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ACTN3 and *Vitamin D receptor* polymorphisms impact in muscle physical properties of Spondyloarthritis patients

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Mestrado em Biologia Molecular e Genética

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2020

Agradecimentos

Em primeiro lugar não posso deixar de agradecer ao meu orientador, Professor Doutor Fernando Pimentel-Santos, não só por me ter acolhido de braços abertos no seu grupo, mas principalmente pela sua preocupação, ajuda, disponibilidade e por arranjar sempre tempo mesmo quando não o tinha. E acima de tudo por me ter mostrado o que é ser uma excelente pessoa, mas também um excelente profissional e orientador.
O meu agradecimento também à Professora Susana Silva que aceitou colaborar connosco e, mesmo não sendo oficialmente minha orientadora, acolheu-me como se fosse sua aluna. Muito obrigada por todo o tempo e trabalho extra que teve comigo. Foram ambos, o meu apoio, às vezes até mais que o meu braço direito, foram as minhas pernas para que esta tese chegasse ao fim. Não poderia ter escolhido melhores orientadores para me acompanharem ao longo deste ano.

Tenho também de agradecer ao meu segundo par de mãos, Hugo Mateus, que partilhou toda esta experiência comigo.
O meu obrigada à Dra. Helena Donato da Universidade de Coimbra e a todos os membros da equipa do Professor Fernando Pimentel-Santos que trabalham/trabalharam no projecto MyoSpA na recolha dos dados clínicos e pela sua partilha, sem os quais esta tese não poderia ter sido realizada.

Agradeço também ao CEDOC por me ter acolhido e à minha orientadora interna, Professora Margarida Gama-Carvalho.

Por último, mas igualmente ou mais importantes, cada um à sua maneira, agradeço aos meus pais e aos meus irmãos por tudo. Um tudo que engloba isso mesmo.
E claro, àqueles que não o sendo, se tornaram família ao longo destes anos todos: Carolina Bulhosa, Sofia Silvestre, Rita Sousa e João Marques.

“50%”

Abstract

Background: Spondyloarthritis is the most common group of chronic inflammatory rheumatic disease affecting about 1.5% of the Portuguese adult population. The most common symptoms are low back pain and enthesitis. Spondyloarthritis is a multifactorial disease with well-known genetic contribution. However, other factors such environment and physical components, might contribute for SpA susceptibility and progression. Muscle physical properties may be involved in the physiopathology process. *ACTN3* and *Vitamin D Receptor* genes seems to influence several muscles properties.

Objectives: Analyse the relation between the most studied polymorphisms of these genes and disease susceptibility/ phenotype and influence in muscle properties.

Methods: Twenty-eight patients with axial Spondyloarthritis (axSpA) and 28 healthy controls (HC) were studied. Clinical, epidemiological and muscle characterization data (strength, stiffness, tone, elasticity, mass, and performance) were collected. *ACTN3* (*ACTN3 R577X*) and *Vitamin D Receptor* gene (*FokI, TaqI* e *ApaI)* single nucleotide polymorphisms were studied. Statistical analysis was performed using SPSS software, version 22.0.

Results: 51 individuals were analysed, 27 of which axSpA patients, 66.7% male with mean age of 36 years old. From total patients, 80.8% were HLA-B27 positive, 18.5% with BASDAI $\geq$ 4, 14.8% with BASFI > 4 and 88.9% showing a BASMI <3. HC and patients' characteristics were very similar. Two different muscles were analysed- Multifidus and Gastrocnemius. Muscle physical properties (tonus, stiffness, and decrement), muscle strength and muscle mass were similar, but patients have shown a decrease in muscle performance. None of the studied SNPs have shown any association with disease susceptibility/ phenotype (BASDAI, BASFI, BASMI), muscle physical properties, strength, or mass. *ACTN3 R577X* and *VDR FokI* seems to be associated with muscle performance.

Conclusion: *ACTN3 R577X* and *VDR FokI* seems to be associated with muscle performance. Since this was a pilot-study, further research is needed to obtain a definitive conclusion.

Key words: Spondyloarthritis, Muscle properties, *ACTN3* gene, *Vitamin D Receptor* gene

Resumo[1]

Este estudo faz parte de um projecto actualmente em curso, "MyoSpA Study", que tem por objectivo o estudo do músculo em doentes com Espondiloartrite (SpA).

A SpA é uma doença etiologicamente multifactorial, consistindo num conjunto de doenças reumáticas inflamatórias crónicas, que afecta cerca de 1,5% da população adulta portuguesa.

Esta doença é caracterizada por inflamação das enteses (entesite), o que a torna distinta de outros tipos de artrites. A presença de dor na coluna vertebral e na articulação sacroilíaca acompanhadas por dor na região lombar inferior constituem as características mais comuns da doença. A SpA pode também afectar as articulações mais periféricas, tais como as pequenas articulações das mãos e dos pés assim como as articulações dos ombros e joelhos. Na maioria dos casos existe um envolvimento das enteses (especialmente do tendão de Aquiles e da inserção da fáscia plantar) e dos sistemas extra- articulares (olhos, pele e intestino).

A sua progressão e gravidade conduzem a um aumento da rigidez muscular com consequente redução na mobilidade. Paralelamente, os processos de erosão e osteogénese com formação de sindesmófitos, com consequente repercussão a nível da coluna vertebral e da articulação sacroilíaca, condicionam uma redução da mobilidade de forma irreversível.

Em 1973, Caffrey e James descreveram o alelo HLA-B27 como sendo o principal gene responsável pela susceptibilidade genética para a Espondilite Anquilosante, actualmente designada por Espondiloartrite axial radiográfica (r-axSpA). Mais recentemente, estudos de associação genómica de larga escala ("GWAS studies") permitiram a identificação de inúmeros SNPs em vários genes, relacionados com a SpA. Contudo, e apesar de todos os esforços, estes apenas conseguem explicar uma pequena percentagem da predisposição genética e do fenótipo da doença.

Tendo isto em conta, os factores ambientais e processos biomecânicos, começaram a ser estudados demonstrando a sua relação com algumas características fenotípicas, susceptibilidade, desenvolvimento e no prognóstico da SpA. A aplicação da tecnologia de "*next generation sequencing*" (NGS) no microbioma intestinal, revelou a presença de disbiose, ou seja, a existência de alterações na microbiota intestinal, como sendo uma característica comum tanto à SpA como a doenças inflamatórias intestinais. Esta relação é suportada por vários estudos em modelos animais, em que se avalia o efeito do ambiente "normal" *vs* "germ-free" em ratos que sobre expressam HLA-B27. Mais recentemente, factores biomecânicos, como a existência de microtraumas (intrínsecos ou extrínsecos) recorrentes, têm vindo a revelar um impacto importante na presença de inflamação crónica assim como no surgimento e progressão da espondiloartrite. Mechelen e Lories, num estudo publicado em 2016, recorrendo a ratinhos susceptíveis para a SpA e com sobre-expressão de factor de necrose tumoral, demonstraram que quando estes se encontravam suspensos pela cauda, ou pelas patas posteriores, não desenvolviam a doença. Tendo concluído que danos localizados/ microtraumas poderiam estimular o surgimento da SpA e ter um impacto considerável na progressão da doença. Adicionalmente, alguns autores têm vindo a defender que as propriedades físicas musculares poderão contribuir de forma significativa para a susceptibilidade e progressão desta doença, uma vez que doentes com SpA parecem apresentar elevados valores de rigidez muscular nos músculos da região axial quando comparados com controlos saudáveis.

[1] A introdução não segue o novo acordo ortográfico

De forma complementar, a sarcopenia pode ser um factor igualmente importante desde os estadios iniciais contribuindo para um diagnóstico mais grave da doença.

Tendo em conta as características musculares e o seu impacto, o presente estudo teve por objectivo analisar a existência de uma relação entre os polimorfismos mais estudados dos genes do *VDR* (Receptor para a Vitamina D) e do *ACTN3*, e o seu potencial impacto a nível do músculo. Adicionalmente, foi estudada a correlação entre estes polimorfismos e alguns parâmetros musculares testados: características físicas musculares, força muscular, massa muscular e a performance muscular- a nível dos músculos Multífidos, localizado na região lombar e do músculo Gastrocnémios, localizado na região posterior da perna.

Foram então analisados quatro SNPs diferentes, um no gene *ACTN3 (ACTN3 R577X*) e três no gene *VDR* (*FokI, TaqI* e *ApaI).*

O gene *ACTN3* é um gene altamente estudado, principalmente em atletas de alta competição, uma vez que a sua variante é responsável por uma melhor performance global, com aumento da força e da velocidade. Tal deve-se ao facto deste gene provocar uma alteração no tipo de fibra muscular, convertendo as fibras tipo I (fibras de contração lenta) em fibras tipo II (fibras de contração rápida).

O gene *VDR* foi também estudado uma vez que a vitamina D para além dos processos mais conhecidos em que está envolvida, como sendo a regulação da homeostasia do cálcio e o metabolismo ósseo, parece possuir também extrema importância no desenvolvimento e remodelação do músculo-esquelético. Qualquer variação indesejada tanto no receptor como na proteína em si poderão ter graves efeitos sistémicos. A deficiência em vitamina D ou quando em níveis extremamente baixas pode ser responsável por certas doenças sendo exemplos a osteomalacia e o raquitismo. É também responsável por um aumento do risco de quedas com consequentes fraturas e aumento de comorbidades.

No presente estudo foram incluídos 56 indivíduos (28 indivíduos saudáveis e 28 doentes com Espondiloartrite axial (axSpA)) agrupados de acordo com o género, idade, nível de atividade física. Contudo, devido à existência de alguns dados em falta, nomeadamente a nível de genotipagem, apenas 51 indivíduos reuniram todos os dados necessários de modo a ser possível a sua análise (27 doentes com espondiloartrite axial e 24 controlos saudáveis).

Dos 27 doentes com axSpA, 66,7% são do género masculino com idade média de 36 anos. Do total de doentes, 80,8% são HLA-B27 positivos, 18,5% apresentam um BASDAI $\geq$ 4, 14,8% apresenta um BASFI > 4 e 88.9% um valor de BASMI <3, traduzindo uma população de doentes com baixa atividade da doença e baixa repercussão funcional. As duas populações (controlos e doentes) são bastantes semelhantes tanto a nível de características físicas como epidemiológicas.

Adicionalmente, foram estudadas algumas características físicas musculares como sendo o tónus muscular, rigidez, "decrement" (inverso da elasticidade), força (teste "*Sit-to-stand 5*"), e massa magra (obtida por bioimpedância), dos dois músculos situados em segmentos corporais distintos, não revelaram alterações. Os doentes mostraram, porém, um compromisso da performance muscular (avaliada por dois testes distintos: "*Sit-to-stand 60*" e velocidade da marcha). Adicionalmente, nenhum dos SNPs dos genes *ACTN3* e *VDR* revelaram uma associação com a susceptibilidade ou interferência nos parâmetros clínicos específicos da espondiloartrite axial (BASDAI, BASMI e BASFI).

Podemos assim concluir, que os factores genéticos em estudo, não são suficientes *per si* para explicar as diferenças nas propriedades musculares entre indivíduos saudáveis e doentes com espondiloartrite

axial. Coloca-se a hipótese de que a sua conjugação com outros factores genéticos associados à existência de microtraumas recorrentes ou alterações na microbiota intestinal (factores intrínsecos), tenham, em conjunto com o genótipo, um papel mais preponderante na susceptibilidade, progressão e prognóstico desta doença.
Este estudo trata-se de um estudo piloto, com uma amostra de tamanho reduzido, sendo assim imprescindível a realização de mais estudos de forma a obter robustez nos resultados e uma conclusão mais definitiva.
Seria ainda importante, dar continuidade a este projecto, com a análise do polimorfismo *BsmI* do gene do *Receptor da Vitamina D*, e confirmação da existência de um haplótipo, descrito na literatura, assim como a quantificação desta vitamina e a sua correlação com as propriedades musculares.
O melhor conhecimento destes mecanismos poderia contribuir para uma melhor avaliação e acompanhamento dos doentes com espondiloartrite axial.

