correlation values are: vertical syndesmophytes (Syndesm. Ver. 0.762) and sacroiliitis and enthesitis (Sacro. Enthesit. 0.660). In this axis there is a slight differentiation between individuals with and without sacroiliitis and enthesitis.

4. Discussion

The high frequency of joint pathologies found in the medieval site of San Miguel de Ereñozar (Ereño, Basque Country, Spain, 13th-15th century), and the possibility to extract DNA from these human bone remains, allows to perform a diagnosis of spondyloarthropathies (SpA) with two approaches: on the one hand, the analysis of key bone manifestations for the differential diagnosis of SpA and, on the other hand, the determination of those *HLA-B* gene alleles that have been traditionally associated with rheumatic diseases. Furthermore, the chronology of the site is in the period known as the Little Ice Age, a cold period that was caused by a decrease of solar irradiation, which could have been one of the environmental factors involved in the development of SpA in this population.

Arthropathies were found in 30% of the individuals (N = 47) recovered in this site (N = 163). The most frequent rheumatic pathologies were SpA (45%), within which 4 cases of ankylosing spondylitis (AS) were found through the analysis of bone manifestations (Fig. 1B). Different rheumatic pathologies other than SpA were also found, such as osteoarthritis (OA) and rheumatoid arthritis (RA). The prevalence of SpA in the population studied is very high (12.9%), with respect to the results obtained in epidemiological studies of current populations, such as populations of North America (6.2%) [54], and archaeological populations of South America (7% in Chile) [55], which are also subjected to adverse environmental factors.

The results obtained through the analysis of the pathological manifestations in the individuals of the site of San Miguel de Ereñozar show the importance of sacroiliitis and enthesitis for the diagnosis of SpA (Fig. 1A and B). The affectation of the sacroiliac joint is a characteristic



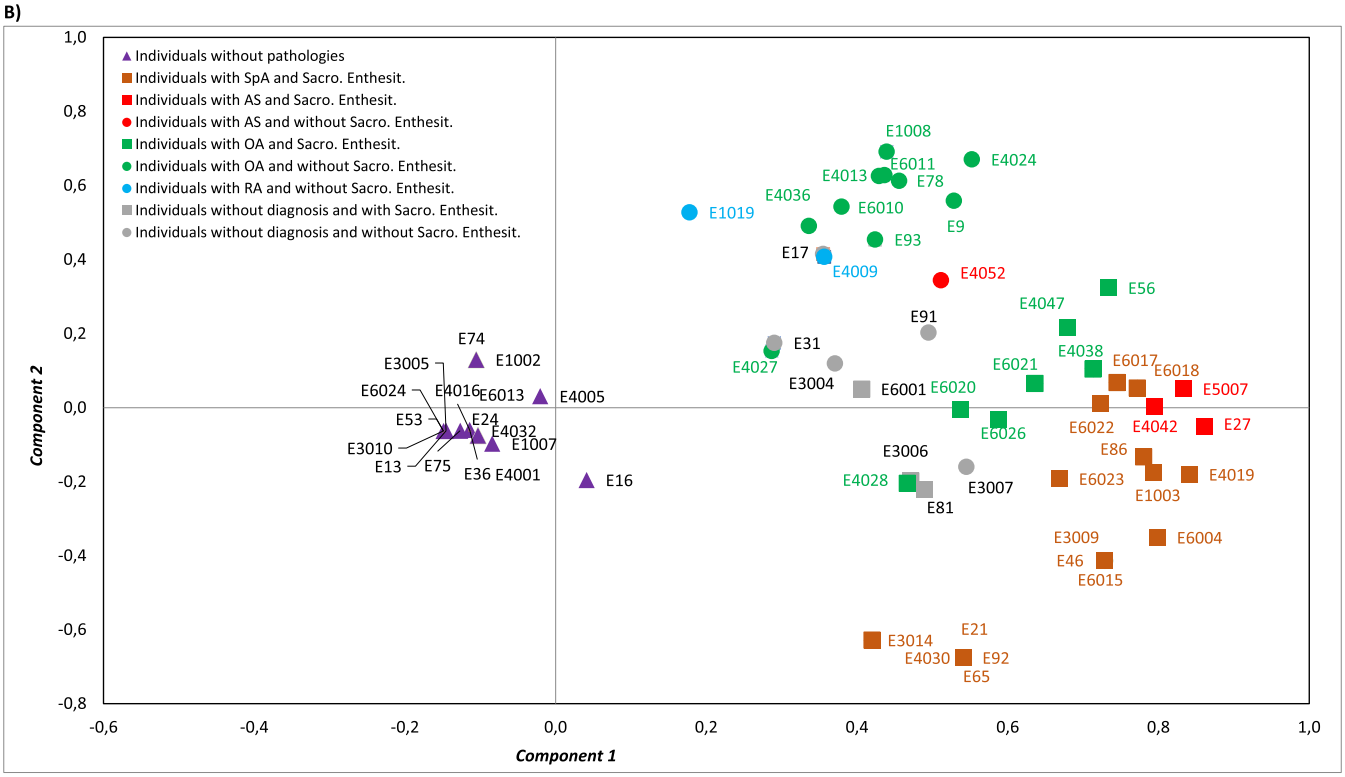


Fig. 5. Principal component analysis of the individuals recovered from the medieval necropolis of San Miguel de Ereñozar. 47 individuals had joint pathologies and 18 individuals did not show joint pathologies (Ereño, Bizkaia, Basque Country, 13th-15th century, N = 65). A) Distribution of the pathological manifestations of SpA and of the alleles of the *HLA-B* gene. B) Distribution of the 65 individuals with and without pathologies. SpA: spondyloarthropaty; AS: ankylosing spondylitis; OA: osteoarthritis; RA: rheumatoid arthritis; Sacro. Enthesit.: Sacroiliitis and enthesitis; Sacro. Indet.: Indeterminate Sacroiliitis; Syndesm. Hor.: Horizontal Syndesmophytes; Syndesm. Ver.: Vertical Syndesmophytes; Syndesm. Sym.: Symmetric Syndesmophytes; Syndesm. Unspe.: Unspecific Syndesmophytes; Syndesm. C.: Syndesmophytes in Cervical Vertebrae; Syndesm. TL.: Syndesmophytes in Torathic and Lumbar Vertebrae; Apoph: Vertebral apophyses.

feature of SpA, although not only of this group of diseases, since in the analysed sample it was also found in the individuals diagnosed with OA (Fig. 1). The age of the individuals may help discriminate between these two entities (SpA and OA), since in OA the affectation of the sacroiliac joint appears in more advanced stages of life (> 40 years). On the other hand, sacroiliitis and enthesitis may contribute to the differentiation of different types of SpA, with these being symmetric in the case of AS and SpA associated with inflammatory bowel disease, and asymmetric in psoriasic arthritis (PsA) and reactive arthritis (ReA).

Despite the great importance of the sacroiliac joint affectation in the diagnosis of SpA, it is necessary to identify other joint lesions in other regions of the skeleton to differentiate the different types of SpA, with spine lesions being crucial, where the location, symmetry and direction of the lesions must be considered. In AS, syndesmophytes are symmetric and vertical, and they usually affect the torathic and lumbar regions; there are severe cases in which the entire spine is affected (bamboo cane) [16]. In PsA and ReA, syndesmophytes are asymmetric, vertical and skip, and they mainly affect the cervical region [17]. Inflammatory bowel disease (IBD) usually causes spine lesions similar to those in AS, and undifferentiated spondyloarthropathies (uSpA) mainly affects the lumbar region [16].

With regard to the *HLA-B* gene variants, allele *HLA-B40* is the most frequent one in this population (18%) (Fig. 2). Ninety percent of the individuals with allele had pathological manifestations, although 10% did not have any arthropathy (Fig. 3). Despite the traditional association of this allele with the development of AS [38], in this study it was only found in one (E27) of 4 diagnosed cases of AS, since in the other 3 cases of AS (E4042, E4052 and E5007) alleles *HLA-B44* and *HLA-B27* were found (Table 1). Allele *HLA-B40* was also found in 5 individuals diagnosed with SpA, in which a more accurate diagnosis could not be established based on the morphological criteria (Table 1). Moreover, this allele appeared in one individual diagnosed with OA (E93) and in one individual without bone manifestations (E26), thus *HLA-B40* could not be considered as a unique allele of AS; however, it is the most frequent allele in individuals with SpA in this study (Table 2). On the other hand, allele *HLA-B27*, which is associated with SpA and a genetic marker of AS par excellence [8,9], is present in this study in individuals with and without pathologies (85.7% vs. 14.3%), including individuals with SpA and OA (Tables 1 and 2, Fig. 3). Therefore, this allele cannot be considered as a marker of AS (Table 1). The third most frequent allele in this study was *HLA-B35*, which despite being involved in susceptibility to SpA in negative patients for allele *HLA-B27* [39], in the present study no differences were observed in the frequencies of *HLA-B35* between the individuals with pathologies and those without pathologies (57% vs 43%) (Fig. 3). Furthermore, *HLA-B35* was found in individuals with different joint pathologies: two diagnosed with OA (E4038 and E4047), one diagnosed with RA (E1019) and one diagnosed with SpA (E6018), thus it cannot be considered a specific allele of SpA as traditionally [39] (Table 1). Likewise, among the individuals with alleles *HLA-B44* and *HLA-B07* the same frequency of cases with and without pathologies was found (50% vs 50%) (Tables 1 and 2, Fig. 3). Allele *HLA-B44*, which is associated with the development of AS and Crohn's disease (CD) [40–43], was found in two individuals with AS (E4042 and E4052) and in one individual with SpA (E3007), but also in individuals without pathologies, which indicates that it is not a specific allele of AS, although it may be associated with the development of CD, which is an inflammatory disease of the intestine and in some cases it causes joint manifestations like AS. Allele *HLA-B07*, which is believed to reduce the risk of developing AS [44], was found in two individuals with OA (E4036 and E6021) and in 3 individuals without pathological bone manifestations, whereas it was not found in any individual with AS, which supports the protecting role of this allele against the risk of developing AS. Allele *HLA-B15*, despite its low frequency in the population analysed (7%) (Fig. 2), was mainly found in individuals with pathological bone manifestations (75% vs 25%); however, it cannot be considered a specific allele of SpA, since it also appeared in individuals without pathologies [45–47].

Considering the pathological and molecular data of the population analysed, some interesting combinations were found. Among the 21 individuals from the site of San Miguel de Ereñozar who were diagnosed with SpA, 14 individuals were found to also have a variant of the *HLA-B* gene that has been suggested to be associated with SpA (E27, E46, E3009, E3014, E4019, E6015, E5007, E6018, E6017, E3007, E4042, E4052, E86 and E6023), which could help establishing a more accurate diagnosis combining the morphological and molecular data.

Individuals E46, E3009, E3014, E4019 and E6015, diagnosed at the morphological level with SpA, had allele *HLA-B40*, which has been associated with the development of AS, increasing the risk of this pathology in 1.5× [38]. Taking into account that this is the most frequent allele in this study among the individuals with pathologies (Fig. 3), the authors believe that in some cases (E3014, E4019 and E6015) the diagnosis can be specified and suggest the presence of AS. Individuals E4019 and E6015 may have had AS in an early stage of development, since they were young individuals, which would explain the presence of asymmetric syndesmophytes in the torathic and lumbar regions. Moreover, individual E4019 lacks some important bone remains for the diagnosis, such as the left coxal bone, which made it impossible to verify the presence of symmetric sacroiliitis, whereas individual E6015 showed symmetric sacroiliitis. Likewise, individual E3014 showed symmetric sacroiliitis characteristic of AS, which could not be verified at the morphological level, as the spine could not be recovered, although the presence of allele *HLA-B40* allows suggesting a diagnosis for AS. However, individuals E46 and E3009, who had typical features of a SpA in early stages, died at an old age (40–60 years). In these cases, the authors believe that the presence of *HLA-B40* does not justify the diagnosis of AS, whose onset takes place at earlier stages (20–30 years). Considering all of the above mentioned, it can be suggested that the presence of allele *HLA-B40*, in combination with other characteristics (arthropathic manifestations and age), can help specify the diagnosis of AS. Although it cannot be considered an exclusive marker of AS, it is the most important allele in the diagnosis of this pathology.

Allele *HLA-B27*, suggested to be associated with SpA [8,9], appeared in 6 of the individuals with pathologies. Individuals E5007 and E6018, diagnosed with AS and SpA, respectively, based on the pathological manifestations, were carriers of this allele. In the case of individual E5007, the diagnosis of AS is supported by the pathological manifestations and the genetic variant, which are characteristic of this disease. Individual E6018 was a mature adult (45–60 years), who had the typical morphological features of a SpA, and allele *HLA-B27* as well. This individual showed sacroiliitis, which is a characteristic of AS, PsA and those SpA associated with inflammatory disease. However, the spine of E6018 presented asymmetric syndesmophytes, which are typical of PsA and ReA. Moreover, the distal interphalangeal joint of the hand of this individual was affected, which is a characteristic feature of PsA. Based on this, the authors suggest that individual E6018 developed PsA. These data, along with the fact that *HLA-B27* was present in 3 individuals with OA and in 14% of the individuals without pathologies, demonstrate the lack of specificity of this allele for the diagnosis of AS, despite the importance given to the susceptibility to develop this disease.

Allele *HLA-B35* has been associated to the development of SpA [39]. In this study, only one individual was found to have SpA and this allele (E6017). This individual had symmetric sacroiliitis, enthesitis, affected apophyseal joints and asymmetric syndesmophytes of the lumbar vertebrae; however, it is neither AS, since the lesions of the spine are asymmetric, nor PsA, as the joints of the hands are not affected. In this case, the molecular analysis did not allow a more accurate characterization within the SpA, since allele *HLA-B35* was found also in two individuals with OA, in one individual with RA and in 42.86% of the individuals without pathologies.

