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The Role of Muscle in Spondyloarthritis

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Dedication

I dedicate this thesis to my family whose endless love, support and inspiration has helped make this possible. Thank you.

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Abstract (Portuguese)

A espondiloartrite axial (axSpA) é uma doença reumática inflamatória, caracterizada principalmente pelo envolvimento da coluna e articulações sacroilíacas, e geralmente apresenta-se como dor crónica nas costas e rigidez. À medida que a doença progride, a mobilidade da coluna vertebral e a função física são prejudicadas podendo afetar as atividades da vida diária. A genética e os fatores ambientais (microbiota e microtrauma) são as causas conhecidas da suscetibilidade e progressão da doença. Esta tese teve como objetivo melhorar o nosso conhecimento atual da fisiopatologia da axSpA, caracterizando as propriedades musculares axiais e periféricas e identificando biomarcadores genéticos e proteicos que possam explicar tais propriedades. Realizamos um estudo transversal com 54 participantes: 27 pacientes com axSpA e 27 controles saudáveis (HC), pareados por idade, sexo e nível de atividade física. Dados epidemiológicos, clínicos e de caracterização muscular (propriedades físicas musculares, força, massa e desempenho) foram registados e comparados entre pacientes com axSpA e HC. Foi ainda colhido sangue periférico para abordagens ómicas. A transcriptómica e a proteómica foram realizadas por sequenciação de RNA e tecnologias de espectrometria de massa, respectivamente. Os nossos resultados indicam que pacientes com axSpA (idade média de 36,5 (DP 7,5) anos, 67% do sexo masculino e duração média da doença de 6,5 (3,2) anos) não apresentaram diferença significativa na rigidez muscular segmentar em comparação com os HC, apesar de apresentarem uma discreta menor rigidez lombar. Pacientes com axSpA, comparados com os HC, apresentaram menor força total, bem como menor força nos membros superiores e inferiores, independentemente das propriedades físicas dos músculos. Os pacientes também apresentaram velocidades de marcha significativamente menores do que o HC. As características da marcha podem representar um potencial biomarcador em pacientes com axSpA. A análise de enriquecimento de genes expressos diferencialmente permitiu revelar vias metabólicas significativas (como sinalização de IL6 e vias do sistema imunológico) com papel patológico para esse grupo de pacientes. Salienta-se ainda que níveis séricos aumentados de várias citocinas pró-inflamatórias em pacientes com axSpA foram observados em correlação adequada com os parâmetros de atividade da doença. Além disso, foram identificados genes expressos diferencialmente associados ao músculo (como *NACA, FRG1* e *ARPC5L*) desempenham papeis no desenvolvimento de miotubos e montagem de actina, afetando eventualmente a força muscular em pacientes com axSpA. Finalmente, a integração dos resultados da transcriptómica e da proteómica mostra que a análise dos genes e proteínas diferencialmente expressos permite obter uma clara

discriminação entre pacientes com axSpA e HCs, possibilitando ainda avançar no diagnóstico, prognóstico e eventuais opções terapêuticas para esse grupo de pacientes. Globalmente, os resultados aqui obtidos permitem oferecer algumas possibilidades interessantes para explicar o papel do músculo na patogénese da axSpA.

Palavras-chave: Espondiloartrite Axial, Músculo, Microtrauma, Transcritómica, Proteómica.

Abstract (English)

Axial spondylarthritis (axSpA) is an inflammatory rheumatic disease, characterized primarily by the involvement of the spine and sacroiliac joints, and usually presenting as chronic back pain and stiffness. As the disease progresses, impaired spinal mobility and physical function may impact activities of daily living. Genetics and environmental factors (microbiota and microtrauma) are the known causes of disease susceptibility and progression. This thesis aimed to improve our current knowledge of axSpA physiopathology by characterizing axial and peripheral muscle properties and identifying genetic and protein biomarkers that might explain such properties. We performed a cross-sectional study on 54 participants: 27 patients with axSpA and 27 healthy controls (HC), matched by age, gender, and level of physical activity. Epidemiological, clinical, and muscle characterization (muscle physical properties, strength, mass, and performance) data were registered and compared between patients with axSpA and HC. Peripheral blood was collected for omics approaches. Transcriptomics and proteomics were performed by RNA-sequencing, RT q-PCR, and mass spectrometry technologies, respectively. Our results indicate patients with axSpA (mean age 36.5 (SD 7.5) years, 67% males, and mean disease duration of 6.5 (3.2) years) had no significant difference in segmental muscle stiffness compared with the HC, despite showing a slight numerically higher lower lumbar stiffness. Patients with axSpA, compared to the HC, had lower total strength as well as lower strength in the upper and lower limbs, independently of muscle physical properties. Patients also had significantly lower gait speeds than the HC. Gait characteristics may represent a potential biomarker in patients with axSpA. Enrichment analysis of differentially expressed genes reveals significant pathways (such as IL6 signaling and immune system pathways) with a pathological role for this group of patients. Notably, increased serum levels of various pro-inflammatory cytokines in patients with axSpA have been observed in proper correlation with disease activity parameters. Moreover, differentially muscle-associated expressed genes (such as *NACA*, *FRG1*, and *ARPC5L*) play roles in the development of myotubes and actin assembly, eventually affecting muscle strength in patients with axSpA. Worth of note, the integration of transcriptomic's and proteomic's results shows number of genes and proteins causes a clear discrimination between patients with axSpA and HCs that may advance diagnosis, prognosis, and therapeutic options for this group of patients. This work, taken together, provides some interesting possibilities to explain the role of muscle in the pathogenesis of axSpA.

Abstract

Keywords: Axial Spondylarthritis, Muscle, Microtrauma, Transcriptomics, Proteomics.

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List of Abbreviations:

5STS: five-times Sit-To-Stand
APOB: Apolipoprotein B
AS: Ankylosing spondylitis
ASAS: Assessment of Spondyloarthritis International Society
axSpA: axial SpA
BASDAI: Bath AS Disease Activity Index
BASFI: Bath Ankylosing Spondylitis Functional Index
BASMI: Bath Ankylosing Spondylitis Metrology Index
BASMI: Bath AS Metrology Index
bDMARD: biologic Disease-Modifying Anti-Rheumatic Drugs
BMD: Bone Mineral Density
BMI: Body Mass Index
CD: Crohn's Disease
cDMARD: conventional Disease-Modifying Anti-Rheumatic Drugs
Cox17: Cytochrome C Oxidase assembly protein 17
CRP: C-reactive Protein
CSA: Cross-Sectional Area
ELISA: Enzyme-linked Immunosorbent Assay
ER: Endoplasmic Reticulum
ERAD: Endoplasmic Reticulum-Associated Degradation
ERAP: Endoplasmic Reticulum Aminopeptidases
ESR: Erythrocyte Sedimentation Rate
ETC: Electron Transport Chain
EULAR: European League Against Rheumatism
EWGSOP: European Working Group on Sarcopenia in Older People
GCP: Good Clinical Practice
GWAS: Genome-Wide Association Studies
HLA: Human Leukocyte Antigen
IBD: Inflammatory Bowel Disease
IDA: Information-Dependent Analysis
IEC: Intestinal Epithelial Cells
IFNG: Interferon Gamma

List of Abbreviations

IPA: Ingenuity Pathway Analysis
IPAQ: International Physical Activity Questionnaire
JAK: Janus Kinases
LPS: Lipopolysaccharide
m-SASSS: modified Stoke Ankylosing Spondylitis Spinal Score
MASES: Maastricht Ankylosing Spondylitis Enthesitis Score
MD Plot: Mean Difference Plot
MHC: Major Histocompatibility Complex
mNYc: modified New York criteria
MRI: Magnetic Resonance Imaging
MRP: Mitochondrial Ribosomal Proteins
MSC: Mesenchymal Stem Cell
N6AMT2: N (6) -adenine-specific DNA Methyltransferase 2
nanoLC: nano Liquid Chromatography
NFκB: Nuclear Factor kappa B
NK: Natural Killer
nr-axSpA: non radiographic-axSpA
NSAID: Nonsteroidal Anti-Inflammatory Drugs
mNYc: modified New York criteria
PBMC: Peripheral Blood Mononuclear Cells
PCA: Principal Component Analysis
PPIA: Peptidylprolyl isomerase A
pSpA: peripheral SpA
PsSpA: Psoriatic Spondylarthritis
r-axSpA: radiographic axSpA
RA: Rheumatoid Arthritis
ROS: Reactive Oxygen Species
RT- qPCR: Reverse Transcription-quantitative Polymerase Chain Reaction
SD: Standard Deviations
SIJ: Sacroiliac Joint
SOCS3: Suppressors Of Cytokine Signalling 3
sEMG: Surface Electromyography
SFB: Segmented Filamentous Bacteria
SMA: Smooth Muscle Actin

List of Abbreviations

SMMHC: Smooth Muscle Myosin Heavy Chain
SNP: Single Nucleotide Polymorphisms
SpA: Spondyloarthritis
sPLSDA: sparse partial least-squares discriminant analysis
SPSS: Statistical Package for Social Science
STAT: Signal Transducer and Activator of Transcription
SWATH-MS: Sequential Window Acquisition of all Theoretical Mass Spectra
TCR: T Cell Receptors
TNF-α: Tumour Necrosis Factor-α
TSA: Total Spinal Ankylosis
UC: Ulcerative Colitis
UPR: Unfolded Protein Response

Chapter 1: Introduction

1.1 Complex Diseases

1.1.1 What are complex diseases?

Complex diseases are defined as medical conditions that arise from a complex interaction of inherited and environmental factors. In fact, the vast majority of diseases are classified as complex diseases, such as Alzheimer's disease, Parkinson's disease, Asthma, Multiple Sclerosis, Psoriasis, Rheumatoid Arthritis and Inflammatory Bowel Disease (1).

In our body, there are not only constant interactions within genetic variants and among environmental factors, respectively, but also continual interactions between host and environment (Figure. 1.1). In a healthy condition, a balanced manner is maintained among these interactions. Nevertheless, dysregulation of the host-environment interplays may activate the onset of complex diseases and persist to play a continuing role during disease progression (2).

Genetic predisposition reflects part of the risk associated with complex disease phenotypes. In comparison with the single gene-controlled diseases following a Mendelian pattern of inheritance, complex diseases are polygenic conditions. where the interactions of diverse genetic susceptibility risk factors may play a more critical role than a single contributory gene (1,2).

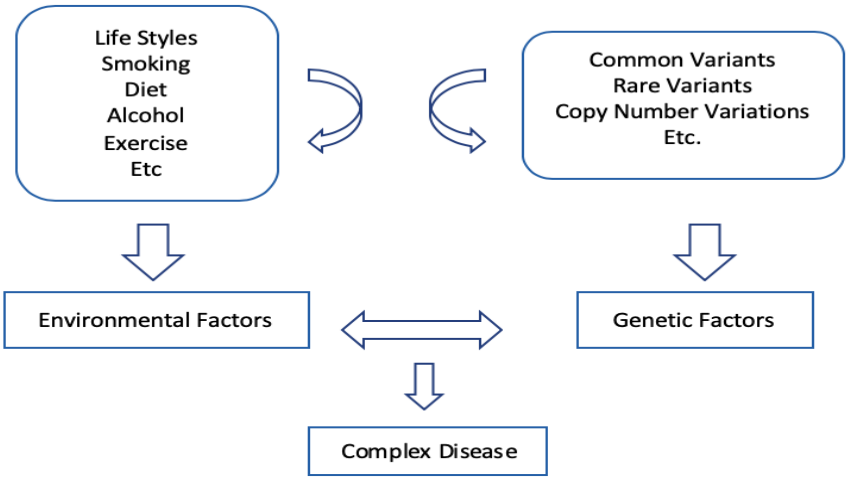


Figure 1.1. Aberrant gene-environment interaction triggers onset of complex diseases. During disease onset and development, there are constant interactions within genetic variants and among environmental factors, respectively. Continual interactions between host and environment also exist.

1.1.2 Causes of Complex Diseases

In general terms, complex diseases are caused by a combination of genetic and environmental factors, the majority of which have yet to be characterised. It is an intriguing task to discriminate against a single causal factor and to define its role in a complex disease due to (1) the effect of a solo factor may be confounded or obscured via other contributing factors; (2) genes and environment both encompass a wide range of variables. Indeed, the complexity of the interaction and combinations of diverse genetic and environmental factors has posed difficulties to investigation on complex diseases (3).

1.1.2.1 Genetics

Genetically, the intricacy of most complex diseases is possibly by reason of contribution and interactions of several susceptible genes. Indeed, diseases that follow the single gene- dominant or single gene-recessive Mendelian patterns of inheritance are infrequent. Since the 19th century, numerous diseases have been determined which do not follow Mendel's rules of inheritance, hence prioritising research of identification of genetic factors that drive the onset and progression of complex genetic diseases (4).

Genomic studies have delivered advantageous insights into the involvement of individual genes/single nucleotide polymorphisms (SNPs) and potential gene-gene interplays to the phenotypes associated with diverse complex diseases (5). The Human Genome Project has exposed that SNPs are greatly prevalent in the human genome (6). SNPs may alter from individual to individual and majority of SNPs have no consequence on health or development. Although, some of them are in association with human disease (6). If a SNP arises in the coding region of a gene which contributes to conferring disease susceptibility, the SNP may interrupt the generation of a functional gene product. Accordingly, there is an elevated likelihood that this SNP will validate a phenotypic effect. More frequently, SNPs are observed in DNA segments between genes, namely in a regulatory region near a gene. If it occurs, the SNPs may impact directly gene-gene interplays, e.g., functioning as enhancers. Accordingly, through SNPs mapping and associating them with a specific phenotypic effect, SNPs can be utilised as genetic markers for evaluating disease risk. For example, if a SNP occurs to be more prevalent in affected subjects than healthy controls, this proposes that the SNP is physically near to the disease-causing mutation or performs a critical task in the expression of the functional genes (7).

Genome-Wide Association Studies (GWAS) have an important role for the understanding of the genetic basis of complex diseases. Pursuing to identify genetic associations with complex diseases, GWAS have conducted wide-ranging analysis of SNPs correlated with complex diseases across the entire human genome, which often deliver insights into molecular pathways that lie beneath the disease. Nevertheless, these investigations are not without downsides. Some investigators claim that GWAS function without a setting for discriminating what is functionally relevant, and henceforth they are of limited value (8,9). Furthermore, the genetic variants observed in GWAS can only describe a minor portion of the disease risk, due to the fact that rare variants (0.01%-0.05% frequencies) with intermediate effects will be neglected by GWAS (9,10). Providentially, the advanced know-hows in sequencing with significant upsurges in speed and volumes are allowing the identification of variants with lower frequencies (11). Despite the given limitations, GWAS still perform dominant tools to discover new sets of genes associated in a diversity of complex diseases (12).

In the past years, remarkable progress has been completed in complex disease research across several omics' layers from genome, transcriptome and proteome to metabolome. There is a growing understanding of the importance of biological interconnections, and much accomplishment has been succeeded through systems biology approaches (13). Nevertheless, due to the usual focus on one particular omics layer at a time, obtainable systems biology outcomes explain merely a modest part of complex disease. Current progress in multi-omics data collection and sharing provide us novel opportunities for exploring complex diseases more broadly, and yet concurrently brings new difficulties considering the data dimensionality and diversity (14).

By reviewing the whole picture, investigators are better positioned to identify causal genes, which may in turn be regulated by environmental factors, and vice versa. Translation of critical findings to the clinical settings proves that GWAS and Omics approaches potentially assist the progress of personalised medicine and optimum clinical care.

1.1.2.2 Environment

By considering genetic risks are of importance for the onset and development of complex diseases, environmental factors are not negligible. Frequently considered environmental triggers comprise lifestyles, smoking, diet, alcohol, exercise, etc. Lately, the gut microbiome is counting as a significant environmental factor in complex disease studies (15).

The human gut is occupied by trillions of microorganisms and healthy status relies on the stability and balance of the interplays between host and intestinal bacteria. In the gastrointestinal tract, the colonisation of gut microbiota is influenced by the host genetic information. Besides, the host immune system is continuously monitoring the potential invasion by pathogens while at the same time tolerating trillions of commensals. Conversely, these microbes dynamically adjust the host immune response in a mutually advantageous relationship. Numerous types of cells in the gut including intestinal epithelial cells (IECs), innate lymphoid cells, B and T lymphocytes and mononuclear phagocytes can be the objects of such microbial alteration (16).

On the one side, the microbiota composition is influenced by host factors, involving physical (epithelial and mucus layers), immunological (IgA and epithelia-associated immune cells) and biochemical (enzymes and antimicrobial proteins), (17). Therefore, host genetic factors perform an essential role in shaping the gut microbiome. The similarity of intestinal

microbiome in twins has established that the host genome acts a role in forming the gut flora (18). Recent studies have revealed that host genetic polymorphisms and deletions lead to alterations in the patterns of bacterial colonisation (19–22). Those researches have associated definite genetic variants with the quality and quantity of some intestinal bacteria. Consequently, in the presence of diverse inherent genetic factors, the composition and abundance of intestinal commensals may alter from individual to individual. Additionally, the maintenance of the gut microbiome mostly relies on available energy in the gut. One important source of energy is from fermentation and sulphate reduction of host carbohydrates, such as intestinal mucus (23,24). A major component of the mucus layer is highly glycosylated Mucin-2, which delivers energy to the gut microbiome (23). Therefore, the survival of microbes in the gut depends on their phenotypic traits changed with the energy availability in the host gut (25). Additionally, composition of gut microbiome can be affected by psychological health (e.g., depression), lifestyle (e.g., smoking and diet) as well as geographical location of the host and living conditions (urban or rural) (26–29).

On the other side, many microbes co-evolved with the host to adjust the immune system in ways that express mutual benefits, either through direct interplay with the host immune system or via the production of metabolites. Namely, microbiota has been known to play a role in determining local population of the effector/regulatory T-cell axis (30). Segmented filamentous bacteria (SFB) can develop Th17 cell expansion, while Bacteroides fragilis and some Clostridia species elevate Treg populations (31–33). Additionally, butyrate and propionate, two bacteria metabolites, have been revealed to induce the differentiation of colonic/extra-thymic Treg cells in mice, proposing an anti-inflammatory function (34,35). Most prominently, the interplay between commensal microbiota and the mucosal immune system is elemental for appropriate immunity.

In addition to the microbiome, physical activity and inactivity can be the causes of most complex diseases. Mechanisms of physical inactivity are reflected anti-parallel, rather than in series (continuum) to physical activity (36,37). Physical activity and inactivity exist in various mechanistic planes and are not simply mirror images of each other as is frequently assumed. Ideal therapies and preventive approaches demand the understanding of underlying mechanisms. Therefore, it is essential to comprehend that some of the mechanisms by which inactivity triggers chronic diseases vary from mechanisms by which exercise plays principal prevention of the same diseases (38).

1.1.3 Evaluation of gene-environment interactions in complex diseases

Reflecting the interaction between gene products and products of environmental insults in complex diseases, separate exploration of genetic and environmental variants is insufficient (39). Cross-disciplinary research in identifying gene-environment interplays is essential to better predict disease onset and screen disease development. There are a number of researches which assess the interactions of these factors.

Twin studies characterise one of the dominant tools to assess the weight of individual hereditary factors in contributing to a complex disease in comparison with environmental factors. Specifically, monozygotic (MZ) twins share identical genetic background although dizygotic (DZ) twins inherit 50% of their genes. In twins, high concordance rates in MZ and DZ twins of a complex disease determine a robust genetic impact, while low concordance rates indicate environmental contribution (40). As an example, the critical genetic involvement of spondyloarthropathies (SpA) is highlighted by the great concordance rate among MZ twins in

this and other studies, with heritability anticipated to be at least 97% (41). Worth of note, all of the disease severity scores are more closely associated with MZ than in DZ twins, implying that disease severity is partially genetically defined. SpA-affected siblings are more comparable in the year of onset than in age at onset, signifying that environmental factor act a greater role in the timing of onset (42). Hence, to better understand complex diseases, interpretation on gene-environment interaction is required rather than a single factor. Other than twin research, commonly performed strategies for gene-environment interplays involve family- and population-based designs, as well as cohort and case-control (43).

In order to unravel the mechanisms underlying complex diseases, investigators must sort out all the pieces of the puzzle from a black box and begin to assemble them one piece at a time. Most significantly, if we reflect every factor (genetic or environmental) as only one piece of a complex jigsaw puzzle, we need to be aware that various combinations of different numbers of pieces could possibly end in the same picture once a puzzle is finalised.

1.1.4 Clinical impact of gene-environment interactions in complex diseases

The most important usage of gene-environment interplays is personalised medicine, both in prevention and treatment.

Personalised preventive medicine can be applied if the effects of an environmental risk intensely depend on a known genetic factor (44). In this view, it is advantageous to assess the impact of an environmental exposure on subjects with different genetic histories and vice versa. Nevertheless, it is still a challenge to identify high-risk individuals even when a robust gene-

environment interplay is observed, due to the fact that many other confounding factors will be contributed in most cases (44,45).

Personalised treatment depends on better understanding of two fields; gene-environment-drug interplays and genetic individualization of drug responses (45). Adverse drug reactions could potentially be because of several factors involving genetic susceptibility, disease determinants and environmental factors. Since drug response may be relied on in genotype and environmental triggers, a test of real clinical value can be demonstrated through characterising the association of host-environment interplays and drug response. Besides, patient care will also be advanced by novel biomarkers that predict treatment response based on host-environment interplays. If biomarkers can classify patients who will respond positively from those who will be unresponsive or will display adverse reactions, personalised treatments will be compensating and will advance health outcomes, involving those linked to complex diseases. Thus, better consideration of gene-environment interplay of complex diseases would have a major impact on clinical care and public health (39).

1.2 Axial Spondyloarthritis

1.2.1 What is Axial Spondyloarthritis?

A group of inflammatory diseases with various clinical phenotypes are categorised under the predominant term – 'spondylarthritis' (SpA) – which is sub-grouped into conditions distressing predominantly the vertebral (axial) and the peripheral (appendicular) musculoskeletal systems. These conditions are named as axial SpA (axSpA) and peripheral SpA (pSpA), respectively (46). They share pathologic and genetic features, might be overlapping, and involve ankylosing

spondylitis, psoriatic arthritis (PsoA), arthritis associated with Inflammatory Bowel Disease (IBD), reactive arthritis and undifferentiated arthritis (47). Features that connect these entities are a connection with *HLA-B27*, a typical pattern of peripheral arthritis that is asymmetric, oligoarticular and predominates in the lower limbs, and possible sacroiliitis, enthesitis, spondylitis and inflammatory eye disease (48).

1.2.2 Axial Spondyloarthritis Classification Criteria

Ankylosing spondylitis (AS) is known as the classic axSpA condition (49). The term is derived from the Greek words 'angkylos' meaning fusion and 'spondylos' meaning vertebral bodies (50), indicating the endpoint of untreated disorder, that is, a spine that is fused in a position of flexed forward deformity. By description (see Figure 1.2), a diagnosis of AS needs at least mild-moderate structural damage of the sacroiliac joints to be observed radiographically on X-ray (51).

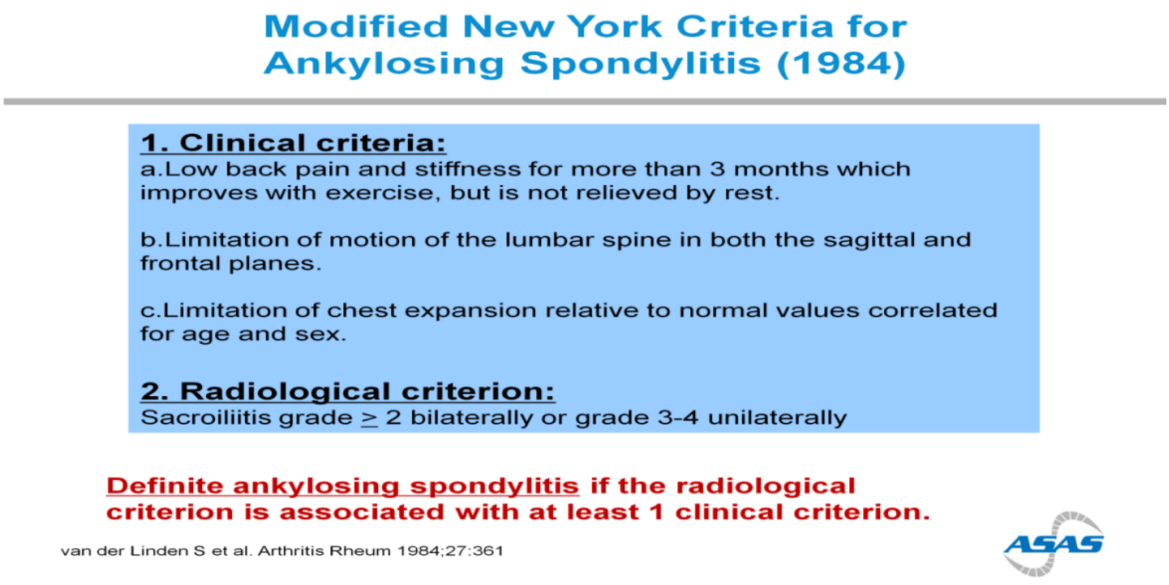


Figure 1.2. Assessment of Spondylarthritis International Society (ASAS) criteria for Ankylosing Spondylitis. Source: ASAS slides downloaded from their website (https://www.asas-group.org/) that originated from scientific paper (51).