Palavras-chave: Espondiloartrite, Propriedades físicas musculares, Polimorfismos, gene *ACTN3*, gene *VDR*

General contents

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Abbreviations list

A- Adenine nucleotide base

a.a residue- Amino acid residue

ACE- Angiotensin I Converting Enzyme

ACTN3- Actinin alpha 3

AS- Ankylosing Spondylitis

ASAS- Assessment of Spondylarthritis International Society

axSpA – Axial Spondyloarthritis

BASDAI- Bath Ankylosing Spondylitis Disease Activity Index

BASFI- Bath Ankylosing Spondylitis Functional Index

BASMI- Bath Ankylosing Spondylitis Metrology Index

bDMARDs- Biological Disease-modifying anti-rheumatic drugs

BIA- Bioelectric impedance analysis

BMI- Body mass index

C- Cytosine nucleotide base

CI- Confidence interval

CRP- C- reactive protein

CT- Computed tomography

dH20- Distilled water

DMARDs- Disease-modifying anti-rheumatic drugs

DNA- Deoxyribonucleic acid

DXA- Dual- energy X- ray absorptiometry

EWGSOP- European Working Group on Sarcopenia in Older People

FAM- Fluorescein amidite

G- Guanine nucleotide base

GWAS- Genome-wide association study

HC- Healthy Control group

HLA – Human Leukocyte Antigen

HWE- Hardy-Weinberg equilibrium

I- Isoleucine

IBP- Inflammatory back pain

IL-17- Interleukin 17

IL-23- Interleukin 23

IPAQ- International Physical Activity Questionnaire

K- Lysine

LNPEP- Leucyl Cystinyl Aminopeptidase

M.D- Muscle decrement (inverse of elasticity)

M.F- Muscle tonus

M.S- Muscle stiffness

MRI- Magnetic resonance imaging

NFQ- Non- fluorescent quencher

NPEPPS- Aminopeptidase Puromycin Sensitive

NSAIDs- Non-Steroidal Anti-Inflammatory Drugs

pB- Pair base

PBS- Phosphate buffered saline

pSpA- Peripheral Spondyloarthritis

R- Arginine

r-axSpA- Radiographic axial Spondyloarthritis

rs- Reference SNP ID number

rt- Room temperature

SD- Standard deviation

SNP- Single nucleotide polymorphism

SpA- Spondyloarthritis

SPPB- Short Physical Performance Battery test

ST- Sit-to-stand test

T- Timine nucleotide base

TE buffer- Tris-EDTA buffer

TNFα- Tumor necrosis factor alfa

TNFRSF- Tumor necrosis factor receptor superfamily

TRADD- Gene that encodes tumor necrosis factor receptor type 1

VDR- Vitamin D (1,25- dihydroxy vitamin D3) receptor

VIC- Victoria probe

Vitamin D- Vitamin D (1,25- dihydroxy vitamin D3)

Introduction

Spondyloarthritis (SpA), is one of the most common group of chronic inflammatory rheumatic disease, affecting about 1.5% of the Portuguese adult population[1–4]. In this context musculoskeletal inflammation of the entheses (enthesitis), i.e the specialized, predominantly extra-articular, localized structures where occurs the insertion of connective tissues such as tendons, ligaments, or joint capsule into the bone, is what makes this type of arthritis distinguishable from the remaining. Achilles tendon and the plantar fascial insertions are the most commonly affected [2,5,6] (**figure 1**).

Tendon/ligament-to-bone entheses play a critical role in the normal function of the musculoskeletal system as this structure allows the transmission of muscle contractile forces into skeletal attachment site and participate in the dissipation of force from the enthesis itself, from tendon into bone [2].

The entheses have a unique immune microenvironment that can be activated trough the combination of several factors (such as mechanical stress, genetic predisposition, and microbiota immune activation triggering), activating prostaglandin E2 release and IL-23-IL17axis [6]. This phenomenon recruits an influx of innate immune cells, promoting entheses inflammation, followed by mesenchymal tissues responses and osteogenesis [6].

These SpA histopathological characteristic distinguish clearly these disease from other types of arthritis [2,3,7].

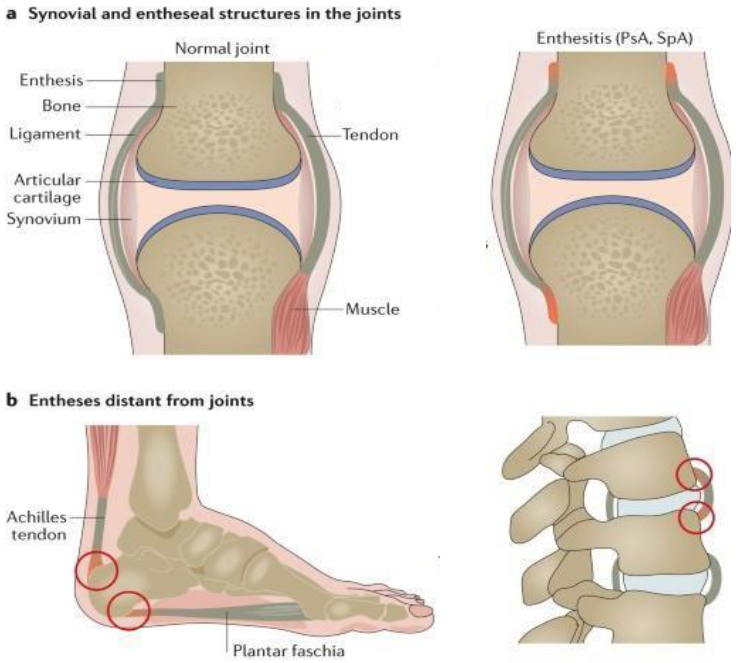

Figure 1- Enthesis anatomic localization and enthesitis. Red circles show entheses inflammation areas. Adapted from (6).

SpA is typically characterized by inflammation of the spine and sacroiliac joints accompanied by pain, stiffness and, in late stages, by reduced mobility. The involvement of peripheral joints (small joints of hands and feets, shoulders, coxo-femoral, knees,…), enthesis and extra-articular systems as eyes (acute anterior uveitis), skin (psoriasis) and gut (inflammatory bowel diseases as Crohn disease or ulcerative colitis) are also common [1,3].

I. Clinical features and radiographic hallmarks

Among the disease characteristic symptoms, inflammatory low back pain is one of the most relevant reported to physicians, affecting about 70% - 80% of patients [5,8] Typically, appears associated with morning stiffness, improving with activity, and gets worse with rest. Usually pain is associated to a good response to non-steroidal anti-inflammatory drugs (NSAIDs) [5].

Throughout the course of the disease, SpA patients may experience some decrease of spinal mobility, loss of lumbar and cervical lordosis and progressively acquire kyphotic deformities. These limitations are initially due to some rachis and sacroiliac joints (sacroiliitis) inflammation and muscle spasm, however, over time begins a process of osteogenesis (new bone formation) with syndesmophytes of the anterior longitudinal ligament and the outer fibres of the annulus fibrosis of intervertebral discs. Later on, the rachis and sacroiliac joints suffers a process of ankylosis. The supra mentioned alterations, syndesmophytes and sacroiliitis, constitute the disease radiographic hallmark of SpA (**figure 2** and **3**) [5,8,9].

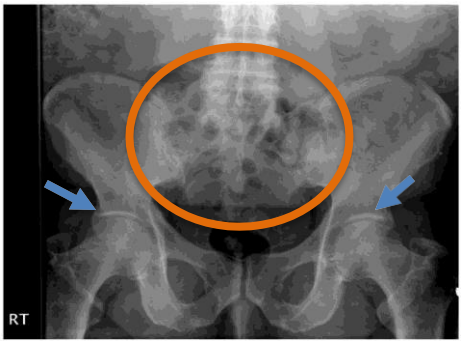

Figure 2 - Radiographic image of the pelvis. Blue arrows indicate a clear coxo- femural joint space narrowing. Orange circle indicate an area of sclerosis, erosion and ankylosis of the sacroiliac joint. Adapted from ref. (5).

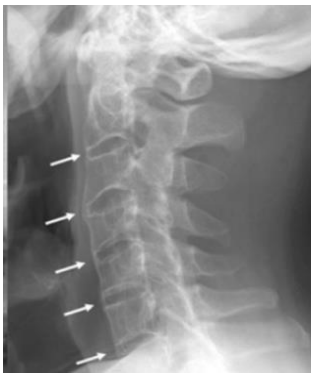

Figure 3- Radiographic image of cervical spine. Exhibition of extensive formation of vertical syndesmophytes that have bridge the anterior vertebral corners causing ankylosis (arrows). Adapted from ref. (9).

II. Spondyloarthritis classification system

Even though SpA is a condition itself, depending on the clinical and radiological findings, the following subsets have been defined [1,3,10]:

Traditional SpA classification system[11]:

- Ankylosing Spondylitis (AS), which is the most common form of SpA;
- Juvenile SpA;
- Psoriatic Arthritis;
- Spondylitis/Peripheral Arthritis associated with Inflammatory Bowel Disease (Ulcerative Colitis, Crohn´s Disease);
- Reactive Arthritis, also known as Reiter´s syndrome;
- Undifferentiated SpA

More recently the former classification fell out of favour and a **new classification system appeared** depending on characteristic pattern of joints involvement according to the *Assessment of SpA International Society* (ASAS) criteria (see **Figure 4, Figure 5** and **Table 1**)[11]**:**

- Axial Spondyloarthritis (AxSpA)
- Peripheral Spondyloarthritis (pSpA)

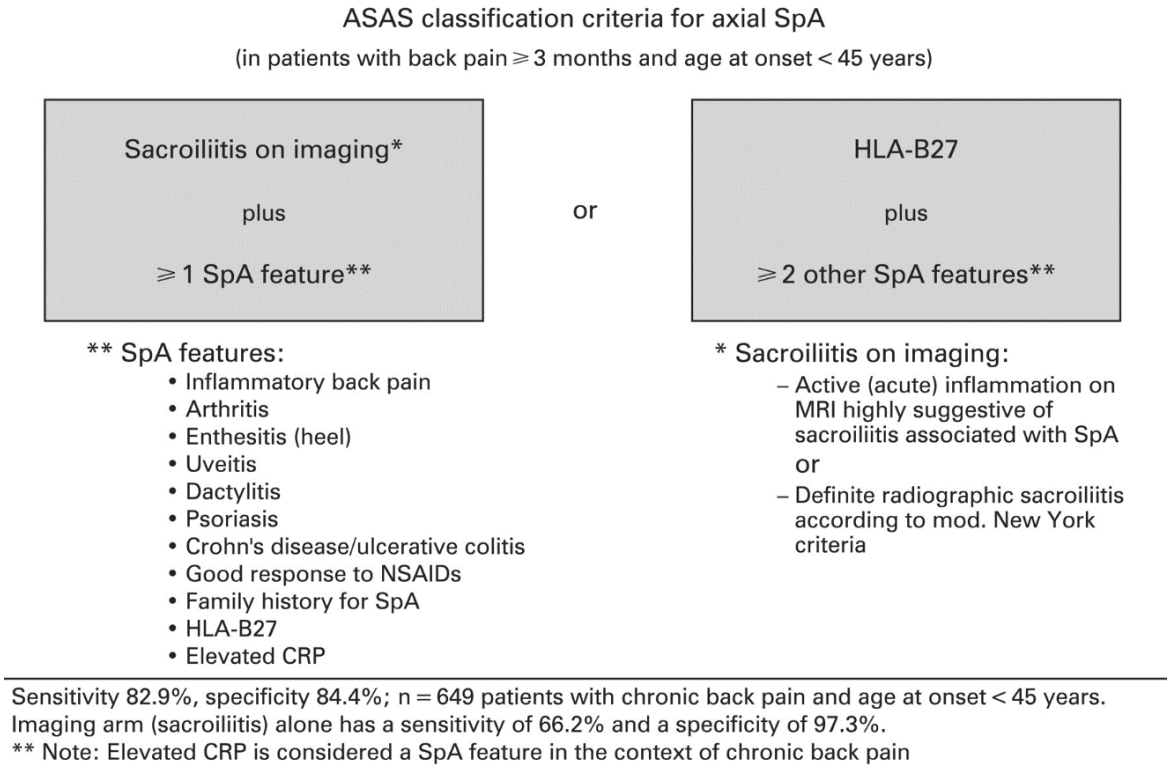

Figure 4- Classification criteria for axSpA according to ASAS classification system. From ref. ([83])