Allele *HLA-B44* was found in individuals E4042 and E4052, both diagnosed with AS, and in individual E3007, diagnosed with SpA. This

allele may favour the development of AS [43] and is also associated with CD [40–42]. Both individuals (E4042 and E4052) had typical bone manifestations of AS, with the exception of sacroiliitis for individual E4052, whose pelvis could not be recovered, although the torathic vertebrae were fused, which allows inferring that he/she developed AS. Individual E3007 had symmetric sacroiliitis, characteristic of AS, although the spine lesion are very mild. This combination of skeletal manifestations, along with the presence of allele *HLA-B44*, could suggest the existence of an intestinal pathology accompanied by joint manifestations of AS. Despite of being an inflammatory disease of the intestine, CD belong to the group of SpA, since CD patients may show a peripheral (type I or II arthritis) or axial arthropathy (isolated sacroiliitis or AS) [16]. There seems to exist a similar common cause for AS and CD, since 5–10% of patients diagnosed with AS have CD, and there is up to 50–60% of patients with AS who develop intestinal lesions similar to CD [56]. The data found in the present study verify the complexity of the association of this allele with SpA and intestinal pathologies. In some cases the rheumatic manifestations are accompanied by some intestinal disease, although it is not possible to discard that some of the individuals without arthropathic manifestations could have developed an intestinal pathology. Therefore, the presence of allele *HLA-B44* could indicate the presence of a disease with not only arthropathic but also intestinal manifestations.

Allele *HLA-B15*, associated with the development of SpA, and more specifically in patients with ReA and uSpA, has a frequency of 7% in this population and is present in individual E86, who was diagnosed with SpA. Due to the presence of symmetric sacroiliitis, it is very improbable that this individual developed ReA, since sacroiliitis is asymmetric in this disease. Regarding uSpA, these make up a group of diseases with common clinical features that prevent them from being classified as a defined disease within the group of SpA. In some case, it may be an early stage of a SpA that could show more symptoms after development, which would allow to frame it within a specific clinical entity, although in other cases they do not develop the complete clinical profile. Individual E86 was a young individual (20–35 years) with symmetric sacroiliitis, enthesitis and vertical and asymmetric syndesmophytes in the lumbar region, thus he/she could have suffered from an uSpA since the age and clinical manifestations are in line with such pathological entity.

With respect to allele *HLA-B37*, associated with PsA [52], in this study it was only found in one individual (E6023), who showed symmetric sacroiliitis and vertical and symmetric syndesmophytes in torathic vertebrae, which are more characteristic of AS than of PsA, due to the symmetry of the lesions of the axial skeleton. It could be a case of AS, although the allele of the *HLA-B* gene in this individual has not been associated with this disease. Likewise, allele *HLA-B*55 has not been associated with SpA and yet it was found in individual E6022, who had bone manifestations of SpA. These data confirm the complexity of the relationship of the *HLA-B* gene variants with SpA.

Allele *HLA-B07*, with a frequency of 11% in this population, and associated with a decreased risk of developing AS [44], was found in 3 individuals with pathologies and 3 without pathologies. Of the 3 individuals with pathologies, 2 had OA (E4036 and E6021) and one (E3006) could not be diagnosed. These results, despite having little significance, do not contradict the association described between allele *HLA-B07* and AS, although it may be present in individuals with different rheumatic diseases other than AS.

On the other hand, there were 12 individuals with rheumatic bone manifestations typical of OA (N = 11) and RA (N = 1), who had those *HLA-B* gene alleles traditionally associated with the development of SpA (*HLA-B40*, *HLA-B27* and *HLA-B35*) (Table 1). These results demonstrate that, although these alleles are mainly in individuals with SpA, they are also shared by other pathological entities such as OA and RA. It is worth highlighting that *HLA-B40*, the most frequent allele in individuals with SpA in this study, only appears in one individual with OA. Therefore, it is not possible to establish a diagnosis of arthropathies based mainly on the alleles of the *HLA-B* gene; it is necessary to reconcile the genetic data with the skeletal manifestations. If the latter are not clear, either due to very mild manifestations or the lack of enough remains, it is more difficult to establish a diagnosis.

Finally, in the case of the individuals without pathologies who had some allele of the *HLA-B* gene associated with SpA (Table 2), it is worth pointing out that the frequency of alleles *HLA-B40* and *HLA-B27* in individuals without pathologies was very (Fig. 3), especially that of *HLA-B40*, which had a frequency of 90% in individuals with pathologies and 10% in individuals without pathologies.

5. Conclusions

Rheumatological paleopathology is a research field that can provide a strong contribution to clarifying pathogenesis of numerous diseases, especially when anthropological studies disclose a high frequency of pathological conditions characteristics of the group of inflammatory and autoimmune diseases as spondyloarthropathies (SpA). However, very few anthropological studies that establish some type of association between environmental and genetic factors and the prevalence of SpA. The methodological advances in the field of ancient DNA allow to analise and genotype nuclear markers of populations from the past; studies in this field were limited to the analysis of the presence/absence of allele *HLA-B27*, traditionally associated with ankylosing spondylitis (AS). The results obtained in the skeletal and molecular analysis of a medieval population of the Basque Country made it possible to determine the different alleles of this gene by means of sequencing, which, along with the usual severity of the lesions shown in these populations, contributes to the establishment of a more accurate diagnosis.

At the morphological level, sacroiliitis and enthesitis, along with spine lesions, are the most helpful bone manifestations when performing a differential diagnosis among the different arthropathies. Moreover, it is important to know the symmetry and topography of the lesions, as well as the age of the individuals, in order to reach a differential diagnosis. At the molecular level, a great variability of *HLA-B* gene alleles was found, some of which had a significantly higher frequency in the individuals with bone pathologies, and even some alleles were shared by different pathological entities. These results indicate that there is not a single *HLA-B* gene allele that is uniquely associated with one rheumatic disease; however, we can attribute a diagnostic character to *HLA-B40*, given its high frequency in the individuals with SpA.

The alleles *HLA-B40*, *HLA-B27* and *HLA-B35*, traditionally described as genetic markers associated with the development of SpA, are the most frequent alleles in the population analysed, with *HLA-B40* and *HLA-B27* being the most prevalent ones in the individuals with pathologies, unlike *HLA-B35*, which did not show differences in frequency between the individuals with pathologies and those without pathologies. Allele *HLA-B40*, associated with the development of AS, although it may not be enough on its own, it can facilitate the differential diagnosis of AS within SpA when considered along with some pathological bone manifestations (sacroiliitis and spinal lesions). Allele *HLA-B27*, traditionally associated with the development of SpA, especially AS, was not determinant for the differential diagnosis of this pathology in this population, although it can contribute to its diagnosis in combination with a series of pathological bone manifestations. Unlike these two alleles, *HLA-B35*, associated with the development of SpA, did not allow discriminating in the differential diagnosis of these pathologies, although some alleles of the *HLA-B* gene identified in the individuals with SpA are shared by other pathological entities found in this study, such as osteoarthritis and rheumatoid arthritis. Nevertheless, bone manifestations along with other epidemiological characteristics of these pathologies allow in some cases a diagnostic differentiation with SpA. It is worth highlighting that *HLA-B40* is the most frequent allele in this study among the individuals with SpA, appearing only in one individual with OA and in one individual without pathologies, which

makes it possible to attribute a diagnostic character to it for SpA.

A wide range of genetic and environmental factors influence the development of SpA, thus these diseases are heterogeneous and complex entities. Despite the strong association proposed between SpA and the *HLA-B* gene, the genetic weight of these diseases lies in different pathogenic routes in which a large variety of genes are involved, whose deficient functioning causes an immune response against the person's own organism, triggering this type of autoimmune pathologies. In view of the results of the present study, the authors suggest that allele *HLA-B40*, along with other SNPs, such as rs30187 from the *ERAP1* gene [44], could be a means of analysis to determine the diagnosis of SpA. The future of this research is aimed at the joint study of a larger number of genetic markers, taking into account the results of the GWAS studies (Genome-wide association study), which, along with the results obtained in this study, will help to better understand the nature of these diseases.

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Article 3

Environmental factors modulated ancient mitochondrial DNA variability and the prevalence of rheumatic diseases in the Basque Country

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Environmental factors modulated ancient mitochondrial DNA variability and the prevalence of rheumatic diseases in the Basque Country

I. M. Laza[1*], M. Hervella[1], M. Neira Zubieta[2] & C. de-la-Rúa[1]

Among the factors that would explain the distribution of mitochondrial lineages in Europe, climate and diseases may have played an important role. A possible explanation lies in the nature of the mitochondrion, in which the energy generation process produces reactive oxygen species that may influence the development of different diseases. The present study is focused on the medieval necropolis of San Miguel de Ereñozar (13th–16th centuries, Basque Country), whose inhabitants presented a high prevalence of rheumatic diseases and lived during the Little Ice Age (LIA). Our results indicate a close relationship between rheumatic diseases and mitochondrial haplogroup H, and specifically between spondyloarthropathies and sub-haplogroup H2. One possible explanation may be the climate change that took place in the LIA that favoured those haplogroups that were more energy-efficient, such as haplogroup H, to endure lower temperatures and food shortage. However, it had a biological trade-off: the increased risk of developing rheumatic diseases.

Various pieces of evidence suggest that mitochondrial dysfunction could influence the pathogeny of some human diseases, including neurodegenerative disorders[1], metabolic diseases[2], rheumatic pathologies[3,4], processes associated to age[5] and cancer[6]. A possible explanation lies in the functionality of the mitochondrion, which is a cellular organelle responsible for supplying most of the energy required for cellular activity, turning nutritional molecules into ATP through oxidative phosphorylation[7]. Mitochondrial metabolism produces Reactive Oxygen Species (ROS) and reactive metabolic intermediates, which are signals that transmit information between the mitochondrion and the nucleus[8,9], and they can modify the expression of nuclear genes, altering numerous cellular processes and metabolic routes that may influence the development of different diseases[10].

The present study is focused on the analysis of rheumatic diseases and the role of mitochondrial lineages in their development and prevalence, given the associations provided by previous works[3,4]. It has been observed that mitochondrial function is altered in chondrocytes of individuals with osteoarthritis (OA)[3,4], producing oxidative stress and increasing chondrocyte apoptosis and cartilage degradation[4,11,12].

To date, some studies have analysed the relationship between mitochondrial haplogroups and rheumatic diseases. It has been stated that haplogroups H and U are significantly related to a higher risk of developing degenerative bone diseases and with greater severity[13–18], whereas haplogroups J and T are significantly related to a decrease in the incidence and progression of OA[13–15,17,19–21], with haplogroup J being also associated with psoriatic arthritis (PsA)[22]. However, no significant association has been found between European mitochondrial haplogroups and rheumatoid arthritis (RA)[18].

The role of haplogroup H in the pathogenesis of rheumatic diseases is due to the fact that, as it shows a high energy efficiency, it generates a greater oxidative stress, increasing the production of ROS, which causes the degradation of the cartilage and the risk of developing degenerative bone diseases[13–18]. On the other hand, the possible protective effect described for haplogroups T and J lies in a lower energy efficiency in the process of converting

[1]Department of Genetics, Physical Anthropology and Animal Physiology, Faculty of Science and Technology, University of the Basque Country (UPV-EHU), 48940, Leioa, Bizkaia, Basque Country, Spain. [2]QarK Arqueología, 01006, Vitoria-Gasteiz, Álava, Basque Country, Spain. *email: imanol.martinl@ehu.eus

calories into ATP, which generates a lower production of ROS and a lower oxidative damage. This mechanism protects the cell from apoptosis and decreases the degeneration of the cartilage involved in the pathogenesis of OA[23,24]. It has been suggested that the differential association of some mitochondrial haplogroups with rheumatic pathologies could be related to an adaptation process of *Homo sapiens* to cold climates, when this species migrated out of Africa[25–27]. Haplogroup H is currently the most common and diverse mitochondrial lineage in Europe (55–40%), which originated 25,000–30,000 years ago in Southwest Asia and entered Europe from the Near East before the Last Glacial Maximum (LGM) (~22,000 years)[28–32]. Haplogroup U, present in 11% of current Europeans, is the oldest maternal lineage in Europe and emerged in Southwest Asia around 55,000 years ago from where it expanded to Europe[28,32–34]. Both haplogroups subsisted in the glacial refugia of Southwestern Europe during the LGM, after which they re-expanded, thus their frequencies were molded by different demographic and cultural factors. Some mitochondrial variants, which were critical for human adaptation in different environments, could have favoured the survival and reproduction of populations that lived in particular climate areas, whereas in other regions they may have not been adaptative[35].

Given the high prevalence of rheumatic diseases found in the medieval necropolis of San Miguel de Ereñozar (Ereño, Bizkaia, Spain, 13th–16th centuries), in the present study we analyse the possible relationship between these diseases and the different mitochondrial lineages, considering the influence of the Earth's temperature decrease during the Little Ice Age (14th–19th centuries). During this period, there was a marked temperature decrease in the Northern Hemisphere, which had negative consequences for survival[36], since the European glaciers advanced in the mountain valleys and the rainfall and floods increased, which caused bad harvests, famine, conflict, epidemics and increased mortality[37–40]. We believe that these conditions could have influenced the energy production process of mitochondria, promoting mitochondrial dysfunction and the development of different rheumatic diseases.

Materials

In the present study, we analysed the human bone remains of 163 individuals recovered from the medieval site of San Miguel de Ereñozar (13th–16th centuries). This necropolis is located in the northeast of the province of Bizkaia (Basque Country, Spain), in the region of Busturialdea-Urdaibai, which is composed of small urban nuclei; the studied necropolis is in one of these urban nuclei.

A total of 47 out of 163 individuals recovered from the necropolis presented rheumatic manifestations. For comparative purposes, another control group of 43 individuals was selected according to the following criteria: (1) adult (>45 years), (2) absence of rheumatic bone manifestations, and (3) well-preserved remains to allow the molecular analysis.

Since this study is focused on rheumatic pathologies, two differentiated groups were established: (1) individuals without pathological bone manifestations (N = 43), and (2) individuals who showed pathological joint manifestations (N = 47), with diagnoses of entities such as SpA, OA and RA; in few cases it was not possible to reach an accurate diagnosis of the rheumatic disease due to the scarcity of the preserved bone remains. To perform the DNA extraction of these 90 individuals, dental pieces were preferably selected, since these provide better results than bones when recovering DNA[41,42]. Only in the cases where no dental pieces were preserved, compact bones were selected, preferentially ribs, as this is the anatomic region with the lowest anthropological interest.