Raised knowledge over the last two decades ends in the recognition of comparable disease developments that are earlier or have less structural damage – henceforth the publication in 2009 of a classification for the SpA related conditions, as presented in Figure 1.3 (52). In this classification, AS is counted as a subset of the broader term 'axSpA', and can likewise be called 'radiographic axSpA' – nevertheless, the AS 'label' remains in common use. Those patients with similar clinical findings, but excluding moderate x-ray alterations, are classified as taking 'non radiographic-axSpA' (nr-axSpA).

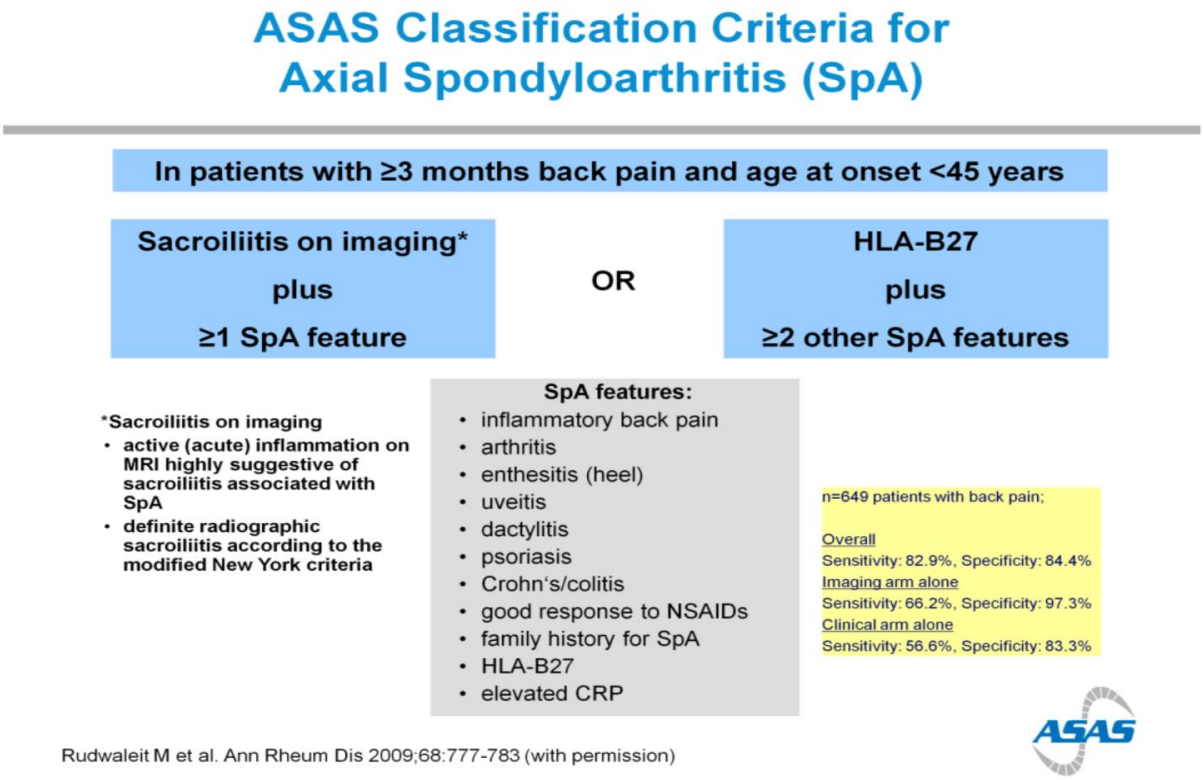


Figure 1.3. Assessment of Spondylarthritis International Society (ASAS) criteria for axial Spondylarthritis. Source: ASAS slides downloaded from their website (https://www.asas-group.org/) that originated from scientific manuscript (53).

1.2.3 Epidemiology of axial Spondyloarthritis

According to the heritable preponderance of AS (further described below), the prevalence of axSpA differs across racial crowds from 0.5 to 1% for AS and up to 1.4% for axSpA (54–56). A recent epidemiological study has reported a prevalence of 1.6% for axSpA in Portugal (57). The frequency of axSpA in females and males is approximately identical, while more males than females are diagnosed with AS (meet the radiographic criteria), in a ratio of 3.1:2 (58). The typical age of onset is in early adulthood, frequently in the third decade of life, while up to 20% might experience their first symptoms before the age of 20 (58). Ancient skeletons with joined spines, belonging to mediaeval Anglo-Saxons and Egyptian pharaohs (59), provide evidence that AS has been affecting humans for significantly longer.

1.2.4. Axial Spondyloarthritis Symptoms and Signs

The major symptoms of axSpA are inflammatory back pain and spinal stiffness (46). Inflammatory back pain is considered according to the following criteria: spinal morning stiffness (lasting more than 30 minutes); nocturnal pain; improvement by exercise and not rest, and chronic back pain (more than three months) with onset earlier than the age of 45 years (60). Additional common manifestations are joint effusions generating swelling (usually lower limb), enthesitis, which derives as tenderness and inflammation at ligament or tendon insertions (e.g., the Achilles tendon) and fatigue. Uveitis, inflammatory bowel disease (including Crohn's) and psoriasis are common extra-articular manifestations of the axSpA (55).

1.2.5. Aetiology and Pathophysiology of Axial Spondyloarthritis

The leading pathological alteration is enthesitis, that is, inflammation at the anatomical zone where tendons, ligaments, or joint capsules connect to the bone (49). The enthesitis arises at multiple zones and may end in osteitis and fatty degeneration of the adjacent bone marrow (61).

Disease localisation shows to be associated with sites of greater biomechanical stress. This involves the sacroiliac joints in over 95% of patients with axSpA (61). The following healing process is assumed to end in amplified bone repair, leading to the characteristic syndesmophyte formation, which can generate a permanent ‘bridge’ over the joint space between one vertebra's bodies (61).

Without treatment, the ongoing disease will lead to a gradual decrease in spinal mobility, often with a progressive kyphosis (flexed forwards) posture, as illustrated in figure 1.4. If spinal ankylosis arises, the impacted joints of the spine will permanently fuse by bony bridging, and when the whole spine is impacted, this ends in ‘total spinal ankylosis’ (TSA): the spine effectively turns to one ‘long bone’ (62).

Figure 1.4. Person with advanced axSpA with total spinal ankylosing and severe thoracic kyphosis. Image shows the spinal ankylosis typical of AS, and these bony/structural changes can mark the evolution of the disease process from nr-axSpA into axSpA.

Source: ASAS slides downloaded from website (https://www.asas-group.org/) 28/03/2018: slides may be used freely therefore specific permission not required.

In addition to considerably limiting mobility, patients with advanced axSpA are susceptible to many disease consequences, leading to a considerably raised all-cause mortality rate, with a risk ratio of 1.6 in comparison to the general population (63). Increased cardiovascular disease is thought to be the leading cause of death (64); however, osteoporosis is the most common comorbidity, it can occur within 10 years of symptom onset (65), and has a prevalence of over 50% (66,67). The combination of osteoporosis and spinal ankylosis increases the risk of both spinal fractures and associated spinal cord injuries (68,69). Therefore, prevention of such sequelae through early diagnosis and optimal management is paramount. However, delays in diagnosis are still common, as diagnosis is made by identifying patterns of symptoms and signs rather than specific or very specific tests, with an average delay of 8 years reported worldwide

(70). Diagnostic delay in patients with axSpA from Portugal is similar to that reported in other developed countries (7.6 ± 0.9 years), (71).

1.2.6. Diagnostic Biomarkers of Axial Spondyloarthritis

The only biomarkers currently widely used in clinical practice are the acute phase reactants c-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), and testing of *HLA-B27* (its expressed protein), (72). Although these tests have noticeable diagnostic and predictive value for response to biological therapies and clinical outcomes, their sensitivity and specificity are not optimal and there is a need for better biomarkers.

So far, axSpA proteomics studies have focused primarily on known inflammatory markers such as cytokine levels in serum samples. Although these markers have some discriminating power in both axSpA diagnosis and disease activity assessment, their performance is generally inadequate for clinical practice (72). Concentrations of several cytokines are elevated in patients with axSpA and are correlated with disease activity (e.g., CXCL8 and CRP), and some particular ones are therapeutic targets in axSpA, such as TNF and IL-17. This correlation proposes that measuring cytokines (either alone or in combinations) can be practical diagnostic assistance and might be predictive of therapy responses. As an example, hypothetically, medicines that target the IL-17 signalling pathway might be less effective in patients who do not have high levels of IL-17, possibly justifying the reported failure in clinical trials of some medications that target this pathway (such as Ustekinumab) (73).

Early studies that proposed the presence of auto-antibodies in axSpA, mostly those targeting epitopes that are cross-reactive with Klebsiella, have not been replicated afterwards (74). A 2014 study revealed that levels of antibodies against the *HLA-B27* (CD74) are elevated in

patients with axSpA in comparison with either patient with chronic back pain or healthy individuals (75). Although, this result has demonstrated challenging to replicate (76). CD74 also acts as a receptor for macrophage migration inhibitory factor, which has increased titres in patients with axSpA and is related to radiographic progression (77).

Serum or faecal calprotectin levels reflect gut and, to a minor extent, skin inflammation, and are broadly used to screen for IBD. In a study in Chinese patients with axSpA and healthy individuals, serum calprotectin presented decent discriminatory power for the diagnosis of axSpA (78). Calprotectin concentrations also correlated with disease activity and with concentrations of pro-inflammatory cytokines (e.g., IL-1 and IL-17), (78).

The RNA profiles of peripheral blood cells from patients with axSpA and related disorders have been screened by quantitative reverse transcriptase PCR (qRT-PCR), microarray and RNA sequencing (RNA-seq), initially to explore the pathogenesis of the axSpA rather than to pursue biomarkers. However, some findings have proposed that RNA profiling might have benefits in the identification of biomarkers for axSpA, but its value has not been established in a strongly replicated approach. An early microarray study established that expression of *RGS1* had great discriminatory power for undifferentiated axSpA compared with chronic back pain (79). In a later study, a three-gene signature (including *NR4A2*, *TNFAIP3* and *CD69*) was known in a discovery set through microarrays that had adequate capacity to distinguish patients with axSpA from healthy individuals in a validation study (80). Recent gene expression assessments in patients with axSpA revealed overexpression of *BMP6*, *PCSK6*, *KREMEN1* and *CTNNAL1* genes, and downregulation of ossification mediators, *SPOCK2*, *EP300* and *PPP2R1A* (81).

The paucity of studies of transcriptomic biomarkers for clinical practice in axSpA hypothetically relates to the difficulties that would contribute to the clinical application of such an approach. To be clearer, when using blood or tissue samples, transcriptomic profiles differ based on the cell type distribution within the sample. The majority part of studies has been performed by using peripheral blood (82–84), while they comprise an extensive spectrum of cell types and are greatly heterogeneous among individuals. Computational techniques to deconvolute diverse cell populations from bulk cell experiments have been suggested (85), but developing techniques using single-cell sequencing would present more insight than bulk cell experiments into the types of cells, including novel cell types, that might characterize axSpA.

1.2.7. Diagnosis and Assessment of Axial Spondyloarthritis

In clinical practice, the diagnosis takes into account many aspects of the patient's clinical findings; in fact, classification criteria are not useful for diagnostic purposes (47). In addition, radiographic sacroiliitis is a prerequisite for AS diagnosis, but this contributes to diagnosis delay. In our days, Magnetic Resonance Imaging (MRI) might be considered for the diagnosis (and monitoring) of axSpA (86). Additional disease monitoring tools include inflammatory blood markers, such as CRP, ESR, and also genetic background (*HLA-B27*). patient-reported outcome measures (PROMS) may give to healthcare practitioners, a perspective about disease activity, the Bath AS Disease Activity Index (BASDAI) functional repercussion Bath AS Disease Functional Index (BASFAI), axial mobility, Bath AS Metrology Index (BASMI), and several others aspects (87,88).

1.2.8. Management of Axial Spondyloarthritis

Ideal axSpA management has long contained a combination of medical and non-medical management. The latter contains exercise, education and other types of physiotherapy, rehabilitation, and patient support groups, as illustrated in Figure 1.5.

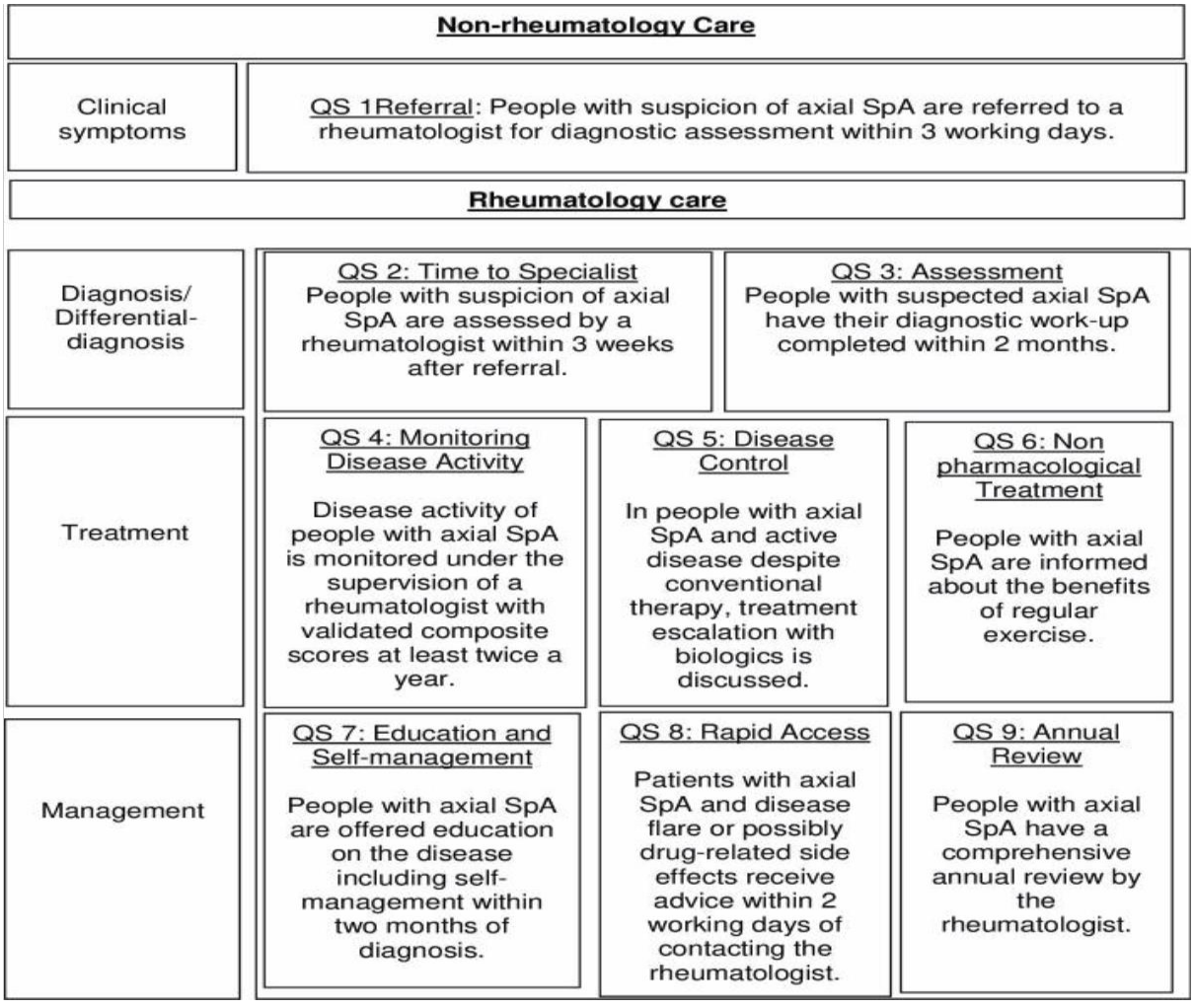


Figure 1.5. Summary of the nine Assessment of SpondyloArthritis International Society quality statements.

SpA: spondyloarthritis.

Source: Kiltz U *et al,* 2020 (89).

1.3. Axial Spondyloarthritis is a Complex Disease

axSpA is a complex disease impacted by both genetic and environmental triggers. The heterogeneity of axSpA roots in the fact that different combinations of genetic triggers and environmental factors may end in the same disease outcome – axSpA (90). In axSpA genetic susceptibility only delivers predisposition for disease development. Environmental factors (e.g., bacterial triggers, microtrauma) are required to trigger the onset of disease and its progression.

1.3.1. Genetics of Axial Spondyloarthritis

1.3.1.1 Early family and twin studies

It has been long considered that there is a robust genetic association with AS from the results of family and twin research. The recurrence risk of siblings with AS has been stated to be as high as 9.2% in comparison with 0.1% in the general population. Likewise, in twin researches, the concordance of AS in monozygotic twins is approximately double that of dizygotic twins. The heritability of AS is assessed to be in excess of 95%, indicating a robust genetic association in AS (91).

1.3.1.2 Role of *HLA-B27* in Axial Spondyloarthritis

In the early 1970s, the detection of a robust association with human leukocyte antigen (HLA)-B27 was the keystone in the genetic research of AS. The first two researches, discovering the association of *HLA-B27* with AS, reported an 88% and 96% of association, respectively. *HLA-*

B27 has been found to be present in 96% of patients with AS, compared with 4% of unaffected cases (92). Worth of note, the prevalence of *HLA-B27* in the general population differs considerably between ethnicities, ranging from almost null in Australian Aborigines to 50% in Haida Indians (93). Nevertheless, the mechanisms of the association of *HLA-B27* with AS remain undetermined. A more systematic investigation of dimerization and misfolding theories is required and definitive relations between the aberrant forms of *HLA-B27* and AS pathogenesis have yet to be identified.

1.3.1.3 Genome-Wide Association Studies (GWAS)

In the current decade, the usage of GWAS is of great assistance to unravel the complicated genetics of AS. Aside from the strong *HLA-B27* association with AS, GWAS have determined 48 non-MHC loci which are potentially causative to AS (53). Relying in their identified functions of the respective proteins, these susceptibility genes have been characterised into groups including DNA methylation, antigen peptide handling, IL-23 cytokine pathways, Nuclear Factor kappa B (NFκB) activation, bacterial sensing in the gut, gut mucosal immunity and TCR signalling (53). Some example genes that were discovered by GWAS are in the following.

With the discovery of IL23R association with AS, trials of IL-17 blockage have been signified in AS. It is remarkable that IL23R, the gene encoding the receptor for the IL-23 cytokine, contributed to the activation of a broad range of pro-inflammatory responses (94). IL23R is a common susceptible factor shared by both IBD and AS. In a phase II study, biologic treatment blocking IL-17 has been revealed to have an advantageous impact similar to TNFi (53). Other genetic risks related with AS that perform a task in IL-23 pathway include *ICOSLG*, *CARD9*,

EOMES, *IL1R1*, *IL1R2*, *IL6R*, *IL7R*, *IL12B*, *IL27*, *PTGER4*, *RUNX3*, *TYK2*, *TBX21* and *ZUMIZI* (53).

The discovery of Endoplasmic Reticulum Aminopeptidases (*ERAP*) 1 and its association with *HLA-B27* by GWAS have shed light on gene-gene interplay in AS. Among the GWAS-identified non-MHC I susceptibility genes, *ERAP1* is the most robust associated gene with *HLA-B27*. *ERAP1* functions as a "molecular ruler" that cleaves peptides down to optimum length for MHC-class I protein presentation to immune effector cells. Hence, *HLA-B27* may perform a task in AS by a mechanism including *ERAP1*, probably by aberrant peptide presentation. *ERAP1* has been related with the development of AS in HLA-B27-positive subjects through interplaying with HLA alleles (46). Nevertheless, there have been controversial results on *ERAP1* impact on *HLA-B27* cell surface expression, it has been revealed that different *ERAP1* variants did change *HLA-B27* FHCs. Therefore, the protective variants of *ERAP1*, which inhibit conformational alterations of the molecule, are essential for accurate peptide cleavage and decrease of abnormal peptide presented in the ER to HLA class I molecule (46).

At this point, there is not adequate evidence to support a convincing theory clarifying the mechanistic interplay of *ERAP1* and *HLA-B27*. Although, it has been distinguished that in the case of influenza infection, generation of preferred B27-epitopes is noticeably dependent on *ERAP1* (95). In the absence of *ERAP*, there is a remarkable decrease in the cytotoxic T cell response to B27/NP383-391 epitope in influenza A-infected HLA-B27tg mice (95).

In addition to *ERAP1*, genetic relations of *ERAP2* and *HLA-B27* have been determined in GWAS studies with AS. Both *ERAP1* and *ERAP2* belong to M1-aminopeptidases which are a

family of metalloproteinases. Both ERAPs share a 50% identical sequence. Similar to *ERAP1*, *ERAP2* has been related with abnormal *HLA-B27* expression and a boosted MHC-I free heavy chains and activation of UPR response. Additionally, *ERAP2* is associated with HLA-B27-negative AS. Loss of function variants of *ERAP2* are protective for AS development the same as *ERAP1*. Nevertheless, *ERAP2* SNPs did not affect *HLA-B27* related ER stress (95).

Another level of gene-gene interplays in AS is presented via the pathogenic effect of interaction between *ERAP1* and *ERAP2* in HLA-B27-related AS through the altered interplays with MHC-I. Acting as an n-terminal aminopeptidase, *ERAP2* may form heterodimers with *ERAP1*. The heterodimers display dissimilar peptide cleavage functions from either *ERAP1* or *ERAP2*. Beside forming heterodimers, *ERAP2* also works with *ERAP1* in trimming peptides, given location and functional overlay of these two molecules. Particularly, inhibition of *ERAP1* or *ERAP2* alone reduced about 10% MHC-I surface expression, although the suppression of both aminopeptidases led to 20% reduction. Yet grander studies are essential to improve our knowledge on ERAP-dependent HLA-B27-related pathogenesis of AS. The absence of *ERAP2* expression in mice has limited studies of this interplay in AS pathogenesis (96).

Despite the abundant data provided by GWAS, the studies do have pitfalls, such as selection bias in SNPs, limitation of cohort size, and lack of specified clinical parameters and well-characterised disease effects. The GWAS-identified susceptibility genes only account for 29% of the heritability of AS, with *HLA-B27* accounting for 25% of total heritability. Hence, there is a large proportion of heritability undetermined in AS. Furthermore, although studies have recognised sexual dimorphism in AS, sex impact has not been taken into consideration in GWAS analysis of AS. Approximately, 15% of quantitative trait loci can be sex-specific (96).

1.3.2 Sexual Dimorphisms in Axial Spondyloarthritis

Being one of the genetic triggers, gender dissimilarity has long been neglected in clinical and biomedical research with both human and animals, which has ended in possible shortcomings in health care and patient management. Namely, from 1997 to 2000, 80% of medicines had to be recalled from the U.S market due to serious risks for females. Despite serious efforts through many funding agencies to mandate gender as a variable in biomedical and pre-clinical studies, females are still under-investigated in clinical trials (97). More advance has to be made to discriminate against such differences in systematic studies.

AS has been considered as a male-dominant disease since the 1600's when Bemard Connor first recognised skeletal variations that resembled AS. In the early 1900's, the most commonly cited gender ratio in AS was ten to one in favour of males (97). This ratio underestimated AS in females. While the reports on gender variance in AS are still inadequate, with rising interest in this aspect, it is now mostly documented that males are generally affected two to three times more often than females. In contrast, there is no existing sexual dimorphism in other subgroups of SpA, such as nr-axSpA and PsA (97).