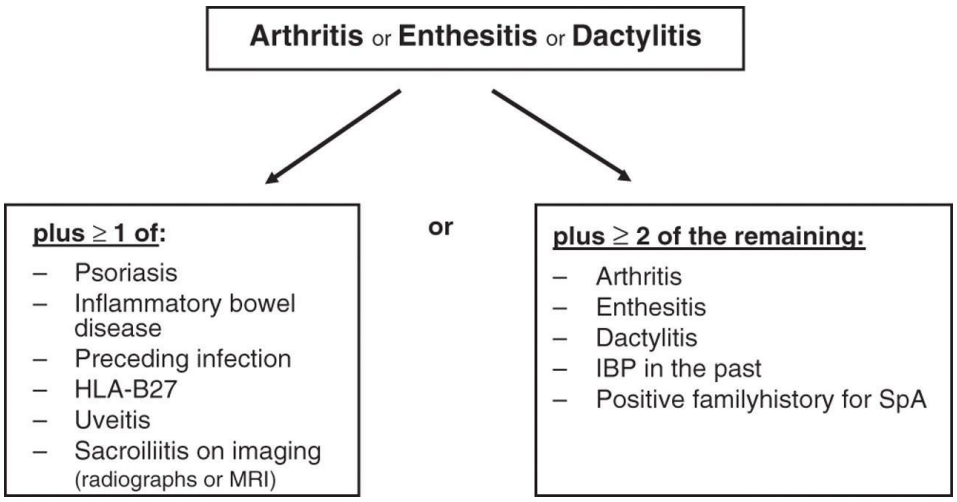

Figure 5- Classification criteria for peripheral spondiloarthritis (pSpA) according to ASAS classification system. From ref. ([84])

Table 1- Definitions of parameters applied in the Assessment of SpA international Society (ASAS) classification criteria for axSpA and pSpA. Adapted from ref. ([12])

Clinical criterion	**Definition**
IBP (inflammatory back pain)	IBP according to experts:14 at least four out of five parameters present: (1) age at onset <40 years; (2) insidious onset; (3) improvement with exercise; (4) no improvement with rest; (5) pain at night (with improvement upon getting up)
Arthritis	Past or present active synovitis diagnosed by a physician
Enthesitis	Heel enthesitis: past or present spontaneous pain or tenderness at examination at the site of the insertion of the Achilles tendon or plantar fascia at the calcaneus
Dactylitis	Past or present dactylitis diagnosed by a physician
Uveitis anterior	Past or present uveitis anterior, confirmed by an ophthalmologist
Psoriasis	Past or present psoriasis diagnosed by a physician
Inflammatory bowel disease	Past or present Chron disease or ulcerative colitis diagnosed by a physician
Good response to NSAIDs	At 24-48h after a full dose of NSAID the back pain is not present anymore or much better
Family history of SpA	Presence in first-degree (mother, father, sisters, brothers, children) or second-degree (maternal and paternal grandparents, aunts, uncles, nieces and nephews) relatives of any of the following: (1) AS; (2) psoriasis; (3) acute uveitis; (4) reactive arthritis; (5) IBD
HLA-B27	Positive testing according to standard laboratory techniques
Elevated CRP	CRP above upper normal limit in the presence of back pain, after exclusion of other causes for elevated CRP elevation
Sacroiliitis by x rays	Bilateral grade 2-4 or unilateral grade 3-4, according to the modified New York criteria
Sacroiliitis by MRI	Active inflammatory lesions of sacroiliac joints with definitive bone marrow oedema/osteitis suggestive of sacroiliitis associated with spondyloarthritis

III. Disease aetiology

III.a. Genetic contribution

SpA is a multifactorial aetiologic disease with a high genetic contribution to disease susceptibility, dominated by the HLA-B27 allele, described in 1973 by Caffrey and James [1,13]. In recent years there were an enormous increase of knowledge, through "*Genome Wide Association Studies"* (GWAS) involving large international databases that enabled a high genotyping data of SNPs with the identification of numerous genetic factors [14]. However, they can only explain a small portion of disease genetic predisposition and phenotype [1]. **Table 2** resumes the different genes found to be associated with SpA aetiology.

Table 2 - Some of the different genes identified by GWAS study to be associated with SpA disease susceptibility [1,15].

GWAS SpA related genes	HLA-B27
	ERAP1
	ERAP2
	LNPEP
	NPEPPS
	IL23 receptor
	TNFRSF1A
	TRADD
	TNFSF15

Besides the direct genetic contribution of HLA-B27 allele and pro-inflammatory genes discovered by GWAS studies, SpA physiopathologic characteristics can be divided in three groups:

1- Genetic contribution (previously explained, sub-chapter III.a)
2- Environmental components (see sub-chapter III.b)
3- Biomechanical factors (recurrent microtrauma vs sarcopenia) (see sub-chapter III.c)

III.b) Environmental components

Inside the environmental components there is a great influence of microbiota. The emerging and influence of next generation sequencing of gut microbiome has described an altered gut microbiota (dysbiosis) as a common component in different inflammatory conditions including SpA and inflammatory bowel diseases (IBD), being also related with an increased development of inflammation at extraintestinal tissues [16,17]. The clinical and pathogenic mechanisms overlap between SpA and IBD reinforce the relationship of both entities where gut dysbiosis, may constitute either a primary or secondary factor in the pathogenesis mechanism [16,17].

This theory was first demonstrated in murine models, where rats that overexpressed HLA-B27 antigen spontaneously developed an inflammatory disease, as for example arthritis and colitis, therefore mimicking the same aspects of human SpA, if living in a normal environment. Animals raised on germ-free environments do not develop a functional adaptative immune system and do not develop articular or systemic manifestations. [16,17]. On the other hand, when these transgenic rats are derived into a germ-free environment, SpA inflammatory symptoms disappear. However, the re- introduction of normal commensal flora in these rats allows the re-establishment of inflammation [16,17].

This strongly demonstrated not only that gut commensals play an important role in the education of immune system but also that disease development is microbiota dependent. The fact that HLA-B27 influence in such way the intestinal microbiome and therefore inflammatory diseases, was proposed in 2011 by Rosenbaum and Davey [18].

III.c) Biomechanical factors

Muscle Physiology- Short review

The muscular system is comprised by all the muscles of the human body, being composed by three different muscle types, namely skeletal, smooth and cardiac striated muscles [19,20].

The voluntary skeletal striated muscle represents about 40% of total muscle mass, being the main effector organ of the musculoskeletal system [19,20]. Its main role is to sustain movement, maintain muscle tone and stabilize bones and other structures [20]. The skeletal muscle is organized in sarcomeres, where the interaction between two filamentous protein, Actin and Myosin, very well organized in a regular pattern, promotes, and give origin to muscle contraction [21]. Actin chains are the most important and abundant protein of skeletal striated muscle since they are the main components of the cell cytoskeleton [21]. Most of the muscles contain a mixture of two different fibres: type I and type II- IIa and IIb, with different contraction times and overall characteristics. [20] The main characteristics of each muscle fibre is summarized in **Table 3**.

Table 3 - Muscle fibres- Physical and contractile characteristics. Adapted from (20).

	Type I	Type II-a	Type II-b
Diameter	Small	Intermediate	Large
Motor Unit Size	Small	Intermediate	Large
Recruitment	Early	Intermediate	Late
Contraction	Slow	Fast	Fast
Twitch	Long	Short	Short

.

The Biomechanical Concept

The biomechanical concept is based on the evidence that abnormal mechanical loading can alter cellular function, and the composition/ arrangement of the extracellular matrix. Therefore, chronically unfavourable loading conditions could lead to tissue or organ pathologies – e.g. enthesopathy [22.] This concept has been defended as an additional contribution to axSpA susceptibility and severity and is supported by different findings:

- Gait-associated microtrauma induced inflammation and osteoproliferation with ankylosis of the spine in animal models of axSpA [23]. Recent studies started to focus on biomechanical factors, such as micro trauma, as having an important impact in the onset and progression of SpA. A study conducted by Mechelen and Lories, 2016, using mice overexpressing tumor necrosis factor, showed that when suspended by the tail, mice did not developed arthritis. Therefore, the suspension of the posterior legs acted has a preventive factor, also reducing osteogenesis after acute arthritis [24].

This study also demonstrated that local damage may act as a trigger for SpA, with loss of stability, potentially giving origin to tissue remodelling with syndesmophytes formation, playing an important role in structural disease progression [24].

- Physically demanding professions potentiate the effect of inflammation in osteoproliferation leading to SpA severity and ankylosis progression [24,25].

Conceptually, microtrauma induced by daily activities or by the muscle itself, should play an important role in entheseal inflammation [26]. In this context, the axial entheses are particularly prone to inflammation as they are subjected to mechanical stress related with posture maintenance [27,28]. This may explain why patients in the early stages, complain about back stiffness without pain or compromise of range of motion [22].

To increase the understanding about the role of muscle as precursor of microtrauma it would be important to evaluate muscle physical properties such as stiffness, elasticity, and tone. In this context, muscle physical properties have been seen as a potential factor contributing for disease susceptibility and/or progression. [1,3,29]. Masi and coworkers identified an increased resting lumbar myofascial stiffness in SpA patients that may supported the role of muscle inducing microtrauma at enthesis level [28].

The sarcopenia concept

SpA patients with long disease duration often exhibit muscle weakness, commonly associated with sarcopenia (skeletal muscle disorder) [30]. Sarcopenia is a progressive and generalised skeletal muscle disease related to an increased probability of falls, fractures, physical disability, and mortality. It is defined by three parameters: low level of muscle strength, muscle quantity/ quality and low measure of physical performance, that can be measured by some tools recommended by EWGSOP [21,30–32]:

- **Muscle Strength**- The force that a muscle needs to exert to overcome a resistance [21,32]. Low muscle strength can predict a decline in overall physical function, performance, and disability [31]. Decrease of muscle strength should allow the suspicion about sarcopenia. There are fewer well-validated techniques to measure muscle strength. Although lower limbs are more relevant than upper limbs for gait and physical function, handgrip strength has been widely used and is well correlated with most relevant outcomes [33].

    - **Grip strength**- Requires the use of a calibrated handheld dynamometer under well-defined test conditions. When measurement of grip is not possible due to hand disability, isometric torque methods can be used to measure lower limb strength [33].

    - **ST5-** *Sit-to-stand 5* test is normally used as a tool to measure lower limb strength, since involves the action of multiple muscles, particularly the knee extensor muscle (quadriceps femoris), and can be useful to quantify functional changes of translational movements [34,35].

- **Muscle mass**- Several studies have reported a low index of muscle mass as a factor to a poor performance status and higher self- reported disability. However, it is not clear if a decline in physical function appears due to a low muscle mass itself or as a result from an association with a loss of muscle strength [31]. The association between decrease muscle strength and muscle mass allow to diagnosis sarcopenia.

  The value of muscle mass is different from person to person and can be estimated by a variety of techniques[36]:

  - Magnetic resonance imaging (MRI) and computed tomography (CT) -considered to be gold standards for non-invasive assessment;

  - Dual- energy X-ray absorptiometry (DXA) and bioelectric impedance analysis (BIA). The two former methods are a more widely available instruments [36].

- **Muscle performance**- It is described as the capacity of the muscle to recruit contractile muscle units (sarcomeres) to generate the necessary energy to origin, maintain and sustain movements. Balance, timing, and sequencing of contraction are as well requested to a functional activity [32]. Adding low muscle performance to decrease muscle strength and mass allow the diagnosis of severe sarcopenia [37].

  Physical performance can be variously measured by gait speed, the Short Physical Performance Battery test (SPPB) and the Timed-Up and Go test (TUG), among others[38].

  - **Gait speed**- Used to evaluate the time needed to walk a 4 meters distance.