Methods

The diagnosis of rheumatic diseases carried out in the 163 individuals recovered from the site of San Miguel de Ereñozar was described in Laza *et al.*[43], following the protocol proposed by Ventades *et al.*[44]. The process of DNA extraction was conducted according to the procedure described in Hervella *et al.*[45,46] and Laza *et al.*[47]. Mitochondrial DNA D-loop hypervariable segment I was sequenced from the aDNA extracts by amplifying six overlapping fragments, each with an approximate length of 100 bp, which finally provided the sequencing between nucleotides 15,995 and 16,399[48]. In the cases in which individuals presented the revised Cambridge Reference Sequence (rCRS) haplotype, a fragment (7F/7R) of mtDNA D-loop Hypervariable Segment II was sequenced, which contains position 73[45,46]. In those individuals who carried polymorphism A073G, PCR-RFLPs were conducted with *Alu*I restriction enzyme to determine the nucleotide in position 7025, with the aim of verifying whether this sequence corresponded to haplogroup H. The mtDNA sequences obtained were aligned with the BioEdit software and then filtered using the databases Haplogrep (https://haplogrep.uibk.ac.at) and Phylotree (https://www.phylotree.org), in order to determine the haplogroup, sub-haplogroup and mitochondrial haplotype of the analysed samples[49,50].

The following were some of the authentication criteria applied in this study: (1) controls of DNA extraction and amplification (extraction blank and negative control of the PCR), (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[51–53].

For the data analysis, several principal component analyses (PCAs) were conducted using the SPSS Statistics v24 software, with the aim of interpreting the covariation of the bone pathologies and mitochondrial lineages in the analysed individuals. The Fst distance and the genetic diversity of different groups were calculated taking into account the frequencies of mtDNA haplogroups and sub-haplogroups using Arlequin 3.5.2.2.

Results

The analysis of mtDNA in 90 adult individuals of the Ereño necropolis allowed identifying 7 different mitochondrial haplogroups (H, U, T, K, R, J, HV) (Table 1). The most frequent haplogroup was H (73.3%), which is also the most frequent haplogroup in the current population of Europe (55–40%) and in the region of Busturialdea-Urdaibai, where the Ereño necropolis is located[28,30,31,54–56]. The Ereño necropolis shows higher frequencies than the region of Busturialdea-Urdaibai for haplogroups T and R, and lower frequencies for HV, J and U; the frequencies for haplogroup K are similar in both populations (Table 1)[56]. The differences between the two populations were statistically significant ($p_{value} = 0.02$).

Haplogroups	% Ereño necropolis (N = 90)	% Busturialdea-Urdaibai (N = 158)
H	73.3	55.1
U	10.0	15.2
T	6.7	4.4
K	3.3	3.2
R	3.3	—
J	2.2	10.1
HV	1.1	7.0
X	—	3.2
I	—	1.3
W	—	0.6

Table 1. Frequency of mitochondrial haplogroups in the medieval necropolis analysed in the present study (Ereño, 13th–16th centuries) and in the current population where the Ereño necropolis is located, i.e., the region of Busturialdea-Urdaibai (Bizkaia, Spain)[56].

Sub-haplogroups	% Ereño necropolis (N = 90)	% Busturialdea-Urdaibai (N = 158)
H2	36.67	31.65
H1	21.11	16.46
H3	6.67	3.16
T2	6.67	3.80
U5	5.56	12.66
H14	3.33	—
K* (K1, K2)	3.33	3.2
R8	3.33	—
H5	2.22	1.27
J1	2.22	8.86
U*	2.22	—
H7	1.11	—
H11	1.11	—
H24	1.11	—
HV*	1.11	10.6
U1	1.11	—
U3	1.11	—
X*	—	3.2
I*	—	1.3
W*	—	0.6

Table 2. Frequency of the mitochondrial sub-haplogroups in the medieval necropolis analysed in the present study (Ereño, 13th–16th centuries) and in the current population where the Ereño necropolis is located, i.e., the region of Busturialdea-Urdaibai (Bizkaia, Spain)[56].

In the present study, 18 mitochondrial sub-haplogroups were defined in the Ereño necropolis. The most frequent sub-haplogroup is H2 (36.7%), followed by sub-haplogroups H1, H3 and T2 (Table 2); all of them have higher frequencies in the analysed necropolis with respect to the current population of Busturialdea-Urdaibai[56]. However, other sub-haplogroups, such as U5, J1 and HV, show lower frequencies in the studied necropolis than in the current population of Busturialdea-Urdaibai (Table 2)[56], with significant differences between the two populations ($p_{value} = 0.045$).

The analysis of the mitochondrial haplotypes allowed identifying the presence of 55 different haplotypes in the 90 individuals analysed from the medieval necropolis of Ereño, which demonstrates the high variability of the population of this site (Supplementary Table S1).

Regarding the rheumatic pathologies of the 90 adult individuals of the Ereño necropolis, 47 of them (52.2%) had some type of joint pathology and 43 (47.7%) did not have any pathological bone manifestations. Of the 7 mitochondrial haplogroups identified (H, HV, J, K, R, T, U), 5 were found in the individuals with rheumatic pathologies (H, J, R, T, U), whereas all 7 haplogroups were identified in the individuals without bone pathologies, with the absence of haplogroups HV and K in the first group (Supplementary Fig. S1). The individuals with rheumatic pathologies showed a lower diversity of mitochondrial haplogroups (0.3691) compared to the individuals without rheumatic pathologies (0.5360), although no statistically significant differences were found between the two groups ($p_{value} = 0.23$).

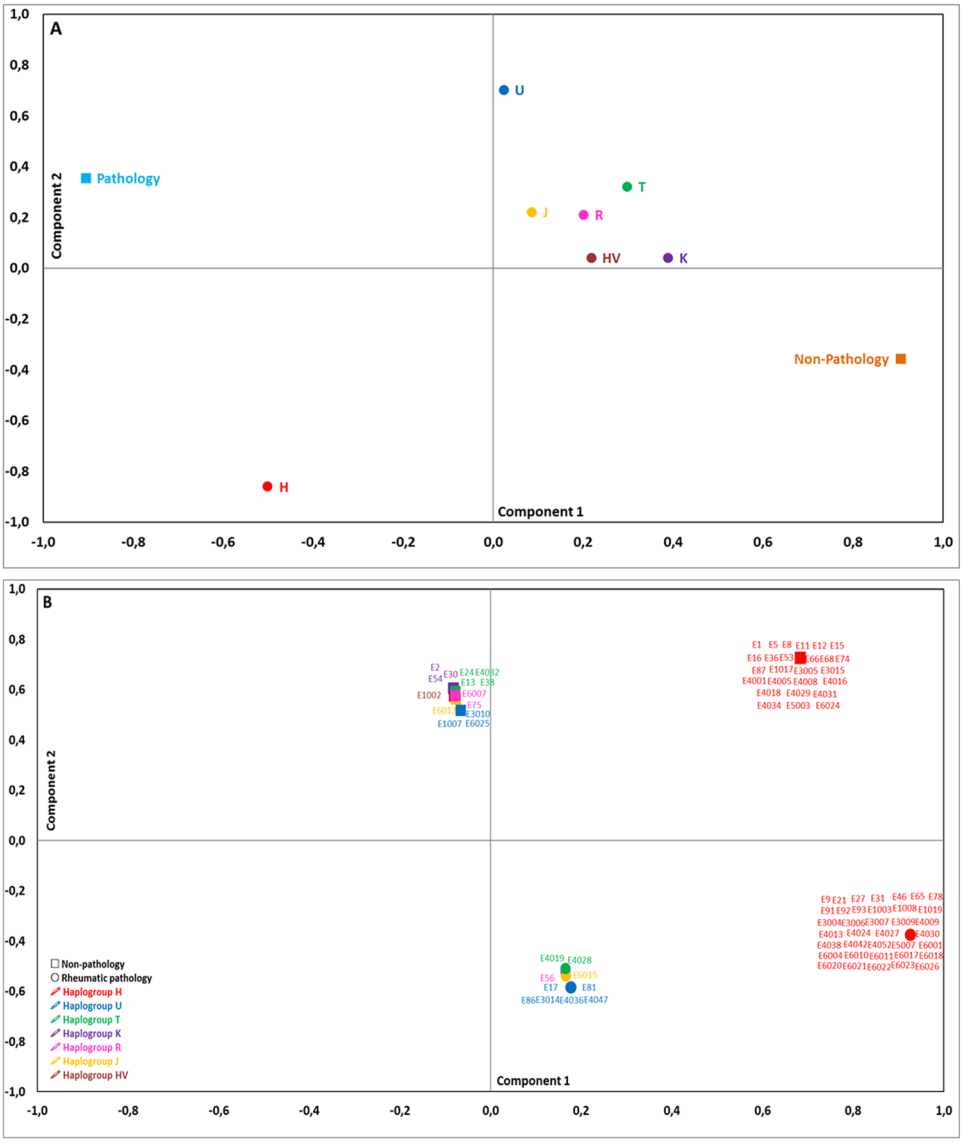

Figure 1. Principal component analysis of the frequency of the mitochondrial haplogroups and the variables pathology and non-pathology of the 90 individuals recovered from the medieval necropolis of San Miguel de Ereñozar (Ereño, Bizkaia, Spain, 13th–16th centuries). (**A**) Distribution of the variables pathology and non-pathology and the mitochondrial haplogroups. (**B**) Distribution of the individuals according to the variables pathology and non-pathology and the mitochondrial haplogroups. Component 1: 50.7% of the variance; component 2: 31.5% of the variance.

The results of the mitochondrial haplogroups regarding joint pathologies show that 56.1% of the individuals who carried haplogroup H had joint lesions, whereas the rest of the individuals (43.9%) did not have signs of arthropathy (Supplementary Fig. S1). Although the other 6 haplogroups had a much lower frequency than haplogroup H, it is worth mentioning that, in the case of haplogroup U (10%) (Table 1), it was observed that 66.7% of the individuals had rheumatic lesions, whereas 33.3% showed no joint bone lesions (Supplementary Fig. S1). Regarding haplogroup T (6.7%) (Table 1), lower frequency values were obtained in the individuals with joint bone pathologies (33.3%) (Supplementary Fig. S1). Haplogroups K, R, J and HV had a very low frequency in this population (<5%); in fact, haplogroups K and HV were not found in individuals with rheumatic pathologies (Table 1, Supplementary Fig. S1).

In order to obtain a more global view of the variability of the individuals of the Ereño necropolis (N = 90), a PCA was conducted considering the mitochondrial haplogroups of both groups, i.e., individuals diagnosed with joint pathologies (N = 47) and without joint pathologies (N = 43), which generated two principal components that explained 82.2% of the variance (Fig. 1A). In PCA axis 1 (50.7% of the variance), the variables with the highest correlation values were: joint pathology (Pathology, −0.906) and absence of joint pathology (Non-Pathology, 0.906). In the second component (31.5% of the variance), the variables with the highest correlation values were: haplogroup H (H, −0.859) and haplogroup U (U, 0.703).

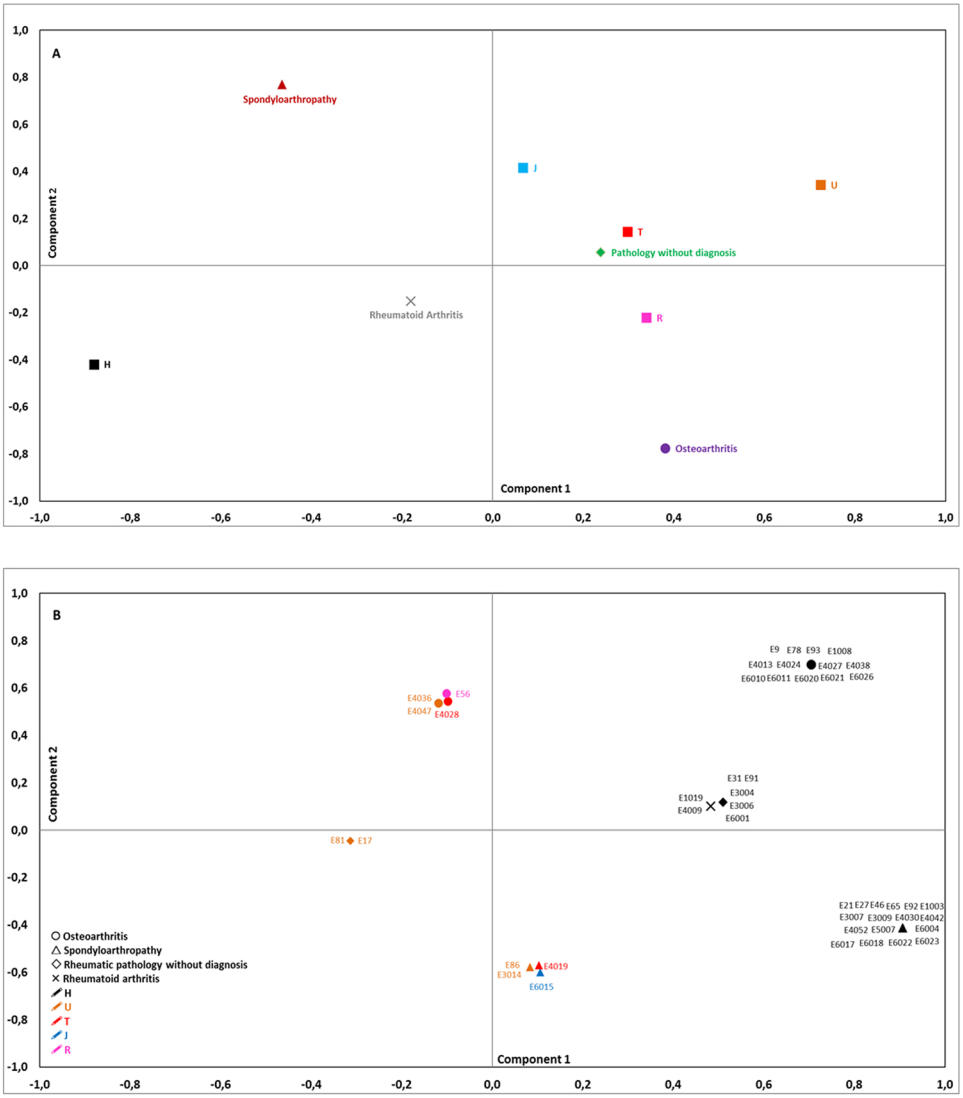

Figure 2. Principal component analysis of the 90 individuals recovered from the medieval necropolis of San Miguel de Ereñozar (Ereño, Bizkaia, 13th–16th centuries). (**A**) Distribution of the variables: rheumatic diseases and mitochondrial haplogroups. (**B**) Distribution of the individuals according to the rheumatic diseases and mitochondrial haplogroups they showed in each case. Component 1: 47.8% of the variance; component 2: 25.4% of the variance.