Several explanations have been suggested for underestimation of AS in females. Firstly, the persistent belief that AS is an exclusively male disease may lead to the under or late diagnosis of this disease in females. Secondly, reluctance to expose female reproductive organs to X-ray can be another justification. The existing developed classification criteria with the operation of MRI offer better opportunities for the screening and diagnosis of female subjects with AS. Last but not least, it is of importance to consider that gender bias in AS is not only present in

prevalence numbers. The alterations in clinical and radiographic features in female vs male AS can play an important role in a longer diagnostic delay (97).

In clinical aspects, AS males have an earlier age of symptom onset and a longer mean disease period at diagnosis than females. Remarkably, females have lower baseline C-reactive protein (CRP) levels (13.1 vs 20.9 mg/l) and a lower proportion of *HLA-B27* positivity (76.3% vs 85.2%) in comparison with males. It is more frequent for males to progress extra-articular manifestations, such as gut inflammation, than females in AS. In contrast, females appear to tolerate more systemic burden present by greater pain scores and disability than males. This is determined via baseline data representing females with more back pain (high BASDAI) and lower quality of life (high ASQoL), (98).

Radiographically, females have been stated to display more structural variations in the cervical spine than men which may lead to the under-diagnosis of females with AS. In contrast, a more rapid rate of radiographic progression and worse radiographic severity has been detected in males (98).

Beside clinical and radiographic alterations of the disease, females also have inferior response rates to treatment. Henceforth it suggests that different genders of patients might contain different mechanistic pathways of disease onset and development, which should be taken into consideration at the time of making treatment decisions.

Despite the gender differences noticed in the AS clinic, the small number of female patients in most researches of AS delivers few mechanistic clues about it. Initially, common hypotheses on changed gender hormone levels and X-linked genes were suggested. Sadly, no direct links

have been established (98). Namely, in male patients, the levels of testosterone were similar in AS vs healthy cases. Likewise, in females, pregnancy or the consumption of oral contraceptives did not impact the disease. Besides, no X-associated genes have been discovered to be associated with AS. Hence, there is no sharp mechanism for the gender-bias of AS. Nevertheless, recent studies aiming at the gender-biased immune response shed some light on some potentials. Male AS subjects are determined to have higher pro-inflammatory cytokines than females (99). Recent research by Gracey et al. exposed that there was sexual dimorphism in the Th17 but not Th1 axis of AS. In male patients, the frequency of *IL-17A* and Th17 cells was boosted in comparison with female AS patients. Remarkably, this skew was independent of *HLA-B27* condition. On the other hand, the fact that male subjects are more susceptible to infection may relate this bias to the shift of gut microbiome in AS. Although sex-biased impacts on gut microbiome in AS are not well-defined, such alterations have already been established in patients with obesity (99). Due to the fact that there is a close relationship between host immunity and gut bacteria, the microbial impact on gender-biased immune response may be another clarification. More studies are still required to unravel the gender difference in AS.

1.3.3. Bacterial Triggers of Axial Spondyloarthritis

The contribution of the environment in the development of AS has long been indicated, while a definitive association is yet to be identified (100). Despite the strangely high heritability in AS, the monozygotic twin concordance rate is 75% (101), demonstrating that environmental factors may serve as a disease trigger.

Recently, there is growing evidence supporting the function of the intestinal microbiome in AS. One study established a dysregulated gut microbial composition in the terminal ileum in

AS subjects in comparison with healthy controls (102). AS subjects had a greater abundance of Lachnospiraceae, Rikenellaceae, Ruminococcaceae, Pophyromonadaceae, and Bacteroidaceae, and a reduction in Veillonellaceae and Prevotellaceae. The gut mycobiota of AS patients was characterized by higher levels of Ascomycota, especially the class of Dothideomycetes, and decreased abundance of Basidiomycota, which was mainly contributed by the decease of Agaricales (103). Additional analysis presented those interplays between these indicator species within the microbial colonisers formed the AS gut microbial community signature. Furthermore, most of the AS subjects with active disease exhibited an increased serum IgA level, indicating the probability of microbial translocation and intestinal barrier failure. The "leaky gut" theory is reinforced via a new observation of adherent and invasive bacteria in the gut of AS subjects with intestinal inflammation, as well as dysregulation of zonulin, a component of the gut vascular barrier (104). Remarkably, it has been noticed that breastfeeding, which is considered to moderate early colonisation of gut microbiota, might protect against the development of AS (105). These outcomes associate a protective role of gut commensals to AS development. Breast milk possibly adjusts the infant's gut flora directly by transmitting maternal bacteria or indirectly by interplay with the infant's immune system.

The growing interest in the association between intestinal bacteria and AS has changed conventional thinking regarding *HLA-B27* pathogenesis of AS. Owing to the fact that more than 95% of HLA-B27 subjects remain healthy, environmental factors appear required in *HLA-B27* pathogenesis. Although the specific interaction of *HLA-B27* and intestinal microbiome in AS is poorly implied, there is evidence of bacteria-driven AS in genetically predisposed individuals and animals. Findings presented that there is a greater tendency of HLA-B27-positive subjects to develop into AS from ReA triggered via intestinal infection with Yersinia, Shigella, Salmonella, and Campylobacter or urogenital infection with Chlamydia (106,107).

Besides, the HLA-B27tg rats were not diagnosed by arthritis when housed in a germ-free environment but developed disease after being transferred to a particular pathogen free environment. This implies the potential interplay of gut bacteria with the host *HLA-B27* in AS development. However, its underlying mechanisms remain doubtful, a distinctive sign of *HLA-B27* on the gut commensals has been exposed by a recent animal research comparing three clusters of bacteria including Proteobacteria, Firmicutes and Akkermansia muciniphila (108). Additional data is required to report the roles of bacterial stress in stimulating HLA-B27-associated AS.

Little is identified in regard to definite functions of gut microbiome in AS at the molecular stages. Nevertheless, microbial researches have delivered clues on the effect of bacteria on immune responses as well as on bone health. The bacterial impact on immune response has been clarified in section 1.1.2.2. Direct evidence of the relationship between gut microbes and bone is the research on bone mineral density (BMD) in germ-free vs. conventionally raised mice (102).

In germ-free animals, greater trabecular bone density, greater rates of bone formation and minor rates of osteoclasts per bone perimeter were detected. These mice also had lower expression of pro-inflammatory cytokines such as IL-6 and TNFα in bone tissues. In line with these outcomes, normalised bone mass and frequency of T-lymphocytes and osteoclast precursor cells were determined in germ-free mice conventionalized with gut microbiota from conventional animals (109). A number of probiotics intervention researches in rat and mouse models additionally established the impact of gut bacteria on bone (66). Numerous mechanisms have been suggested for the relation between gut microbiome and bone involving gut-derived

serotonin, maturation of the immune system within development, and lipopolysaccharide (LPS)-induced systemic inflammation (110).

To date, no distinct bacteria has been identified to be causative of AS, although, this does not exclude the possibility of a combination of various commensals/pathogens that would end in disease. Besides, in a complex disease such as AS, merely considering aberrant host immune response or only dysbiosis is not sufficient. There is a continuous and dynamic interplay between the host and gut microbiome during disease onset and development. Mediators which can expose such interplays might be of significance.

With better understanding of the association between gut microbiota and AS, there is a developing interest in novel AS therapies pointing to the gut microbiome. These potential treatments involve antibiotics, prebiotics, probiotics, dietary manipulation, and fecal microbial transplantation (111). Given limited records of clinical trials, indication underlying the advantage of these new therapies is still questionable in the topic of AS and related diseases, such as inflammatory bowel disease (112). Among these therapies aiming at the gut microbiome, fecal transplants appear to be the most effective. Nevertheless, there are actual concerns about the safety of its application. Many numbers and various combinations of bacteria, parasites and viruses in the intestinal content may not be risky to the donor, whereas potential pathogens as well as healthy microbes may raise unpredicted outcomes in recipients. Further findings are essential to elucidate the accurate composition of the ecosystem being administered which may accordingly develop the total safety of this approach. Besides, with the existence of various inherent genetic triggers, the composition and abundance of intestinal commensals may alter from individual to individual, as does the efficacy of therapy interventions aiming the gut microbes (102,113) To investigate the possibility of counting

microbes as a new therapy target, outsized cohorts of patients and controls with matched genetic, clinical and microbiome data will be required.

1.3.4. Mechanical trigger in Axial Spondyloarthritis

Similar to many autoimmune diseases (114), the pathogenesis of axSpA is multi-factorial and comes from a complex interplay between genetic predisposition and environmental triggers (81). Novel clinical and animal model data support that biomechanical factors act a role in the onset and progression of axSpA (115). Bringing together these insights with the progress made in the understanding of the immune-pathogenesis and genetic susceptibility of axSpA may deliver new opportunities for better disease management.

It was proposed that the inflammatory responses characteristic of axSpA are triggered at enthesis sites (in genetically susceptible individuals) through a combination of anatomical factors which ends to higher levels of tissue microtrauma (81,116). Several lines of evidence indicate that the localization of disease in the skeleton of patients with axSpA, significantly depends on bone stressing. Worth of note, paediatric HLA-B27-related arthritis, which is characterised by enthesitis and oligoarthritis in the midfoot and lower limbs, provides insight into the essential significance of skeletal biomechanics (117). With growing age and variations in muscle structure and Body Mass Index (BMI), it is possible to observe paediatric disease migration with further topographic SIJ, and subsequent spinal contribution, reflecting the more frequent pattern of adult-onset disease (117).

With the multifaceted pattern of skeletal pathology apparent in tissues from patient samples with later phases of axSpA, the pre-eminence of enthesis in axSpA was not completely understood (118). In the 1990s, MRI findings indicated that not clinically diagnosed enthesitis was not rare in synovitis joints, and that enthesitis was correlated with neighbouring osteitis

(119). These observations led to the current understanding that other joint structures comprising fibrocartilaginous joints (e.g., SIJ) share comparable histological and matching patterns of mechanical stress. As a consequence of these findings, a theoretical enthesis-based biomechanical model for every axSpA feature was suggested (81). The hypothesis that mechanical stress and microtrauma may be involved in inflammatory enthesitis as an initial driver in axSpA, is further supported by studies from La Cava and co-workers where sports injury-associated enthesopathy seems to be related to an inflammatory reaction (characterised by fibrosis and calcification arising at the enthesis) in reflex to continuously recurring microtrauma (120).

Although the hypothesis of a mechanically based origin for axSpA is simple to grasp on a conceptual level, formal proof from human studies is difficult to come by. Animal models have recently been used to investigate the mechanical stress paradigm for the development of axial and peripheral axSpA. Using the TNF^{DARE} model for axSpA it was possible to determine that mechanical stress contributes to the appearance of Achilles tendon enthesitis (121), (Figure 1.6). In this model, deletion of adenylate-uridylate (AU)-rich elements in the *TNF* gene results in deregulated *TNF* expression. This deregulation is believed to be associated with systemic inflammation, axial and peripheral arthritis, and enthesitis (121). Additionally, it has been previously shown that joint inflammation initiated in the Achilles tendon enthesis and extended to the surrounding tissues and, moreover, the enthesis fibroblasts are considered as early disease initiations (122).

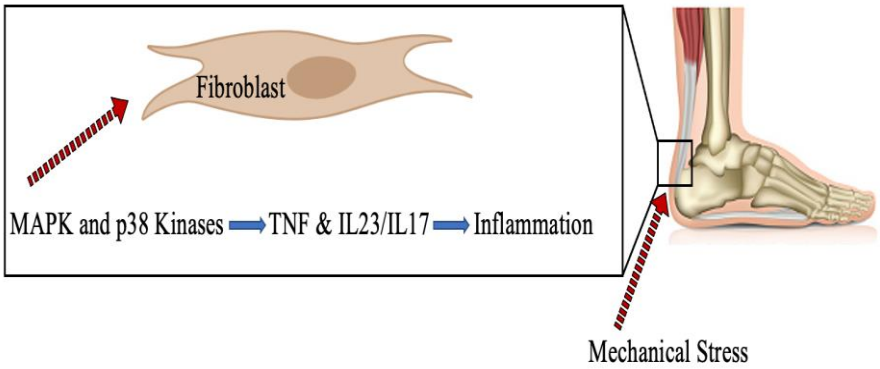


Figure 1.6. Overexpression of *TNF* and *IL-23* in experimental animals has been linked to the development of SpA symptoms that begin at the Achilles tendon enthesis. The MAPK and p38 kinases may be activated by mechanical stress. These kinases subsequently activate the *TNF* and IL23/IL17 pathways. The existence of a unique population of innate lymphoid-like cells at the enthesis also appears to be required for *IL-23* upregulation. The figure is adapted from Jacques P *et al* (121).

In terms of biomechanical characterization of axSpA, P. Jacques and co-workers eloquently showed evidence for a critical role of biomechanical stress as an inducer of enthesitis and new bone formation in a murine experimental axSpA model (123). However, the molecular mechanisms underlying this observation have never been established (124). Sofia Ramiro and her co-workers investigated the complex relationship between inflammation, mechanical stress and radiographic progression in patients with axSpA using job type as a proxy for continuous mechanical stress (125). They noticed blue-collar workers (physically demanding labour) with axSpA, experience faster disease progression compared with white-collar workers (sedentary labour), (125). Therefore, physically demanding jobs may amplify the potentiating effects of inflammation on bone formation in axSpA.

In axSpA, the axial entheses seem to be prone to inflammation when subjected to repetitive biomechanical stress forces transmitted by muscles, ligaments and tendons (126–128). Mechanical properties of spinal muscles of axSpA patients (Lumbar and cervical muscles) exhibit greater linear elastic properties and lower viscoelastic properties, which are related with age, clinical and psychophysiological features of axSpA (129). Masi and his co-workers suggested innate axial myofascial hypertonicity reflects basic mechanobiological principles in human function, tissue reactivity, and pathology. The proposed physical mechanisms likely interact with recognized immunobiological pathways. The structural biomechanical processes and tissue reactions might perhaps precede the initiation of more related pathways (130).

In addition to Masi and co-workers, we investigated muscle physical properties, strength, mass, physical performance in patients with axSpA compared to the healthy controls (131). We noticed patients with axSpA had reduced physical performance, lower muscle strength and higher lumbar myofascial stiffness compared to the healthy controls, despite normal muscle mass, suggesting a possible muscle dysfunction (131). However, the role of myofascial stiffness as a source of microtrauma with impact at the entheses level, inducing inflammation and osteoproliferation, remains to be demonstrated. Research in the combined structural mechanobiology and immunobiology processes promises to improve understanding of the initiation and perpetuation of axSpA than prevailing concepts. The combined processes might better explain characteristic enthesopathic and inflammatory processes in axSpA.

Aims and Objectives

The overall aim of this study is to assess the role of muscle in susceptibility and progression of axSpA and, consequently, to enhance the understanding of the underlying mechanisms of its physiopathology, contribute to improve diagnosis, and identify therapeutic targets.

Specific objectives to meet this aim include:

1. Test novel hypothesis of increased resting lumbar myofascial stiffness/tone in patients with axSpA compared to healthy controls.

2. Characterise the muscle physical characteristics (stiffness, tone, elasticity, strength), muscle mass, and physical performance in patients with axSpA.

3. Establish gene and protein signatures in axSpA, and additionally try to establish signatures that associate with muscle properties such as muscle stiffness.

This will allow the identification of new serological biomarkers potentially relevant for diagnosis of the disease and as well as preventive/therapeutic approaches, to be tested in future studies.

Chapter 2: Methodology

The current study was submitted and approved by the ethical committees of NOVA Medical School, NOVA University of Lisbon and Centro Hospitalar de Lisboa Ocidental, Hospital de Egas Moniz, EPE. The study was conducted following the International Conference on Harmonisation of Good Clinical Practice (GCP) and the Declaration of Helsinki (132,133). Furthermore, voluntary written informed participants' consent was obtained from all subjects before starting the study procedures.

It was decided that the pipeline for our investigation comprehends the use of muscle characterization measurements and transcriptomics and proteomics approaches (Figure 2.1). The study protocol of this research is already published (134), (Appendix 1. Paper 1).

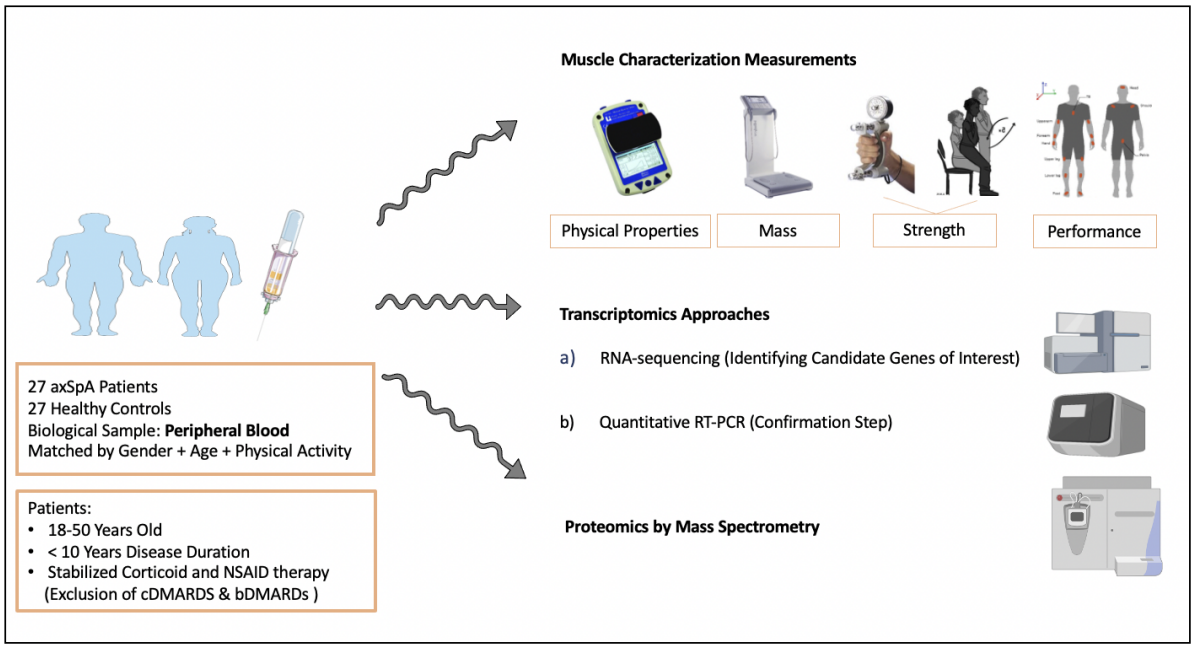


Figure 2.1. Experimental Study Approach; 1. Muscle Characterization Measurements (Physical Properties, Mass, Strength and Physical Performance). 2. Transcriptomics Approach (a) RNA-sequencing, b) Quantitative RT-PCR). 3. Proteomics by Mass Spectrometry.

cDMARD: conventional disease-modifying anti-rheumatic drugs. bDMARD: biologic disease-modifying anti-rheumatic drugs.

2.1. Patients and Samples

Two sample power analyses for a 5% two-tailed test with 80% power, were selected based on our pre-liminary study and considering a 10% drop-out rate (a sample size of 27 subjects per group was calculated to detect differences in gait speed of 0.5 m/s between the two groups). Thus, for this cross-sectional study, 27 young (< 50 years to remove the effect of age) patients with axSpA (according to ASAS classification criteria (52)), with symptoms duration < 10 years and 27 healthy controls matched by age, gender, and level of physical activity were considered (Appendix 2: Supplemental Material, Table S1) according to the pre-specified inclusion/exclusion criteria:

Inclusion Criteria:

- Patients classified with axSpA according to the Assessment of Spondyloarthritis International Society (ASAS) classification criteria (52);
- Age between 18-50 years;
- Symptom duration < 10 years;
- Ability to provide informed consent;
- Corticosteroid therapy allowed (equivalent to ≤10 mg prednisone) and/or nonsteroidal anti-inflammatory drugs (NSAID), in a stable dose within 4 weeks before study enrolment.

Exclusion Criteria:

- History of rheumatic disorder other than axSpA;
- BMI≥35kg/m^2; Any uncontrolled medical condition (e.g., diabetes mellitus, ischemic heart disease);
- Malignancy (except for completely treated squamous or basal cell carcinoma);
- Positive serology for hepatitis B or C, or human immunodeficiency virus;

- Infections requiring hospitalisation or intravenous treatment with antibiotics within 30 days or oral treatment within 14 days before enrolment;
- Previous treatment with conventional DMARDs (cDMARDs) or biologic DMARDs (bDMARDs);
- Intra-articular or periarticular injections within 28 days before screening;
- Ankylosis of the spine (syndesmophytes at all levels from T12 to S1 on the lateral view radiograph);
- Current pregnancy or breastfeeding.

Eligible patients were recruited in the Spondyloarthritis Clinic of CHLO, Hospital de Egas Moniz, and the CORPOREA national database (71). Healthy controls, subjects without any lumbar pain during the last year or previous history of lumbar surgery, were identified by the patients (e.g., work colleagues).

2.2. Clinical and Epidemiological Characterization and Biological Samples Collection

All participants were characterised through a standardised questionnaire available in a specific area of the Rheumatic Diseases Portuguese Register (Reuma.Pt), (135). The Reuma.pt is a web based online system developed by the Portuguese Society of Rheumatology with the aim of prospectively record data from patients with various rheumatic diseases, including axSpA. In the questionnaire, the following variables were collected from all participants: age, gender, height, weight, handedness, marital status, level of education and level of physical activity, assessed with the International Physical Activity Questionnaire (IPAQ), (136).

For patients with axSpA, the following information were additionally collected: disease duration (time between the onset of first symptoms and study's enrolment), extra-articular

manifestations (enthesitis, dactylitis, uveitis, psoriasis, inflammatory bowel diseases, others), disease activity (assessed by Bath Ankylosing Spondylitis Disease Activity Index (BASDAI)) and functional status (assessed by Bath Ankylosing Spondylitis Functional Index (BASFI)).

All participants have been submitted to a detailed clinical examination to obtain an extensive muscle characterization. Additionally, patients with axSpA were assessed for enthesitis (by Maastricht Ankylosing Spondylitis Enthesitis Score (MASES)) and metrology (using Bath Ankylosing Spondylitis Metrology Index (BASMI)). Blood samples were collected from all participants to allow biochemical, genetic, transcriptomic, and proteomic studies.

2.3. Muscle Characterization of Study Subjects

To test the hypothesis of greater resting lumbar myofascial stiffness in patients with axSpA, extensive muscle characterization was performed for the first time in three body segments: upper limbs, lower limbs and lumbar region. All measurements in each task were performed by a single trained and experienced investigator. The different features of the muscle studied include:

a) Muscle physical properties, in particular, stiffness, tone and elasticity, which were measured by a non- invasive, hand-held myotonometer, the Myoton- PRO®. This device quickly releases a mechanical impulse by applying a constant pressure via a probe to the skin and tissue layers directly above the muscle being measured. Consequently, an impulse is transmitted to the muscle below. The muscle responds to the exterior mechanical impulse with a damped natural oscillation, which is recorded by an accelerometer in the form of an acceleration sign, with subsequent computation and quantification of muscle properties (137). The muscles to be tested after a 10-minutes rest were the multifidus and longissimus dorsi muscles (assessed at

L3-L4 level), the lateral gastrocnemius (assessed at a point 15 cm distal to the knee lateral flexion line, in the bulk of the muscle) with the patient in the prone position and the brachioradialis muscle (assessed at a point, 6 cm distal to the lateral epicondyle of the elbow with the patient in the back position). Measurements of the left and right sides were performed.

b) Muscle Strength was measured by: 1. a hand-held dynamometer, the Lafayette Manual Muscle Tester and five-times sit-to-stand (5STS) test, that measures the time a patient takes to stand five times from a sitting position, as quickly as possible, with- out using his/her arms. Both measures will be used as a proxy of total body strength, as suggested by EWGSOP2 (138); 2. Through a resisted lumbar spine hyperextension (dynamometer placed in the midline over the dorsal area), leg extension (dynamometer placed proximal to the ankle joint) and forearm flexion (dynamometer placed in the middle of anterior forearm), with the participant in a sitting position. These measurements aim to reproduce the anatomical areas evaluated for muscle physical properties as strength evaluation for specific muscles is challenging.

c) Muscle Mass, which were measured by: 1. Bioimpedanciometry, using an octopolar multifrequency bioelectrical impedance analysis device (In- Body770®) and 2. In case of the axial region, also MRI of the lumbar spine, through quantification of the cross-sectional area (CSA) of paravertebral muscles.

d) Physical Performance will be measured by the "Gait Speed Test" (138). Gait analysis were performed by a tri-dimensional full-body kinematic model (Kinetikos technology®), fed by 15 wireless inertial sensors placed in the head, arms, trunk, pelvis, thighs, shanks, and feet to collect several spatiotemporal gait parameters (e.g., gait speed, stance/swing time, step length, step frequency), to allow participant's movement characterization.