  - **ST60**- *Sit-to-stand 60* test, is based in the same principals than ST5, however ST60 consists in the number of repetitions of sit and stand that a person can successfully complete in 60 seconds.

Although, its underlying mechanism remain unrevealed, sarcopenia may be caused by the complex interplay between genetic factors, environmental and biomechanical stress aspects (such as intestinal microbiota and micro traumas) as stated above [30]. These interactions may be responsible for an aberrant overexpression of immune responses, with the activation of several pro-inflammatory cytokines (for example IL-23- IL-17 axis and TNFα). Chronic inflammation is then thought to induce anorexia, muscle loss, among other consequences, with an impairment of the contractile, metabolic, or endocrine function of skeletal muscles [6,30].

The Human genome comprises at least 214 variant gene sequences and genetic markers linked to individual´s physical performance and health-related phenotypes. In this work our objective was to study the ones linked to muscle properties and possible relation with axSpA, which lead us to the gene polymorphisms described below.

IV. *Alpha-actinin 3* (*ACTN3*)

Human *ACTN3* gene is located on the long arm of chromosome 11, position 13.2 (11q13.2) and encodes the alpha-actinin-3 protein which is associated with physical fitness and/ or performance and muscular efficiency [29,39,40]. For a long time *ACTN3* have been called "the gene for speed" due to the promotion of fast twitch fibres and quick contraction characteristics. ACTN3 protein is a fast-twitch-specific isoform uniquely expressed in type-II muscle fibres (see **table 4**) and is associated with speed and power phenotypes. These characteristics makes it of great importance in the generation of contractile forces at high speeds [20,34].

These gene displays a common polymorphism at position 1747, located in exon 16, leading to a Cytosine (C) to Timine (T) nucleotide base substitution, which consequently results in the replacement of an arginine (R) residue by a premature stop codon (X), at amino acid 577 [29,39,41], causing the absence of a detectable protein product in the muscles of individuals with *ACTN3* null genotype, homozygous for the X allele [5,7] (**figure 6**). X allele homozygotes (R577X) are then deficient in ACTN3 protein, which can be related to a lower percentage of fast twitch fibres, leading to lower muscle strength and performance [41].

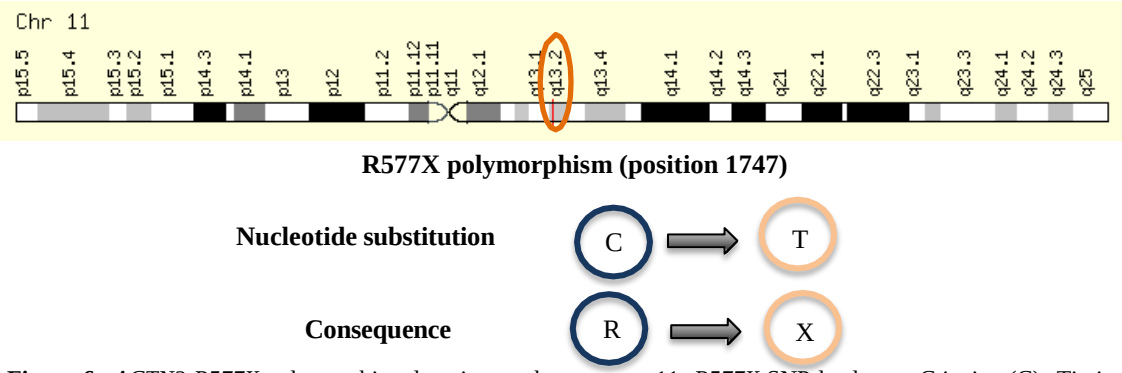

Figure 6- *ACTN3 R577X* polymorphism location at chromosome 11. *R577X* SNP leads to a Ctiocine (C)- Timine (T) nucleotide base substitution and consequently a change in protein amino acid from an arginine (R) residue to a premature stop codon (X). Adapted from ([40]).
).

On **Table 4** is showed the possible genotypes and their impact on protein expression [29]:

Table 4 - *ACTN3* possible genotypes and their impact in one's protein expression level.

Possible genotypes	Effect
Homozygous null (XX)	Protein total absence
Heterozygous (XR)	Protein normal expression level
Homozygous (RR)	Maximum expression level of protein

Several studies state that the null genotype (XX) can be responsible for a shift in the properties of the fast type-II fibres towards slow type-I fibres, promoting also a higher oxidative enzymes and baseline calcineurin activity [29].

The presence of one or both X alleles have been found to be related to a release of anabolic or catabolic molecules from either skeletal muscles or bone cells, low physical fitness and with an abnormal osteoblast/osteoclast activity therefore, contributing to the establishment of sarcopenia and osteoporosis [39].

The null genotype can be beneficial for endurance performance due to muscle fibre shift however it have a negative impact in speed and/or strength of the individuals [29]. Athletes are a commonly group used to study the effect of different genotypes, being recognized that elite speed, power, and strength athletes have a significantly higher frequency of the R allele when compared with normal controls. Beside this improvement effect, *ACTN3* R577X polymorphism may have an impact in exercise recovery, injury risk and training adaptation [41].

V. Vitamin D and Vitamin D Receptor gene

Vitamin D is produced in human skin due to the influence of ultraviolet-B (UVB) radiation which will activate a cascade of reactions ending in the conversion of 7-dehydrocholesterol into 25-hydroxy vitamin D (25(OH)D) in the liver. Further hydroxylation, in kidneys, produce its active form, 1,25 hidroxy vitamin D (1,25(OH)2D) [42]. It can also be ingested in diet, in the form of vitamin D2 (ergocalciferol), which pass through the same hydroxylation process described previously. Although 1,25(OH)2D obtained by this way is much less [42].

Vitamin D plays an important role in the regulation of calcium homeostasis, bone metabolism and is essential for many tissues metabolism including the skeletal muscle [42,43]. However, and despite its benefit effect in human health, vitamin D deficiency is increasing worldwide, being now considered an epidemic problem [44].

Severe deficiency of this vitamin causes rickets, in children's, and it is responsible for the establishment of osteomalacia in adulthood [43]. It is also implicated in osteoporosis and stress-related fractures [44]. It can also cause muscle weakness with proximal myopathy, being characterized by a difficulty of stand from a chair without armrests and walking on stairs, muscle wasting and a gait disturbance frequently characterized as waddling ("penguin gait") [42,43]. Several cohort and cross-sectional studies have described an association between vitamin D status and muscle strength, body balance and physical performance, being important for normal skeletal muscle development [43].

Additionally, skeletal muscle tissue biopsies of adults with vitamin D deficiency have shown a predominance of muscle fibres type- II atrophy, enlarged interfibrillar spaces and infiltration of fat, fibrosis, and glycogen granules [43].

On the other hand, studies have demonstrated that high levels of Vitamin D enhance muscle function in individuals with debilitated muscles [30]. With this in mind, it would be of interest to study the influence of *Vitamin D receptor* gene polymorphisms and their impact in muscle characteristics.

VDR gene is located on the long arm of chromosome 12 (12q13.11), and the codified protein is expressed in muscle cells nuclei and seems to affect muscle contractility. This gene exhibits different polymorphisms being the most studied and relevant, *BsmI*, *FokI*, *ApaI* and *TaqI* SNPs, which are thought to be associated with functional outcomes, such as muscle strength, postural balance and muscle performance [42–44].

Figure 7 shows *VDR* gene and localization of the different SNPs mentioned above.

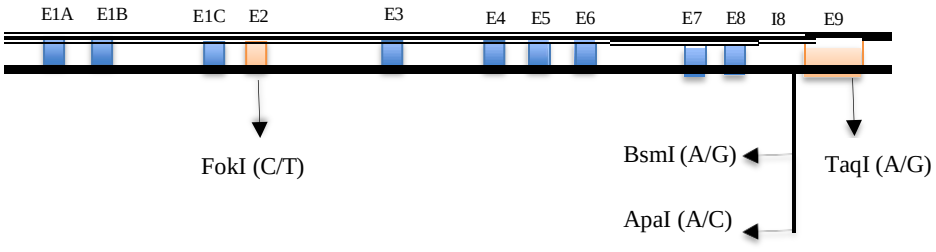

Figure 7 - Vitamin D receptor gene and some of the most studied polymorphism. E - means exon, I - stands for intron.

***BsmI* polymorphism**

BsmI is a restriction fragment length polymorphism located at the 3´end of intron 8 [42,44].

This SNP has been associated with muscle characteristics. The bb allele, i.e the presence of restriction site on both alleles, is thought to be associated with an higher VDR activity, promoting lower fat-free mass and hamstring strength when compared with individuals carrying BB alleles or showing an heterozygote genotype [42,44].

***FokI* polymorphism**

FokI polymorphism involves a T/C nucleotide transition in exon 2, resulting in a shorter (424 pb) amino acid. The T allele, is associated with enhanced VDR transcription factor ability, possibly improving muscle strength. On the other hand, C allele, can be associated with reduced fat-free mass and quadriceps muscle strength, being also associated with variations in bone mineral density [42,44].

***ApaI* and *TaqI* polymorphism**

Both are restriction enzymes. *ApaI* is located at intron 8 and promotes an A/C transition however it does not affect *VDR* activity. *TaqI* is a silent/synonymous polymorphism located on exon 9 promoting an A/G transition [45].

Synonymous SNPs do not cause any change in the protein however they can affect messenger RNA splicing, stability, structure and protein folding, having a considerable effect in protein function, changing also cellular responses to therapies [46]. Depending on the different SNP they can have different impact in muscle characteristics.

Aims of the project

The aims of the present project are:

1- To characterize the association of the *ACTN3* and *VDR* genes SNPs with axSpA susceptibility;
2- To analyse the association of these SNPs with muscle physical properties in the axSpA context.

As secondary outcomes:

1- The association of the studied SNPs with disease characteristics namely, disease activity (BASDAI), functional repercussion (BASFI), axial and peripheral joint movement amplitude (BASMI), or age at disease onset.
2- The association of the studied SNPs and muscle characteristics namely muscle strength, mass, and performance.

Methods

1. Patient and Healthy control population

This study enrolled 56 individuals. Of these, 28 patients, previously diagnosed with axSpA according to ASAS classification criteria (ref. 12), and 28 healthy controls, matched by gender, age and level of physical activity. Eligible axSpA participants were aged between 18 to 50 years old and have a symptom duration under 10 years. Previous exposure to synthetic disease-modifying anti-rheumatic drugs (**DMARDs**) or biological disease-modifying anti-rheumatic drugs (**bDMARDs**) were not allowed. Inclusion and exclusion criteria can be found with more detail in **table 5**. Protocol was submitted and approved by both ethical committees of NOVA Medical School|Faculdade de Ciências Médicas and Centro Hospitalar Lisboa Ocidental, Hospital de Egas Moniz, EPE. The study was conducted in accordance with International Conference on Harmonization good clinical practices and the Declaration of Helsinki. Voluntary written informed participants consent was obtained from all subjects before start study procedures.

Table 5 - Healthy controls and patients inclusion/ exclusion criteria for the present study

Inclusion criteria	Exclusion criteria
Clinical diagnosed axSpA (meeting the ASAS classification criteria) with <10 years of evolution since symptoms begin	Body mass index (BMI) ≥ 35kg/m^2
Age 18-50 years old	Previous treatment with conventional or biological DMARDs
Being able to conscious and voluntary give informed consent	Pregnancy or present breast feeding
NSAIDs and/or corticosteroids therapeutics (equivalent to ≤ 10 mg of prednisone), in stable doses 4 weeks before screening was allowed	Infection that required hospital stay, intravenous or antibiotic treatment in 30 days previously or oral antibiotic treatment in 14 days before screening
	Neoplastic disease (except squamous cells or basal cells carcinomas successfully treatment)
	Any non-treated conditions (ex. diabetes mellitus, ischemic heart disease)
	Intra or peri-articular extra-axial injections and in tendon sheaths in the 28 days previously to screening
	Previous rachis surgery
	Rachis ankylosis (with syndesmophytes in all levels from D12 to S1, observable on profile radiography)

Even though the study started with 56 enrolled participants, in equal number of HC and patients, due to missing data in some physical measures and in individuals polymorphisms identification, we ended up adjusting total sample population to 51 individuals (24 healthy controls and 27 axSpA patients).