When representing the individuals on the two principal components of the PCA (Fig. 1B), we found that in the most positive end of component 1 there is a group characterised for having rheumatic pathologies and carrying haplogroup H. In the most positive end of the second component of the PCA there are the individuals who had no rheumatic lesions, and in the most negative end there are the individuals that did show joint lesions, regardless of the mitochondrial haplogroup carried by any of these two groups. Considering the 2 axes of the PCA, it is worth highlighting that the most differentiated individuals were those who had rheumatic pathologies and carried haplogroup H, which indicates that haplogroup H would be more related to those individuals who had a joint pathology. Therefore, the next step was to analyse the distribution of the haplogroups in the individuals who had a particular rheumatic disease (spondyloarthropathy, osteoarthritis, rheumatoid arthritis and rheumatic pathology without an accurate diagnosis).

When analysing the mitochondrial variability only in the individuals with rheumatic diseases (N = 47), 5 different haplogroups were identified (H, U, T, R, J), with H being the most frequent one, followed by U; the rest of the haplogroups (T, R, J) showed frequency values below 5%. With respect to the type of rheumatic disease, in the present study it was observed that almost half of the individuals with haplogroup H had spondyloarthropathies (SpA), whereas the other 4 haplogroups represented a small sample size which did not allow any statistical valuation (Supplementary Fig. S2).

A second PCA was carried out in order to analyse the variability of the mitochondrial haplogroups only in the individuals who had rheumatic diseases, which produced two principal components that explained a variance of 73.2% (Fig. 2). The variables with the highest correlation values in PCA axis 1 (47.8% of the variance) were

haplogroup H (H, −0.811) and haplogroup U (U, 0.724), and in PCA axis 2 (25.4% of the variance), the variables with the highest correlation values were osteoarthritis (OA, −0.776) and spondyloarthropathy (SpA, 0.770) (Fig. 2A).

When representing the individuals on the two principal components (Fig. 2B), we found the individuals who had SpA and haplogroup H in the most positive end of axis 1. In axis 2, the individuals with OA were in the most positive end and in the most negative end there were those diagnosed with SpA, with both groups carrying different mitochondrial haplogroups. Considering the PCA axes, it is worth highlighting that the most differentiated individuals were those with SpA and haplogroup H, which indicates the covariance of haplogroup H and SpA.

18 different mitochondrial sub-haplogroups were identified in this study (H2, H1, H3, T2, U5, H14, R8, H5, J1, K1, U*, H7, H11, H24, HV*, K2, U1, U3) (Table 2), 8 of which were shared by individuals with and without rheumatic pathologies (H2, H1, H3, T2, U5, H14, R8, J1) (Supplementary Fig. S3).

The most frequent mitochondrial sub-haplogroup found in this necropolis was H2 (36.7%) (Table 2), in which 66.7% of the individuals showed joint lesions (Supplementary Fig. S3). The rest of the mitochondrial sub-haplogroups showed low frequency values, preventing any statistical valuation (Table 2, Supplementary Fig. S3). When we analysed the distribution of the mitochondrial sub-haplogroups in the different rheumatic diseases, we found that the most frequent sub-haplogroup (H2) appeared in 57% of the individuals with SpA, a very high frequency compared to other rheumatic pathologies (Supplementary Fig. S4).

Discussion

The genetic heritage of the current European populations is the result of numerous demographic and cultural episodes that took place since the arrival of the anatomically modern *Homo sapiens* to Europe around 45,000 years ago[57]. Regarding the mitochondrial lineages, haplogroup H is the most common and diverse in the current European population (55–40%)[28,30,31,54], and haplogroup U is the oldest European lineage, with a current frequency of 11%[28,32–34]. Both lineages survived in the glacial refugia of Southwestern Europe during the Last Glacial Maximum (LGM)[29–32], from where they re-expanded to Northern and Eastern Europe.

Among the causes that would explain the distribution of haplogroup H in the current European population, the climate change that took place during the LGM seems to have played an important role. Moreover, it has been described that the demographic changes that occurred during the Neolithic were a key factor for the diversification of haplogroup H and for the increase of its frequency, becoming the main haplogroup at present in Western Europe (55–40%)[28,30,31,58]. The highest frequency has been reported in the populations of the Northern Iberian Peninsula (Basque Country, Asturias and Galicia), decreasing toward Northern and Eastern Europe, with the exception of Wales, where this haplogroup has a frequency of over 50%[54,55].

Furthermore, some authors have suggested the existence of a relationship between mitochondrial DNA (mtDNA) and rheumatic diseases, based on the critical role that the mitochondrion plays in cellular function and in survival against oxidative stress[59,60]. In the energy generation process, via oxidative phosphorylation, the mitochondrion produces Reactive Oxygen Species (ROS), whose increase causes damage to lipids, proteins and DNA, promoting mitochondrial dysfunction and inflammation in different pathologies[11,59,61,62].

Different studies have shown that some mitochondrial haplogroups can increase the risk of developing degenerative joint diseases, whereas other haplogroups constitute a protection factor[19,59,63–67]. Haplogroup H could be related to a higher risk of developing degenerative bone diseases[13–18], whereas other haplogroups, such as J and T, would be associated to a lower risk of developing osteoarthritis (OA) and psoriatic arthritis[13–15,17,19–21].

In this context, the population recovered from the medieval necropolis of San Miguel de Ereñozar (Ereño, Bizkaia, Basque Country, Spain, 13th–16th centuries), allowed us to evaluate the possible relationship between mtDNA and rheumatic diseases, since this population presents three relevant characteristics: (i) a high frequency of mitochondrial haplogroup H, (ii) a chronology that overlaps with an unfavourable climate period (Little Ice Age, LIA; 14th–19th centuries), and (iii) a high prevalence of rheumatic diseases (30%), which were diagnosed as spondyloarthropathies (SpA) (45%), osteoarthritis (OA) (36%) and rheumatoid arthritis (RA) (4%), with a 15% frequency of rheumatic pathologies that could not be accurately diagnosed.

In the present study, we analysed the mitochondrial variability of 90 adult individuals of the medieval necropolis of Ereño, finding 7 haplogroups (H, U, T, K, R, J and HV) and 18 different sub-haplogroups (Tables 1 and 2). The most frequent mitochondrial haplogroup was H (73.3%), whose frequency is higher than that reported in the current population of Busturialdea-Urdaibai (Table 1)[56]. These two populations showed statistical significant differences due to the high frequency of haplogroup H in the necropolis of Ereño.

The analysis of the mitochondrial haplogroups revealed the existence of 55 different haplotypes among the 90 individuals, with haplotype rCRS presenting a very high frequency in the necropolis (≃24%) (Table S1), although similar to the values found in the current populations of the Basque Country (27-21%)[55,56]. Therefore, we can discard the existence of endogamy among the individuals of the necropolis.

When analysing the relationship between haplogroup H and rheumatic diseases, it was observed in the present study that 56% of the individuals that carried this haplogroup had rheumatic lesions, compared to 44% of the individuals who did not show these bone pathologies, which suggests that carrying haplogroup H can pose a greater risk of developing rheumatic diseases (Supplementary Fig. S1). This relationship would be supported by the hypothesis that haplogroup H would be related to a greater oxidative stress, greater cartilage degradation and higher risk of developing degenerative bone pathologies[13–18]. In the present study, it was observed that haplogroup H was more frequent among the individuals who had bone manifestations characteristic of SpA (81%) (Supplementary Fig. S2, Fig. 2B), although this haplogroup is also present with lower frequencies in individuals diagnosed with other rheumatic diseases, such as OA and RA (Supplementary Fig. S2). Regarding the H sub-haplogroups identified in the Ereño necropolis, H2 (the most frequent one, 36.7%), showed differences in the individuals with joint manifestations with respect to those who did not have these manifestations (Supplementary

Fig. S3). This result suggests that this sub-haplogroup would be strongly related to a higher risk of developing rheumatic diseases. Moreover, H2 was identified in 57% of the individuals diagnosed with SpA (Supplementary Figure S4), suggesting that sub-haplogroup H2 increases the risk of developing SpA.

The other mitochondrial haplogroups (U, T, K, R, J, HV) showed a small sample size, which does not allow determining their role in the susceptibility of developing rheumatic diseases (Table 1, Supplementary Fig. S1).

The proposed relationships between these mitochondrial haplogroups and their protective or non-protective role in different pathologies are based on the nature of the mitochondrion, in which, by means of the Oxidative Phosphorylation System (OXPHOS), energy is generated to produce heat and thus maintain the body temperature, as well as ROS (Reactive Oxygen Species)[25,27]. Different studies have suggested that European haplogroups would have been molded by selective mechanisms related to low temperatures during the last glacial period, when *Homo sapiens* dispersed over Europe[25–27,68]. In the present study, we propose that the temperature decrease that took place in the Little Ice Age (LIA) was an environmental factor that influenced the selection of some mitochondrial haplogroups over others, favouring those haplogroups that were more efficient at obtaining energy and heat from food to endure lower temperatures and food shortage.

Haplogroup H, one of the most energy-efficient mitochondrial lineages, has a high frequency in the medieval population of this study that lived through the LIA, a period in which energy demands would have risen. However, this haplogroup produces a high oxidative stress, cell damage and cartilage degeneration, promoting the development of degenerative bone diseases[24,27,69,70]. In this study, we observed that the individuals who carried haplogroup H had a significantly high frequency of rheumatic pathologies, more specifically spondyloarthropathies, which was also verified for sub-haplogroup H2.

Conclusions

From the evolutionary perspective, the high frequency of haplogroup H found in the population of San Miguel de Ereñozar (Basque Country, Spain) could be considered as a result of a biological adaptation related to adverse environmental conditions, such as the ones that took place during the Little Ice Age, since this mitochondrial haplogroup is more energy-efficient at enduring and surviving these conditions. However, this would imply a biological trade-off, increasing the risk of developing rheumatic diseases.

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Author contributions

I.M. Laza, M. Hervella and C. de-la-Rúa conceived and designed the project. I.M. Laza designed and developed the DNA extraction, molecular analysis and analysed the data. M. Hervella contributed laboratory experimental steps. M. Hervella and C. de-la-Rúa contributed analytical steps. M. Neira Zubieta provided the contextual information of the necropolis. C. de-la-Rúa obtained financial support for the project. I.M. Laza wrote the manuscript. All authors discussed the article and gave comments.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to I.M.L.

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Article 4

Clinical and genetic study of axial spondyloarthritis

Clinical and genetic study of axial spondyloarthritis

Laza IM[1]*, Hervella M[1], Izagirre N[1], Blanco Madrigal JM[2],

Galíndez E[2], Rivera NA[2], Garcia Vivar ML[2], de-la-Rúa C[1]

1 Department of Genetics, Physical Anthropology and Animal Physiology, Faculty of Science and Technology, University of the Basque Country (UPV-EHU); Leioa; Biscay; Basque Country; 48940; Spain.

2 Department of Rheumatology, Basurto University Hospital, Montevideo Avenue 13-18, 48013 Bilbao, Spain

*Correspondence to imanol.martinl@ehu.eus

Research Square

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Abstract

Objective

The aim of the present study was to evaluate whether ankylosing spondylitis (AS) and non-radiographic axial spondyloartritis (nr-axSpA) are subsets of a single disease from a genetic perspective.

Materials and Methods

We analyzed from a clinical and genetic perspective 62 patients from the University Hospital of Basurto (Bilbao, Basque Country, Spain) diagnosed with axial spondyloarthritis. Forty three SNPs previously associated with spondyloarthritis (SpA) were selected. The DNA samples were genotyped through SNP Type Assay, using the *BioMark HD* platform of *Fluidigm*. The statistical analysis was carried out through the Student's T-test and Fisher's exact test. Moreover, Heatmap and Principal Component Analysis were carried out to show the correlation between patients and genetic markers.

Results

Regarding clinical characteristic we found statistically significant differences between the patients with AS and nr-axSpA in the age at diagnosis, the disease duration, the presence of syndesmophytes and the BASMI index. In relation to genetic markers, we only found statistically significant differences in *HLA-B27* allele frequency between the patients with AS and those with nr-axSpA. Regarding the frequencies of the set of risk SNPs associated with SpA, no statistically significant differences were found in this study between the two groups. Despite the high genetic heterogeneity observed among the analyzed patients, it is worth highlighting that some of the most important risk SNPs associated with the pathogenesis of AS, located in several genes

(*ERAP1*, *ERAP2*, *IL-23R*, *GPR25*) and intergenic region (2p15), appeared at high frequencies in all the patients of this study.

Conclusion

In the present study, we have observed that AS and nr-axSpA have a common genetic background associated with the pathogenic development of these diseases. Among the genetic factors, the present study shows the importance of genes involved in the pathogenesis of AS, such as *HLA-B27*, *ERAP1*, *ERAP2*, *IL-23R*, *GPR25* and intergenic region 2p15, whose role may influence the onset, development and severity of the disease. However, the pathogenesis of autoimmune diseases, such as SpA, is very complex, indicating the involvement of environmental factors such as smoking and obesity, in the triggering of the disease, so that patients with different genotypes would have the same pathogenic phenotype.

Keywords

Ankylosing spondylitis, non-radiographic axial spondyloarthritis, SNP, *HLA-B27* allele, pathogenesis

Introduction

Spondyloarthritis (SpA) are a group of rheumatic, inflammatory diseases that share a series of characteristics, such as affected axial skeleton [spine and sacroiliac joints (SIJs)], and peripheral and extra-articular manifestations[1]. SpA traditionally include ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis, undifferentiated spondyloarthritis, juvenile spondyloarthritis and inflammatory bowel disease-associated spondyloarthritis (IBD) (Crohn´s disease and ulcerative colitis)[2].

The classification criteria for the diagnosis of SpA developed by the Assessment of Spondyloarthritis International Society (ASAS) **(Table 1)**, were created and published in 2009[3,4], due to the diagnostic limitations of the previous criteria [modified New York criteria[5] and European Spondyloarthropathy Study Group criteria[6]]. Based on the ASAS criteria, SpA can be classified as axial (axSpA) or peripheral[3,4]. Within axSpA, there are patients with radiographic changes in SIJs (AS) and other patients without radiographic manifestations (nr-axSpA).

The ASAS classification criteria (image branch) use magnetic resonance imaging (MRI) to diagnose nr-axSpA by detecting the active inflammation (bone marrow edema). However, in the case of AS, conventional x-ray imaging shows advanced and irreversible changes of the sacroiliitis[7], although several years are necessary for these changes to take place, which makes the diagnosis delay years after the beginning of the symptoms[7], thus decreasing the quality of life of the patient.