2.4. Expression Data Collection

We screened peripheral blood to identify gene/protein signatures and biological pathways that may potentially be related to the observed muscle properties in axSpA.

2.4.1. Global Transcriptomic Analysis by RNA-sequencing

2.4.1.1. Blood Sample Storage: PAXgene® blood RNA tubes were used by the physicians according to the manufacturer's guidelines (139). In brief, peripheral blood samples were collected, tubes were mixed gently by inverting them 8-10 times, stored upright for about 2 hours at room temperature and then frozen at -80°C.

2.4.1.2. mRNA Isolation: Blood samples (stored in PAXgene blood tubes) were equilibrated to room temperature for 2 hours prior to isolation. Whole blood RNA isolation was performed using PAXgene Blood miRNA Kit following the standard protocol (140). In brief, RNA was eluted with 40 µl Buffer 5 directly onto the spin column membrane at 20,000 g twice. RNA was denatured by incubating for 5 min at 65 °C and then stored at −80 °C. The purity (OD260/280 ratio) and concentration (OD260) of extracted total RNA was measured using a spectrophotometer NanoDrop ND-2000 according to the manufacturer's procedure (141).

2.4.1.3. RNA Processing and Sequencing Analysis: The qualities of the libraries were assessed by Fragment Analyzer with the method of DNF-474-22 - HS NGS Fragment 1-6000bp (142). Libraries were also quantified by fragment analyser with the same method for QC and quality final libraries. RNA processing has been carried out using TruSeq stranded mRNA library preparation kit, with 100ng of total RNA as input (143). Sequencing libraries

were prepared with the NextSeq 500/550 High Output Kit and read mode of 75PE [39] (average of 40 million reads per sample), (144).

Raw reads were aligned to the human GRCh38 genome reference using the STAR aligner, followed by the generation of a table of gene counts with the feature counts software (145,146). Counts were processed with the edgeR and Limma-Voom packages in the R software to perform normalization, sparse partial least-squares discriminant analysis (sPLSDA) and differential gene expression analysis comparing the different groups of subjects (147–149). sPLSDA analysis was performed by expert from FCT-Nova (Dr Daniel Sobral). Gene ontology (GO) enrichment analysis was performed with STRING (https://string-db.org) and Gene Set Enrichment Analysis (GSEA) was performed using the fgsea R package (150–152).

2.4.2. Confirmation of Candidate Genes' Expression by RT-qPCR

2.4.2.1. cDNA Preparation: The first strand cDNA was synthesized using the NZY First Strand cDNA Synthesis Kit according to manufacturer's guidelines (MB125) (153). In brief, first strand cDNA was synthesized by mixing up to 5 µg of RNA with 2 µL of NZYRT enzyme mix and 10 µL of NZYRT 2× Master Mix. The reaction was performed in 3 steps: priming (10 min, 25°C), elongation (30 min, 50°C) and reverse transcriptase inactivation (5 min, 85°C). The cDNA sample was stored at −80°C before quantitative PCR analysis.

2.4.2.2. RT-qPCR: RT-qPCR reactions were performed with IQ SYBR Green supermix in 96-plates and were amplified and quantified in CFX Connect Real-Time PCR Detection System (Manufactured by BioRad) under the following conditions: Reactions were carried out in a final volume of 20 µL with primer's concentration of 400 nM and either 20 ng of RNA starting quantity (RT negative control, NRT), different amounts of cDNA (2 pg to 20 ng) or DEPC

water (no template control, NTC). The cycling conditions of 40 cycles were as follows: denaturation for 10 s at 95°C, annealing for 30 s at 58°C, and extension for 15 s at 72°C after a 3-min hot start at 95°C. Discrimination of specific products from non-specific products and primer dimers was performed by a melt curve gained by a gradual increase in temperature from 65°C to 95°C at a rate of 0.25°C/s. The list of candidate genes and their primer sequences used in the confirmation step by RT-qPCR is presented in the Appendix 2: Supplemental Material, Table S5. Primers have been designed using Ensembl Genome and NCBI primer-BLAST browsers (154,155).

2.4.3. Proteomic Analysis by SWATH - MS

2.4.3.1. Sample Preparation for LC-MS/MS

As mentioned earlier, we performed LC-MS/MS analysis for the same group of samples as transcriptomics. A first spiking step was introduced when beta-galactosidase (*E. coli*) was added to a final concentration of 200 fmol/µL to the raw serum. Due to sample complexity and high dynamic range, samples were immunodepleted of their top-14 most abundant proteins through a Hu-14 multiple affinity removal system (MARS, Agilent Technologies, Palo Alto, CA, USA) column. The resulting F1 and F2 fractions from each sample were pooled together. A second spiking step was performed by adding chicken ovalbumin to a final concentration of 200 fmol/µL before depleted samples were buffer exchanged into 50 mM ammonium bicarbonate (AmB) pH 8.4 using centrifugal ultrafiltration (3000 molecular weight cut-off) to a final volume of 500 µL. Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL, USA). Samples were deglycosylated through incubation with the peptide: N-glycosidase F (PNGase F) enzyme for 2h at 37 °C. One-dimensional

polyacrylamide gels (1D-PAGE) were performed in the high-resolution pre-cast gel system XCell SureLock™ Mini-Cell and NuPAGE® Novex® Bis-Tris using pre-casted 4-12% gels (Invitrogen, Carlsbad, CA, USA) at a constant voltage of 150 V for approximately 10 min. After staining with SimplyBlue™ SafeStain, gel images were acquired. A gel band containing the entire sample was excised and destained in a solution of 50% ACN in 50 mM AmB. Reactive cysteine residues were reduced via rehydration of gel bands in 10 mM DTT and 25 mM AmB followed by incubation at 56 °C for 45 min and alkylated via incubation in 55 mM iodoacetamide for 30 min at ambient temperature in the dark. Bands were then dehydrated with acetonitrile, rehydrated with sequencing grade porcine trypsin (Promega, Madison, WI, USA) in 25 mM AmB, and digested at 37 °C for 16h. Peptide digests were extracted with 70% acetonitrile, 5% formic acid, dried by vacuum centrifugation, and stored at -80 °C until further analysis. A 10 µL aliquot was removed and used to perform a quantitative colorimetric peptide assay (Pierce, Rockford, IL, USA) before LC-MS/MS analysis.

2.4.3.2 Mass Spectrometry Analysis in Information-dependent Acquisition (IDA) Mode to Generate the Spectral Library

Nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) analysis was performed on an ekspert™ NanoLC 425 cHiPLC® system coupled with a TripleTOF® 6600 with a NanoSpray® III source (Sciex). Peptides were separated through reversed-phase chromatography (RP-LC) in a trap-and-elute mode. Trapping was performed at 2 µl/min on a NanoLC Trap column (Sciex 350 µm x 0.5 mm, ChromXP C18-CL, 3 µm, 120 Å) with 100% A for 10 min. The separation was performed at 300 nl/min, on a NanoLC column (Sciex 75 µm x 15 cm, ChromXP 3C18-CL-120, 3 µm, 120 Å). The gradient was as follows: 0-1 min,

5% B (0.1% formic acid in acetonitrile, Fisher Chemicals, Geel, Belgium); 1-91 min, 5-30% B; 91-93 min, 30-80% B; 93-108 min, 80% B; 108-110 min, 80-5% B; 110-127 min, 5% B. Peptides were sprayed into the MS through an uncoated fused-silica PicoTip™ emitter (360 µm O.D., 20 µm I.D., 10 ± 1.0 µm tip I.D., New Objective, Oullins, France). The source parameters were set as follows: 15 GS1, 0 GS2, 30 CUR, 2.5 keV ISVF, and 100 ºC IHT. The samples used to generate the spectral library were acquired in three m/z ranges: 400–642.0, 641.0–840.0, and 839.0–2,000. The 50 most intense precursors were selected for subsequent fragmentation and the MS/MS were acquired in the range of 150-1,800 m/z, in high sensitivity mode for 40 msec, for a total cycle time of 2.3 s. The selection criteria for parent ions included an intensity of greater than 125 cps and a charge state ranging from +2 to +5. Once an ion had been fragmented through MS/MS, its mass was excluded from further MS/MS fragmentation for 12 s. The ions were fragmented in the collision cell using rolling collision energy, and CES was set to 5. Individual samples from each condition were pooled before being analysed in IDA mode. The acquired raw files were subjected to database search in unison using ProteinPilot software v. 5.0 (Sciex, Framingham, US) with the Paragon algorithm to generate the Spectral Library. The search was performed against the protein sequences of Homo sapiens, retrieved from the Uniprot database (20,368 entries, accessed on 21/01/2020). The following search parameters were set: Iodoacetamide, as Cys alkylation; Trypsin, as digestion; TripleTOF 6600, as the Instrument; Gel-based ID, as Special factors; Biological modifications, as ID focus; Thorough, as search effort; and an FDR analysis enabled. Only the proteins with <1% FDR were considered.

2.4.3.3. Protein Quantification by SWATH-MS

Three technical replicates of each individual (n=53) were analyzed by sequential window acquisition of all theoretical fragment ion spectra (SWATH-MS), using the instrument setup described for the IDA runs. The mass spectrometer was set to operate in cyclic data-independent acquisition (DIA), similarly to the previously established method (156). SWATH-MS data were acquired in SWATH acquisition mode using a set of 64 overlapping variable SWATH windows covering the precursor mass range of 400–1,800 m/z. The variable SWATH windows were calculated using the SWATH Variable Window Calculator V1.0 (Sciex, Framingham, US) based on a reference sample. At the beginning of each cycle, a 50 ms survey scan (400-1,600 m/z) was acquired, and the subsequent SWATH windows were collected from 400 to 1,800 m/z for 50 ms, resulting in a cycle time of 3.3 s. The collision energy for each window was set using rolling collision energy, and the collision energy spread was set to 5.

Data processing was performed using a SWATH processing plug-in for PeakView 2.2 (Sciex, Framingham, MA USA). First, the spectral library was imported. The maximum number of proteins to import was set to 176, corresponding to a protein FDR <1% Global FDR. Shared peptides were set not to be imported. Next, the RT calibration was performed by selecting peptides that covered the entire LC gradient. The calibration curve was manually inspected before proceeding with the RT calibration. Once the RT calibration was performed, manual inspection was performed for random peptides to check the quality of the data before data processing, and ions were edited when required. Data were processed using the following criteria: Number of peptides per protein: 6; Number of transitions per peptide: 6; Peptide confidence threshold: 98% (corresponding to a peptide FDR <1% Global FDR from fit); False discovery rate threshold: 1%; Exclude modified peptides: No; Fix rank: No; XIC extraction window: 6 min; XIC width: 20 ppm. Data were directly exported to Markerview 1.3.1 (Sciex, Framingham, MA USA) and normalized using total area sums to obtain the final quantification

values. MarkerView was also used to perform the PCA and t-test statistical tests. Protein quantification estimates were used to perform similar analyses as the transcriptomics, namely differential expression, sPLSDA, GO enrichment and GSEA analysis. sPLSDA analysis was performed by expert from FCT-Nova (Dr Daniel Sobral).

2.4.4. Integration of Gene Expression and Protein Abundance with Clinical Data

We integrated gene expression values and protein abundances in a single dataset (as independent variables), followed by sPLSDA analysis in the combined dataset, similarly to what was done before for each of the omics datasets independently. sPLSDA analysis was performed by experts from FCT-Nova (Dr Daniel Sobral). We also performed correlation analysis (using spearman correlation) between gene expression / protein abundance values and clinical parameters such as muscle strength.

Chapter 3: Results and Findings

3.1. Characteristics of the Cohort Studied

The participants had a mean age of 36.5 (SD 7.5) years and were predominantly males (67%). The patients with axSpA had mean disease duration of 6.5 (3.2) years, with BASDAI and BASFI of 2.7 (2.3) and 0.9 (3.1), respectively. Subject characteristics are shown in Table 3.1.1.

Table 3.1.1. Demographic and clinical characteristics of patients with axSpA and healthy controls.

	Patients (*n*=27)	**Controls (*n*=27)**	***p*-value**
Age*	37 (7)	36 (8)	0.79
Gender (male), *n* (%)	18 (67)	18 (67)	0.99
Body height (cm)	170 (164 – 177)	173 (165 – 178)	0.52
Body weight (Kg)	73 (67 – 86)	70 (65 – 80)	0.35
BMI (Kg/m^2)	25 (23 – 30)	24 (23 – 26)	0.3
IPAQ (%)			0.8
Low	29	21	
Moderate	38	42	
High	33	38	
BASDAI*	3 (2)	-	-
BASFI*	1 (3)	-	-
Disease duration*	7 (3)	-	-

Values are presented as median (25th - 75th percentiles), except otherwise indicated.

***** Mean (SD).

BMI: Body Mass Index. IPAQ: International Physical Activity Questionnaire. BASDAI: Bath AS Activity Index. BASFI: Bath AS Functional Index.

3.2 Muscle Physical Properties

Regarding muscle physical properties, there was no significant difference in muscle stiffness, tone or decrement (inverse of elasticity) in any of the three regions between the patients with axSpA and the HC (Table 3.2.1). However, patients with axSpA showed a numerically higher trunk muscle stiffness than the HC [246.5 (230.5–286.5) *vs.* 232.5 (211.0–293.5), p=0.38]. This numerical difference was more pronounced in the dominant side [261.0 (232.0–312.0) *vs.* 241.0 (204.3–303.0), p=0.28]. Our results have been published in more details (157), (Appendix 1. Paper 2).

Table 3.2.1. Muscle stiffness (expressed in Nm) in patients with axSpA and control subjects, stratified for body segment.

	Patients (n=27)	**Controls** (n=27)	***p*-value**
Trunk			
Average	246.5 (230.5–286.5)	232.5 (211.0–293.5)	0.38
Dominant side	261.0 (232.0–312.0)	241.0 (204.3–303.0)	0.28
Non-dominant side	242.0 (219.0–291.0)	232.0 (209.3–288.0)	0.32
Upper Limb			
Average	288.0 (266.0–320.0)	292.0 (265.0–307.5)	0.6
Dominant side	282.0 (266.0–334.0)	292.0 (254.8–311.8)	0.8
Non-dominant side	283.0 (267.0–313.0)	290.0 (266.0–313.0)	0.96
Lower Limb			
Average	293.5 (277.0–329.5)	289.0 (265.0–325.0)	0.75
Dominant side	299.0 (257.0–349.0)	298.0 (271.0–325.0)	0.91
Non-dominant side	295.0 (269.0–321.0)	290.0 (263.5–314.3)	0.81

Values are presented as median (25th-75th percentile). “Average” refers to the mean of right and left sides of each segment, while “dominant” and “non-dominant” sides refers to the handedness of individuals.

Table 3.2.2 shows the comparison of strength, body composition, and physical performance between both groups. No participants fulfilled the definition of sarcopenia, since none of the patients or controls had simultaneously low muscle strength and low muscle mass. Low muscle strength was found in 8.3% (n=2) of patients *vs.* 0% of the HC (p=0.15). Skeletal muscle mass was reduced in the other 8.3% (n=2) of patients *vs.* 4.2% (n=1) of the HC (p=0.55).

Nonetheless, although patients with axSpA had significantly lower median total muscle strength, evaluated by 5STS, than the HC [7.0 (5.9–8.9) *vs.* 5.5 (5.0–6.9), p=0.01], these values were still in the normal range in both groups (cut-off of 15 seconds). Regarding the strength of different body segments, evaluated by dynamometry, patients with axSpA, compared to the HC, also had lower median values in the upper limbs [47.6 (40.2–73.2) *vs.* 71.8 (51.9–80.5), p=0.02] and lower limbs [51.0 (38.5–57.1) *vs.* 59.8 (54.6–64.5), p=0.01], but not in trunk.

There were no differences in total or segmental lean mass and body water, between both groups. Total fat mass was higher in the patients than in the HC [19.8 (12.1–29.1) *vs.* 15.7 (10.1–22.2), p=0.04], but no differences were registered in segmental body evaluation.

As a surrogate marker of physical performance, low gait speed was found in 55% of the patients *versus* 22% of the HC (p=0.02). In addition, median gait speed values were lower in patients compared to the HC [0.8 (0.7–0.9) *vs.* 0.9 (0.8–1.0), p=0.02]. Our results have been published in more details (157), (Appendix 1. Paper 2). More detailed information can be seen in Appendix 2: Supplemental Material, Table S2.

Table 3.2.2. Comparison of muscle strength, body composition and physical performance between patients with axSpA and healthy controls.

	Patients (*n*=27)	**Controls** (*n*=27)	***p*-value**
Strength			
Trunk (Nm/s)	56.3 (37.6 – 67.2)	57.3 (51.2 – 63.0)	0.67
Upper Limb (Nm/s)	47.6 (40.2 – 73.2)	71.8 (51.9 – 80.5)	**0.02**
Lower Limb (Nm/s)	51.0 (38.5 – 57.1)	59.8 (54.6 – 64.5)	**0.01**
Total - 5STS (seconds)	7.0 (5.9 – 8.9)	5.5 (5.0 – 6.9)	**0.01**
Lean Mass (Kg)			
Trunk	24.9 (21.9 – 27.0)	25.3 (20.4 – 27.6)	0.92
Upper Limb	3.1 (2.56 – 3.5)	3.1 (2.3 - 3.5)	0.81
Lower Limb	8.0 (7.2 – 9.5)	9.2 (7.5 – 10.0)	0.15
Total	50.1 (44.5 – 57.8)	54.1 (43.2 – 60.2)	0.59
Fat Mass (Kg)			
Trunk	10.3 (6.3 – 15.9)	8.1 (5.1 – 11.1)	0.05
Upper Limb	1.3 (0.6 – 2.2)	0.9 (0.5 – 1.5)	0.05
Lower Limb	2.9 (1.9 – 4.0)	2.5 (1.6 – 3.4)	0.21
Total	19.8 (12.1 – 29.1)	15.7 (10.1 – 22.2)	**0.04**
Body water (L)			
Trunk	19.6 (17.1 – 21.3)	18.8 (14.4 – 21.1)	0.84
Upper Limb	2.4 (2.0 – 2.7)	2.3 (1.6 – 2.7)	0.38
Lower Limb	6.5 (5.8 – 7.4)	6.5 (5.1 – 7.5)	0.82
Total	39 (34.6 – 44.9)	42.1 (33.5 – 46.8)	0.58
Physical Performance ∓			
Gait speed (m/s)	0.8 (0.7-0.9)	0.9 (0.8-1.0)	**0.02**
Low gait speed, n (%)	12 (54.5%)	5 (21.7%)	**0.02**

Values are median (25^{th} –75^{th} percentiles). Mann-Whitney U-test was used for continuous variables and Fisher's exact test or the chi-square test were used for categorical variables.

ɵ Available for 48 subjects (24 patients and 24 HC).

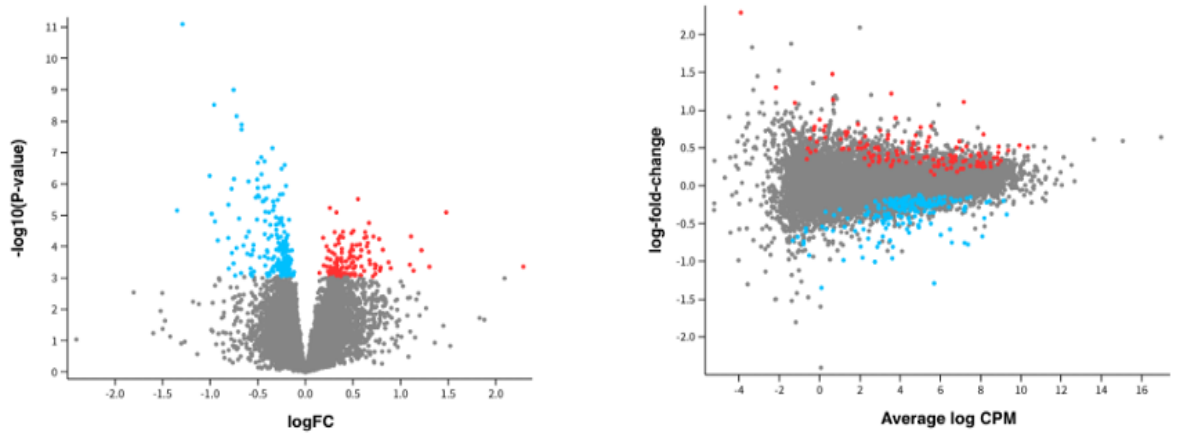
Ŧ Available for 45 subjects (22 patients and 23 HC).

In model 1 of multivariable analysis (Table 4.1.4), i.e., without muscle physical properties, patients with axSpA, compared to the HC, had lower total strength, reflected by a higher 5STS (B=2.00, 95% CI 0.59–3.42), as well as lower strength in the upper [B= -14.85, 95% CI -25.05– (-4.66)] and lower limbs [B=-11.83, 95% CI -18.67– (-4.98)], independently of muscle mass. Likewise, patients had significantly lower gait speed than the HC [B= -0.1, 95% CI -0.212– (-0.006)], adjusted for muscle mass and strength. When muscle physical properties (stiffness, tonus and decrement) were added to the model (model 2), the same results were found.

3.3. Biomarkers Associated with Muscle in axSpA

3.3.1. Differentially Expressed Genes Between Patients and Healthy Controls

In total, 15520 differentially expressed genes (DEGs) were identified between the paired groups of axSpA patients and their matched HC. Only 76 were significant DEGs, including 17 upregulated and 59 downregulated expressed genes (cut-off adjusted *p-value* ≤ 0.05), (Figure 3.3.1.1). These genes are involved in immune systems, interleukin-6 signalling and interleukin-10 signalling pathways that may play an important role within the context of axSpA. The top 10, upregulated and downregulated, DEGs are expressed in Table 3.3.1.1 (Full list can be seen in Appendix 2: Supplemental Material, Table S3). Among the top 10 differentially expressed genes (Table 3.3.1.1), we can also find *SOCS3*, a highly potent and specific inhibitor of IL-6 family cytokines, which was found to be upregulated in axSpA patients (158). Interestingly, *LDHB*, an exercise-inducible lactate dehydrogenase regulating mitochondrial function in muscle was also identified as one of the top downregulated genes in axSpA(159).



A. Volcano Plot of Patients with axSpA-Healthy Controls

B. MD Plot of Patients with axSpA-Healthy Controls

Figure 3.3.1.1. Volcano Plot (A) and MD plot of patients with axSpA and healthy controls.

In the volcano plot the top genes (by adjusted *p*-value) are highlighted. The MD Plot highlighted genes are significant at an adjusted *p*-value threshold of 0.05 and exhibit log2-fold-change of at least 0.

Table 3.3.1.1. Top 10 DEGs, upregulated or downregulated, in patients with axSpA *vs* healthy controls.

Gene reference	Gene name	logFC	*p*-value	Regulation Status
RPS23P8	Ribosomal Protein S23 Pseudogene 8	-1.306	0.0000464	Down
RPL36AL	Ribosomal Protein L36a Like	-1.236	0.0000001	Down
RPL37P2	Ribosomal Protein L37 Pseudogene 2	-0.982	0.0000051	Down
MRPL51	Mitochondrial Ribosomal Protein L51	-0.897	0.0000019	Down
NDUFA1	Ubiquinone Oxidoreductase Subunit A1	-0.893	0.0000306	Down
RPL37	Ribosomal Protein L37	-0.817	0.0000031	Down
LEXM	Lymphocyte Expansion Molecule	-0.78	0.0000602	Down
NDUFS5	Ubiquinone Oxidoreductase Subunit S5	-0.748	0.0002120	Down
LDHB	Lactate Dehydrogenase B	-0.717	0.0001770	Down
NPM1	Nucleophosmin 1	-0.717	0.0000201	Down

TG	Thyroglobulin	0.573	0.0001738	Up
TLR5	Toll Like Receptor 5	0.582	0.0001103	Up
GK-AS1	GK Antisense RNA 1	0.595	0.0000601	Up
*TR*IM9	Tripartite Motif Containing 9	0.67	0.0001336	Up
SLC7A11	Solute Carrier Family 7 Member 11	0.706	0.0002127	Up
ALDH1A2	Aldehyde Dehydrogenase 1 Family Member A2	0.717	0.0001031	Up
MMRN1	Multimerin 1	0.726	0.0000406	Up
SOCS3	Suppressor Of Cytokine Signalling 3	0.74	0.0000518	Up
HLA-H	Major Histocompatibility Complex, Class I, H	1.239	0.0000255	Up
PF4V1	Platelet Factor 4 Variant 1	1.438	0.0000311	Up

sPLSDA analysis was used to group the subjects based on the detected DEGs generated by RNA-sequencing (Figure 3.2.1.2). As observed in the bi-plot, DEGs showed a clear discrimination between patients with axSpA and healthy controls (Figure 3.3.1.2. A). Moreover, loading plots of components 1 and 2 (Figure 3.3.1.2.B and 3.3.1.2.C respectively) represents the genes contributing to this discrimination, including *RPL36AL*, *NACA*, *OSFT1*, and *MICA*.