In **Figure 8** is schematized in more detail the sample size adjustment.

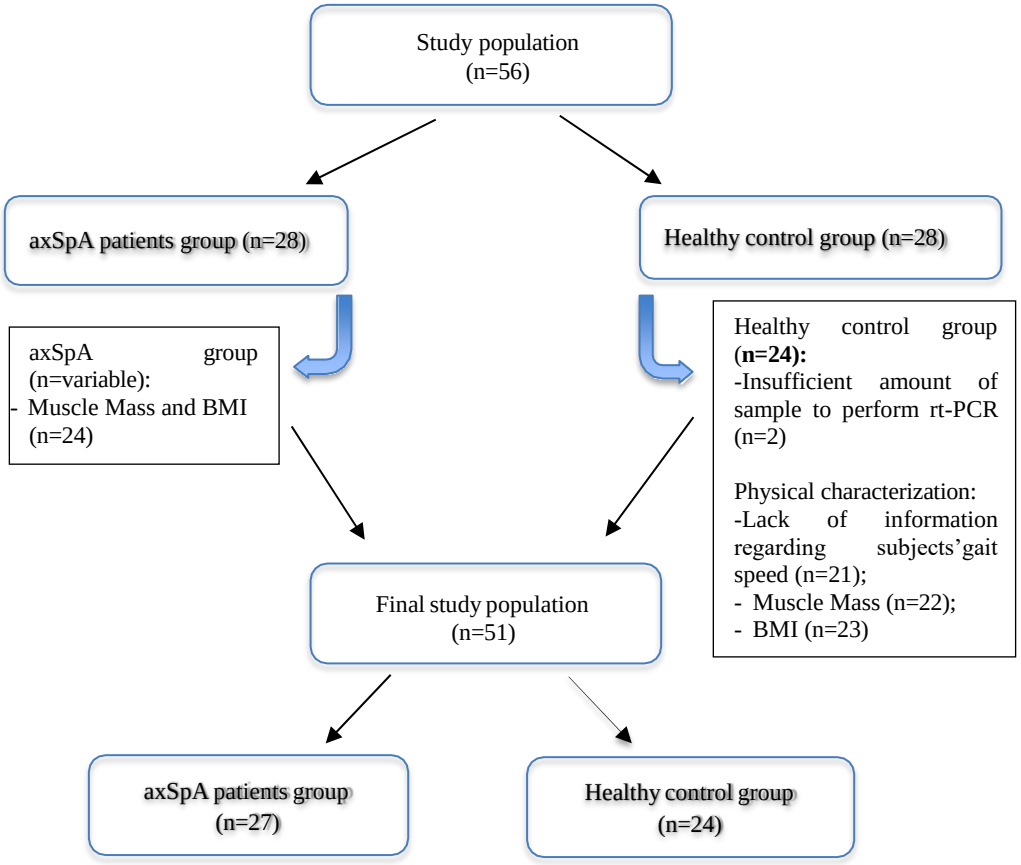

Figure 8- Sample size adjustment process

2. Clinical protocol

All 56 participants were submitted to a standardized protocol for an extensive epidemiologic and physical characterization.

All participants were evaluated in terms of **physical activity**, assessed accordingly to the International Physical Activity Questionnaire (IPAQ) (please consult reference ([47])). Additionally, whole body and segmental myofascial characterization, were performed using different approaches:

1. Muscle physical properties (stiffness, tone, and elasticity), assessed by a non-invasive device, the MyotonPro®, focusing on torso (axial multifidus and longissimus dorsi muscles) and lower (gastrocnemius lateralis muscle) limbs. The participants were in a prone position and measurements were taken after a 10 min rest;

There are several devices to measure these parameters. One of the most convenient is MyotonPro®, that consists on a non-invasive digital device, that enables the measurement of superficial skeletal muscles, tendons, ligaments, skin and other soft tissues, recording damped natural oscillation of soft tissues, in the form of an acceleration signal induced by an exterior low force quick release of a mechanical impulse under constant pre-load, state of tension, and both biomechanical and viscoelastic

properties [48]. Natural oscillation frequency provides us information about muscle tone, dynamic stiffness and decrement constituting the biomechanical properties. Viscoelastic properties give information about mechanical stress relaxation time and Creep (ratio deformation/relaxation time) [48]. All these parameters are strictly related to the type of muscle.

2. Muscle strength, measured using the Lafayette Manual Muscle Testing System for torso and knee extension; global strength evaluated through Sit to Stand test 5 times [49];

For *ST5* test, participants had to stand up from and sit down on a 43cm high armless chair as quick as possible for 5 consecutive times. Arms folded across the chest, completely isometric knee extension and a firm contact when sitting were required in order to successfully complete the test. There are no normal range definition for female population [34].

3. Muscle performance: through a Sit-to-Stand test, 60 seconds (*ST60*) and walking speed. Gait analysis were conducted using a 3D full-body kinematic model (Kinetikos® Coimbra, Portugal) fed by 15 wireless inertial sensors placed in the head, arms, trunk, pelvis, thighs, shanks, and feet. The primary outcomes comprise the gait spatiotemporal parameters, 3D joints kinematics and dynamics during self-paced walking; *ST60* test is dependent on the individuals' gender and age. In this work we followed the values stablished for Swiss population [50].

4. Muscle Mass assessed by bioimpedance, using direct segmental 8-point multifrequency bioelectric impedance analysis (InBody770, InBody Co., Ltd, Seoul, Korea).

In addition, patients completed a questionnaire to allow a full disease characterization including:

1. Disease activity, through Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) ([51]) - Assesses fatigue, spinal pain, peripheral joints involvement, entheses, morning stiffness (intensity and duration). Normally a cut-off of 4 (score) is used to define disease activity.
2. Disease functional repercussion, trough Bath Ankylosing Spondylitis Functional Index (BASFI) ([52]) - Gives to the clinicians a self- sense of how well the patient can perform activities of the daily living.
3. Disease metrological repercussion, trough Bath Ankylosing Spondylitis Metrology Index (BASMI) ([52]) - Indicates the range of motion of several bodily segments, mainly axial rachis
4. Bath Ankylosing Patient Global Score (BASG), one week and 6 months.
5. Quality of life evaluated by 36 item short form survey (SF-36) and Euro-Qol 5 dimensions (EQ-5D).

It is also important to highlight that due to the absence of already stablished reference values for several variables considered in the study related to muscle physical properties we had to stablish categories (low, intermedium and high) without taking into account the differences between genders because of the reduced size of our sample.

In addition, a whole blood sample was collected from each individual included in the study for posterior analysis (see next topic).

3. Laboratorial analysis

3.1. DNA extraction

51 whole blood samples were screened and genotyped for both *ACTN3* and *VDR* polymorphisms; *R557X* (rs1815739) and *ApaI* (rs7975232), *FokI* (rs2228570), *TaqI* (rs731236), respectively.

DNA extraction was performed using PureLink™ Genomic DNA Mini Kit from Invitrogen (Catalog number: K182001) (See ref. ([53])). Extraction was performed according to the Mini kit protocol for Blood Lysates with the following modifications:

a) In 2 mL Eppendorf's tubes with blood clots, 200µL of PBS were added in order to allow the collection of the right amount of blood sample.
b) 100% ethanol was added to the lysate.

On **figure 9** is represented a schematically experimental overview.

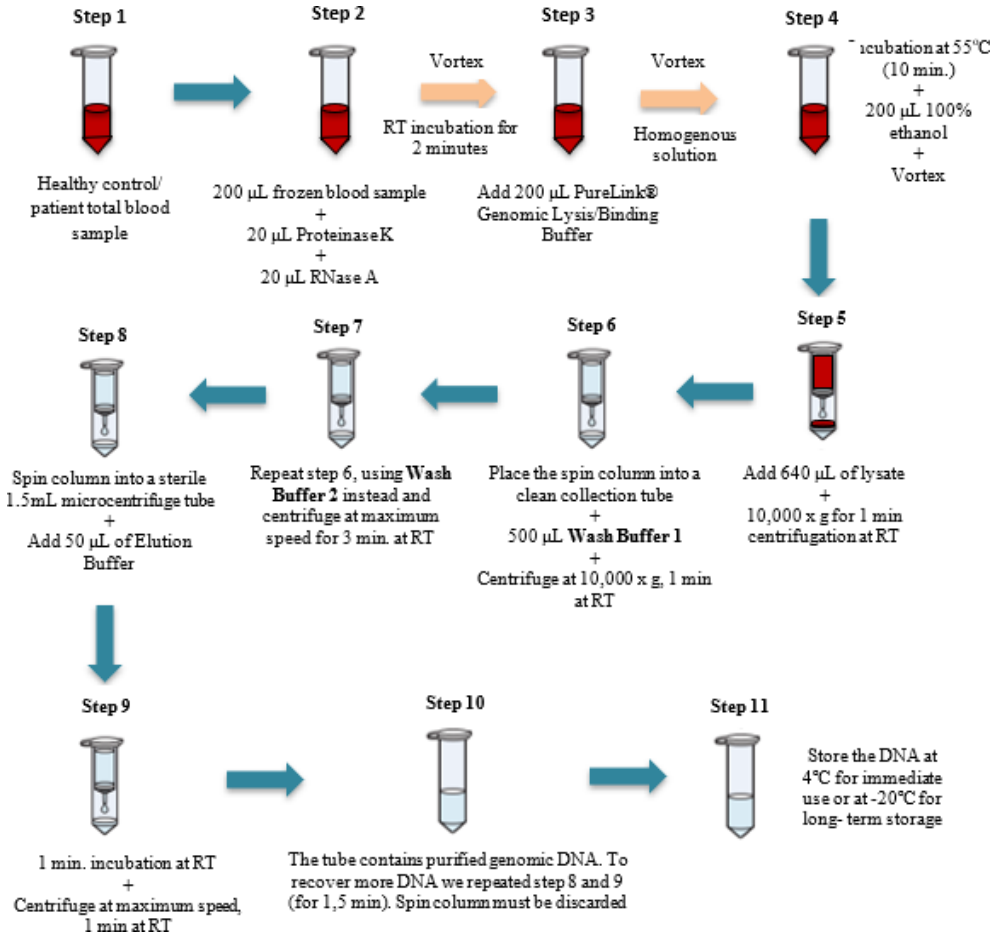

Figure 9 - Experimental protocol scheme overview. Note: **Wash Buffer 1** and **Wash Buffer 2** were previously prepared as indicated in manufacturer's protocol, by adding 100% ethanol. For the complete protocol please consult ref. ([53]).

3.2. Sample genotyping

After DNA extraction from all blood samples (patients and healthy subjects), all samples were genotyped for the polymorphism of interest separately. To perform these steps, we used the TaqMan SNP Genotyping Assay from *ThermoFisher Scientific - UK*, combined with *real-time* PCR (RT-PCR) technique.

3.3. TaqMan SNP Genotyping Assay – Principles

Applied Biosystems™ TaqMan® SNP genotyping assay commercially available in a 40X concentration solution were optimized to be used at a 20X concentration by making a 1:2 dilution with TE buffer when preparing the reactional mix. **Table 6** shows the four different assays performed and elucidates about some important information of each gene, gene sequence and SNP.

Table 6 - Genes and related SNPs.

GENE	SNP ID	SNP NAME	ALELLE 1 (VIC)	ALLELE 2 (FAM)	CONTEXT SEQUENCE [VIC/FAM]	SNP TYPE	A.a residue
ACTN3	rs1815739	**R577X**	T	C	**CAAGGCAACAC TGCCCGAGGCT GAC[T/C]GAGA GCGAGGTGCCA TCATGGGCAT**	Nonsense	R / *
VDR	rs2228570	**FokI**	A	G	**GGAAGTGCTGG CCGCCATTGCC TCC[A/G]TCCCT GTAAGAACAGC AAGCAGGCC**	Missense	K / R
VDR	rs731236	**TaqI**	A	G	**TGGACAGGCGG TCCTGGATGGC CTC[A/G]ATCAG CGCGGCGTCCT GCACCCCAG**	Silent	I / I
VDR	rs7975232	**ApaI**	A	C	**AAGGCACAGGA GCTCTCAGCTG GGC[A/C]CCTCA CTGCTCAATCC CACCACCCC**	Intron	

Legend: a.a residue– Aminoacid residues R- Arginine, K- Lysine, I- Isoleucine, * - absence of a.a. Alleles: T- Timine; A- Adenine; C- Cytosine; G- Guanine. In gene sequence, SNP alleles are included in brackets. The order of the alleles corresponds always to the same association with the probe reporter dye, i.e, allele 1 is labelled with VIC dye, and allele 2 with FAM dye. Each assay is specific for the DNA sequence we want to analyse.