Sacroiliitis on Imaging + ≥1 SpA Feature OR *HLA-B27* + ≥2 Other SpA Features	
SpA Features	**Sacroiliitis on Imaging**
Inflammatory back pain Arthritis Enthesitis (heel) Uveitis Dactylitis Psoriasis Crohn´s disease/ulcerative colitis Good response to NSAIDs Family history for SpA *HLA-B27*$^{+}$ Elevated CRP	Active (acute) inflammation on MRI highly suggestive of sacroiliitis associated with SpA OR Definitive radiographic sacroiliitis according to modified New York criteria

Table 1. The ASAS classification criteria for axial spondyloarthritis in patients with back pain 3 months or more and age at onset younger than 45 years[3,4]. ASAS, Assessment of

Spondyloarthritis International Society; SpA, spondyloarthritis; NSAIDs, non-steroidal anti-inflammatory drug; *HLA-B27*, human leukocyte antigen-B27; CRP, C-reactive protein, MRI, magnetic resonance imaging.

There is current debate on whether nr-axSpA is a different form of AS[8,9], an early form of AS[10,11], or whether both are two expressions of the same disease[12,13]. Some patients with nr-axSpA will develop AS after years of disease[14-17], which occurs in 10% of patients with over 2 years of follow-up, and in over 20% of patients after 2 years with high levels of C-reactive protein (CRP) or an active inflammation detected by MRI[15]. However, other patients with nr-axSpA will suffer the disease for decades, and probably for life, without any evidence of radiographic damage[18], whereas remission will take place in others, either spontaneously or through the administration of drugs.

Patients with AS and nr-axSpA share some clinical characteristics, such as peripheral manifestations (arthritis, enthesitis, dactylitis) and extra-articular manifestations [uveitis, IBD, psoriasis (Ps)][10-12], with uveitis being slightly more prevalent in AS[19]. Different researchers believe that intestinal dysbiosis plays a fundamental role in the genesis of SpA[20]; in fact, SpA have been associated with IBD, since approximately 5-10% of patients with SpA develop IBD, and approximately 70% may have a subclinical intestinal disease[21]. Furthermore, common genes and loci have been identified for these two diseases[22], as well as the influence of specific alterations in the composition of the gut microbiota with several immune-mediated disorders[23]. Moreover, 10% of patients with AS suffer from Ps, whereas in the case of Ps, 7-42% of patients develop SpA-type axial affectation[24].

Other common characteristics among patients with AS and nr-axSpA are related to the clinical activity, damage, functional deterioration, quality of life[10,12,15,25], similar response to anti-*TNF-α* treatment and similar average age in the appearance of symptoms, with small differences in the duration of the disease.

Regarding the differences between AS and nr-axSpA, we can highlight the presence of structural changes in the spine and SIJs, which limit the mobility of patients with AS, whose functionality is influenced by inflammation and the formation of new bone material[26]. Furthermore, several authors have reported a greater number of inflammatory lesions and high levels of CRP in patients with AS[27]. This could be due to the fact that patients with axSpA and high levels of CRP or positive MRI progress more rapidly to radiographic sacroiliitis and, therefore, there may be a larger percentage of patients with radiographic progression if there are signs of inflammation (CRP and MRI)[28]. It has been described that smoking increases the pace and severity of the

progression to the radiographic form of SpA[29]. With respect to gender, there is a larger proportion of women among the population of patients with nr-axSpA and a larger percentage of men among patients with AS[10,16,30]. In studies with Asian cohorts, the prevalence of nr-axSpA was twice as high in women with respect to men[31,32], whereas in European and North American cohorts the prevalence of nr-axSpA was similar in both gender[8,11,12]. Therefore, basal radiographic damage, high levels of CRP, smoking and being male are factors that could predict the future radiographic damage in patients with early SpA[33].

The etiopathogenesis of SpA results from the complex interaction between genetic and environmental factors[34]. Although some studies report that genetic factors have a great influence on the susceptibility to develop AS (up to 80-90% in studies with relatives and twins[34]), this type of disease is very complex from the genetic perspective. It has been suggested that allele *HLA-B27* contributes to 20-25% of the genetic inheritance of this disease[35], and the single nucleotide polymorphisms (SNP)s found in genome-wide association studies (GWASs) seem to contribute to 3-7%[36]. These data suggest that there may still be many unknown genetic variants that are part of the background of the disease.

Among the SpA, AS has the strongest association with allele *HLA-B27*[37,38], since this allele has been found in almost 90% of patients with AS[38]. However, in the general population, only 5% of *HLA-B27*(+) individuals will eventually develop this disease, with the peculiarity that homozygous for this allele have a greater risk of suffering from AS compared to heterozygous[39], although not all subtypes of *HLA-B27* have a predisposition for the disease[40]. Despite the reported association between *HLA-B27* and susceptibility to AS, it is currently unknown how *HLA-B27* contributes to the development of AS[41]. Different studies have stated that *HLA-B27* does not play a direct role in the formation of new bone and cartilage[42,43], and that it is indirectly involved in the inflammatory process[43]. It has been reported that *HLA-B27*(+) patients have more severe radiographic damage, a younger disease onset age and a shorter delay in the diagnosis than *HLA-B27*(-) patients[30,44]. Furthermore, it has been suggested that other alleles of the *HLA-B* system increase or decrease the susceptibility to develop SpA, such as alleles *HLA-B07, HLA-B13, HLA-B40, HLA-B47, HLA-B51* or *HLA-B57* among others[45]. In recent years, thanks to GWAS, alleles of different genes, both inside and outside of the Major Histocompatibility Complex (MHC), have been associated with the susceptibility to develop AS[34,36,46-49].

Some studies indicate that the prevalence of *HLA-B27* is similar in patients with nr-axSpA and in those with AS[8,10,11,27,50-52]. However, others indicate that patients with AS have a greater frequency of *HLA-B27*[34,36,46-49].

Considering the hypothesis that AS and nr-axSpA are subsets of a single disease with common clinical characteristics and a similar disease burden, the genetic background could be expected to be similar for the two entities. The aim of the present study was to evaluate this hypothesis, through the analysis of a set of risk SNPs localized in genes involved in the pathogenesis of AS, Ps, PsA and IBD in a sample of patients with axSpA.

Material and methods

Patients

DNA was obtained from blood samples of 62 patients of the University Hospital of Basurto (Bilbao, Basque Country, Spain). These samples were supplied through the Basque Biobank for Research (Biobanko, Bioef), along with the informed consent of the patients, following the protocols of the Drug Research Ethics Committee of Euskadi (CEImE, PI2017141).

This study included patients over 18 years of age who suffered from inflammatory back pain and met the New York modified criteria for AS or the ASAS classification criteria for axSpA. Moreover, clinical, demographic and laboratory data were also included (CRP, ESR, *HLA-B27*). The functional state, the activity of the disease and the spinal and pelvic mobility were evaluated through Bath Ankylosing Spondylitis Functional Index (BASFI), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Bath Ankylosing Spondylitis Metrology Index (BASMI), respectively.

Pelvic plain radiography was evaluated by three rheumatologists and the existence of sacroiliitis, defined according to the New York modified criteria [5], was decided by consensus with the positive opinion of at least 2 of the 3 rheumatologists. The patients fulfilling criteria with radiographic sacroiliitis were diagnosed with AS, whereas those without radiographic criteria, were diagnosed with nr-axSpA by MRI, according to ASAS image branch criteria [3,4].

Selection of SNPs associated with rheumatic diseases

For the selection of SNPs, a search was carried out in different bibliographic databases: 1) PubMed (*http://www.ncbi.nlm.nih.gov/pubmed/*), 2) *dbSNP* (*http://www.ncbi.nlm.nih.gov/snp/*) of *NCBI* (*National Center for Biotechnology Information*, USA), and 3) databases that gather the results obtained in different GWAS analyses (*GWAS Catalog*, *GWAS Central*, *Ensembl Genome Browser*), which identify those allele variations of SNPs associated with the susceptibility to developing a disease.

In this study, we selected those SNPs that had been previously associated with the diagnosis, prognosis and treatment of AS, Ps, PsA and IBD **(Supplementary table)**. These SNPs are located

in genes that are involved in different routes and mechanisms that could partially explain the pathogenesis of the disease: (1) Genes that encode for endoplasmic reticulum aminopeptidases (*ERAP1*, *ERAP2* and *NPEPPS*), which are proteins that cut peptides to an adequate length (8-9 amino acids) in order for the latter to be presented by HLA molecules, such as *HLA-B27*, to T-lymphocytes. The association between *ERAP1* and AS has only been described in *HLA-B27*(+) individuals, unlike *ERAP2*, whose association has been reported in both *HLA-B27*(+) and *HLA-B27*(-) individuals. (2) Genes involved in the route of interleukin-23 (*IL-23*), which is a key cytokine involved in the differentiation of $CD4^+$ T-lymphocytes into Th17, which produce *IL-17*, *IL-6*, *IL-22*, *TNF-α* and other similar proinflammatory cytokines. Some of these genes are: *CARD9*, *EOMES*, *ICOSLG*, *IL1R1*, *IL1R2*, *IL6R*, *IL7R*, *IL12B*, *IL27*, *PTGER4*, *RUNX3*, *TBX21*, *TYK2* and *ZMIZ1*. (3) Genes involved in the differentiation of T cells (*EOMES*, *IL7R*, *RUNX3*, *ZMIZ1*, *BACH2* and *SH2B3*) and G protein-coupled receptors (*GPR25*, *GPR35*, *GPR37* and *GPR65*). (4) SNPs located in intergenic regions with high frequency among individuals diagnosed with AS. (5) Other genes involved in the inflammatory response (*NOD2*, *ATG16L1*, *TRAF3IP2*).

For the selection of the SNPs, the following criteria were applied: (1) GWAS described in Caucasian populations, and (2) the p_{value} of the association between the SNP and the disease had to be lower than $5*10^{-6}$. Moreover, the SNPs that were in linkage disequilibrium ($r^2 > 0.8$) were excluded from the study.

Genotyping

The DNA samples were genotyped through SNP Type Assay, using the *BioMark HD* platform of *Fluidigm* (96.96 Dynamic Array™ IFC, Fluidigm, South San Francisco, CA, USA). The genotyping was carried out at the Sequencing and Genotyping Department of the SGIker General Genomic Services of the University of the Basque Country (UPV/EHU, Bizkaia, Spain). All the SNPs that did not obtain a call rate greater than 0.90 were discarded from the analysis. The genotyping of *HLA-B27* was conducted using tagSNP rs11688202 in the mentioned platform. The sensitivity and specificity of this SNP is >98% for the typing of *HLA-B27*[49]. The rest of the alleles of the *HLA-B* gene were analyzed through sequencing, following the methodology proposed by Laza et al., 2016[56].

Statistical analysis

The continuous variables, expressed as mean and standard deviation, were compared using the Student's T-test, and the results were shown as mean and standard deviation. Regarding the categorical variables, a statistically significant $p_{value}<0.05$ was considered for the clinical and laboratory variables, as well as for the allele and genotype frequencies, which were compared using the Fisher's exact test (applying the Bonferroni correction for multiple comparisons). These

analyses were carried out using *SPSS Statistics software v25* [57]. The Hardy-Weinberg equilibrium test for the frequencies of the SNPs was conducted using the *HardyWeinberg* package of *RStudio*[58,59], considering that there was no equilibrium in those with $p_{value}<0.05$ (applying the Bonferroni correction for multiple comparisons). To carry out the Principal Component Analysis (PCA) and Heatmap, we used the *FactoMineR*[60] and *Pheatmap*[61] packages of *RStudio*[59], respectively.

Results

In the present study, 62 patients diagnosed with axSpA were analyzed, all of whom had a history of inflammatory back pain. Of these, 49 patients (79%) met the radiographic criteria being diagnosed with AS, and 13 (21%) were diagnosed with nr-axSpA using MRI. Table 2 shows the demographic and clinical characteristics and laboratory parameters of the patients of both groups. The patients of the AS group showed a higher inclusion age and a significantly longer duration of the disease with respect to those of the nr-axSpA group ($p_{value}<0.05$), although the symptoms onset age was lower, the diagnostic delay was shorter and the diagnosis age was significantly lower ($p_{value}<0.05$). In the group of patients with AS there was a predominant proportion of males (77.55% males *vs* 22.45% females), whereas in the nr-axSpA group the proportions of males and females were similar (53.85% *vs* 46.15%). The prevalence of smokers (including ex-smokers) was larger in the AS group compared to the nr-axSpA group **(Table 2)**. The presence of syndesmophytes in the spine was statistically higher in the AS group ($p_{value}<0.05$), confirmed by the BASMI index ($p_{value}<0.05$), which measures the mobility of the spine and SIJs **(Table 2)**. In the present study, no statistically significant differences were found for peripheral and extra-articular manifestations, although dactylitis, peripheral arthritis and uveitis were mostly diagnosed in the AS group **(Table 2)**.

Regarding the laboratory variables analyzed in this study, the levels of CRP were higher among the patients with AS. It is worth highlighting the significantly higher prevalence of allele *HLA-B27* in individuals with AS compared to those with nr-axSpA (87.8% *vs* 38.5%) **(Table 2)**. In the patients who did not have allele *HLA-B27* (N=14), the sequencing revealed the presence of alleles *HLA-B40* (N=5), *HLA-B55* (N=4), *HLA-B56* (N=2), *HLA-B07* (N=1), *HLA-B39* (N=1) and *HLA-B47* (N=1), and no statistically significant differences were found between AS and nr-axSpA patients, since 4 of these alleles were detected in patients with AS [*HLA-B40* (N=3), *HLA-B55* (N=2), *HLA-B56* (N=1), *HLA-B07* (N=1)), and 5 in those with nr-axSpA (*HLA-B40* (N=2), *HLA-B55* (N=2), *HLA-B56* (N=1), *HLA-B39* (N=1), *HLA-B47* (N=1)].