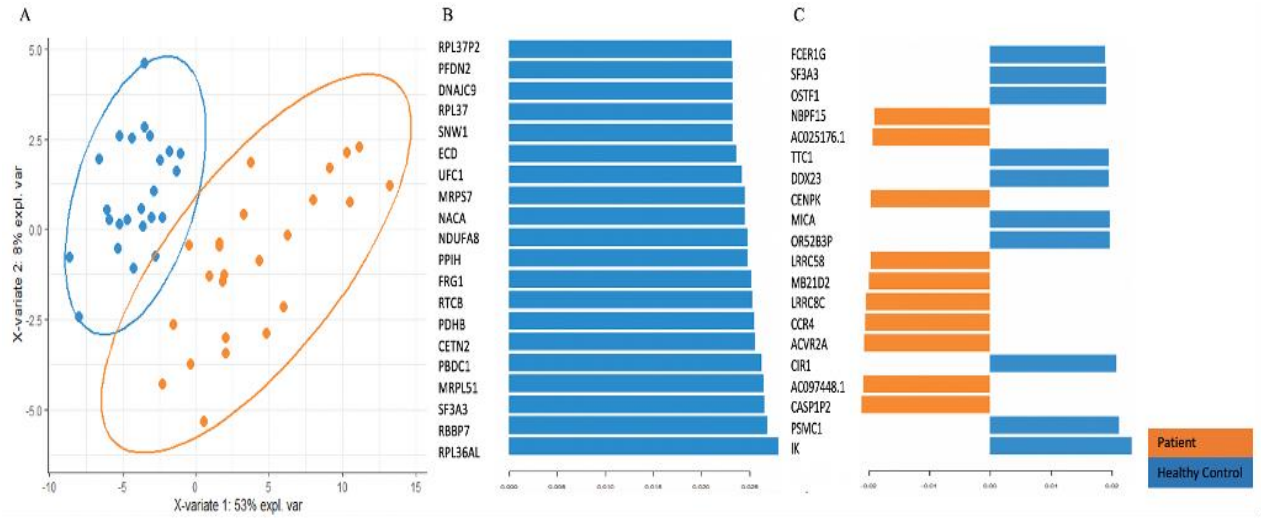


Figure 3.3.1.2. A. Multivariate sPLSDA of DEGs between patients with axSpA and matched healthy controls. B. The contribution loading plot for component 1 of the sPLSDA plot that discriminates these 2 groups. C. The contribution loading plot for component 2 of the sPLSDA plot. Bar length indicates the loading coefficient weight of selected genes contribute in the plot discrimination. Orange and blue bars represent patients with axSpA and healthy controls, respectively.

Abbreviations: sPLSDA: Sparse partial least-squares discriminant analysis; DEG: differentially expressed gene.

3.3.2. Enrichment Analysis

We utilised STRING to perform GO enrichment analysis for the 76 DEGs which revealed predominant association with a range of biological processes, including mitochondrial electron transport, mitochondrial ATP synthesis, mitochondrial respiratory chain complex assembly and cellular respiration (Figure 3.3.2.1). To complement these results, we also performed Gene Set Enrichment Analysis (GSEA), which allowed identifying immunity-related biological pathways in patients with axSpA that were altered when compared with HC (Figure 3.3.2.2) including an increased expression of genes associated with the complement cascade, IL6 and IL10 signalling, and decreased expression of genes associated with rRNA processing and mitochondrial translation.

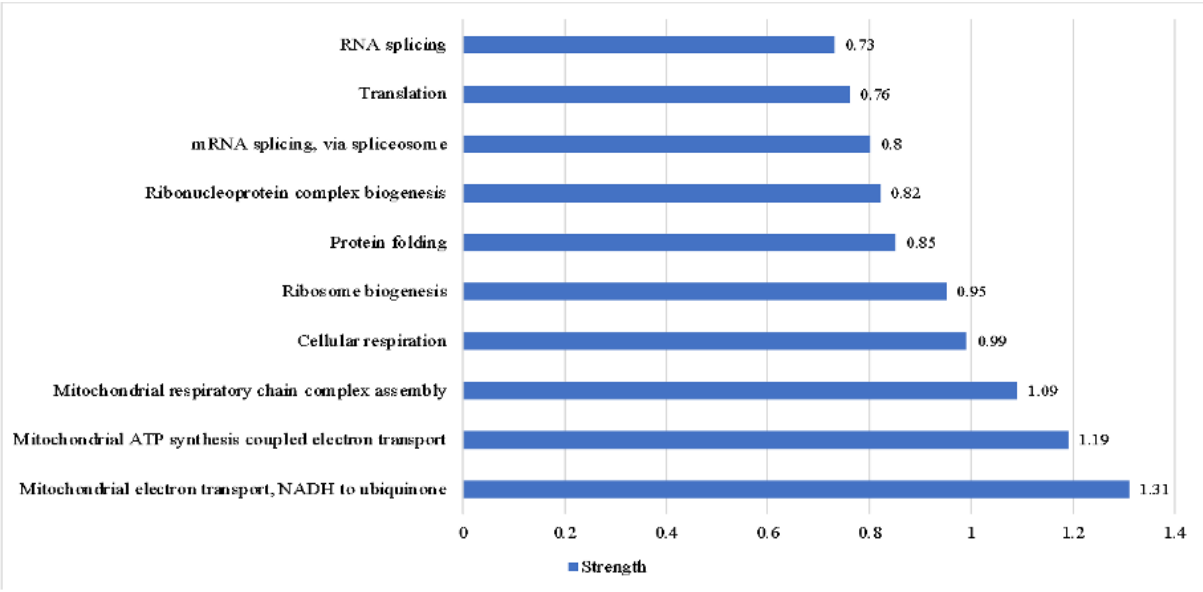


Figure 3.3.2.1. GO enrichment analysis results for DEGs. The top 10 significant pathways are presented. Strength indicates the magnitude of the enrichment effect. All pathways carry a significant *p*-value (cut-off ≤ 0.05).

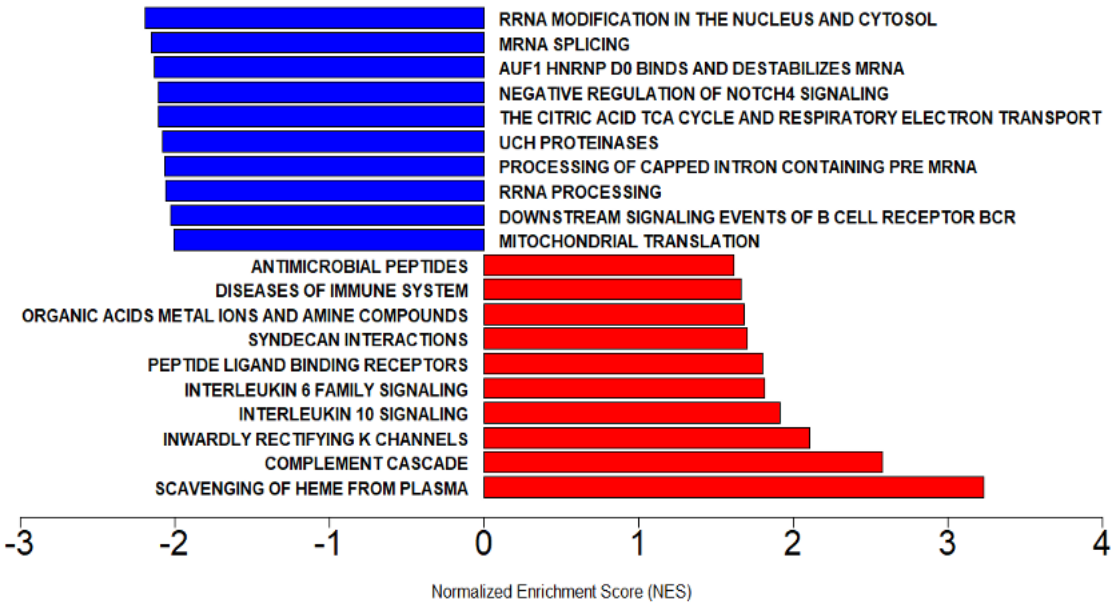


Figure 3.3.2.2. GSEA pathway enrichment of differentially expressed genes between axSpA patients and healthy controls. The top 10 significantly enriched biological pathways are represented. Red and blue bars correspond to patients with axSpA and healthy controls, respectively. All pathways carry a significant p-value (cut-off ≤ 0.05). Abbreviation: GSEA: Gene Set Enrichment Analysis.

3.3.3. Confirmation of Selected Candidate Genes by RT q-PCR

From the 76 DEGs, 15 genes were selected for confirmation by RT-PCR, based upon *p*-value, fold-change and their biological relevance to muscle function and structure, osteogenesis or innate immunity (Appendix 2: Supplemental Material, Table S4). Compared with the HC group, RT-qPCR detected numerically lower levels of expression for all selected genes except *LRRFIP1* and *SOCS3*, in the group of patients with axSpA which were consistent with our RNA-sequencing results (Figure 3.3.3.1). Worth of note, 4 genes (*SOCS3*, *NACA*, *PPIA* and *N6AMT2*) were differentially significantly expressed with the same trend as RNA-sequencing results (Detailed results can be seen in Appendix 2: Supplemental Material, Table S5).

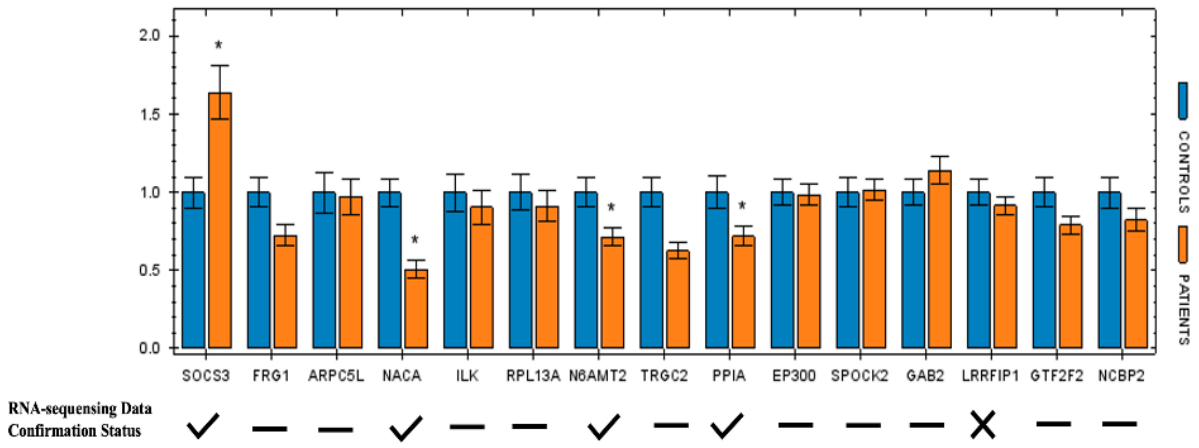


Figure 3.3.3.1. Relative normalised expression of candidate genes in healthy controls and patients with axSpA by RT-qPCR. Error bars represent standard deviations.

* Indicates *p*-value < 0.05; ✔ Indicates genes significantly differentially expressed in axSpA vs HC, in agreement with RNA-sequencing results; − Indicates genes expressed in the same trend (non-significant *p*-value) with RNA-sequencing results. × Indicates genes expressed non-significantly the opposite trend with RNA-sequencing results.

Abbreviation: RT-qPCR: Real Time Quantitative Polymerase Chain Reaction.

3.3.4. Screening of Differentially Expressed Proteins

Serum samples were obtained from 11 patients with axSpA and their matched healthy controls (According to the adequacy and eligibility of biological samples in experimental steps). From these, 170 proteins were identified and quantified with 1% FDR. From these, we identified 87 proteins that were significantly (adjusted $p<0.05$ from a differential expression analysis - see methods) up- or down-regulated in patients with axSpA compared with HC. Among the up-regulated proteins were several acute-phase proteins such as C reactive protein (CRP), complement proteins, APCS and SERPIN3A. Table 3.3.4.1 represents the top 10 significantly up-regulated and top 10 down-regulated proteins with the highest fold changes in patients with axSpA compared to healthy controls (Full list can be seen in Appendix 2: Supplemental Material, Table S6).

Table 3.3.4.1. Top 10 significantly up-regulated and top 10 down-regulated proteins in patients with axSpA compared to healthy controls.

Protein	Gene	*p*-value	Log (Fold Change)
C-reactive protein	*CRP*	2.80E-07	0.75
Coagulation factor XI	*F11*	5.68E-14	0.74
Immunoglobulin lambda constant 3	*IGLC3*	4.18E-03	0.72
Serum albumin	*ALB*	4.48E-02	0.61
T-complex protein 1 subunit eta	*CCT7*	1.80E-04	0.52
60 kDa heat shock protein, mitochondrial	*HSPD1*	2.61E-03	0.5
Exportin-2	*CSE1L*	1.41E-06	0.5
Mitogen-activated protein kinase 4	*MAP3K4*	8.06E-08	0.45
Elongation factor 2	*EEF2*	2.09E-03	0.44
CDK5 regulatory subunit-associated protein 2	*CDK5RAP2*	4.20E-04	0.42

Hemoglobin subunit alpha	*HBA1*	2.84E-03	-0.23
Keratin, type I cytoskeletal 14	*KRT14*	5.78E-03	-0.24
Keratin, type I cytoskeletal 10	*KRT10*	1.03E-03	-0.24
Mannan-binding lectin serine protease 2	*MASP2*	2.49E-02	-0.28
Corticosteroid-binding globulin	*SERPINA6*	1.09E-03	-0.28
Apolipoprotein A-I	*APOA1*	3.14E-03	-0.3
Tenascin	*TNC*	3.10E-06	-0.32
Fibronectin	*FN1*	2.27E-05	-0.33
Alpha-1-antitrypsin	*SERPINA1*	5.43E-08	-0.39
Serotransferrin	*TF*	2.57E-08	-0.47

We then performed sPLSDA analyses in an attempt to find proteins that discriminate patients with axSpA from HC. To do this, we used the R packages and the results are depicted in Figure 3.3.4.1. The patients group seems to be divided into 2 upper and lower sub-groups. The incorporation of clinical data revealed that patients in the upper group of the sPLSDA have higher muscle strength (Figure 3.3.4.1.A). Among the most discriminating proteins (Figure 3.3.1.2), we found inflammation-associated proteins CRP, C9, C4A upregulated in axSpA, and several SERPIN and IG proteins downregulated in the same group. Another example of discriminating protein is RBP4, the major transport component of Vitamin A, which has multiple roles in muscle function and bone remodelling.

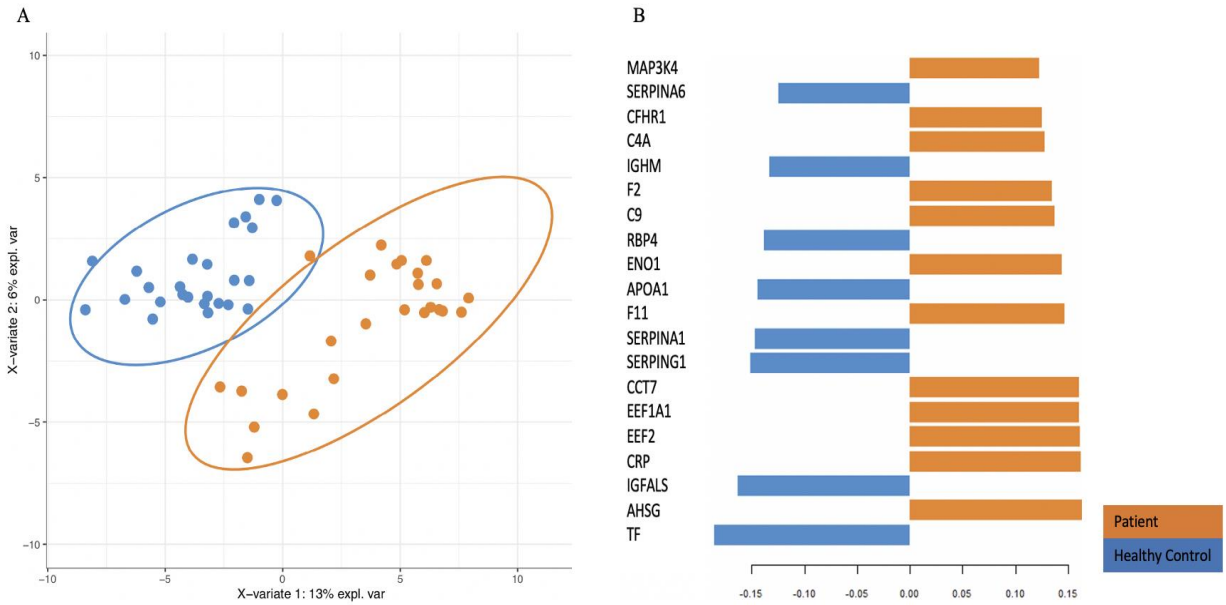


Figure 3.3.4.1. A. Multivariate sPLSDA differentially expressed proteins between patients with axSpA and healthy controls. B. Contribution plot indicating contributing weight of component 1 of the sPLSDA plot that discriminates these 2 groups. Bar length indicates the loading coefficient weight of selected protein. Orange and blue bars represent patients with axSpA and healthy controls.

Abbreviation: sPLSDA: sparse partial least-squares discriminant analysis.

Additionally, we utilised the STRING software (https://string-db.org) to perform GO enrichment analysis for the 176 differentially expressed proteins. GO generates sets of explicitly defined, structured vocabularies that describe biological processes, molecular functions and cellular components of gene products in both a human- and computer-readable manner. GO analysis placed them into 341 subclasses; Figure 3.3.4.2 represents the top 10 subclasses with a significant *p*-value (< 0.05). Most have roles in innate immunity and/or the acute-phase inflammatory response.

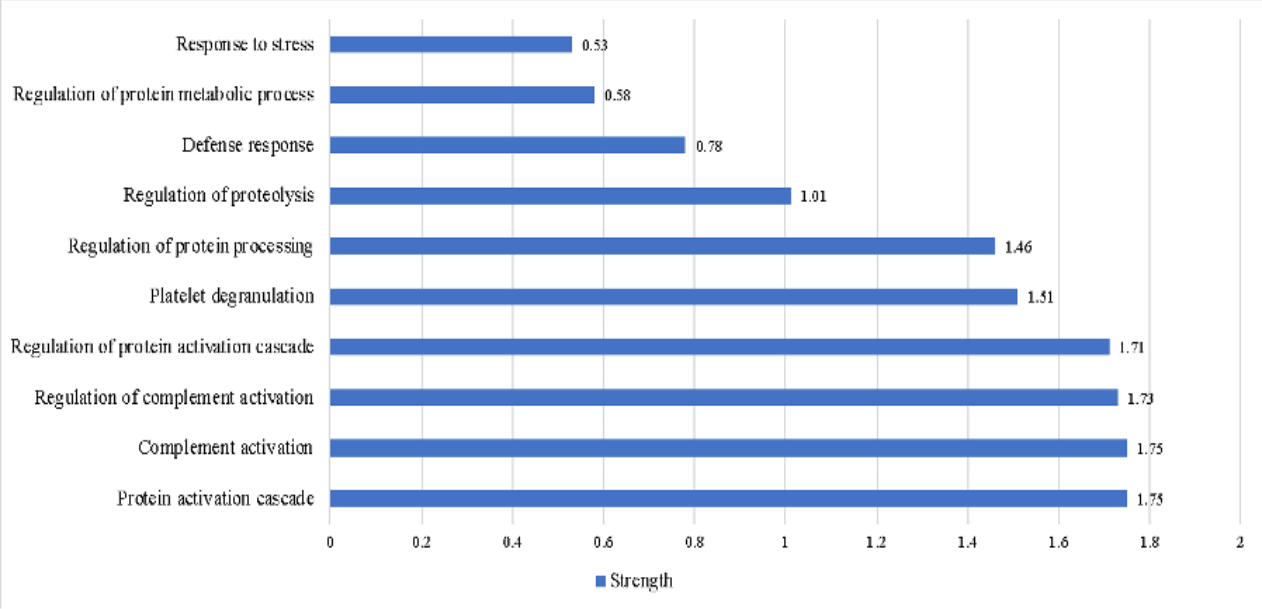


Figure 3.3.4.2. GO enrichment analysis results for differentially expressed proteins. The top 10 significant pathways are presented.

Strength measure describes how large the enrichment effect is. All the pathways carry significant *p*-value (cut-off ≤ 0.05).

3.3.5. Integrated Analysis of Transcriptomics and Proteomics Data

We combined transcriptome and proteome data and performed a multivariate sPLSDA analysis to evaluate the combined discrimination power of DEGs and differentially expressed proteins between patients with axSpA and HCs. As shown in Figure 3.3.5.1A, the sPLSDA score plot revealed the goodness of fit and high predictability of the model, demonstrating good separation between patients with axSpA and HCs, better than only using transcriptome or proteome individually. Interestingly, as it is shown in Figure 3.3.5.1B, the most informative variables in the distribution plot were transcriptome variables, with 18 genes and only 2 proteins appearing. All of these genes were found to be highly expressed in HCs, while the 2 proteins appeared more abundant in the patients' sera (Figure 3.3.5.1B).

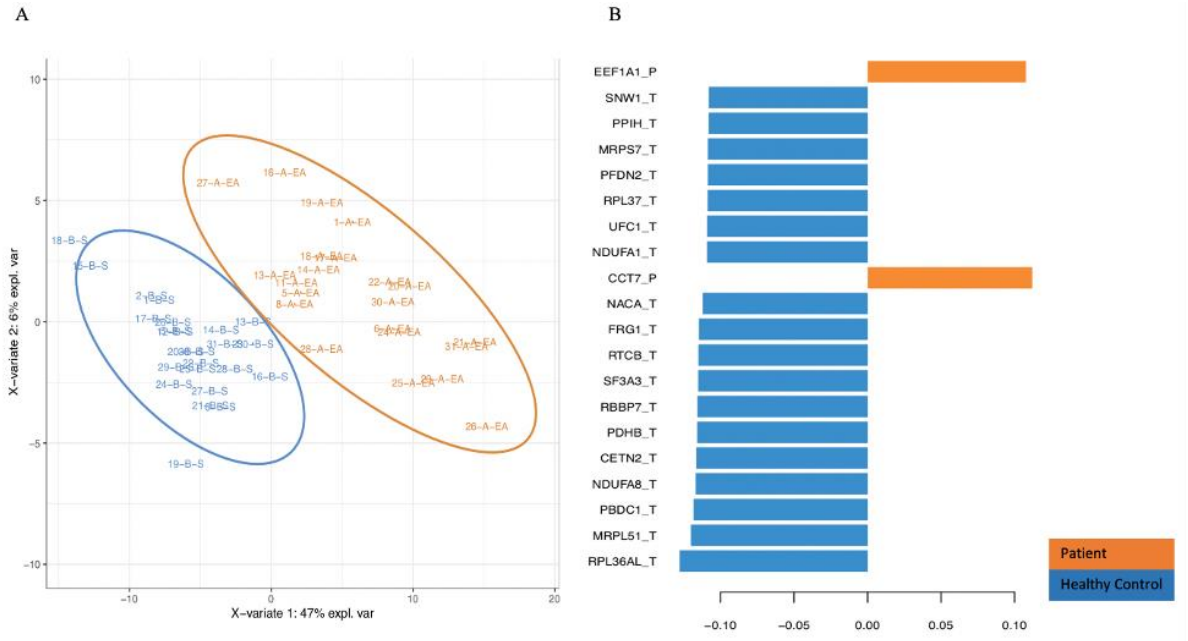


Figure 3.3.5.1. A. Multivariate sPLSDA of transcriptomics and proteomics results between patients with axSpA and matched HC. B. Contribution plot indicating genera contributing to component 1 of the sPLSDA plot that discriminate these 2 groups.

sPLSDA: sparse partial least-squares discriminant analysis.