TaqMan SNP genotyping assay occurs through the following steps (**figure 8**):

1- DNA double strand denatures, giving origin to two complementary strands;
2- TaqMan probes hybridize to the target DNA between the two unlabelled PCR primers;
3- Signal from the fluorescent dye is quenched by the reporter NFQ;

4- During RT-PCR, polymerase enzyme extends the unlabelled primers using the template strand as model;
5- The Taq polymerase cleaves the TaqMan™ probe molecule during each extension cycle. This causes the separation of the dye from the quencher and the emission of the fluorescence by the free reporter dye;
6- The plate runs in the RT-PCR machine, which will detect fluorescence emitted from the unquenched free FAM or VIC dye, depending on the SNP present in the gene sequence.

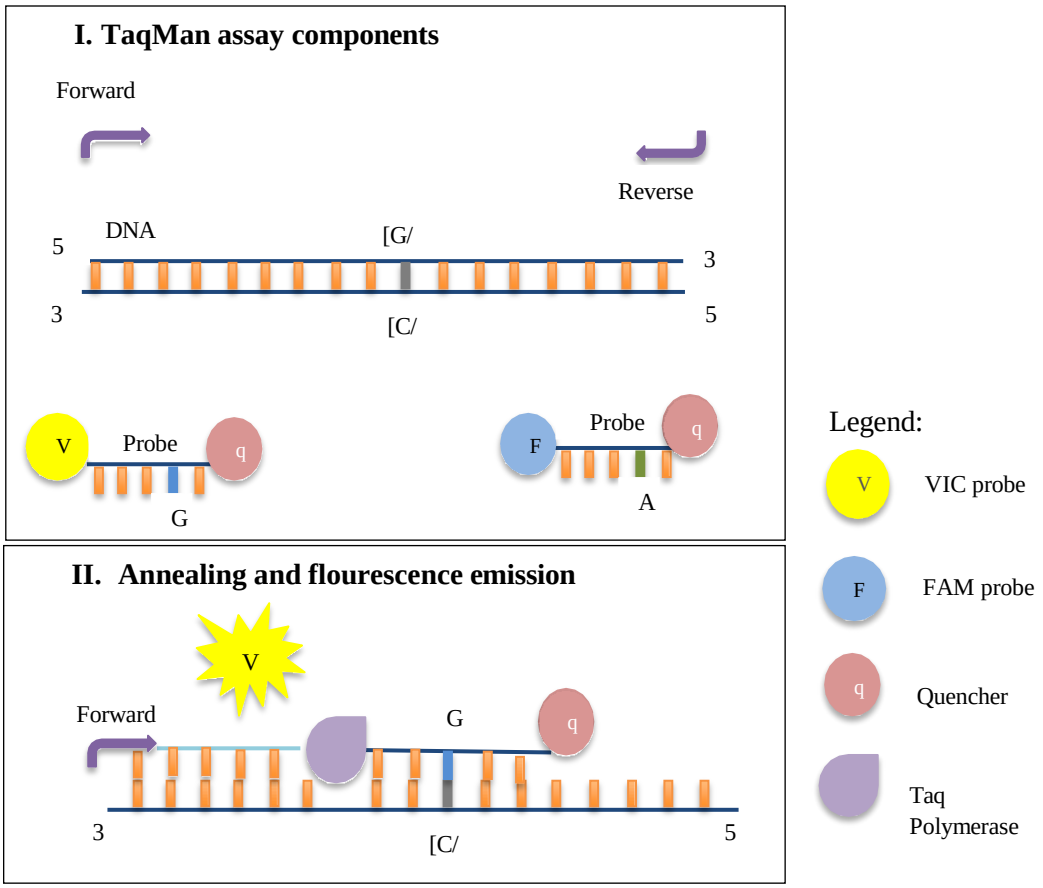

Figure 10 - TaqMan SNP Genotyping Assay. I - In image I there are present, in a simplify way, all the components needed to perform this assay for one polymorphism; as we can see there is a quench of the reporter (VIC fluorescent probe).
II - Taq Polymerase cleaves the TaqMan probe in each extension cycle, leading to the emission of the fluorescence by the free reporter dye (VIC)

3.4. Protocol

In order to perform the TaqMan® SNP genotyping assay we used a 96-well plate for each SNP (rs1815735; rs7975232; rs2228570; rs731236). For a single SNP assay, we made a 1:2 dilution of the Master Mix (TaqPath™ ProAmp™ Master Mix) to obtain a 20x final concentration and used the reagents in **table 7.**

Table 7 - Reagents and volume of reaction (µL) for a single reaction. TE- buffer.

Components	Volume/ reaction (µL)	Final concentration
Master mix	5	20X
DNA	4	---------
dH2O	0.5	---------
TaqMan Assay	0.5: 0.25 µL assay + 0.25 µL TE 1X	---------

The dH20 used went through autoclave twice being in between filtered using a syringe filter of 0.22µm, to ensure the maximum purification avoiding a potential source of contamination.

The reaction mix was prepared according the instructions on table 8, a total volume of 6 µL per well was distributed for the number of samples we wanted to analyse after which were added 4 µL of each DNA sample in corresponding well. In the last column of the 96 well-plate we added 6 (around 10%) totally blind samples that were used as duplicate reactions and two wells as non-template control (NTC) – negative control.

After these steps, the plate went through RT-PCR using a QuantStudio™ 5 Real-Time PCR Instrument (96-well 0.2ml Block) from *Applied Biosystems* by *Thermo Fisher Scientific*. All the results were analysed using ThermoFishers´software available.

4. Statistical analysis

Data analysis was performed using the Statistical Package for the Social Sciences for Windows 22.0 version (SPSS, Inc.). All genotypes were coded accordingly in order to proceed with the statistical analysis. The analysis of Hardy-Weinberg frequencies for all alleles present in patients' populations was carried out using exact probability tests available using the SNPStat software (http://bioinfo.iconcologia.net/SNPstats). Differences in genotype frequency between both populations were evaluated by the Chi-Square ($\chi 2$) test.

Since this is not a conclusive final study but an exploratory one on the role of selected polymorphisms in *ACTN3* and *VDR* genes, and the data to be obtained should be looked at as proof of concept, the Bonferroni adjustment was deemed as not necessary as it is too conservative. Logistic regression was used to estimate the risk of muscle properties modification when associated with each genotype: risk estimates were calculated under the codominant model and expressed as crude odds ratios (OR) and corresponding 95% confidence intervals (CI). Results were considered significant when the corresponding two-tailed *p*-values were <0.05. The most common homozygous genotype was considered the reference classes for such calculations.

Results

1. Epidemiological characterization

In our study we involved 27 axSpA patients, 66.7% male, mean age of 36 years old with a mean of 2.6 ± 2.0 years of disease duration. From the total of patients, 80.8% were HLA-B27 positive, 18.5% with active disease (BASDAI ≥ 4) and 14.81% with functional repercussion (BASFI > 4). The majority of patients do not exhibit a considerable reduction in movement amplitude, 88.89% (BASMI <3). Patients and Healthy control groups were largely similar regarding epidemiological and characteristics.

Baseline characteristics are shown in detail, in **table 8**, were all data comparison between patients and controls can be found.

Table 8 - Study population characterization. Cases group (n=27) and Healthy control group (n=24)

Characteristics	Cases *n* (%)	Mean ± SD	Controls *n* (%)	Mean ± SD	P-value*
Age 0-29 30- 39 40- 50	 3 (11.1) 14 (51.9) 10 (37.0)	36.81 ± 7.10	 4 (16.7) 10 (41.7) 10 (41.7)	36.79 ± 7.66	0.728
Gender Male Female	 18 (66.7) 9 (33.3)	-	 15 (62.5) 9 (37.5)	-	0.756
Physical Activity level Low High	 22 (81.5) 5 (18.5)	-	 21 (87.5) 3 (12.5)	-	0.555
HLA.B27 Status Positive Negative	 21 (80.8) 5 (19.2)	-	-	-	-
Body height (cm)	-	170.40 ± 7.38	-	171.08 ± 8.79	
Body mass (kg)	-	75.86 ± 12.68	-	71.82 ± 12.85	
BMI low height normoponderal overweight obesity	 1 (4.2) 11 (45.8) 7 (29.2) 5 (20.8)	26.15 ± 4.34	 1 (4.3) 13 (56.5) 8 (34.8) 1 (4.3)	24.48 ± 3.71	0.410
BASDAI <4 ≥4	 22 (81.48) 5 (18.52)	2.99 ± 2.01	-	-	-
BASMI <3 ≥3	 24 (88.89) 3 (11.11)	1.07 ± 1.33	-	-	-
BASFI ≤ 4 >4	 23 (85.19) 4 (14.81)	2.21 ± 2.68	-	-	-

*P- value determined by χ^2 test

2. Clinical data- physical characteristics analysis

After the clinical and epidemiological characterization of both populations, our primary objective was to see if any of the muscular characteristics measured were different between cases and controls.

For that we registered and analysed the results obtained from muscle physical properties (Muscle tonus (M.F), Muscle decrement, i.e the inverse of elasticity (M.D) and Muscle stiffness (M.S) measurements from two distinct segmental areas: trunk (Multifidus muscle) and lower limbs (Gastrocnemius muscle) (**Table 9**). The regression analysis was performed individually for each characteristic (crude analysis).

Table 9 – Multifidus muscle and Gastrocnemius muscle characterization according to the criteria's used to categorize each muscle characteristic.

Characteristics	Cases *n* (%)	Controls *n* (%)	P-value*	OR crude[a] (95% CI)
Multifidus muscle				
Muscle tonus				
Low	6 (22.2)	9 (37.5)		1.000 (Reference)
Intermediate	9 (33.3)	10 (41.7)	0.185	1.350 (0.343- 5.315)
High	12 (44.4)	5 (20.8)		3.600 (0.829- 15.628)
Muscle decrement				
Low	6 (22.2)	9 (37.5)		1.000 (Reference)
Intermediate	12 (44.4)	7 (29.2)	0.406	2.571 (0.640- 10.338)
High	9 (33.3)	8 (33.3)		1.687 (0.414- 6.878)
Muscle stiffness				
Low	5 (18.5)	11 (45.8)		1.000 (Reference)
Intermediate	12 (44.4)	6 (25.0)	0.099	**4.400 (1.041- 18.599)†**
High	10 (37.0)	7 (29.2)		3.143 (0.751- 13.159)
Gastrocnemius muscle				
Muscle tonus				
Low	10 (37.0)	7 (29.2)		1.000 (Reference)
Intermediate	8 (29.6)	9 (37.5)	0.790	0.622 (0.160- 2.416)
High	9 (33.3)	8 (33.3)		0.788 (0.203- 3.057)
Muscle decrement				
Low	12 (44.4)	6 (25.0)		1.000 (Reference)
Intermediate	6 (22.2)	10 (41.7)	0.235	0.300 (0.073- 1.227)
High	9 (33.3)	8 (33.3)		0.563 (0.143- 2.206)
Muscle stiffness				
Low	9 (33.3)	9 (37.5)		1.000 (Reference)
Intermediate	9 (33.3)	7 (29.2)	0.936	1.286 (0.332- 4.972)
High	9 (33.3)	8 (33.3)		1.125 (0.298- 4.241)

* p-value $\chi 2$ test. † $p < 0.05$ for regression logistics. Abbreviations: [a] ORs and 95% CI for specific categories were calculated using logistic regression models; OR, odds ratio; CI, confidence interval.