Characteristic	AS (N = 49)	nr-axSpA (N=13)	p_{value}
Current age, mean ± S.D. (years)	51.3 ± 13.5	47.5 ± 12.2	0.360
Male gender (N, %)	38 (77.6%)	7 (53.9%)	0.176
Female gender (N, %)	11 (22.5%)	6 (46.2%)	0.176
Family history (N, %)	15 (30.6%)	5 (38.5%)	0.838
Inclusion age mean ± S.D. (years)	49.7 ± 13.4	45.9 ± 12.0	0.355
Age at diagnosis mean ± S.D. (years)	33.3 ± 13.0	42.2 ± 13.4	0.039*
Age at symptom onset mean ± S.D. (years)	28.6 ± 12.5	32.7 ± 9.9	0.276
Disease duration mean ± S.D. (years)	17.9 ± 13.3	4.4 ± 4.3	2E-07*
Diagnostic delay mean ± S.D. (years)	5.00 ± 5.3	9.7 ± 11.1	0.180
HLA-B27(+) (N, %)	43 (87.8%)	5 (38.5)	0.0007*
CRP mean ± S.D. (mg/L)	21.7 ± 43.1	9.7 ± 10.1	0.367
ESR mean ± S.D. (mm/h)	22.2 ± 21.7	24.1 ± 19.2	0.795
BMI mean ± S.D. (kg/m^2)	26.7 ± 4.4	27.0 ± 5.4	0.395
Enthesitis (N, %)	2 (4.1%)	2 (15.4%)	0.401
Dactylitis (N, %)	6 (12.2%)	0 (0%)	0.424
Peripheral arthritis (N, %)	12 (24.5%)	1 (7.7%)	0.348
Uveitis (N, %)	14 (28.6%)	2 (15.4%)	0.542
Psoriasis (N, %)	3 (6.1%)	3 (23.1%)	0.190
IBD (N, %)	2 (4.1%)	3 (23.1%)	0.096
Obesity (N, %)	11 (22.5%)	4 (30.8%)	0.796
Smoker (N, %)	34 (69.4%)	7 (53.9%)	0.470
Presence of syndesmophytes (N, %)	28 (57.1%)	1 (7.7%)	0.004*
Cardiovascular disease (N, %)	12 (24.5%)	3 (23.1%)	0.796
BASDAI mean ± S.D. (score)	2.5 ± 2.1	3.53 ± 2.5	0.138
BASFI mean ± S.D. (score)	2.6 ± 2.4	3.16 ± 2.7	0.448
BASMI mean ± S.D. (score)	1.6 ± 0.5	1.25 ± 0.3	0.0005*

Table 2. Clinical and laboratory parameters of patients diagnosed with AS and nr-axSpA included in the present study, as well as the p_{value} of the comparison between the two patient groups (*p_{value}<0.05). AS, ankylosing spondylitis; nr-axSpA, non-radiographic axial spondyloarthritis; *HLA-B27*, human leukocyte antigen-B27; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; BMI, body mass index; IBD, inflammatory bowel disease; BASDAI, bath ankylosing spondylitis disease activity index; BASFI, bath ankylosing spondylitis functional index; BASMI, bath ankylosing spondylitis metrology index.

The analysis of the 28 risk SNPs located in genes involved in the pathogenesis to develop AS according to the existing GWAS studies, showed that over 75% of the patients of this study shared 60% of the risk alleles **(Figure 1)**. However, no statistically significant differences were found in the frequencies of the risk alleles of these 28 SNPs between the individuals with AS and those with nr-axSpA, except for allele *HLA-B27*, whose frequency was significantly higher in the AS group (p_{value}<0.05) **(Table 2)**.

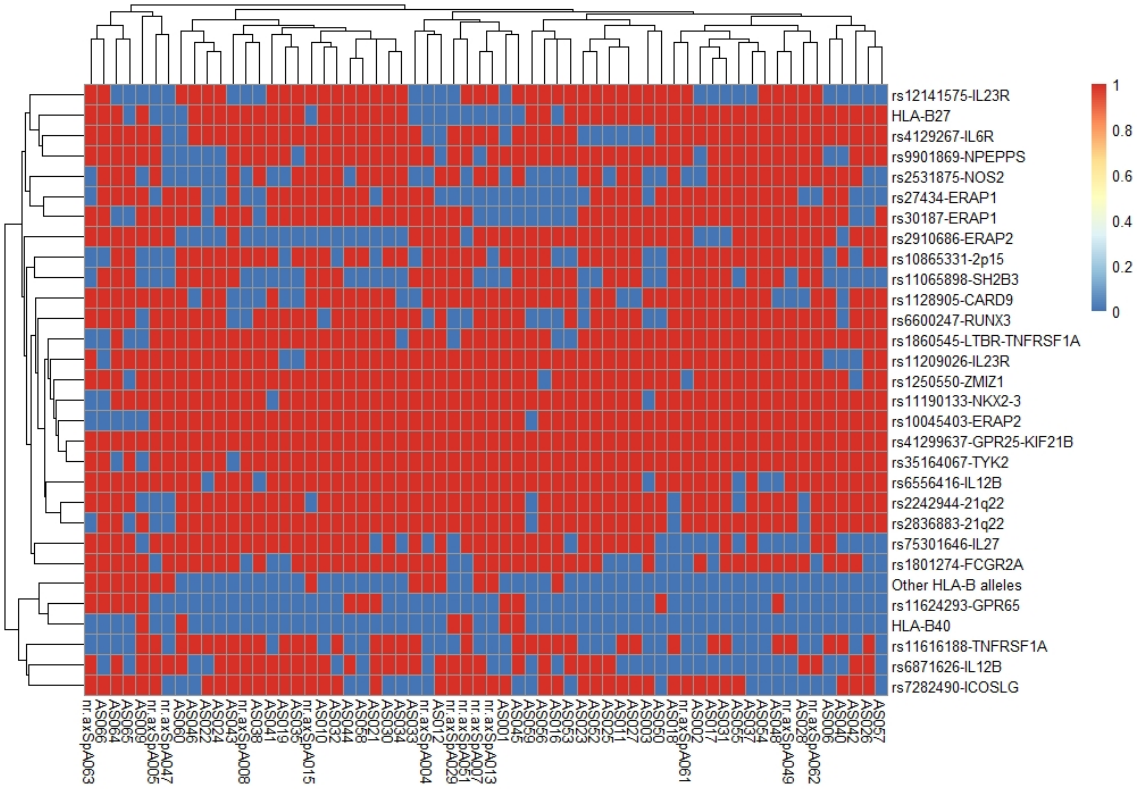

Figure 1. Heatmap of the allele frequencies of the SNPs associated with the susceptibility to develop AS in patients diagnosed with AS and nr-axSpA analysed in the present study. Red: presence of the risk allele; blue: absence of the risk allele. AS, patients diagnosed with ankylosing spondylitis; nr.axSpA, patients diagnosed with non-radiographic axial spondyloarthritis.

Considering that some SNPs showed a dominance relationship, thus the existence of a single copy of that allele increases or decreases the risk of developing AS, we analyzed the different genotypes of the 28 SNPs associated with AS.

Regarding the genotypic frequencies of these SNPs, no statistically significant differences were found in this study between the patients with AS and those with nr-axSpA. However, Figure 2 shows that some risk alleles in homozygous or heterozygous patients (in the case of showing a dominance relationship) relative to genes involved in important pathogenic routes, such as *ERAP1*, *ERAP2*, *GPR25*, 2p15 or *IL-23R*, appear at high frequencies in all the individuals of this study. Nevertheless, there is no defined pattern in any of the two pathological entities in terms of risk genotypes, which shows the great genetic heterogeneity underlying these two entities **(Figure 2)**.

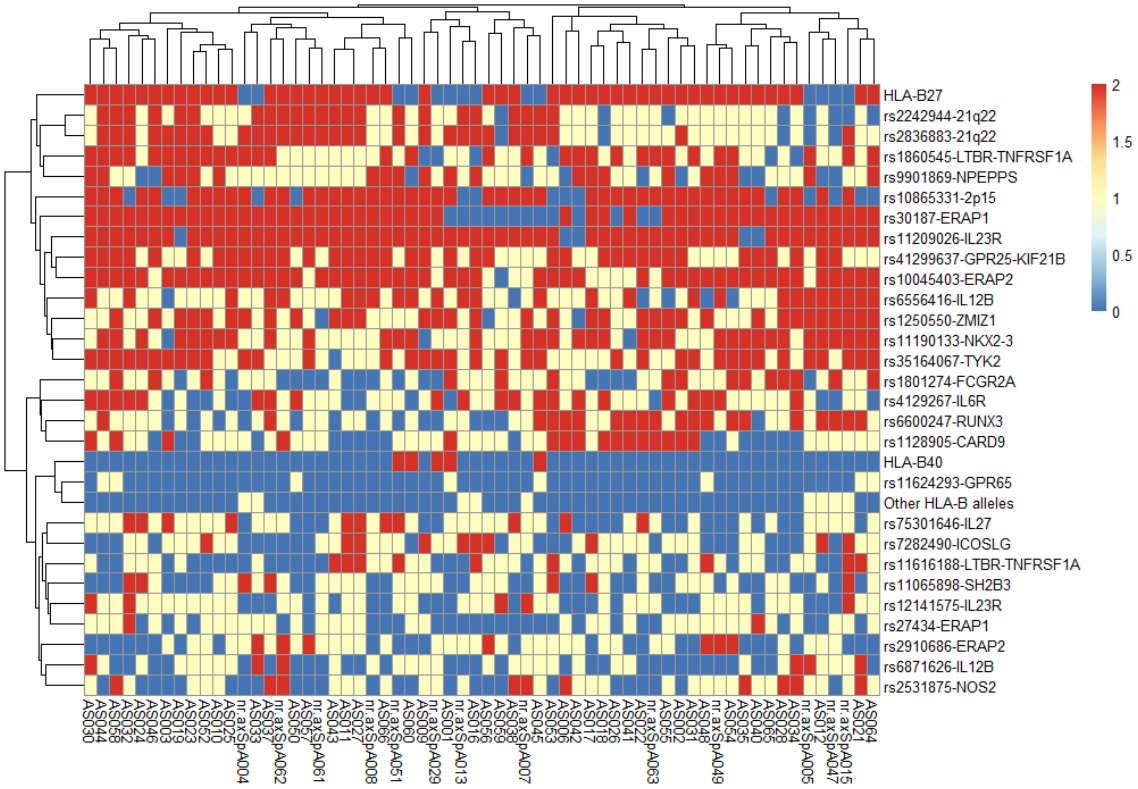

Figure 2. Heatmap of the genotypic frequencies of the selected SNPs in the patients diagnosed with AS and nr-axSpA in the present study. Red: presence of the risk allele in homozygous or in heterozygous patients when such allele is dominant; yellow: presence of the risk allele in heterozygous patients; blue: absence or presence of the risk allele in heterozygous patients when such allele is recessive. AS, patients diagnosed with ankylosing spondylitis; nr.axSpA, patients diagnosed with non-radiographic axial spondyloarthritis.

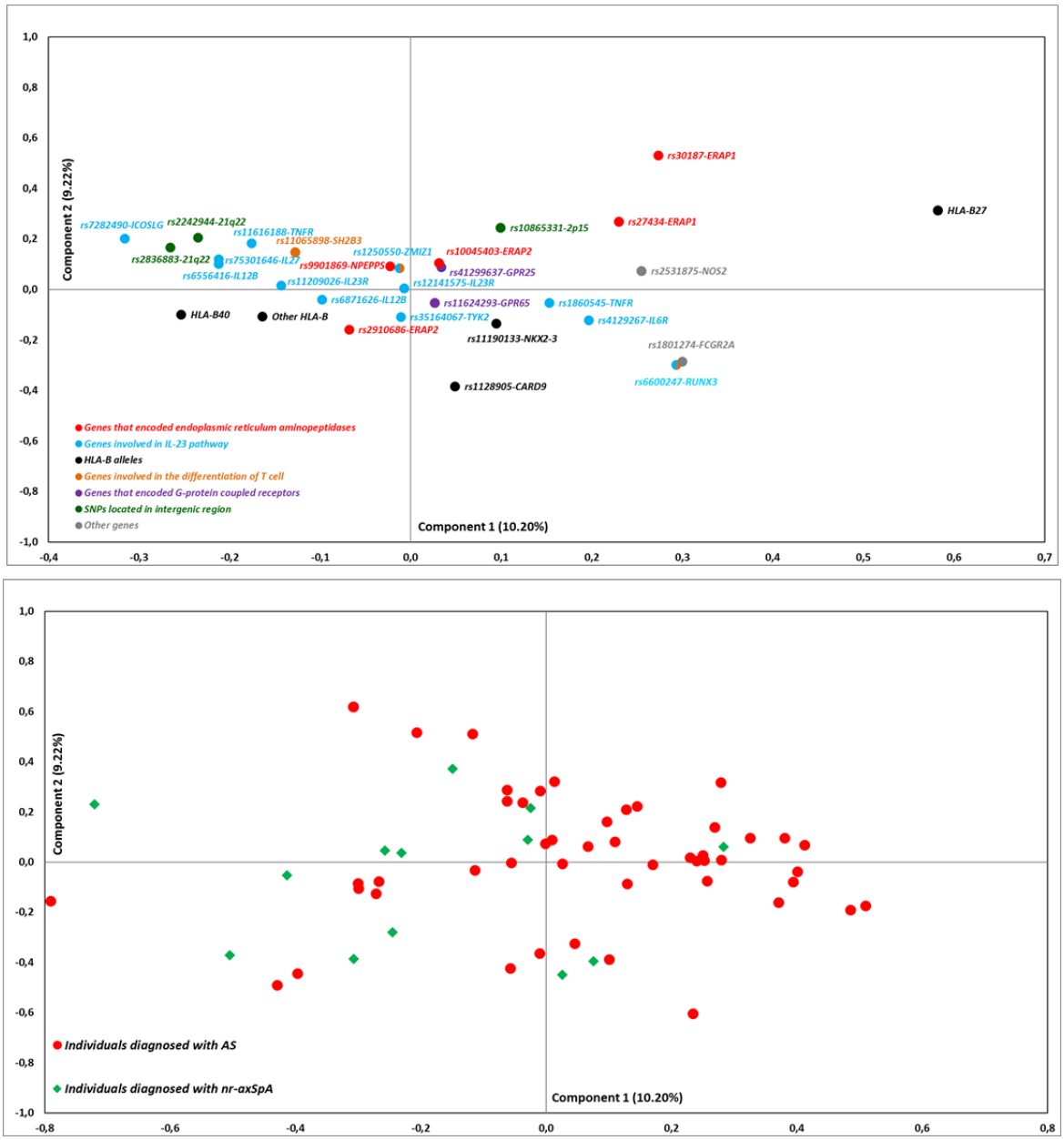

Figure 3. Principal component analysis of the genotypic frequency of the SNPs associated with the susceptibility to develop AS and of the alleles of *HLA-B* gene in the 62 individuals analysed. A) Distribution of the genetic variables analysed. B) Distribution of the patients. AS, ankylosing spondylitis; nr-axSpA, non-radiographic axial spondyloarthritis.