T stands for a gene identified in the Transcriptomic screening and P stands for a protein identified in the Proteomic screening. Orange and blue colours correspond to patients with axSpA and healthy controls, respectively.

3.3.6. Integrated Analysis of Omics Data and Muscle Strength

Moreover, we also evaluated the correlation of omics data with muscle strength in patients with axSpA (Table 3.3.6.1). Interestingly, proteins with the highest values of muscle strength correlation (such as *IGHA1*, *IGKV3-20, and IIGLL5*) are related to the immunoglobulin domain, suggesting a potential role for adaptive immunity in muscle strength. Worth noting, these proteins are all downregulated in patients with axSpA samples.

Table 3.3.6.1. DEGs that correlate with muscle strength.

Gene Name	Correlation with Muscle Strength	*p*-value	adj *p*-value
IGHA1	0.63	6.10E-15	1.03E-12
IGKV3-20	0.59	9.54E-13	1.61E-10
IIGLL5	0.05	2.57E-10	4.32E-08
IGHH3	0.53	3.76E-10	6.28E-08
IgA2HC	0.51	1.47E-09	2.44E-07
IgG1HC	0.49	9.36E-09	1.54E-06
IgKLC	0.46	9.05E-08	1.48E-05
APOB	0.42	1.42E-06	2.31E-04
PON1	0.41	2.44E-06	3.96E-04
GPX3	-0.37	1.73E-05	2.79E-03
C1R	-0.35	5.14E-05	8.22E-03
SERPINA3	-0.35	6.03E-05	9.59E-03

Chapter 4: Discussion

This study aimed at shedding light on the role of muscle in the susceptibility and progression of axSpA. To overcome the lack of information regarding muscle properties, we proposed a broad, multidisciplinary, and innovative design study that allows an extensive muscle characterization, including physical properties, strength, mass and performance in different body segments (i.e., trunk, upper and lower limbs), together with the establishment of transcriptomic and proteomic signatures, which was performed in the same group of participants. Identification of gene/protein signatures in axSpA may represent a key contribution to explaining muscle properties and unravelling the subsequent underlying physiopathologic mechanisms of the disease. It may also provide an opportunity for the development of new diagnostic tools and preventive/therapeutic approaches, with relevance to clinical practice.

We aimed to identify specific muscle characteristics in an axSpA context and to understand their systemic or local expression. Masi et al. have documented an increase in axial (lumbar) muscle stiffness in a group of patients with AS (160). It would be of interest to expand from this observation by analysing peripheral muscles to consider any, general or local, inflammatory effect. If the specific muscle characteristics point to a systemic involvement, meaning that changes in axial muscles, where muscles are under the effect of local inflammation, will be also reproduced in peripheral muscles, where this effect is absent, a genetic/molecular subjacent background should be pursued.

Furthermore, the selection of young patients (under 50 years old) with short disease duration (without cDMARDs or bDMARDs and only low doses of systemic corticoids allowed to avoid bias in muscle and peripheral features of the disease) allowed speculating about the possible impact of muscle in disease susceptibility. Conceptually, microtrauma induced by daily

activities or by the muscle itself should play an important role in entheseal inflammation. Entheses are specialized interfaces where the integration of tendon into bone occurs (161) and they can be subjected to repetitive biomechanical stress forces applied during the action of normal muscle and other periarticular structures. In axSpA, entheses are known as the initiating sites of musculoskeletal inflammation. In this context, the axial entheses are particularly prone to inflammation as they are subjected to mechanical stress related to posture maintenance (162,163).

In our study, relatively young patients with axSpA, with a mean disease duration of 6.5 years, presented similar segmental muscle stiffness, tone and elasticity as healthy subjects. There was, however, an asymmetry in muscle stiffness between lumbar and appendicular muscles. Although the underlying mechanism for the numerically higher trunk stiffness in axSpA patients (even though the difference does not reach statistical significance) is unknown, we hypothesise that it may result from the local effect of inflammation. These data are in line with a previous study conducted by Andonian et al., in which 24 patients with r-axSpA presented higher lumbar myofascial stiffness than 24 age- and sex-matched control subjects (this difference is statistically significant), measured by the same myotonometry device as ours (163). Importantly, these results may also support the hypothesis that abnormalities in biomechanical pathways might be implied in the clinical course of axSpA, as these patients had established disease with a mean disease duration of 12.7 years. However, it is difficult to speculate whether these changes are the cause or consequence of the disease. The low scores for BASDAI and BASFI in our patients, reflect low disease activity and functional impairment.

In our study, all patients except 8% (2 out of 27), had values of general muscle strength and muscle mass in the range of the normality but presented low levels of physical performance, which suggests a possible muscle dysfunction. Although we cannot fully explain this

observation, we can hypothesise that a possible genetic determinism may be evoked and should be further investigated in future research.

Despite the normal values for total strength in patients, a deeper analysis showed a significant reduction of general and appendicular (but not in the trunk) muscle strength in the patients with axSpA patients compared to the HC. These results also raise questions about the existing reference values for strength and their applicability to our population, for whom they have not been validated. However, previous studies have also reported lower appendicular strength in patients with r-axSpA (164), even in the absence of peripheral joint involvement (164). Various potential factors may justify a decrease in muscle strength, including systemic inflammation or fatigue (164). Inactivity or disuse is also associated with loss of strength, but in our study, the patients were matched with the HC also according to the levels of physical exercise to control for this influential effect.

Reduced appendicular strength has been associated with loss of appendicular lean mass in patients with longstanding r-axSpA (165). A major known determinant of strength loss is indeed the loss of muscle mass (166). However, in our study, the reduced appendicular strength was independent of muscle mass. Since our patients had a mean disease duration of 6.5 years, we can consider that muscle mass loss may still occur in a later phase of the disease. Despite being a different age group, in older people, the strength decline has been proved to be faster than the concomitant loss of muscle mass (166). An intriguing result was the absence of decreased muscle strength in the axial muscles. The distinct physiological role of axial and peripheral muscles, the former being responsible for maintaining posture and the latter for generating strength may represent a possible explanation to be explored.

Several studies on body composition in axSpA have found inconsistent results that may be explained by differences in the disease duration and levels of physical activity, and also, by

discrepancies in the methods used to estimate muscle mass. In agreement with our data, two previous studies did not observe differences in total lean mass or even skeletal muscle mass index, as measured by dual-energy x-ray absorptiometry or bioelectrical impedance, between patients with axSpA (disease duration 6–10 years) and controls (167)

Regarding physical performance, gait in patients with longstanding r-axSpA has long been referred to as "walking gingerly", as they walk slower and have a shorter stride length than healthy individuals, which can be attributed to the increased rigidity of the spine (168). In our cohort, we showed that young patients with axSpA also have significantly lower gait speed than the HC, independently of muscle mass, strength or muscle physical properties. In this context, gait characterisation (including speed and other parameters) could be considered a marker with potential interest in axSpA, eventually for diagnosis and, for disease monitoring.

We conducted this research to establish gene and protein signatures in axSpA related to specific muscle properties. We could successfully identify DEGs that contribute to the translation and mitochondrial pathways (e.g., *RPL36AL*) and are essential for muscle metabolism(169). Transcriptomic's data further supports the idea of an active role of muscle in the pathogenesis of axSpA, as we identified muscle-associated differentially expressed genes (e.g., *NACA*) in our results. We also identified elevated levels of inflammatory proteins (e.g., CRP), that are indicative of disease activity in patients with axSpA (170), (Table 3.3.4.1). Interestingly, the expression of some of these differentially expressed proteins (e.g., APOB) has a significant correlation with muscle strength (Table 3.3.6.1).

Our findings corroborate the work of Lee and co-workers, who highlighted Mitochondrial Ribosomal Proteins (MRP) and cyclooxygenase (*COX*) genes as some of the most down-regulated genes in patients with axSpA in their meta-analysis study (171). Interestingly, we also identified genes belonging to MRP and COX families in our results, such as Mitochondrial

Ribosomal Protein S7 (MRPS7) and Cytochrome C Oxidase assembly protein 17 (CXO17) (Appendix 2: Supplemental Material, Table S3). It has been shown that MRPs play roles in ribosome biogenesis that have emerged as an important regulator of skeletal muscle growth and maintenance by altering the translational capacity of the mitochondria in the cells (172). It is also suggested that different isoforms of COX catalysis the prostaglandins synthesis that may regulate muscle regeneration, since they modulate inflammation and are involved in various stages of myogenesis (173).

GSEA results (Figure 3.3.2.2.) suggest that significant pathways such as IL6 signalling and immune system, in particular innate immunity, seem to play a role in axSpA patients since it was only found in patients with axSpA. Increased serum levels of various pro-inflammatory cytokines in patients with axSpA, namely increased levels of IL-6 and TNF-α have been observed in the serum of patients with axSpA in a study completed by Gratacos and co-workers (174). Inflammatory proteins such as CRP, APOA and SERPINA1 in our proteomic's data strengthen the role of inflammatory pathways in patients with axSpA (Table 3.3.4.1). Our findings agree with those of Park and co-workers (175), who reported that patients with axSpA have significantly raised serum levels of IL-6 correlating significantly with CRP and disease activity (BASDAI), while non-significantly correlated with ESR. Another detected inflammatory protein, APOA, exerts an anti-inflammatory property in inhibiting lymphocyte cells' migration by decreasing the expression of adhesion molecules (176). It is also suggested that SERPINA1 may be a potential biomarker for the diagnosis of AS and evaluation of the efficacy of treatment by influencing inflammation.

Our transcriptomics's and proteomic's findings further support the role of muscle in the pathogenesis of axSpA. CRP and albumin are two differentially expressed proteins found to be associated with low physical performance, muscle strength or muscle mass (170). MAP3K4,

also known as MEKK4, is another detected protein that plays a role in skeletal muscle atrophy (177). The transcriptomic's result further provides evidence of different expressions of muscle-associated genes, namely *NACA, FRG1* and *ARPC5L*, in patients with axSpA. These genes have roles in skeletal muscle growth and regeneration, muscle development and function, and regulation of actin polymerization, respectively (178–180). Our RT-qPCR results match those observed in RNA-sequencing, confirming the downregulation of these muscle-associated genes (*NACA*, *FRG1* and *ARPC5L*) in patients with axSpA in comparison with HC.

Microtrauma of variable degrees in muscle tissue is reflected in temporary and repairable damage through the immune system (181). Muscle contraction raises calcium and promotes pro-inflammatory cytokines release ultimately ending in the attraction of neutrophils, lymphocytes, monocytes and other cells to the damaged site (182,183). Indeed, both innate and adaptive immune systems are activated after microtrauma. Hence, this microtrauma and its consequent inflammation may be the reason for the appearance, in our results, of the immune and inflammatory pathways, such as IL-6 family and IL-10 signalling pathways, and the inflammation-related proteins (such as CRP and APOA1).

Our gene expression assessment results revealed several interesting genes with potential roles in axSpA pathogenesis. One of the remarkable genes is Suppressor of Cytokine Signalling 3 (*SOCS3*). Previous studies have shown that the SOCS family was involved in the pathogenesis of ankylosing spondylitis and elevated level of *SOCS3* was negatively correlated with serum inflammatory cytokine IL6 in patients with axSpA (184). *SOCS3* was shown to inhibit the catalytic activity of Janus Kinases (JAKs) that initiate signalling within the cell[61]. JAK and Signal Transducer and Activator of Transcription (STAT) proteins are central transmitters of pro- and anti-inflammatory signals in immune regulation (158). Currently, JAK inhibitors are a new therapeutic class for the treatment of axSpA(177). Worth of note, genetic deletion of

SOCS3 delays the expression of myogenic factors critical for myogenesis (178). *SOCS3* overexpression in our patients with axSpA is consistent with the results of another RNA-sequencing experiment performed by Chen and his co-workers in 53 patients with axSpA (184). This consistency supports the robustness of our RNA-sequencing and RT-qPCR results.

Another noticeable gene among our DEGs is Nascent Polypeptide-Associated Complex and co-regulator α (*NACA*, *αNAC*), which is down-regulated in patients with axSpA in comparison with matched healthy controls. *NACA* is a 215–amino acid transcriptional co-factor engaged in the regulation of AP-1 transcription (185–187). *NACA* prevents short recently synthesized (i.e., nascent) ribosome-associated polypeptides from inappropriate interactions with cytosolic proteins. *NACA* binds nascent-polypeptide domains emerging from ribosomes unless it contains a signal peptide which is fully exposed (188). Skeletal and heart muscle exclusive isoforms of *NACA* (*skNACA*) may function to regulate the expression of genes that contribute to the development of myotubes (189). It has a significant role in ventricular cardiomyocyte expansion and adjusts postnatal skeletal muscle development and regeneration. It likewise contributes to the organized assembly of thin and thick filaments of myofibril sarcomeres. In addition, *skNAC* regulates myoblast migration and sarcomere structure in a calpain-dependent manner (189).

FSHD region gene 1 (*FRG1*) is also a muscle-associated gene in our DEGs and is down-regulated in patients with axSpA when comprised with matched healthy controls. FRG1 is a dynamic cytoplasmic and nuclear shuttling protein that is also localized to the sarcomere in skeletal muscle (190). In the nucleus, *FRG1* is located in nucleoli and Cajal bodies. It is also associated with transcribed chromatin (191,192), where it is thought to participate in RNA splicing (193) and regulate the activity of the histone methyltransferase SUV4-20H1(194). Notably, *FRG1* overexpression is believed to cause muscle stem cell defects (195). and the

appearance of FSHD-like phenotypes in mice (196). There is evidence for a direct *FRG1* and *DUX4* interaction in development of FSHD muscular dystrophy (197). Furthermore, it has been shown that *FRG1* gene silencing contributes to develop muscle mass and strength in FRG1(-high) mice (198). This gene is expressed in our patients' samples (represents 3.4 Average Expression value, Appendix 2: Supplemental Material, Table S3) that may contribute to observation of low muscle strength in our patients with axSpA.

The Arp2/3 complex was formerly discovered in Acanthamoeba and contains seven proteins (complex subunits; ARPC1–5 and actin-related proteins; Arp2 and Arp3, and Arp2/3) that are conserved in every eukaryote, except for some microsporidia, algae, and protists. The complex plays a critical role in an extensive variety of cellular processes, including endocytosis, phagocytosis, and lamellipodia-mediated cell migration, due to its capability to participate in generation of branched actin filament nets (199). It has been observed that Arp2/3 complexes including *ARPC5L* are remarkably better at promoting actin assembly than the complexes with *ARPC5*, both and in vitro and in vivo (200). Actin assembly is vital for muscle contraction and force generation (199). Interestingly, our results indicate that *ARPC5L* is slightly less expressed (- 0.2-fold change) in patients with AS (Appendix 2: Supplemental Material, Table S3) which may reflect low muscle strength in patients with axSpA (Table 3.3.1).

Our RT-qPCR results yielded consistent results when confirming downregulations in N (6)-adenine-specific DNA Methyltransferase 2 (*N6AMT2*) and peptidylprolyl isomerase A (*PPIA*) genes (Figure 3.3.3.1). *N6AMT2* is an isoform of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. It plays a role in positive regulation of Interferon Gamma (*IFNG*) transcription in T-helper 1 cells as part of an IFNG promoter-binding complex (179). The other gene, *PPIA* encodes a member of the peptidyl-prolyl cis-trans isomerase (PPIase) family. PPIases catalyse the cis-

trans isomerization of proline imidic peptide bonds in oligopeptides and accelerate the folding of proteins. The encoded protein is a cyclosporin binding protein and may play a role in cyclosporin A-mediated immunosuppression (180).

Our analysis found shreds of evidence for the correlation of differentially expressed proteins with muscle strength (Table 3.3.6.1). We observed several positively correlated proteins that have essential roles in the adaptive immune system (e.g., IGHA1 and IGA). The findings of this study mirror those of previous studies that have examined the elevated total IgA levels in patients with axSpA (201). It was suggested that the adaptive immune system targets the inflammation in the joints, while autoinflammatory stimuli in the intestines and the enthesis are also essential muscle strength (202). Moreover, we also observed the positive correlation of Apolipoprotein B (APOB) protein expression with muscle strength in accordance with earlier studies (203). Indeed, overexpression of APOB decreases skeletal muscle lipid accumulation and affects overall muscle functionality. These observations are consistent with our current knowledge about patients with axSpA that experience high muscle fat metaplasia (203).

The most important feature of this study is that it represents the first multivariable investigation on patients with axSpA. We explored the muscle role in terms of clinical characteristics (muscle properties) and omics data. Clinical and laboratory results were merged and integrated to identify possible reliable diagnosis biomarkers axSpA, thus enhancing our understanding of disease mechanisms. Finally, several important limitations need to be considered. Firstly, the number of patients and controls is relatively small. And secondly, ideally, tissue biopsies are more appropriate samples to investigate muscle associated biomarkers since many factors may affect genes expression and protein production in peripheral blood.

Chapter 5: Conclusions and Future Perspectives

Muscular involvement may have prognostic significance in patients with axSpA. Several approaches have supported muscle strength reduction and muscle mass reduction, in association with physical performance compromise, favouring the diagnosis of sarcopenia, from the early stages of the disease.

Overall, we intended to establish a pattern of muscle properties in axSpA and depict the triggers involved in entheses inflammation. We hypothesized that muscle properties, such as stiffness, may contribute to a continuous endogenous microtrauma and subsequent inflammation. We also intended to establish gene/protein signatures in axSpA that may represent a key contribution to explaining muscle properties and unravelling the subsequent underlying physiopathologic mechanisms of the disease. It may also provide an opportunity for the development of new diagnostic tools and preventive/therapeutic approaches, with relevance to clinical practice.

Our clinical findings suggest that muscle physical properties were not different between axSpA patients and HC. These results cannot be extrapolated for patients with a longstanding disease (e.g., superior to 10 years of disease duration). Young axSpA patients with a relatively short disease duration presented similar segmental muscle physical properties as the HC. Patients with axSpA had reduced physical performance and lower strength compared to the HC, despite normal muscle mass, suggesting a possible muscle dysfunction.

We conducted this research to establish gene and protein signatures in axSpA that are related to specific muscle properties. Strong shreds of evidence have been found in our results. We could successfully identify DEGs that contribute to the electron transport chain, mitochondrial respiratory chain complex, cellular respiration and RNA splicing pathways. These pathways

are essential for muscle metabolism. Complementary enrichment analysis also revealed significant pathways such as IL6 signalling and the immune system that seem to have a role in axSpA patients.

Our transcriptomics and proteomics findings further support the role of muscle in the pathogenesis of axSpA. CRP and Albumin are two differentially expressed proteins found to be associated with low physical performance, muscle strength or muscle mass. MAP3K4 which is known as MEKK4 is another detected protein that plays a role in skeletal muscle atrophy. The transcriptomics result further provides evidence of different expressions of muscle associated genes namely, *NACA*, *FRG1* and *ARPC5L* in patients with axSpA. These genes have roles in skeletal muscle growth and regeneration, muscle development and function, and regulation of actin polymerization, respectively. Our RT-qPCR results match those observed data in RNA-sequencing confirming the downregulations of these muscle-associated genes (*NACA*, *FRG1* and *ARPC5L*) in patients with axSpA in comparison with HC. We also have identified the elevated level of inflammatory proteins (e.g., CRP) that is a measure of disease activity in patients with axSpA. Interestingly, the expression of some of these differentially expressed proteins (e.g., APOB) has a positive correlation with muscle strength. Noteworthy, the correlation of transcriptomics and proteomics results shows a clear separation between patients with axSpA and controls that may contribute to improving the diagnosis, prognosis, and therapeutic options for this disorder.

Our analysis found shreds of evidence for the correlation of differentially expressed proteins with muscle strength. We observed several positively correlated proteins that have essential roles in the adaptive immune system (e.g., IGHA1 and IGA). It is suggested that the adaptive immune system targets the inflammation in the joints, while autoinflammatory stimuli in the intestines and the enthesis are also essential. We also observed the positive correlation of

APOB protein with muscle strength. Indeed, overexpression of APOB decreases skeletal muscle lipid accumulation and affects overall muscle functionality and we already know that patients with axSpA experience high muscle fat metaplasia.

Noteworthy, the correlation of transcriptomics and proteomics results shows clear discrimination between patients with axSpA and HC that may contribute to improving the diagnosis, prognosis, and therapeutic options for this disorder.

There is abundant room for further progress in determining the role of muscle in axSpA. Further research in the field is needed to identify and validate the role of muscle in patients with axSpA. Diagnosis biomarkers that can accurately and reliably predict axSpA evolution, differentiate patients from controls and assess disease activity might have functions in muscle associated pathways. Future research can be conducted with a higher number of study subjects to deliver more reliable data. Furthermore, ideally, tissue biopsies are more appropriate samples to investigate muscle associated biomarkers since many factors may affect genes expression and protein production in peripheral blood. Indeed, single-cell omics approaches will provide more precise data.

Chapter 6: References

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Appendix

Appendix 1: Published Manuscripts

Appendix 1. Paper 1

CLINICAL PRACTICE

The role of muscle in the susceptibility and progression of axial Spondyloarthritis: The MyoSpA study protocol

Sardoo AM[1,2], Neto A[1,3], Pinheiro Torres R[1,3], Rodrigues-Manica S[1,3], Domingues L[1,4], Lage Crespo C[1], Lagoas-Gomes J[1,3], Mascarenhas V[5], Mendes CS [1], Galzerano A[6], Fernandes de Almeida S[7], Sepriano A[1,3], Ramiro S[8], Masi AT[9], Nair K[10], Costa J[11], Alexandre BM[12], Vassilevskaia T[2], Cunha CV[2], Sobral D[13], Branco JC[1,3], Gomes-Alves P[12], Pimentel-Santos FM[1,3]

ACTA REUMATOL PORT. 2021;46:342-349

ABSTRACT

Background: Axial Spondyloarthritis (axSpA) is a chronic, inflammatory rheumatic disease that affects the axial skeleton, causing pain, stiffness, and fatigue. Genetics and environmental factors such as microbiota and microtrauma are known causes of disease susceptibility and progression. Murine models of axSpA found a decisive role for biomechanical stress as an inducer of enthesitis and new bone formation. Here, we hypothesize that muscle properties in axSpA patients are compromised and influenced by genetic background.
Objectives: To improve our current knowledge of axSpA physiopathology, we aim to characterize axial and peripheral muscle properties and identify genetic and protein biomarker that might explain such properties.
Methods: A cross-sectional study will be conducted on 48 participants aged 18-50 years old, involving patients with axSpA (according to ASAS classification criteria, symptoms duration < 10 years) and healthy controls matched by gender, age, and levels of physical activity. We will collect epidemiological and clinical data and perform a detailed, whole body and segmental, myofascial characterization (focusing on multifidus, brachioradialis and the gastrocnemius lateralis) concerning: a) Physical Properties (stiffness, tone and elasticity), assessed by MyotonPRO®; b) Strength, by a dynamometer; c) Mass, by bioimpedance; d) Performance through gait speed and 60-second sit-to-stand test; e) Histological and cellular/ molecular characterization through ultrasound-guided biopsies of multifidus muscle; f) Magnetic Resonance Imaging (MRI) characterization of paravertebral muscles. Furthermore, we will perform an integrated transcriptomics and proteomics analysis of peripheral blood samples.
Discussion: The innovative and multidisciplinary approaches of this project rely on the elucidation of myofascial physical properties in axSpA and also on the establishment of a biological signature that relates to specific muscle properties. This hitherto unstudied link between gene/protein signatures and muscle properties may enhance our understanding of axSpA physiopathology and reveal new and useful diagnostic and therapeutic targets.

Keywords: Spondyloarthritis; Muscle; Biomarkers; Precision medicine

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BACKGROUND

Spondyloarthritis (SpA) is a group of chronic, inflammatory, rheumatic diseases characterized by overlapping clinical symptoms and genetic background[1]. Axial SpA (axSpA), a subgroup of SpA, mainly affects the spine and the sacroiliac joints (SIJs), being the

symptom onset typically before the age of 45 years[2]. A recent epidemiological study has reported a prevalence of 1.6% for axSpA in Portugal[3]. The disease spectrum clusters patients as having either radiographic axSpA (r-axSpA), also named ankylosing spondylitis (AS), whether they fulfil the 1984 modified New York criteria (mNYc)[4] , or as having non-radiographic axSpA (nr-axSpA) in the lack of explicit SIJs alterations on a plain radiograph[5].