No significant differences in muscle physical properties were globally identified between patients and controls. However, it seems that patients have a tendency to express higher levels of stiffness in multifidus muscle (trunk). Due to the small size of our study population and low number of female individuals, we did not take into account possible gender differences in any of our analysis.

Next, we wanted to elucidate the existence (or not) of differences regarding strength and performance properties among HC and patients.

Results can be found in the table below (**table 10**)

Table 10- Muscle mass, Body mass index, Strength and performance measures

Characteristics	Cases *n* (%)	Controls *n* (%)	P-value*	**ORcrude (95% CI)**
Muscle Mass				
Muscle Mass low normal range above	 1 (4.2) 19 (79.2) 4 (16.7)	 1 (4.5) 19 (86.4) 2 (9.1)	0.724	N.D.
Muscle Strength				
ST5 Normal Reduced	 25 (92.6) 2 (7.4)	 22 (100.0) 0 (0.0)	0.192	N.D.
Muscle Performance				
ST60[#] low intermedium high	 9 (33.3) 8 (29.6) 10 (37.0)	 0 (0.0) 7 (31.8) 15 (68.2)	**0.008**	N.D.
Gait Speed good low	 6 (27.3) 16 (72.7)	 12 (57.1) 9 (42.9)	**0.047**	 1.000 (reference) 3.556 (0.993 – 12.733)‡

*P- value determined by $\chi 2$ test; #- Swiss reference values were used. ‡borderline effect (P-value = 0.051).

Global Strength measured trough *ST5* and muss mass did not show any difference between patients and controls. However, muscle performance, measured trough *ST60* and Gait speed, showed a significant reduction in patients.

In summary, patients seem to have muscle performance decreased but muscle physical properties, strength and mass did not reveal any difference in comparison with healthy controls.

3. Polymorphisms genotype analysis

3.1 TaqMan RT-PCR Assay results

In **figure 11** is shown a typical graphical display of the results obtained for genotyping distribution (the example shown is for *ACTN3* gene polymorphism). Briefly, each spot represents one single sample. On vertical axis we detect the homozygous samples for allele 2 (FAM fluorophore); on horizontal axis are represented the homozygous for allele 1 (VIC fluorophore), while in the diagonal we can identify the heterozygous samples.

After genotyping analysis, the SNPs distribution was performed for each SNP individually and their genetic contribution to the development of the disease was evaluated through logistic regression (**table 11**). To perform the following correlations, we found important to verify if the four SNPs were in Hardy-Weinberg Equilibrium (HWE). All the populations follow HWE except for TaqI SNP (**p-value = 0.021**).

***ACTN3 R557X* (rs1815739)**

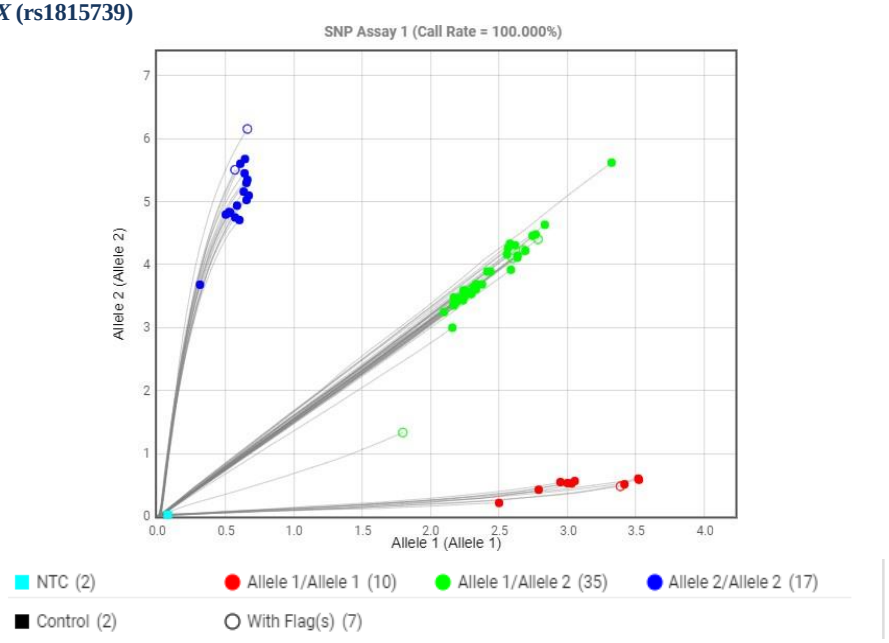

Figure 11 - *ACTN3* samples expression profile. Allele 1 corresponds to Timine, Allele 2 to a Citocine. NTC- Non-template control

Considering that three SNPs from the same gene (*VDR*) were analysed, we also evaluated the possibility of establishing a haplotype, as referred in literature. However, the allele combination obtained for our populations did not revealed a statistically significant combination to be correlated with the disease development. After a multiple-SNP analysis, the results showed the existence of *Linkage Disequilibrium* between ApaI and TaqI SNPs from *VDR* gene (D' = 0.9382, P-value ≤0.001).

After muscular characterization and correlation between groups, we wanted to analyse polymorphism genotypes among the two populations to see if there was any considerable difference between each group genotype expression. For that we studied every possible genotype (homozygous, heterozygous and homozygous for the variant in case), and applied, through logistic regression models, a dominant model for the variant genotype for each gene polymorphism, to see if the dominance of the variant SNP exhibit differences between HC and patients group (**table 11**).

According to the results obtained, we could state that the SNPs individually could not be associated to an increased risk to the development of axSpA disease. However, we still had to correlate *ACTN3* and *VDR* gene polymorphisms with established clinical criteria (BASFI, BASDAI and BASMI) parameters specific for axSpA disease (**table 13**).It is important to highlight that those parameters are only applied to patients, since that we could not perform the logistic regression, the allelic distribution between each parameter was evaluated by the Chi-Square ($\chi 2$) test.

Nevertheless, we hypothesize how relevant might be the genetic background influencing muscle performance parameters identified as statistically significant (*ST60* and Gait Speed).

To understand the hypothetical effect of single SNP in each one of the physical parameters identified as different between both populations, we applied the regression logistic model adjusted to the presence of the genetic component, the results were presented in table below (**table 12**).

Table 11- Genotype distribution and axial Spondyloarthritis (axSpA) for *ACTN3* and *VDR* polymorphisms: rs1815739; rs2228570, rs731236 and rs7975232, respectively

Genotype	Cases *n* (%)	Controls *n* (%)	P-value*	OR[a] (95% CI)
ACTN3				
C/C	8 (30.8)	6 (25.0)	0.463	1.000 (reference)
C/T	15 (57.7)	12 (50.0)		0.375 (0.066 – 2.145)
T/T	3 (11.5)	6 (25.0)		0.938 (0.255 – 3.449)
C/T + T/T	18 (69.2)	18 (75.0)		0.750 (0.216 – 2.602)
VDR - FokI				
A/A	4 (15.4)	2 (8.3)	0.339	1.000 (reference)
A/G	12 (46.2)	16 (66.7)		0.375 (0.059 – 2.397)
G/G	10 (38.5)	6 (25.0)		0.833 (0.115 – 6.013)
A/G + G/G	22 (84.6)	22 (91.7)		0.500 (0.083 – 3.017)
VDR - TaqI				
A/A	11 (42.3)	7 (30.4)	0.599	1.000 (reference)
A/G	7 (26.9)	9 (39.1)		0.495 (0.126 – 1.945)
G/G	8 (30.8)	7 (30.4)		0.727 (0.181 – 2,914
A/G + G/G	15 (57.7)	16 (69.6)		0.597 (0.183 – 1.943)
VDR - ApaI				
C/C	7 (28.0)	3 (12.5)	0.400	1.000 (reference)
C/A	9 (36.0)	10 (41.7)		0.351 (0.070 – 1.761)
A/A	9 (36.0)	11 (45.8)		0.386 (0.076 – 1.959)
C/A + A/A	18 (72.0)	21 (87.5)		0.367 (0.083 – 1.633)

* p-value $\chi 2$ test. Abbreviations: [a] ORs and 95% CI for specific categories were calculated using logistic regression models; OR, odds ratio; CI, confidence interval.

Table 12- Impact of *ACTN3* and *FokI* association on muscle stiffness and gait speed parameters.

Characteristics	OR crude (95% CI)	OR adjusted to *ACTN3 R377X* (95% CI)	OR adjusted to *VRD* – Fok1 (95% CI)
Muscle Stiffness			
Low	1.000 (Reference)	1.000 (Reference)	1.000 (Reference)
Intermediate	**4.400 (1.041- 18.599)[a]**	**4.436 (1.032 - 19.064)[b]**	**4.378 (1.022 – 18.749)[c]**
High	3.143 (0.751- 13.159)	2.595 (0.599 – 11.235)	2.816 (0.656 – 12.088)
Gait Speed			
good	1.000 (reference)	1.000 (reference)	1.000 (reference)
low	3.556 (0.993 – 12.733)[d]	**3.911 (1.044 – 14.658)[e]**	**3.785 (1.025 – 13.982)[f]**

ORs and 95% CI for specific categories were calculated using logistic regression models; OR, odds ratio; CI, confidence interval. [a] P-value = 0.044; [b] P-value = 0.045; [c] P-value = 0.047; [d] P-value = 0.051 (borderline effect); [e] P-value = 0.043; [f] P-value = 0.047.

Data shown only relates the information for *ACTN3 R377X* and *VDR FokI* SNPs, since the logistic regression of other two SNPs did not show statistically significant results.

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Table 13- Clinical criteria and its correlation with *ACTN3* and *VDR* genes polymorphisms

SNPs	BASFI			BASDAI			BASMI			
	Cases n (%)		*p*-Value*	Cases n (%)		*p*-Value*	Cases n (%)		*p*-Value*	
	No functional repercussion	Functional repercussion		No active disease	Active Disease		Mild Score	Severe Score		Controls n (%)
ACTN3										
C/C	6 (27.3)	2 (50.0)	0.633	6 (28.6)	2 (40.0)	0.700	8 (34.8)	0 (0.0)	0.398	6 (25.0)
C/T	13 (59.1)	2 (50.0)		12 (57.1)	3 (60.0)		12 (52.2)	3 (100.0)		12 (50.0)
T/T	3 (13.6)	0 (0.0)		3 (14.3)	0 (0.0)		3 (13.0)	0 (0.0)		6 (25.0)
VDR – FokI										
A/A	3 (13.6)	1 (25.0)	0.247	3 (14.3)	1 (20.0)	0.534	4 (17.4)	0 (0.0)	0.188	2 (8.3)
A/G	9 (40.9)	3 (75.0)		9 (42.9)	3 (60.0)		9 (39.1)	3 (100.0)		16 (66.7)
G/G	10 (45.5)	0 (0.0)		9 (42.9)	1 (20.0)		10 (43.5)	0 (0.0)		6 (25.0)
VDR – TaqI										
A/A	11 (50.0)	0 (0.0)	0.323	9 (42.9)	2 (40.0)	0.810	10 (43.5)	1 (33.3)	0.886	7 (30.4)
A/G	5 (22.7)	2 (50.0)		5 (23.8)	2 (40.0)		6 (26.1)	1 (33.3)		9 (39.1)
G/G	6 (27.3)	2 (50.0)		7 (33.3)	1 (20.0)		7 (30.4)	1 (33.3)		7 (30.4)
VDR – ApaI										
C/C	7 (31.8)	0 (0.0)	0.415	6 (28.6)	1 (25.0)	0.692	6 (26.1)	1 (50.0)	0.528	3 (12.5)
C/A	8 (36.4)	1 (33.3)		7 (33.3)	2 (50.0)		9 (39.1)	0 (0.0)		10 (41.7)
A/A	7 (31.8)	2 (66.7)		8 (38.1)	1 (25.0)		8 (34.8)	1 (50.0)		11 (45.8)

* P-value $\chi 2$ test

Discussion

This study, involving young axSpA patients with short disease duration, have not shown any difference in muscle physical properties, compared to HC. However, an asymmetry for muscle stiffness would be evident between lumbar and appendicular lower limb muscles. In addition, no differences in global muscle strength or mass were registered, but a reduction in muscle performance through two different approaches, *ST60* and Gait speed. It would be of interest to know if genes related with muscle performance, as *ACTN3* and *VDR*, may contribute to explain such differences.