A PCA was carried out considering the genotypic frequencies of such markers in the individuals with AS and those with nr-axSpA, obtaining two principal components that explain 19.42% of the variance **(Figure 3A)**. In the first component (10.20% of the variance), the genetic markers with greater correlation were: HLA-B27 (HLA-B27, 0.582), rs7282490 (rs7282490-ICOSLG, -0.316) and rs1801274 (rs1801274-FCGR2A, 0.3). In the second component (9.22% of the variance), the variables with greater correlation were: rs30187 (rs30187-ERAP1, 0.531),

rs1128905 (rs1128905-CARD9, -0.383), HLA-B27 (HLA-B27, 0.314) and rs6600247 (rs6600247, RUNX3, -0.3).

When the 62 individuals were represented on the first two components of the PCA **(Figure 3B)**, there was no significant spatial distribution of the individuals of the two entities regarding the genotypic frequencies. On the other hand, there were some common genotypes of SNPs among the analysed individuals. Likewise, no common genetic pattern was found in the patients of each group, which indicates the existence of great intragroup variability.

Given that the presence of SNPs associated with the susceptibility to develop Ps and IBD has been described in patients with AS, which suggests the existence of a common pathogenetic mechanism, we analyzed in the patients of this study, 6 SNPs associated with the susceptibility to develop Ps and PsA and 9 SNPs associated with IBD. The analysis of the genotypic frequencies of these SNPs did not reveal any statistically significant difference between the two patient groups analyzed in the present study (AS and nr-axSpA).

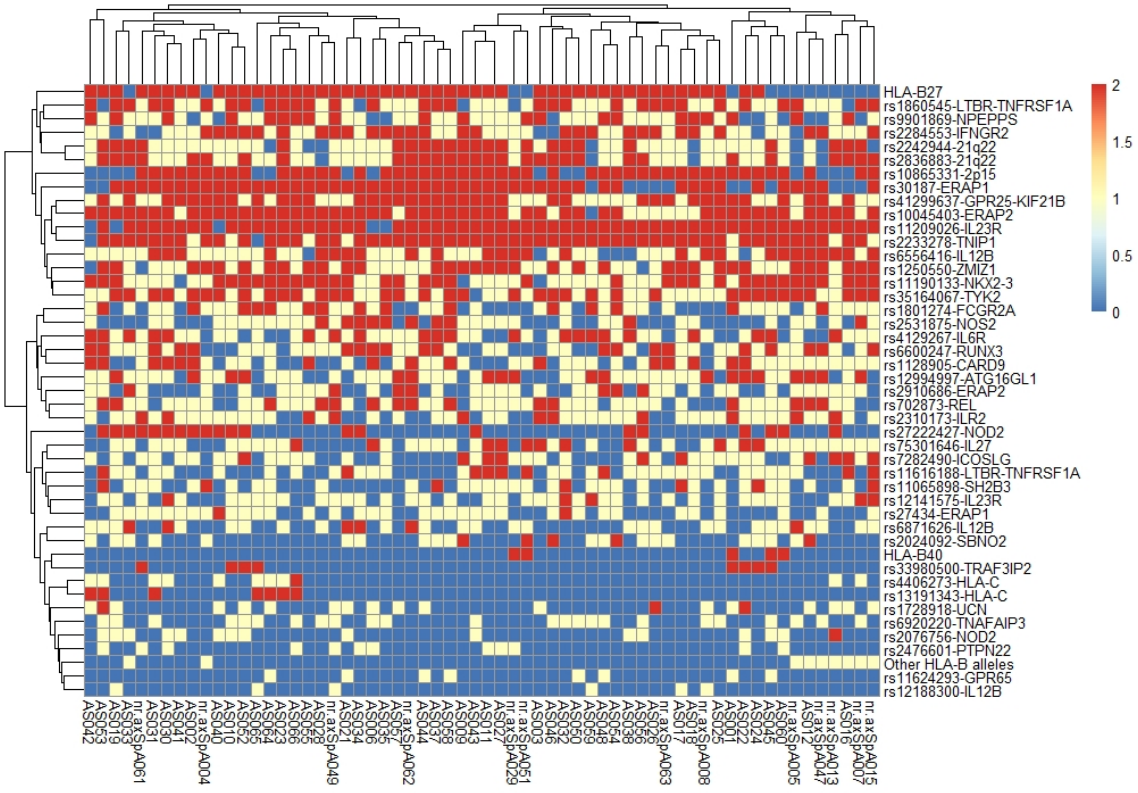

Figure 4. Heatmap of the genotypic frequencies of the SNPs associated with the susceptibility to develop AS, Ps, PsA and IBD in the patients diagnosed with AS and nr-axSpA analysed in the present study. Red: presence of the risk allele in homozygous or heterozygous patients when such allele is dominant; yellow: presence of the risk allele in heterozygous patients; blue: absence or presence of the risk allele in heterozygous patients

when such allele is recessive. AS, patients diagnosed with ankylosing spondylitis; nr.axSpA, patients diagnosed with non-radiographic axial spondyloarthritis.

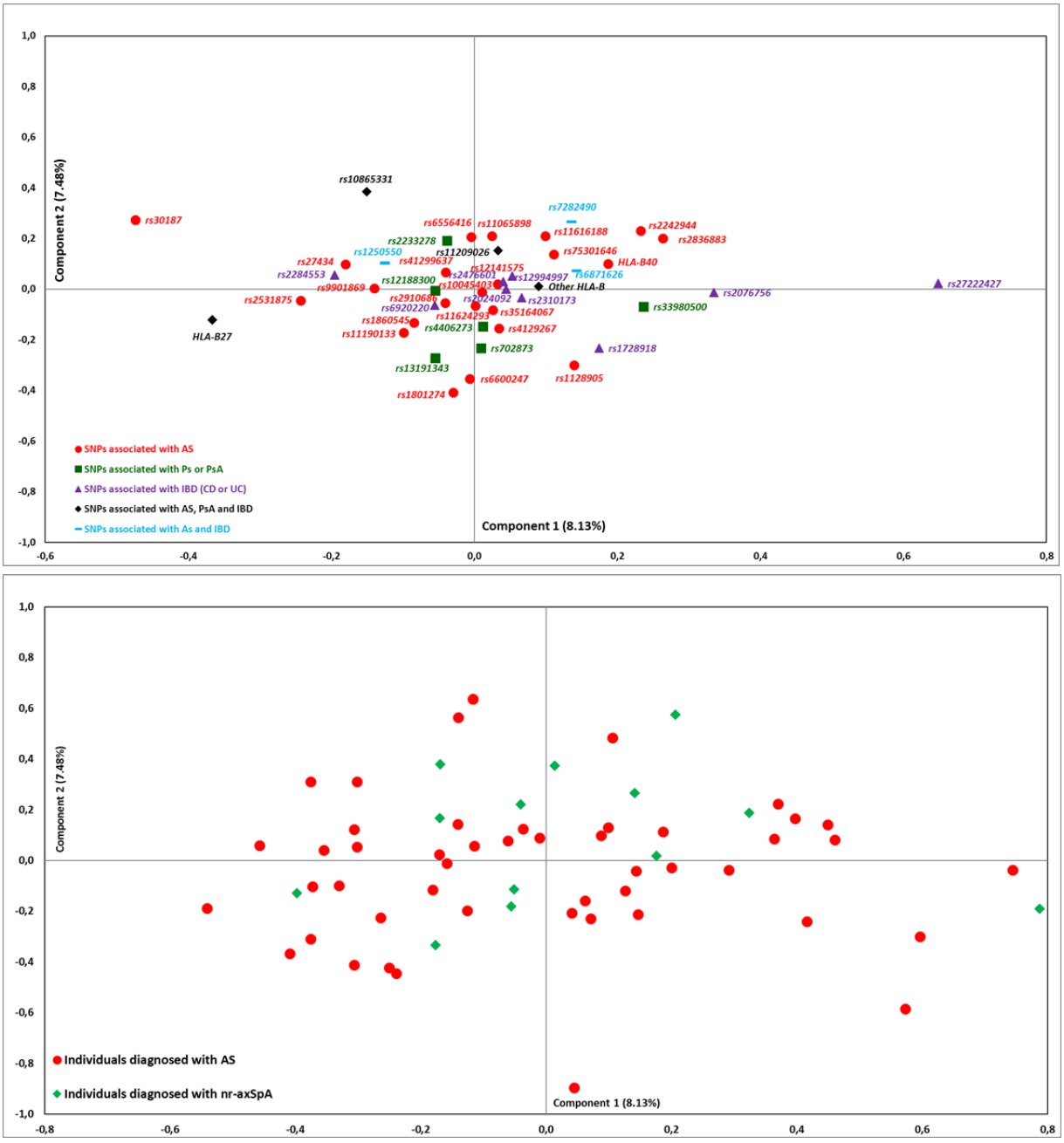

Figure 5. Principal component analysis of the genotypic frequency of the SNPs associated with the susceptibility to develop AS, Ps, PsA and IBD and the genotypic frequency of the alleles of gene *HLA-B* in the 62 individuals analysed. A) Distribution of the genetic variables. B) Distribution of the patients. AS, ankylosing spondylitis; nr-axSpA, non-radiographic axial spondyloarthritis.

A second PCA was conducted considering the genotypes of the SNPs associated with the susceptibility to develop AS, Ps, PsA and IBD, as well as the alleles found for the *HLA-B* gene,

obtaining two principal components that explain 15.61% of the variance **(Figure 5A)**. In the first component of this PCA (8.13% of the variance), the genetic markers with greater correlation were: rs27222427 (rs27222427-NOD2, 0.648), rs30187 (rs30187-ERAP1, -0.474), HLA-B27 (HLA-B27, -0.366) and rs2076756 (rs2076756-NOD2, 0.335). In the second component (7.48% of the variance), the variables with greater correlation were: rs1801274 (rs1801274-FCGR2A, -0.409), rs10865331 (rs10865331-2p15, 0.384), rs6600247 (rs6600247, RUNX3, -0.355) and rs1128905 (rs1128905-CARD9, 0.320).

When the 62 individuals were represented on the first 2 components **(Figure 5B)**, there were no patterns based on the genotypic frequencies of the SNPs associated with AS, Ps, PsA an IBD. Figure 5 shows what was already observed in the first PCA, regarding the genetic heterogeneity underlying these two entities. However, it is worth highlighting two variables that showed a higher correlation values with respect to axis 1; these are two SNPs of the *NOD2* gene (rs27221427 and rs2076756), which is a gene that shows a stronger association with CD and whose association with AS has not been described to date **(Figure 5A)**. Moreover, the combination of the risk genotypes of these two SNPs of the *NOD2* gene is present in 16 individuals, almost exclusively in the AS group (N=15, 30.61%), appearing only in one nr-axSpA individual (7.69%). These results suggest an association of these alleles with a greater radiographic progression of the disease, although no statistically significant differences were detected between the two groups (p_{value}>0.0001) due probably to the sample size.

Discussion

There is current debate on whether nr-axSpA is a different disease from AS[8,9], an early form of AS[10,11] or whether both are two expressions of the same disease[12,13]. In the present study, we analyzed the clinical and demographic characteristics, laboratory markers and SNPs associated with the susceptibility to develop AS, Ps, PsA and IBD in 62 patients of the University Hospital of Basurto (Bilbao, Spain), with the aim of determining whether these two entities, apart from sharing clinical characteristics, had a common genetic background.

It was observed that the patients with AS had a lower symptom onset age, shorter diagnostic time and longer duration of the disease compared to the patients with nr-axSpA **(Table 2)**. This could be due to the fact that AS can be more aggressive than nr-axSpA, with a faster radiographic progression, which would imply an earlier symptom onset and diagnosis compared to nr-axSpA. Regarding peripheral manifestations (arthritis, enthesitis, dactylitis), our data confirm those obtained in other studies related to the prevalence of enthesitis in the two patient groups[10-12]. However, the prevalence of peripheral arthritis and dactylitis was greater in AS patients[10-12] **(Table 2)**. With respect to extra-articular manifestations, we found a greater prevalence of uveitis

among the patients with AS, as has been reported in other studies[19], which could be due to the fact that uveitis is associated with the duration of the disease, which is longer in AS[62] **(Table 2)**. Nevertheless, the prevalence of Ps and IBD is greater among individuals diagnosed with nr-axSpA, which is in contrast to what has been reported in the literature to date[10-12] **(Table 2)**.

The BASMI index is statistically higher among individuals diagnosed with AS[11,12,25,32] **(Table 2)**, due to the decreased mobility of the axial skeleton as a consequence of the formation of syndesmophytes and even bone bonds and changes in the SIJs[26]. The BASFI and BASDAI indices, which measure the clinical activity, damage and functional deterioration, would be expected to be higher in individuals with AS, given their greater severity and progression[10,11,32]; however, in the present study, higher values of these indices were found in those individuals with nr-axSpA **(Table 2)**. In this study, high levels of CRP were more frequent in patients with AS **(Table 2)**, which could be due to the fact that axSpA patients with high CRP values tend to progress more rapidly to radiographic sacroiliitis[28].

Regarding the gender of the patients, our results confirmed the predominance of males among the individuals diagnosed with AS (77.55%) **(Table 2)**, which could be due to the fact that these patients have a faster progression than females, as well as more structural changes, which in turn, may cause a more severe disability[28]. Another possible influencing factor is physical activity, which, in the case of men usually involves greater mechanic stress, which would increase the inflammatory activity[63]. However, in the group of nr-axSpA patients, the proportion of women (46.15%) was very similar to that of men (53.85%) **(Table 2)**, as has been described in other studies conducted in European and North American cohorts[8,11,12]. The explanation suggested for the differential prevalence between the two entities in terms of gender is that women have a lower radiographic damage and a slower progression to a radiographic state, remaining in the non-radiographic form for longer periods of time[64], suggesting the possible differential role of hormones in the formation of new bone material in patients with SpA.