A growing body of literature has pointed out the relevance of genetics in susceptibility and progression of axSpA[6,7]. Since 1973, an association with the human leukocyte antigen B27 (*HLA-B27*) locus has been established[6,8]. Later on, 12 additional loci have been associated with AS in Europeans (ANTXR2, ERAP1, CARD9, IL12B, IL23R, KIF21B, PTGER4, RUNX3, TBKBP1, TNFRSF1A and chromosomes 2p15 and 21q22)[9,10], and, more recently, 2 additional loci have been identified in Han Chinese populations (HAPLN1-EDIL3 and ANO6)[11]. Nevertheless, these genetic loci can only account up to 50% for the axSpA susceptibility[6], suggesting the contribution of other critical factors for disease pathogenesis, such as environmental factors, including microbiota[12] and biomechanical mechanisms[13,14].

Regarding the biomechanical features of SpA, P. Jacques and co-workers elegantly provided evidence for a decisive role of biomechanical stress as an inducer of enthesitis and new bone formation in a murine experimental SpA model[15]. Nevertheless, the explicit mechanisms have never been established[16]. In axSpA, the axial entheses seem to be prone to inflammation when subjected to repetitive biomechanical stress forces transmitted by muscles, ligaments and tendons[13,17–19]. Moreover, Masi and col. have demonstrated an increase of the lumbar myofascial stiffness in AS[19]. However, a link between myofascial stiffness as a source of microtrauma with impact at the entheses level, inducing inflammation and osteoproliferation, remains to be demonstrated. This represents the main hypothesis subjacent to this project.

The analysis of synovial tissue from patients with SpA has provided an explanation linking inflammation, biomechanical stress and bone remodelling in SpA. Using an expression microarray approach, a robust overexpression of genes (*ACTA1, MYH2, MYH7,* and *ACTN2*) associated with myofibroblasts cells was found[20]. Classically, myofibroblasts represent activated fibroblasts that release large amounts of collagens and express stress fibres as well as contractile proteins. Interestingly, it was observed that none of the myofibroblast genes was downregulated by TNF blockers, despite significant downregulation of markers of inflammation[20]. The presence in these locations of mesenchymal stem cells (MSC) could be a triggering factor for the increase of fibrotic tissue[21] and/or bone formation[22].

OBJECTIVES

The overall aim of this study is to assess the role of muscle in susceptibility and progression of axSpA and, consequently, to enhance the understanding of the underlying mechanisms of its physiopathology and reveal new diagnostic and therapeutic targets.

Specific objectives to meet this aim include:

1. Test novel hypothesis of increased resting lumbar myofascial stiffness/tone in patients with axSpA compared to healthy controls.
2. Characterize the muscle strength, muscle mass, and physical performance in patients with axSpA to assess the prevalence of sarcopenia.
3. Establish gene and protein signatures in axSpA, trying to establish an association to specific muscle properties. This will allow the identification of new serological biomarkers potentially relevant for preventive/therapeutic approaches, to be tested in future studies.

METHODS

The current study was submitted and approved by the ethical committees of NOVA Medical School, NOVA University of Lisbon and Centro Hospitalar de Lisboa Ocidental, Hospital de Egas Moniz, EPE. The study will be conducted following the International Conference on Harmonization of Good Clinical Practice (GCP) and the Declaration of Helsinki[23,24]. Furthermore, voluntary written informed participants' consent will be obtained from all subjects before starting the study procedures.

It was decided that the pipeline for our investigation comprehends the use of muscle characterization measurements and transcriptomics and proteomics approaches (Figure 1).

PATIENTS AND SAMPLES

Two sample power analyses for a 5% two-tailed test

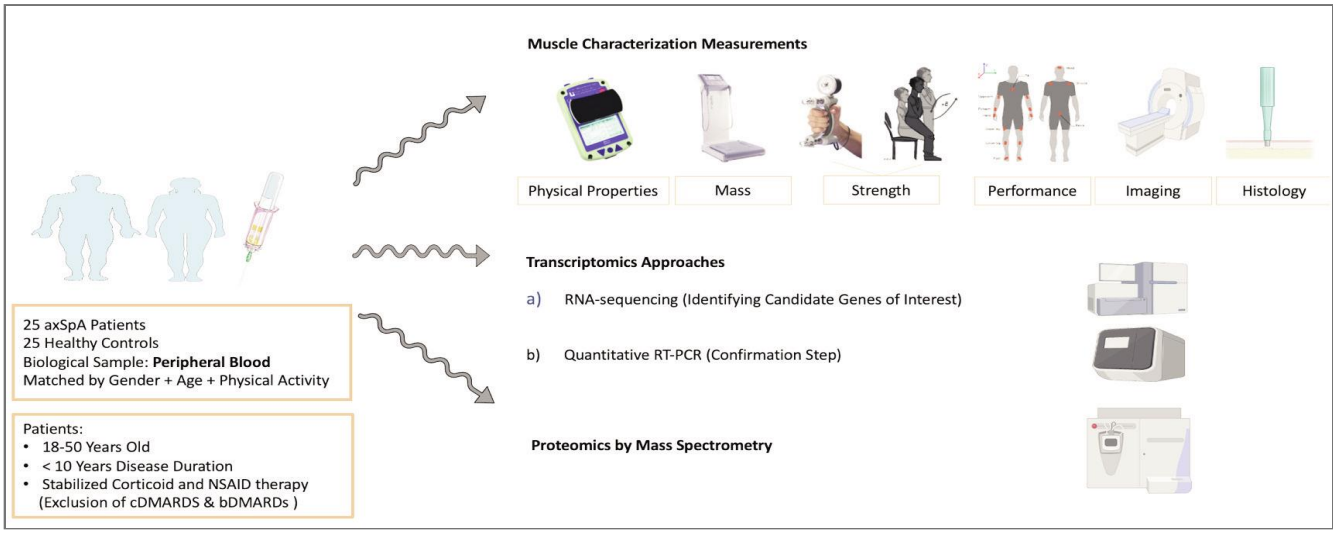

FIGURE 1. Experimental Study Approach; 1. Muscle Characterization Measurements (Physical Properties, Mass, Strength, Physical Performance, Imaging and Histology). 2. Transcriptomics Approach (a) RNA-sequencing, b) Quantitative RT-PCR). 3. Proteomics by Mass Spectrometry.
cDMARD: conventional disease-modifying anti-rheumatic drugs. bDMARD: biologic disease-modifying anti-rheumatic drugs

with 80% power, will be selected based on our preliminary study and considering a 10% drop-out rate (a sample size of 25 subjects per group was calculated to detect differences in gait speed of 0.5 m/s between the two groups). Thus, for this cross-sectional study, 25 young (< 50 years to remove the effect of age) patients with axSpA (according to ASAS classification criteria (2)), with symptoms duration < 10 years and 25 healthy controls matched by age, gender, and level of physical activity will be considered according to the pre-specified inclusion/exclusion criteria:

INCLUSION CRITERIA

- Patients classified with axSpA according to the Assessment of Spondyloarthritis International Society (ASAS) classification criteria[2];
- Age between 18-50 years;
- Symptom duration < 10 years;
- Ability to provide informed consent;
- Corticosteroid therapy allowed (equivalent to ≤10 mg prednisone) and/or nonsteroidal anti-inflammatory drugs (NSAID), in a stable dose within 4 weeks before study enrollment.

EXCLUSION CRITERIA

- History of rheumatic disorder other than SpA;
- BMI≥35kg/m^2;
- Current pregnancy or breastfeeding;
- Any uncontrolled medical condition (e.g., diabetes mellitus, ischemic heart disease);
- Malignancy (except for completely treated squamous or basal cell carcinoma);
- Positive serology for hepatitis B or C, or human immunodeficiency virus;
- Infections requiring hospitalization or intravenous treatment with antibiotics within 30 days or oral treatment within 14 days before enrolment;
- Previous treatment with conventional DMARDs (cDMARDs) or biologic DMARDs (bDMARDs);
- Intra-articular or peri-articular injections within 28 days before screening;
- Ankylosis of the spine (syndesmophytes at all levels from T12 to S1 on the lateral view radiograph).

Eligible patients will be recruited in the Spondyloarthritis Clinic of CHLO, Hospital de Egas Moniz, and the CORPOREA national database[25]. Healthy controls, subjects without any lumbar pain during the last year or previous history of lumbar surgery, will be identified by the patients (e.g., work colleagues).

CLINICAL AND EPIDEMIOLOGICAL CHARACTERIZATION AND BIOLOGICAL SAMPLES COLLECTION

All participants will be characterized through a stan-

dardized questionnaire available in a specific area of the Rheumatic Diseases Portuguese Register (Reuma.Pt)[26]. The Reuma.pt is a web based online system developed by the Portuguese Society of Rheumatology with the aim of prospectively record data from patients with various rheumatic diseases, including axSpA. In the questionnaire, the following variables will be collected from all participants: age, gender, height, weight, handedness, marital status, level of education and level of physical activity, assessed with the International Physical Activity Questionnaire (IPAQ)[27].

For patients with axSpA, the following information will be additionally collected: disease duration (time between the onset of first symptoms and study's enrollment), extra-articular manifestations (enthesitis, dactylitis, uveitis, psoriasis, inflammatory bowel diseases, others), disease activity (assessed by Bath Ankylosing Spondylitis Disease Activity Index (BASDAI)) and functional status (assessed by Bath Ankylosing Spondylitis Functional Index (BASFI)).

All participants will be submitted to a detailed clinical examination to obtain an extensive muscle characterization (see section 2). Additionally, patients with axSpA will be assessed for enthesitis (by Maastricht Ankylosing Spondylitis Enthesitis Score (MASES)) and metrology (using Bath Ankylosing Spondylitis Metrology Index (BASMI)). Blood samples will be collected from all participants to allow biochemical, genetic, transcriptomic, and proteomic studies. In a subgroup of patients and after the appropriate consent signature, an ultrasound-guided needle biopsy of the lumbar muscle (Multifidus) will be performed for histological and molecular characterization. All participants will perform radiographs (cervical and lumbar lateral and anteroposterior pelvic) and an MRI of the whole spine and paravertebral muscles.

MUSCLE CHARACTERIZATION OF STUDY SUBJECTS

To test the hypothesis of greater resting lumbar myofascial stiffness in patients with axSpA, an extensive muscle characterization will be performed for the first time in three body segments: upper limbs, lower limbs and lumbar region. All measurements in each task will be performed by a single trained and experienced investigator. The different features of the muscle to be studied include:

a) Muscle physical properties, in particular, stiffness, tone and elasticity, which will be measured by a non-invasive, hand-held myotonometer, the MyotonPRO®. This device quickly releases a mechanical impulse by applying a constant pressure via a probe to the skin and tissue layers directly above the muscle being measured. Consequently, an impulse is transmitted to the muscle below. The muscle responds to the exterior mechanical impulse with a damped natural oscillation, which is recorded by an accelerometer in the form of an acceleration sign, with subsequent computation and quantification of muscle properties[28]. The muscles to be tested after a 10-minutes rest are the multifidus and longissimus dorsi muscles (assessed at L3-L4 level), the lateral gastrocnemius (assessed at a point 15 cm distal to the knee lateral flexion line, in the bulk of the muscle) with the patient in the prone position and the brachioradialis muscle (assessed at a point, 6 cm distal to the lateral epicondyle of the elbow with the patient in the back position). Measurements of left and right sides will be performed.
b) Muscle Mass, which will be measured by: 1. Bioimpedanciometry, using an octopolar multifrequency bioelectrical impedance analysis device (InBody770®) and 2. In case of the axial region, also MRI of the lumbar spine, through quantification of the cross-sectional area (CSA) of paravertebral muscles.
c) Muscle Strength, which will be measured by: 1. a hand-held dynamometer, the Lafayette Manual Muscle Tester and five-times sit-to-stand (5STS) test, that measures the time a patient takes to stand five times from a sitting position, as quickly as possible, without using his/her arms. Both measures will be used as a proxy of total body strength, as suggested by EWGSOP2[29]. 2. Through a resisted lumbar spine hyperextension (dynamometer placed in the midline over the dorsal area), leg extension (dynamometer placed proximal to the ankle joint) and forearm flexion (dynamometer placed in the middle of anterior forearm), with the participant in a sitting position. These measurements aim to reproduce the anatomical areas evaluated for muscle physical properties as strength evaluation for specific muscles is challenging.
d) Physical Performance will be measured by the "Gait Speed Test"[29]. Gait analysis will be performed by a tri-dimensional full-body kinematic model (Kinetikos technology®), fed by 15 wireless inertial sensors placed in the head, arms, trunk, pelvis, thighs, shanks, and feet to collect several spatiotemporal gait parameters (e.g., gait speed,

stance/swing time, step length, step frequency), to allow participant's movement characterization.

e) Muscle Histology, with the specimens being obtained by an ultrasound-guided biopsy (using a 14G semiautomatic guillotine biopsy needle) at the L4 level. The histological analysis will be performed under the dissecting microscope and the tissue samples will be snap-frozen in liquid nitrogen-cooled isopentane.
   - To allow differential diagnosis between adipose metaplasia and fibrous tissue: cryostat sections will be prepared and stained with haematoxylin-eosin and Gomori's trichrome for better visualization of collagen. In addition, immunohistochemical staining with monoclonal mouse anti-human -smooth muscle actin (-SMA) (clone 1A4; Dako), monoclonal mouse anti-human -actin (clone AC-40; Sigma-Aldrich), and monoclonal mouse anti-human smooth muscle myosin heavy chain (SMMHC) (clone SMMS-1; Dako) for fibers type distinction[5].
   - To analyse infiltration of different cellular types, double-staining will be performed with markers related to inflammation/immunity and mesenchymal stem cells: biotinylated monoclonal antibodies against macrophages (CD68, clone Y1/82A and CD163, clone GHI/61; both from BioLegend), T lymphocytes (CD3, clone UCH-T1; Thermo Scientific Pierce), B lymphocytes (CD120, clone plasma cells (CD138 clone B-A38; Abcam), leukocytes (CD45, clone HI30; BioLegend), pericytes (CD146; clone P1H12; Abcam), fibroblasts/myo- fibroblast (CD90, clone 5E10 [BioLegend], vimentin [D21H3] XP rabbit monoclonal antibody [Cell Signaling Technology], and prolyl 4-hydroxylase ß, rabbit polyclonal [Abcam]) and endothelial cells (von Willebrand factor, rabbit polyclonal; Dako).

f) Muscle imaging: All participants will perform cervical and lumbar lateral and anteroposterior pelvic radiographs to characterize disease severity through the modified Stoke Ankylosing Spondylitis Spinal Score (m-SASSS)[30] and the New York classification system for sacroiliitis[31]. MRI of the whole spine and paravertebral muscles will be performed, focusing on a) the muscle cross-sectional area or volume, b) the percentage of intramuscular fat and c) the muscle water on T2-weighted scans, which quantify muscle trophicity, chronic fatty degenerative changes and oedema (or more broadly, "disease activity"), according to the ASAS/Outcome Measures in Rheumatology (OMERACT) MRI Group[32]. The MRI muscle protocol will follow recommendations by the TREAT-NMD group[33] on a 1.5T MRI. T1w imaging depicts muscle fat, muscle volume, fascia and subcutaneous fat, serving as a qualitative assessment of fat infiltration. The 4-point Dixon technique quantifies fat and water in the muscle[34] and allows to monitor small changes in muscle fat. The qualitative and quantitative assessment of oedema (a surrogate for inflammation) is possible on T2w imaging and on the "only-water" sequence of the 4-point Dixon. T1w images will be graded according to the semiquantitative scale of Mercuri *et al.* [35,36]. Quantitative fat fraction and T2 mapping of the muscle based on the Dixon sequence will be obtained and analysed by regions of interest in muscles. This evaluation will be performed in a selected and experienced Imaging Centre. Disease duration, structural severity and clinical activity will be considered for data analysis; the impact on extensor (multifidus, longissimus dorsi) and flexors (psoas) muscles will be considered.

EXPRESSION DATA COLLECTION

We will screen peripheral blood and muscle biopsy samples to determine gene/protein signatures. Such data will aim to elucidate biological pathways that may explain muscle properties.

GLOBAL TRANSCRIPTOMIC ANALYSIS BY RNA-SEQUENCING

This method allows an unbiased overview of the full mRNA population in biological samples, at the nucleotide level. Raw reads will be aligned to the human genome reference using the STAR aligner, followed by the generation of the table of gene counts with the feature counts software[37,38]. Counts will be processed with the edgeR and limma-Voom packages in the R software to perform normalization, principal component analysis (PCA) and differential gene expression analysis comparing the different groups of subjects, taking into account the information from patients and their matched controls[39,40]. Confirmation of transcriptomic results will be performed by real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and the data will be analysed using CFX Maestro qPCR Analysis Software[41].

PROTEOMIC ANALYSIS BY SWATH MASS SPECTROMETRY

SWATH-MS (Sequential Window Acquisition of all THeoretical Mass Spectra) will be used to perform a

high-throughput differential proteomic analysis to screen differences in serum protein levels between axSpA patients and controls. To assess less abundant proteins, serum will be depleted of the 14 most abundant proteins (equivalent of 94% of serum total protein content), using immunoaffinity kits before analysis[42,43]. Protein samples will be digested and each sample will be separated using reversed-phase nano liquid chromatography (nanoLC). Before the SWATH analyses, IDA (Information-Dependent Analysis) runs will be used to obtain information on protein identity and to generate a spectral library. Protein identification will be performed using the ProteinPilot™ software (v5.0 AB-Sciex) with the Paragon algorithm. For the relative quantification, each sample will be subjected to 3-5 SWATH runs. Peptide and protein identification, spectral alignment and targeted data extraction will be performed using the PeakView v.2.2 software with SWATH™ 2.0 Acquisition MicroApp (Sciex)[44]. Statistical analysis will be performed using MarkerView v1.2 (Sciex), namely PCA and t-test. Data may be queried from a global perspective, focusing on abundance variations between proteomes or from a targeted perspective, focusing on specific proteins/pathway variations (Ingenuity® Pathway Analysis software).

Ingenuity Pathway Analysis (IPA) software, String, and other bioinformatic's tools will be also used to provide a pathway-centric mechanistic view of the data. Abundance variations of interesting protein targets will be further confirmed by Western Blotting and when possible quantified by Enzyme-linked Immunosorbent Assay (ELISA).

INTEGRATION OF TRANSCRIPTOMICS AND PROTEOMICS RESULTS

We will explore bioinformatics tools for integration of transcriptomic and proteomic results, including: 1) multivariate statistical platforms like Perseus (maxquant.net/perseus/) which enables high-dimensional omics data analysis; 2) functional annotation enrichment analysis tools like DAVID (david.ncifcrf.gov/) and/or Panther (pantherdb.org/); 3) pathway analysis using IPA as described above.

DISCUSSION

This study aims at shedding light on the role of muscle in the susceptibility and progression of axSpA. To overcome the lack of information regarding muscle properties, we propose a broad, multidisciplinary and innovative design study that allows an extensive muscle characterization, including physical properties, strength, mass, performance, histological and imaging (x-rays and MRI) features, in different body segments (i.e., trunk, upper and lower limbs), together with the establishment of transcriptomic and proteomic signatures, which will be performed in the same group of participants.

We aim to identify specific muscle characteristics in an axSpA context and to understand their systemic or local expression. Masi *et al.* have documented an increase of axial (lumbar) muscle stiffness in a group of patients with AS[19]. It would be of interest to expand from this observation by analyzing peripheral muscles to consider about any, general or local, inflammatory effect. If the specific muscle characteristics point to a systemic involvement, meaning that changes in axial muscles, where muscle are under the effect of local inflammation, will be also reproduced at peripheral muscles, where this effect is absent, a genetic/molecular subjacent background should be pursued.

Along with increased stiffness, patients with axSpA may present a decrease in global or segmental strength and mass, as well as reduced gait speed. Assuming that these results are confirmed, they can potentially contribute to establish a muscle and gait pattern in order to predict the disease behavior at relatively early stages. In addition, imaging and histopathology will reveal muscle structural changes and inflammatory markers, respectively, clarifying the link between muscle dysfunction and inflammation. This study will allow to understand the relationship between muscle characteristics and different stages of disease progression through the correlations with radiographic changes/new bone formation (mSASS and MRI) and loss of mobility (BASMI). In future studies, a prospective design will allow the confirmation of potential associations and to establish a causality effect.

Furthermore, the selection of young patients (under 50 years old) with short disease duration (without cDMARDs or bDMARDs and only low doses of systemic corticoids allowed to avoid bias in muscle and in peripheral features of the disease) allows speculating about the possible impact of muscle in disease susceptibility. Conceptually, microtrauma induced by daily activities or by the muscle itself should play an important role in entheseal inflammation. Entheses are specialized interfaces where the integration of tendon into bone occurs[20] and they can be subjected to repetitive

biomechanical stress forces applied during the action of normal muscle and other periarticular structures. In axSpA, entheses are known as the initiating sites of musculoskeletal inflammation. In this context, the axial entheses are particularly prone to inflammation as they are subjected to mechanical stress related to posture maintenance[17,19].

Overall, we intend to establish a pattern of muscle properties in axSpA and depict the triggers involved in entheses inflammation. We hypothesize that muscle properties, such as stiffness, may contribute to a continuous endogenous microtrauma and subsequent inflammation, that should be tested in future studies.

Finally, the identification of gene/protein signatures in axSpA may represents a key contribution to explain muscle properties and unravel the subsequent underlying physio-pathologic mechanisms of the disease. It may also provide an opportunity for the development of new diagnostic tools and preventive/therapeutic approaches, with relevance for clinical practice.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The current study was submitted and approved by the ethical committee of University of Lisbon and Centro Hospitalar de Lisboa Ocidental, Hospital de Egas Moniz, EPE (Reference Number: 20170700050). The study will be conducted in accordance with the International Conference on Harmonization Good Clinical Practice (GCP) and the Declaration of Helsinki. Furthermore, voluntary written informed participants' consent will be obtained from all subjects before the start of the study procedures.

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AUTHORS' CONTRIBUTIONS

Study concept and design: F. P, A.S, S.R, A., K.N, P.G.
Direction of the global study coordination: F.P, L.D
Laboratorial research direction: F.P, C.C, C.V.C, S.A, P.G.
Acquisition of clinical data team: F.P, A.N, R.T, S.R, L.D, J.L, J.B.
Performance of laboratorial experiments team: A.S, C.C, T.V, A.G, B.A.
Analysis and interpretation of data team: D.S, A.S, P.G, F.P
Writing of the protocol: A.S, A.N, D.S, F.P.
Critical revision of the protocol for important intellectual content: All authors
All authors had access to the text, commented on the report drafts, and approved the final
submitted version.

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Appendix 1. Paper 2

Muscle dysfunction in axial spondylarthritis: the MyoSpA study

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Abstract

Objective

We aimed to investigate muscle physical properties, strength, mass, physical performance, and the prevalence of sarcopenia in patients with axial spondylarthritis (axSpA) compared to the healthy controls (HC).

Methods

We performed a cross-sectional study on 54 participants: 27 patients with axSpA and 27 HC, matched by age, gender, and level of physical activity. Muscle physical properties (stiffness, tone and elasticity), muscle strength (five-times sit-to-stand [5STS] test), muscle mass, physical performance (measured through gait speed) and sarcopenia were compared between the groups. Linear regression models were conducted allowing adjustment for relevant variables.

Results

Patients with axSpA (mean age 36.5 (SD 7.5) years, 67% males, mean disease duration 6.5 (3.2) years) had no significnt difference in segmental muscle stiffness, tone or elasticity, compared with the HC, despite showing a slight numerically higher lower lumbar (L3-L4) stiffness [*median 246.5 (IQR 230.5–286.5) vs. 232.5 (211.0–293.5), p=0.38*]. *No participants presented sarcopenia. Patients with axSpA, compared to the HC, had lower total strength [B=1.88 (95% CI 0.43;3.33)], as well as lower strength in the upper (B= -17.02 (-27.33;-6.70)] and lower limbs [B= -11.14 (-18.25;-4.04)], independently of muscle physical properties. Patients had also significantly lower gait speed than the HC [B= -0.11 (-0.21;-0.01)], adjusted for muscle mass, strength and muscle physical properties.*

Conclusion

Young axSpA patients with a relatively short disease duration presented similar segmental muscle physical properties as the HC and had no sarcopenia. Patients with axSpA had reduced physical performance and lower strength compared to the HC, despite normal muscle mass, suggesting a possible muscle dysfunction. Gait characteristics may be a potential biomarker of interest in axSpA.