As expected, due to our selection criteria, both groups (SpA patients and HC) are largely similar, regarding age, gender, levels physical activity (**table 8**). When analyzing disease related characteristics, the majority of patients exhibit low disease activity, low functional and low metrological repercussion (**table 8**).

Most of our patients are HLA-B27 positive (80.8%) which is in line with the percentages already found for Portuguese population [54].

The MyoSpA study allows for the first time an extensive muscle characterization regarding muscle physical properties (stiffness, elasticity and tone) in different corporal segments and simultaneously, parameters such as muscle strength, muscle mass and muscle performance, that allow sarcopenia diagnosis and sarcopenia severity classification [28].

Contrary to our expectations no differences in muscle physical properties were registered in different body segments studied. Curiously, a slight tendency for higher values of muscle stiffness were registered in lumbar area which is in agreement to previous published results [28]. A speculative explanation for this asymmetry, between axial and peripheral muscles, might be related with local inflammatory process in the rachis. This also possibly explain the stiffness, as the main symptom in parallel of pain, in this group of patients.

In our population, no sarcopenia diagnosis was established, neither in SpA patients nor in HC. This result might be explained by younger age of patients and HC and short disease duration and low levels of disease activity and severity of the included patients. If no differences in global muscle strength or muscle mass were registered, muscle performance has shown to be significantly reduced in axSpA, when assessed by *ST60* or gait speed. Data evaluating muscle performance using the recommended EWGSOP tools [29] are still scarce for SpA patients [55,56]. The most commonly tool used is gait speed and published studies have shown similar reductions in muscle performance [55,56].

In this study we selected several SNPs of well-known genes – *ACTN3* [57,58] and *VDR* [58] - related with muscle performance to evaluate their influence in axSpA susceptibility, axSpA phenotype and their impact in muscle properties.

None of the four markers showed significant single-locus disease associations ($p > 0.05$), suggesting that neither *ACTN3* nor *VDR* were a major determinant of axSpA susceptibility in our population. No association has been identified with these genes in genome wide association studies (GWAS) in SpA to date [59,60]. No association was observed between these SNPs and specific clinical parameters, which means that *ACTN3* and *VDR* polymorphisms do not markedly influence axSpA disease activity, functionality, and severity, as measured by BASDAI, BASFI and BASMI respectively. However, some *VDR* polymorphisms were linked to some musculoskeletal diseases as idiopathic scoliosis susceptibility or curve severity, herniation and spinal tissues degeneration and rheumatoid arthritis [61–64]. In addition,

in experimental models of *VDR* null mutant mice document diffused muscle fiber abnormalities and severe changes in muscle cell differentiation or fiber development/ maturation [65,66]

However, these genes have been established as contributing to variation in lean body mass and bone mass density contributing to understand the molecular bases of sarcopenia and osteoporosis [39,67–69]. In 2003, Yang et al. demonstrated a significant association between *ACTN3* genotype and athletic performance. They found that both male and female elite sprint athletes have significantly higher frequencies of the 577R allele compare to controls. Later on several papers have consistently reported a strong association between RR genotype and elite power performance [57,70–73]. Similar evidence were documented for *VDR* polymorphisms [74,75]. In this context it was our interest to study these genetic variants in the axSpA context looking for lower frequencies of the variants related with high performance in the group of patients and simultaneously evaluate any association with muscle physical properties, strength, and muscle mass.

No association was established with the studied SNPs and muscle physical properties, namely stiffness, tone, or elasticity. To the best of our knowledge this association was never performed.

Again, no association for overall strength (*ST5*) was registered in our cohort. However, in several studies *ACTN3 577R* allele and *VDR* was associated with higher levels of strength. The rs540874 polymorphism of *ACTN3* gene was associated with the muscle function of lower limb (women with G allele were likely to be higher strength compared with A allele) but not higher limb, in postmenopausal women. Interestingly, in the same study, the improvement of muscle strength after intervention (exercise and Vitamin D supplementation) were possibly correlated with rs540874, rs618838 and rs2229456 polymorphisms [76]. A significant association between *VDR* genotype and quadriceps (23% difference) and grip (7% difference) strength was observed in non-obese elderly women [77].

In elite Italian soccer players, an interaction of two polymorphisms (*ACE* and *ACTN3*) predicted explosive leg-muscle strength. However the contribution of genetic factors was only 23.92% [78].

The analysis of these genetic markers regarding lean muscle mass did not show any difference between both groups. It is well-known that muscle performance is heavily influenced by basal muscle mass and its dynamic response to training. Genetic factors account for approximately 50-80% of inter-individual variation in lean body mass, with impacts detected on both 'training-naive' muscle mass and its growth response [75].

However, in one study involving older Caucasian men whole body and thigh non-skeletal lean mass were independent of *ACTN3* R/X polymorphisms [79]. Contrary, VDR expression decreases with age and *VDR* genotype seems to be associated with fat-free mass in elderly men and women [75].

The MyoSpA study data, have shown a clear reduction in axSpA muscle performance without changes in muscle physical properties, strength, and mass. This observation allows us to speculate about a possible muscle dysfunction that should be explored in future studies. As genetic has a strong influence in disease overall SpA susceptibility, it is of main interest to investigate a possible genetic base to explain this change compromise in muscle performance. Our results were speculative but potentially indicate that *ACTN3* 577R and *VDR FokI* SNPs might influence Gait Speed [OR, 3.911; 95% CI (1.044 – 14.658) and OR, 3.785; 95% CI (1.025 – 13.982), respectively]. Our understanding on the influence of those genes will be helpful not only for clinicians as diagnostic tools but also to identify patients for whom exercise should be really helpful.

Furthermore, we also evaluate the possibility to establish a specific haplotype for *VDR* gene SNPs. The sample size was the main limitation not allowing the haplotype characterization. Also, we hypothesize, based on literature, that the study of *VDR Bsml* polymorphism would be of utmost importance to stablish this link, since the VDR haplotype is constituted by *BsmI*, *ApaI* and *TaqI* SNPs.

A multiple- SNP analysis revealed that *TaqI* polymorphism do not follow HWE (p-value= 0.021), meaning that its frequency it is not constant. There is also a *Linkage Disequilibrium* between *ApaI* and *TaqI* SNPs. This corroborate some studies [80] and means that both SNPs are always transmitted in block [81] (data not shown).

This study, at our knowledge, is the very first including the genetic susceptibility analysis for muscle performance in the axSpA context. So, the scarce of bibliographic data, made it not a conclusive final study but an exploratory one that should be regarded as 'proof of concept'.

Conclusion

With this study we can conclude that genetic component related with *ACTN3* and *VDR* genes cannot explain entirely the differences in muscle performance identified in the axSpA context. None of the four SNPS produce any impact in disease susceptibility nor in disease characteristics, measured through BASDAI, BASMI and BASFI.

Since this was a pilot study, all the results should be cautiously interpreted.

Future perspectives

It would be interesting to continue this project, increase sample size with more heterogeneous patients to give us more robust results.

A population quantification of vitamin D levels would be necessary for a better interpretation of *VDR* variants.

A prospective study design will allow to answer to the main question- muscle changes are a cause or a consequence of the disease. This approach would fulfill a gap in this area and could lead to results that could be useful to diminish the psychological, social, and physical burden of Spondyloarthritis.

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Annex section

I. Physical performance measure cut- points

Taking into account the clinical knowledge, we defined the following cut-offs:

BMI - Using the current classification, defined by World Health Organization (WHO), for BMI. Adapted from ref. ([82])

Table S15- Classification of body mass index accordingly to WHO criteria.

Low weight	<18.5
Normoponderal	18.5 – ≤ 24.9
Overweight	25- ≤ 29.9
Obesity	≥ 30

Age- Since the mean age for the beginning of the symptoms is around 40 years old and considering our population mean age and inclusion criteria, we defined the following age cut-offs:

Table S16- Age cut-off points

0-29 years old
30-39 years old
40-50 years old

BASDAI

Table S17- BASDAI clinical cut off points

0-3.99	non-active disease
≥ 4	active disease

BASFI

Table S18- BASFI clinical classification scale

0-4	No functional repercussion
>4	Disease functional repercussion

BASMI

Table S19 - BASMI clinical cut-off points

0-2	No perceptible range of movements limitation
≥3	Impact on range of movements

Multifidus trunk muscle L3-4 evaluation- For the following muscle parameters: M.F, M.D, M.S, cut off was calculated using only reference values of HC and terciles (P33-P66). It is important to highlight that although it is known that tonus and rigidity exhibit differences between both genders (male and female), due to the small size of our sample, this fact was no taken into account for the cut off values definition and analysis.

M.Fmean – Muscle tonus

Table S20- Muscle tonus cut-off

0-13.72	Low
13.73-14.76	Intermedium
≥14.77	High

M.Dmean – Muscle Decrement

Decrement corresponds to the inverse of elasticity, which is in fact the property we want to study. Values above the 95% CI were considerate as having low elasticity level and values bellow the CI as having a high elasticity level. Therefore:

Table S21- Muscle decrement cut-off points

0-1.06	Higher elasticity level
1.07-1.24	Intermedium elasticity
≥1.25	Lower elasticity level

M.Smean- Muscle Stiffness

Table S22- Muscle stiffness cut- off points

0-240.48	low muscle stiffness
240.49-275.88	Intermedium
≥275.89	high muscle stiffness

Gastrocnemius (inferior limb) muscle evaluation

For the following muscle parameters: G.F, G.D, G.S, cut off was calculated using only reference values of HC and terciles (P33-P66). It is important to highlight that although it is known that tonus and rigidity exhibit differences between both genders (male and female), due to the small size of our sample, this fact was no taken into account for the cut off values definition and analysis.

G.Fmean – Muscle tonus

Table S23- Muscle tonus cut- off points

0- 15.60	Low tonicity
15.61- 16.80	Intermedium
≥ 16.81	High tonicity

G.Dmean – Muscle Decrement

Table S24- Muscle Decrement cut- off points

0-1.16	High elasticity
1.17- 1.36	Intermedium
≥1.37	Low elasticity

G.Smean- Muscle Stiffness

Table S25- Muscle Stiffness cut-off points

0-280.50	low rigidity
281-311	Intermedium
≥ 312	High rigidity

Strength measurements

ST5- measured in seconds. Can be used as a strength estimative. Due to small size of our population the value was not measured considering the differences for female gender.

Table S26- Sit to stand standard cut-off point

0-15	Absence of sarcopenia
>15	Evidence of sarcopenia

Performance measurement

ST60- number of repetitions/minute. We used the reference values for Swiss population for CI 25%-75% (see table S12)

Table S27- CI 95%, P25-P50 values for ST60 test in Swiss population. Adapted from ref. ([50])

Age group (years)	Number of STS repetitions									
	Men					Women				
	p2.5	p25	p50	p75	p97.5	p2.5	p25	p50	p75	p97.5
20–24	27	41	50	57	72	31	39	47	55	70
25–29	29	40	48	56	74	30	40	47	54	68
30–34	28	40	47	56	72	27	37	45	51	68
35–39	27	38	47	58	72	25	37	42	50	63
40–44	25	37	45	53	69	26	35	41	48	65
45–49	25	35	44	52	70	25	35	41	50	63
50–54	24	35	42	53	67	23	33	39	47	60
55–59	22	33	41	48	63	21	30	36	43	61
60–64	20	31	37	46	63	20	28	34	40	55
65–69	20	29	35	44	60	19	27	33	40	53
70–74	19	27	32	40	59	17	25	30	36	51
75–79	16	25	30	37	56	13	22	27	30	43

Speed of march- In meters/second. This parameter cut off point was calculated based in the terciles (P33 and P66) for HC population. CI 95%. Differences between gender and age were not considered due to reduced population size.

Table S28- Speed of march cut off point

>0.8 m/s	good performance
≤ 0.8 m/s	low performance

Lean Mass- This parameter diverges from person to person. The evaluation was performed for each individual accordingly to the results given by bioimpedance device.