Some studies carried out in relatives and twins indicate that the genetic component has a fundamental role in the immunopathogenesis of SpA, although the primary trigger is still unknown[34]. Allele *HLA-B27*, which has been traditionally granted considerable relevance, could contribute to up to 25% of the total inheritance of AS[35]. In the present study, *HLA-B27* showed a significantly greater frequency among the patients with AS compared to those with nr-axSpA (87.8% *vs* 38.5%), which is in line with the results obtained in other studies[12,28,53-55] **(Table 2)**. Furthermore, this greater frequency of *HLA-B27* in the AS group confirms the hypothesis that associates the presence of *HLA-B27* with more severe radiographic damage, a lower onset age that characterize the AS and an earlier diagnosis[30,44] **(Table 2)**.

Apart from allele *HLA-B27*, 6 additional alleles of the *HLA-B* gene were found in the patients of this study (*HLA-B39*, *HLA-B40*, *HLA-B47*, *HLA-B55*, *HLA-B56* and *HLA-B07*). Alleles *HLA-B39*, *HLA-B40* and *HLA-B47* have been previously associated with AS[65-67] and, in the case of *HLA-B39*, with PsA[68]. However, allele *HLA-B07* is associated with a decrease in the risk of developing AS[67] and alleles *HLA-B55* and *HLA-B56* are not associated with AS. Although the proportion of individuals with other alleles of the *HLA-B* gene different from *HLA-B27* is greater in the group with nr-axSpA with respect to the AS group (61.5% *vs* 12.2%), both entities share some alleles (*HLA-B40*, *HLA-B55* and *HLA-B56*). According to these data, it can be stated that there is a predominance of allele *HLA-B27* in patients with AS (87.8%); however, the patients with nr-axSpA showed a greater heterogeneity regarding the alleles of the *HLA-B* gene. Nevertheless, patients with axSpA can have alleles of the *HLA-B* gene associated or unassociated with AS, or even AS-protective *HLA-B* alleles.

GWAS studies have revealed a considerable number of genes or gene regions that contribute to the susceptibility to develop AS. In our study, we analyzed the allele frequencies of 28 risk SNPs, and found no statistically significant differences between the AS and nr-axSpA patients. It was observed that 75% of the individuals of both groups shared a large number of risk alleles (60%) **(Figure 1)**. These results indicate that both pathological entities have a common genetic background, at least at the level of risk SNPs.

At the genotype level, no statistically significant differences were found between the two groups of patients for risk SNPs located in genes involved in the pathogenesis of AS. Some SNPs showed similar genotypes in most of the individuals of the two groups, e.g., *ERAP1* (rs30187), *ERAP2* (rs10045403), *IL-23R* (rs11209026), *GPR25* (rs41299637), and in intergenic region 2p15 (rs10865331) **(Figure 2)**. Outside of the MHC, it is worth highlighting the *ERAP1* gene for its strong association with the susceptibility to develop AS. *ERAP1* is an aminopeptidase involved in the cleavage of peptides to a length of 8-9 amino acids, in order for the new peptide segments to be presented by MHC class I molecules, such as *HLA-B27*[47]. The SNPs located in the *ERAP1* gene only appear to be associated with AS in *HLA-B27*(+) and *HLA-B40*(+) patients [65,67]. The *ERAP2* is another aminopeptidase, whose association with AS has been described in *HLA-B27*(+) and *HLA-B27*(-) patients[49]. The high frequency of risk SNPs of the *ERAP-1* and *ERAP-2* genes found in the analyzed patients could suggest the alteration of the correct functioning of both genes[69], thus influencing the supply of optimum peptides for MHC class I molecules, such as *HLA-B27*, which could explain their relationship with the development of SpA[48,70].

The *IL-23R* gene codes for *IL-23* receptor. *IL-23* is a key cytokine involved in the differentiation of naive $CD4^+$ T-lymphocytes into Th17 lymphocytes, which produce *IL-17*, *IL-6*, *IL-22*, *TNF-α* and other similar proinflammatory cytokines. Furthermore, other genes of the *IL-23* route have been associated with AS, which shows that this route is an important pathway in the pathogenesis of AS[46,48,49]. There are polymorphisms of *IL-23R*, such as rs11209026, associated with AS[46], IBD[71], Ps[72] and PsA[73]. The A variant of this SNP leads to a nonsynonymous substitution of amino acid residue R381Q, which considerably weakens *IL-23R* function and the production of several proinflammatory cytokines[74]. Besides, R381Q can exert a protective effect against AS inflammation and development[74]. In the present study, the G variant of rs11209026 showed a very high frequency among the patients of two groups (AS and nr-axSpA), **(Figure 2)**. These results confirm the evidence that *IL-23* and its entire pathogenic route are involved in the susceptibility to develop axSpA, granting inflammation a main role in the triggering of the disease.

GWAS studies have shown the association of SNPs of intergenic region 2p15 (rs10865331) with AS, although their role in the pathogenesis of AS is unknown. It has been hypothesised that this region has non-coding RNA species or protein-coding genes unknown to date, which could be involved in the susceptibility to develop AS[47]. G-protein coupled receptor 25 (*GPR25*), which is associated with AS[49] is strongly expressed in memory T-cells and NK-cells and involved in the positive regulation of B-cell proliferation[75], suggesting a potential mechanism in different autoimmune diseases. Although it is still unknown how the SNPs located in 2p15 (rs10865331) and *GPR25* (rs41299637) may influence the pathogenesis of AS, their high prevalence in our study could suggest a key role in the onset and development of axSpA **(Figure 2)**.

The results obtained for SNPs located in genes (*ERAP1*, *ERAP2*, *IL-23R*, *GPR25)* and gene regions (2p15), along with the different *HLA-B* gene subtypes, demonstrate their importance in the pathogenesis of axSpA, probably with an essential role in the onset of both AS and nr-axSpA. Despite the fact that the individuals who suffer from different axSpA have common genotypes for some SNPs of genes that are important in the pathogenesis of AS, we observed great genetic heterogeneity within each group and between the two pathological entities (AS and nr-axSpA), which demonstrates the complex nature of this type of diseases **(Figure 3)**.

Moreover, given that AS, IBD and Ps could have a common pathogenic mechanism, we analysed 15 SNPs associated with the susceptibility to develop IBD, Ps and PsA, finding no statistically significant differences for any of these SNPs between the two groups of patients (AS and nr-axSpA). However, the combination of the risk genotypes of 2 SNPs located in the *NOD2* gene associated with CD was found mainly in individuals with AS (AS: 30.7% ***vs*** nr-axSpA: 7.7%), which could indicate that these two SNPs are associated with a greater progression of this disease

(Figures 4 and 5). The *NOD2* gene was identified as the most relevant risk factor of CD[76], although its association with AS had not been described to date. *NOD2* is an intracellular pathogen recognition receptor[77] and plays a role in the immune response to bacterial lipopolysaccharides, since it regulates the response of Th17 cells for the elimination of bacteria by inducing the secretion of cytokines *IL-23* and *IL-1B*, which appear in individuals with CD who have a mutation in *NOD2*[78]. The results suggest that individuals with these two SNPs of the *NOD2* gene will be at greater risk of developing intestinal lesions characteristic of IBD throughout their lives **(Figure 4)**. Furthermore, the presence of these and other SNPs located in genes associated with AS and IBD would confirm the hypothesis about the relevance of intestinal dysbiosis in the genesis of axSpA[20].

Between 20 and 25% of the known inheritance of AS is attributed to allele *HLA-B27*, and 3-7% to SNPs identified in GWAS studies[35,36], thus approximately 70% of the inheritance of this pathology could be related to genetic variants that have not been described to date. Missing heritability is a common issue in complex genetic diseases, and it may be caused by multiple factors. It has also been suggested that general heritability is overestimated, with the probability that epigenetic factors, especially environmental factors (smoking and obesity), may be more relevant in the susceptibility to develop AS.

Smoking is an important environmental factor in the inflammatory process of rheumatic diseases, including SpA, and it is a risk factor of cardiovascular disease. In the present study, 66.1% of the patients were smokers or ex-smokers, with a higher proportion of smokers in the individuals diagnosed with AS (69.4%) with respect to those diagnosed with nr-axSpA (53.9%) **(Table 2)**. This suggests that smoking could contribute to increasing the progression to the radiographic form of SpA[29], promoting spine damage and an earlier onset of the inflammatory back pain, which characterizes AS[29].

Obesity and overweight are an increasing issue worldwide due to their influence on the development of metabolic, cardiovascular and rheumatic diseases, which increases the mortality and morbidity of patients[79,80]. In our study, it was observed that 61.3% of the patients were obese (BMI>29.99 kg/m^2) or overweight (BMI>24.99 kg/m^2), with BMI and the prevalence of obesity being similar between patients with AS and those with nr-axSpA **(Table 2)**. Excess adipose tissue in overweight and obese individuals may have immunomodulating properties that affect the course of the disease[81], which could be associated with an increase in the production of proinflammatory cytokynes[80]. Previous studies have reported a greater prevalence of obesity and overweight[81] and a significantly higher BMI in AS patients compared to the healthy population[83]. It has been described that overweight and obese patients with AS have greater functional

limitations, greater activity of the disease and a lower response to anti-*TNF-α* therapies[81,82,84], which would contribute to increasing physical inactivity and, thus, gaining weight[83,84]. The high prevalence of smoking and obesity or overweight among the individuals analyzed in the present study would suggest the relationship between these life habits and the development of these diseases, with inflammation playing a key role in their triggering.

Conclusions

In the SNPs analyzed in the present study, we observed that AS and nr-axSpA have a common genetic background associated with the pathogenic development of these diseases; therefore, from the genetic perspective, it could be suggested that the two entities share the main pathogenic pathways described for AS. However, the pathogenesis of autoimmune diseases, such as SpA, is very complex, and it is the result of intricate mechanisms interaction where the genetic and environmental factors are involved. Among the genetic factors, this study shows the importance of genes and intergenic regions involved in the pathogenesis of AS, such as *HLA-B27*, *ERAP1*, *ERAP2*, *IL-23R*, *GPR25* and 2p15, whose role may influence the onset, development and severity of the disease. Nevertheless, we also observed great genetic heterogeneity among individuals who had the same clinical diagnosis, which could indicate the involvement of exogenous factors in the triggering of the disease, thus patients with different genotypes would have the same pathogenic phenotype. The most relevant of these external factors are: infections by pathogens that alter the intestinal microbiota, endocrine alterations such as overweight and obesity, and unhealthy life habits (e.g., smoking). Numerous studies have granted great relevance to the genetic component in the development of AS; however, this relevance may be overestimated, since environmental factors could be more important than reported to date in the triggering of the disease. Our results should be confirmed in larger observational studies with a larger nr-axSpA population.

Supplementary material

Locus	Gene	SNP	Associated disease
1p13	*PTPN22*	rs6679677	IBD, CD[22]
1q21	*IL6R*	rs4129267	AS[49]
1q23	*FCGR2A*	rs1801274	AS[49]
1p31	*IL23R*	rs11209026	AS, Ps, PsA, IBD[22,48,49,73,85-87]
1p31	*IL23R*	rs12141575	AS[49]
1q32	*GPR25-KIF21B*	rs41299637	AS[49]
1p36	*RUNX3*	rs6600247	AS[49]
2q11	*IL1R2*	rs2310173	IBD, UC[87]
2p15	Intergenic	rs10865331	AS, Ps, IBD[22,47,48,89]
2p16	*REL*	rs702873	Ps, PsA[85,90]
2p23	*UCN*	rs1728918	IBD, CD[22]
2q37	*ATG16L1*	rs12994997	IBD, CD[22,91]
5q15	*ERAP1*	rs27434	AS[47]
5q15	*ERAP1*	rs30187	AS[48,49]
5q15	*ERAP2*	rs2910686	AS[49]

5q15	*ERAP2*	rs10045403	AS[49]
5q33	*IL12B*	rs6556416	AS[48,49]
5q33	*IL12B*	rs6871626	AS[49]
5q33	*IL12B*	rs12188300	Ps, PsA[89,92]
5q33	*TNIP1*	rs2233278	Ps[89]
6p21	*HLA-B27*	rs116488202	AS[49]
6p21	*HLA-C*	rs4406273	Ps[89,93]
6p21	*HLA-C*	rs13191343	PsA[88]
6q21	*TRAF3IP2*	rs33980500	Ps, PsA[89,90,92,93]
6q23	*TNAFAIP3*	rs6920220	IBD, UC[22,91]
9q34	*CARD9*	rs1128905	AS[49]
10q22	*ZMIZ1*	rs1250550	AS, IBD[49,94]
10q24	*NKX2-3*	rs11190133	AS[49]
12p13	*LTBR-TNFRSF1A*	rs1860545	AS[49]
12p13	*LTBR-TNFRSF1A*	rs11616188	AS[48]
12q24	*SH2B3*	rs11065898	AS[49]
14q31	*GPR65*	rs11624293	AS[49]
16p11	*IL27*	rs75301646	AS[49]
16q12	*NOD2*	rs2076756	IBD, CD[86,94]
16q12	*NOD2*	rs17221417	IBD, CD[88]
17q11	*NOS2*	rs2531875	AS[49]
17q21	*NPEPPS-TBKBP1-TBX21*	rs9901869	AS[49]
19p13	*TYK2*	rs35164067	AS[49]
19p13	*SBNO2*	rs2024092	IBD, CD[22,91]
21q22	Intergenic	rs2242944	AS[47]
21q22	Intergenic	rs2836883	AS[49]
21q22	*ICOSLG*	rs7282490	AS[49]
21q22	*IFNGR2*	rs2284553	IBD, CD[22,91]

Supplementary table. SNPs associated with ankylosing spondylitis (AS), psoriasis (Ps), psoriatic arthritis (PsA) and inflammatory bowel disease (IBD), Crohn disease (CD), ulcerative colitis (UC).

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Author contributions

All authors were involved in drafting the manuscript or revising it critically for important intellectual content, and all authors approved the final version to be published. IML, MH, JMBM, EG, NAR, MLGV and CdR had full access to all of the data in the study and take responsibility for the integrity of the data. IML, MH and CdR carried out the statistical analysis and the accuracy of the data analysis. IML, MH, MLGV and CdR contributed to the study conception and design. IML, MH, JMBM, EG, NAR, MLGV and CdR contributed to the analysis and interpretation of data.

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Ethics declarations

Ethics approval and consent to participate

This study was conducted with the approval of the Drug Research Ethics Committee of Euskadi (CEImE, PI2017141). All patients gave their signed informed consent to participate in the study.

Consent for publication

No individual person´s data are present in this manuscript. All data are completely anonymized. All patients gave their signed informed consent to participate in the study.

Competing interests

The author declares that they have no competing interests.