Key words

spondylarthritis, sarcopenia, body composition, muscle strength, physical performance

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Introduction

Axial spondylarthritis (axSpA) is an inflammatory rheumatic disease, characterised primarily by the involvement of the spine and sacroiliac joints and usually presenting as chronic back pain and stiffness (1). As the disease progresses, impaired spinal mobility and physical function may impact activities of daily living (2).

Despite extensive research in the last decade, the precise aetiopathogenesis of axSpA remains unknown, although it is thought to likely result from a complex interplay of genetic and environmental factors (3). The most important known genetic risk factor is the human leukocyte antigen B27 (HLA-B27), which explains approximately 20% of the disease heritability (4). Environmental factors, such as microbiota and biomechanical stress, may also be predisposing contributors to disease susceptibility (3). In particular, the link between biomechanical stress and axSpA has been suggested, as enthesitis is a hallmark of the disease, and entheses are sites of high mechanical stress due to repetitive forces of contracting muscles applied during movement (5). Passive axial myofascial stiffness has been proposed to contribute to chronic mechanical overload and increased stress and microinjury at enthesis sites in the spine (6). Accordingly, it has also been reported that strenuous physical activities may amplify the effects of inflammation on bone formation measured through radiographic progression in patients with radiographic axSpA (r-axSpA) (7). On the other hand, regular exercise, either as an individual home-based exercise or supervised physiotherapy, has been shown to have beneficial effects on pain and physical function of patients with axSpA (1, 8).

Sarcopenia is a generalised disorder of the skeletal muscle associated with an increased risk of falls and fractures, worse quality of life, and increased mortality (9-11). According to its revised definition by the European Working Group on Sarcopenia in Older People (EWGSOP2), sarcopenia is diagnosed when there is primarily low muscle strength associated with low muscle quantity. The additional presence of poor physical performance is used to identify severe sarcopenia (11). Although frequently attributable to ageing, sarcopenia can occur in younger ages due to various causes, including inflammatory processes (12). Pro-inflammatory cytokines, particularly tumour necrosis factor-α (TNF-α), can hypothetically induce anorexia, resting energy expenditure and muscle loss (13). However, data on sarcopenia in axSpA are still scarce.

Therefore, the aims of this study were to investigate muscle physical properties, and also muscle strength, muscle mass, and physical performance (allowing to determine the prevalence of sarcopenia) in patients with axSpA contrasting them to the healthy controls (HC). We hypothesised that patients with axSpA display general changes in muscle (axial and peripheral) physical properties, namely, increased stiffness and tone. Additionally, patients with axSpA may present reduced muscle strength and/or mass and deterioration of physical performance, having criteria for sarcopenia at young ages.

Methods

Study design and population

A cross-sectional study was conducted on 54 participants: 27 patients diagnosed with axSpA according to their rheumatologists and 27 HC, matched by gender, age and level of physical activity. The patients were recruited from a Rheumatology Outpatient Clinic at Hospital de Egas Moniz in Lisbon, Portugal and the HC from the local community (mostly co-workers).

All patients with axSpA were aged between 18 and 50 years, met the Assessment of SpondyloArthritis international Society (ASAS) classification criteria and had a symptom duration of ≤10 years. Exclusion criteria were as follows: Body Mass Index (BMI) ≥35kg/m^2 (above this value, myotonometry measures are not accurate (14); previous exposure to synthetic disease-modifying anti-rheumatic drugs (DMARDs) or biological disease-modifying anti-rheumatic drugs (bDMARDs); current pregnancy or breastfeeding; infections requiring hospitalisation or intravenous antibiotics within 30 days or oral anti-

biotics within 14 days prior to screening; malignancy (except for completely treated squamous or basal cell carcinoma); any uncontrolled non-treated medical condition (*e.g*. diabetes mellitus, ischaemic heart disease); intra or peri-articular extra-axial injections within 28 days prior to screening; spine ankylosis, with syndesmophytes in all levels from the lumbar spine, on lateral spine radiograph.

This study was approved by the Ethics Committees of Centro Hospitalar Lisboa Ocidental (National Registry for Clinical Studies (RNEC), no. 20170700050), and conducted according to the Declaration of Helsinki, and written informed consent was obtained from all participants before study inclusion.

Data collection and measurements

The following information was collected from all participants: age, gender, height, weight, BMI, and level of physical activity, assessed with the International Physical Activity Questionnaire (IPAQ) (15). For patients, disease duration (time since diagnosis, at study enrolment) was also registered. Disease activity and function were assessed by the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Bath Ankylosing Spondylitis Functional Index (BASFI), respectively.

In order to obtain a detailed muscle characterisation, a set of measurements was performed on all participants by a single investigator, according to a standardised protocol.

Muscle physical properties (stiffness, tone, decrement) were measured using a non-invasive, hand-held myotonometer, the MyotonPRO®. The measurement with MyotonPro® consists of applying a constant pre-load by a probe to the skin surface above the muscle being measured. A mechanical impulse is then transmitted to the underlying muscle and the subsequent dampened oscillation of the muscle is recorded in the form of an acceleration signal, followed by computation of parameters of interest. This device has been previously used in other studies, to measure properties of peripheral muscles in patients with Parkinson's disease (16) or subacute stroke (17), as well as axial muscles in patients with r-axSpA (17). In our study, measurements were made in the prone position after a 10-minute resting period, in three different body segments: trunk (low lumbar myofascial tissue), upper limbs (Extensor Digitorium, 5 cm below the lateral epicondyle) and lower limbs (Gastrocnemius, 10 cm below the lateral side of the knee), considering the muscle bulk. For each segment, measurement of left and right sides was performed, and the mean value calculated. Values were recorded according to the dominant and non-dominant sides of the participants' handedness. Total stiffness, tone and decrement were calculated using the sum of the values of each body segment. Decrement is the direct measure given by the myotonometer to characterise elasticity and should be interpreted as to its inverse (the lower the decrement, the higher the elasticity).

Isometric muscle strength of three different body segments (trunk, upper and lower limbs, on both sides) was quantified with a dynamometer, the Lafayette Manual Muscle Test System. In the sitting position, maximal resisted lumbar spine extension (dynamometer placed in the midline over the dorsal area), leg extension (dynamometer placed proximal to the ankle) and flexion (dynamometer placed in the middle of the anterior forearm) were performed. Thus, strength of torso extension, knee extension and elbow flexion was registered. The mean strength of right and left, upper and lower limbs, was calculated and used in the analysis. Five-times sit-to-stand (5STS) test was used as a measure of total strength, as suggested by Bohannon (18). This test measures the time a patient takes to stand from the sitting position five times as quickly as possible, without using his/her arms (12). The longer the duration, the lower the total strength.

Body composition was measured by an octopolar multifrequency bioelectrical impedance analysis device (InBody770®). Total and segmental lean mass, fat mass, and body water were recorded.

Physical performance was measured through gait speed (12), using a 3D full-body kinematic model (Kinetikos®) fed by 15 inertial sensors placed in the head, arms, trunk, pelvis, thighs, shanks, and feet. Low physical performance was defined as gait speed <1.0 m/s for both genders (12).

Low skeletal muscle mass was defined as low strength (evaluated by 5STS >15 seconds) for both genders and low skeletal muscle mass (according to the equipment's inbuilt and personalised reference values) (12).

Statistical analysis

Categorical variables are shown as frequencies and percentages. Continuous variables are presented as means and standard deviations (SD), or medians and interquartile ranges for variables with skewed distributions. Normal distribution was assessed by graphical inspection and additionally using the Kolmogorov-Smirnov test. Chi-square test or Fisher's exact test (for categorical variables), and Student's t-test (for continuous variables) were used to compare differences between patients and controls, as appropriate. A completers analysis was performed without missing data imputation.

Linear regression was used to investigate differences in muscle parameters, namely muscle strength and physical performance, between the patients with axSpA and the HC. To correct for possible confounding effects, two multivariable linear regression models were developed: model 1 was adjusted for muscle mass for all outcomes, and for physical performance additionally adjusted for total strength. Model 2 was adjusted for the same covariates plus the muscle physical properties, namely stiffness, tone, and decrement. Standard assumptions for linear regression were met.

Statistical significance was defined as a *p*-value of less than 0.05. Statistical Package for Social Science (SPSS) version 23 was used.

Results

The participants had a mean age of 36.5 (SD 7.5) years and were predominantly males (67%). The patients with axSpA

had mean disease duration of 6.5 (3.2) years, with BASDAI and BASFI of 2.7 (2.3) and 0.9 (3.1), respectively. Subject characteristics are shown in Table I.

Regarding muscle physical properties, there was no significant difference in muscle stiffness, tone or decrement in any of the three regions between the patients with axSpA and the HC (Table II and Supplementary Tables S1-2). However, patients with axSpA showed a numerically higher trunk muscle stiffness than the HC [246.5 (230.5–286.5) *vs.* 232.5 (211.0–293.5), p=0.38]. This numerical difference was more pronounced in the dominant side [261.0 (232.0–312.0) *vs.* 241.0 (204.3–303.0), p=0.28].

Table III shows the comparison of strength, body composition, physical performance and the proportion of sarcopenia between both groups. No participants fulfilled the definition of sarcopenia, since none of the patients or controls had simultaneously low muscle strength and low muscle mass. Low muscle strength was found in 8.3% (n=2) of patients *vs.* 0% of the HC (p=0.15). Skeletal muscle mass was reduced in other 8.3% (n=2) of patients *vs.* 4.2% (n=1) of the HC (p=0.55).

Nonetheless, although patients with axSpA had significantly lower median total muscle strength, evaluated by 5STS, than the HC [7.0 (5.9–8.9) *vs.* 5.5 (5.0–6.9), p=0.01], these values were still in the normal range in both groups (cut-off of 15 seconds). Regarding the strength of different body segments, evaluated by dynamometry, patients with axSpA, compared to the HC, also had lower median values in the upper limbs [47.6 (40.2–73.2) *vs.* 71.8 (51.9–80.5), p=0.02] and lower limbs [51.0 (38.5–57.1) *vs.* 59.8 (54.6–64.5), p=0.01], but not in trunk.

There were no differences in total or segmental lean mass and body water, between both groups. Total fat mass was higher in the patients than in the HC [19.8 (12.1–29.1) *vs.* 15.7 (10.1–22.2), p=0.04], but no differences were registered in segmental body evaluation.

As a surrogate marker of physical performance, low gait speed was found in 55% of the patients *versus* 22% of the HC (p=0.02). In addition, median gait speed values were lower in patients compared to the HC [0.8 (0.7–0.9) *vs.* 0.9 (0.8–1.0), p=0.02].

In model 1 of multivariable analysis (table 4), *i.e.* without muscle physical properties, patients with axSpA, compared to the HC, had lower total strength, reflected by a higher 5STS (B=2.00, 95% CI 0.59–3.42), as well as lower strength in the upper [B= -14.85, 95% CI -25.05–(-4.66)] and lower limbs [B=-11.83, 95% CI -18.67–(-4.98)], independently of muscle mass. Likewise, patients had significantly lower gait speed than the HC [B= -0.1, 95% CI -0.212–(-0.006)], adjusted for muscle mass and strength. When muscle physical properties (stiffness, tonus and decrement) were added to the model (model 2), the same results were found.

Table I. Demographic and clinical characteristics of patients with axSpA and healthy controls.

	Patients (n=27)	Controls (n=27)	p-value
Age (years)*	37 (7)	36 (8)	0.79
Gender (male), n (%)§	18 (67)	18 (67)	0.99
Body height (cm)	170 (164 – 177)	173 (165 – 178)	0.52
Body weight (kg)	73 (67 – 86)	70 (65 – 80)	0.35
BMI (kg/m^2)	25 (23 – 30)	24 (23 – 26)	0.30
IPAQ (%)§			
Low	29	21	
Moderate	38	42	0.80
High	33	38	
BASDAI*	3 (2)	-	-
BASFI*	1 (3)	-	-
Disease duration* (years)	7 (3)	-	-
HLA-B27 positivity, n (%)§	22 (81.5)	-	-

Values are presented as median (25th –75th percentiles), assessed by Mann-Whitney U-test, except otherwise indicated.
*Mean (SD), assessed by independent t-test, §Chi-square test or Fisher's exact test.
BMI: Body Mass Index. IPAQ: International Physical Activity Questionnaire. BASDAI: Bath Ankylosing Spondylitis Activity Index. BASFI: Bath Ankylosing Spondylitis Functional Index.

Table II. Muscle stiffness (expressed in Nm) in patients with axSpA and control subjects, stratified for body segment.

	Patients (n=27)	Controls (n=27)	p-value
Trunk			
Average	246.5 (230.5–286.5)	232.5 (211.0–293.5)	0.38
Dominant side	261.0 (232.0–312.0)	241.0 (204.3–303.0)	0.28
Non-dominant side	242.0 (219.0–291.0)	232.0 (209.3–288.0)	0.32
Upper limb			
Average	288.0 (266.0–320.0)	292.0 (265.0–307.5)	0.60
Dominant side	282.0 (266.0–334.0)	292.0 (254.8–311.8)	0.80
Non-dominant side	283.0 (267.0–313.0)	290.0 (266.0–313.0)	0.96
Lower Limb			
Average	293.5 (277.0–329.5)	289.0 (265.0–325.0)	0.75
Dominant side	299.0 (257.0–349.0)	298.0 (271.0–325.0)	0.91
Non-dominant side	295.0 (269.0–321.0)	290.0 (263.5–314.3)	0.81
Total	859.5 (774.0-904.5)	847.0 (778.0-884.0)	0.32

Values are presented as median (25th-75th percentile). Mann-Whitney U-test was used in the analysis. "Average" refers to the mean of right and left sides of each segment, while "dominant" and "non-dominant" sides refer to the handedness of individuals.

Discussion

In our study, relatively young patients with axSpA, with mean disease duration of 6.5 years, presented similar segmental muscle stiffness, tone and elasticity as healthy subjects. There was, however, an asymmetry in muscle stiffness between lumbar and appendicular muscles. Although the underlying mechanism for the numerically higher trunk stiffness in axSpA patients (even though the difference does not reach statistical

Table III. Comparison of sarcopenia, muscle strength, body composition and physical performance between patients with axSpA and healthy controls.

	Patients (n=27)	Controls (n=27)	*p*-value
Sarcopenia, n (%) §	0	0	-
Low muscle strength (5-times sit-to-stand >15s), n (%)	2 (8.3%)	0	0.15
Low skeletal muscle mass, n (%)	2 (8.3%)	1 (4.2%)	0.55
Strength			
Trunk (Nm)	56.3 (37.6–67.2)	57.3 (51.2–63.0)	0.67
Upper limb (Nm)	47.6 (40.2–73.2)	71.8 (51.9–80.5)	**0.02**
Lower limb (Nm)	51.0 (38.5–57.1)	59.8 (54.6–64.5)	**0.01**
Total - 5STS (seconds)	7.0 (5.9–8.9)	5.5 (5.0–6.9)	**0.01**
Lean mass (kg)			
Trunk	24.9 (21.9–27.0)	25.3 (20.4–27.6)	0.92
Upper limb	3.1 (2.56–3.5)	3.1 (2.3–3.5)	0.81
Lower limb	8.0 (7.2–9.5)	9.2 (7.5–10.0)	0.15
Total	50.1 (44.5–57.8)	54.1 (43.2–60.2)	0.59
Fat mass (kg)			
Trunk	10.3 (6.3–15.9)	8.1 (5.1–11.1)	0.05
Upper limb	1.3 (0.6–2.2)	0.9 (0.5–1.5)	0.05
LowerlLimb	2.9 (1.9–4.0)	2.5 (1.6–3.4)	0.21
Total	19.8 (12.1–29.1)	15.7 (10.1–22.2)	**0.04**
Body water (L)			
Trunk	19.6 (17.1–21.3)	18.8 (14.4–21.1)	0.84
Upper limb	2.4 (2.0–2.7)	2.3 (1.6–2.7)	0.38
Lower limb	6.5 (5.8–7.4)	6.5 (5.1–7.5)	0.82
Total	39 (34.6–44.9)	42.1 (33.5–46.8)	0.58
Physical performance §§			
Gait speed (m/s)	0.8 (0.7–0.9)	0.9 (0.8–1.0)	**0.02**
Low gait speed, n (%)	12 (54.5%)	5 (21.7%)	**0.02**

Values are median (25^{th}–75^{th} percentiles). Mann-Whitney U-test was used for continuous variables and Fisher's exact test or the chi-square test were used for categorical variables.
§Available for 48 subjects (24 patients and 24 HC).
§§Available for 45 subjects (22 patients and 23 HC).

significnce) is unknown, we hypothesise that it may result from the local effect of inflamation. These data are in line with a previous study conducted by Andonian *et al.,* in which 24 patients with r-axSpA presented higher lumbar myofascial stiffness than 24 age- and sex-matched control subjects (this difference being statistically significant), measured by the same myotonometry device as ours (18). Importantly, these results may also support the hypothesis that abnormalities in biomechanical pathways might be implied in the course of axSpA, as these patients had established disease with a mean disease duration 12.7 years. However, it is difficult to speculate whether these changes are the cause or consequence of the disease. Furthermore, we did not show a higher prevalence of sarcopenia in these relatively young patients according to the revised EWGSOP2 definition. The low scores for BASDAI and BASFI in our patients, whichreflect low disease activity and functional impairment, might explain the absence of sarcopenia. Nonetheless, we examined the three determinants of sarcopenia in detail: muscle strength, muscle mass, and physical performance.

In our study, all patients except 8% (2 out of 27), had values of general muscle strength and muscle mass in the range of the normality, but presented low levels of physical performance, which suggests a possible muscle dysfunction. Although we cannot fully explain this observation, we can hypothesise that a possible genetic determinism may be evoked and should be further investigated in future research.

Despite the normal values for total strength in patients, a deeper analysisa showed a significnt reduction of general and appendicular (but not in the trunk) muscle strength in the patients with axSpA patients compared to the HC. These results also raise questions about the existing reference values for strength and their applicability to our population, for whom they have not been validated. However, previous studies have also reported lower appendicular strength in patients with r-axSpA (19-21), even in the absence of peripheral joint involvement (19, 20). Various potential factors may justify a decrease in muscle strength, including systemic inflammation or fatigue (19). Inactivity or disuse is also associated with loss of strength, but in our study, the patients were matched with the HC also according to the levels of physical exercise to control for this influntial effect.

Reduced appendicular strength has been associated with loss of appendicular lean mass in patients with longstanding r-axSpA (21). A major known determinant of strength loss is indeed the loss of muscle mass (22). However, in our study, the reduced appendicular strength was independent of muscle mass. Since our patients had a mean disease duration of 6.5 years, we can consider that muscle mass loss may still occur in a later phase of the disease. Despite being a different age group, in older people, the strength decline has been proved to be faster than the concomitant loss of muscle mass (22). An intriguing result was the absence of decreased muscle strength in the axial muscles. The distinct physiological role of axial and peripheral muscles, the former being responsible for maintaining posture and the latter for generating strength, may represent a possible explanation to be explored.

Several studies on body composition in axSpA have found inconsistent results that may be explained by differences in the disease duration and levels of physical activity, and also, by discrepancies in the methods used to estimate muscle mass. In agreement with our data, two previous studies did not observe differences in total lean mass or even skeletal muscle mass index, as measured by dual-energy x-ray absorptiometry or bioelectrical impedance, between patients with axSpA (disease duration 6–10 years) and controls (23, 24).

Table IV. Differences in muscle strength and physical performance between patients with axSpA and HC.

	Univariable analysis	Multivariable analysis	
	Regression (95% CI)	Model 1 (without properties) Regression (95% CI)	Model 2 (adjusted properties) Regression (95% CI)
Outcome: Upper limb strength			
AxSpA *vs.* controls	**-14.8 (-25.8; -3.8)**	**-14.9 (-25.1; -4.7)**	**-17.0 (-27.3; -6.7)**
	14.7 (6.4; 23.0)	**14.0 (6.3; 21.7)**	**13.1 (5.4; 20.9)**
Outcome: Lower limb strength			
AxSpA *vs.* controls	**-11.2 (-17.9; -4.5)**	**-11.8 (-18.7; -5.0)**	**-11.1 (-18.3; -4.0)**
Muscle mass of LL	0.9 (-1.5; 3.2)	0.0 (-2.1; 2.2)	0.2 (-2.0; 2.5)
Stiffness of LL	-0.0 (-0.1; 0.0)	-	-0.1 (-0.2; 0.1)
Tonus of LL	-0.6 (-2.4; 1.1)	-	0.3 (-4.1; 4.8)
Decrement of LL	3.3 (-8.0; 14.6)	-	2.4 (-8.6; 13.4)
Outcome: Trunk strength			
AxSpA *vs.* controls	-4.3 (-12.0; 3.5)	-4.2 (-12.2; 3.8)	-6.1 (-14.2; 2.1)
Muscle mass of T	0.9 (-0.2; 1.9)	0.8 (-0.2; 1.8)	0.8 (-0.2; 1.8)
Stiffness of T	-0.0 (-0.1; 0.1)	-	-0.1 (-0.3; 0.0)
Tonus of T	-0.0 (-2.3; 2.2)	-	4.3 (-1.1; 9.6)
Decrement of T	5.0 (-8.3; 18.3)	-	17.3 (-0.5; 35.1)
Outcome: Total strength (5STS)			
AxSpA *vs.* controls	**1.8 (0.5; 3.1)**	**2.0 (0.6; 3.4)**	**1.9 (0.4; 3.3)**
Muscle mass (total)	0.0 (-0.1; 0.0)	0.0 (-0.1; 0.1)	0.0 (-0.1; 0.1)
Total stiffness	0.0 (-0.0; 0.0)	-	0.0 (-0.0; 0.0)
Total tonus	0.1 (-0.2; 0.3)	-	-0.2 (-0.7; 0.3)
Total decrement	0.3 (-0.4; 1.0)	-	0.3 (-0.5; 1.1)
Outcome: Physical performance			
AxSpA *vs.* controls	-0.1 (-0.2; 0.0)	**-0.1 (-0.2; -0.1)**	**-0.1 (-0.2; -0.0)**
Muscle mass (total)	-0.0 (-0.0; 0.0)	-0.0 (-0.0; 0.0)	-0.0 (-0.0; 0.0)
Total strength	-0.0 (-0.0; 0.0)	0.0 (-0.0; 0.0)	0.0 (-0.0; 0.0)
Total stiffness	0.0 (-0.0; 0.00)	-	0.0 (-0.0; 0.0)
Total tonus	-0.0 (-0.0; 0.0)	-	0.0 (-0.0; 0.0)
Total decrement	0.0 (-0.0; 0.1)	-	0.0 (-0.0; 0.0)

Model 1 (without muscle physical properties): adjusted for muscle mass and, in case of physical performance, also total strength.
Model 2 (with muscle physical properties): adjusted for the same covariates as model 1 plus stiffness, tonus, and decrement.
Independent variables (particularly, muscle mass, stiffness, tonus, decrement, and strength) refer to the 54 participants.
In the "axSpA *vs.* controls" variable, HC are the reference group.
p-values<0.05 are shown in bold.

On the other hand, muscle atrophy and/or increased intramuscular fat, evaluated by CT or MRI, have been described in patients with longstanding disease or advanced radiographic changes (24, 25).
cantly higher in patients than in control subjects, suggesting that even if mass loss is not detected, signs of muscle degeneration may already be present. Notably, we underscore the importance of assessing not only muscle quantity, but also muscle quality, a new term that underlines the micro- and macroscopic changes in muscle architecture and composition (12). Imaging techniques and anatomopathological evaluation would be of interest to clarify the physiopathological mechanisms involved.
Regarding physical performance, gait in patients with longstanding r-axSpA has long been referred to as "walking gingerly", as they walk slower and have a shorter stride length than healthy individuals, which can be attributed to the increased rigidity of the spine (13). In our cohort, we showed that young pa-
lower gait speed than the HC, independently of muscle mass, strength or muscle physical properties. In this context, gait characterisation (including speed and other parameters) could be considered a marker with potential interest in axSpA, eventually for diagnosis and, in particular, for disease monitoring.
Limitations of our study include the small sample size and the cross-sectional design that precludes causal inferences. Also due to its small sample size should this study be seen as a pilot study, pioneer in gaining insight into muscle properties in patients with axSpA and characterisation of different body regions, and which should be followed by exclude the possibility of residual confounding, since other variables, such as dietary intake, were not determined and comes. Measurements were performed by one assessor only and future studies should consider at least 2 assessors and some reliability analyses. Furthermore, sarcopenia criteria according to the people. Additionally, the Myoton device is capable of measuring the biomechanical properties of muscles covered by subcutaneous fat up to a depth of 20 mm, and therefore measurements from the deeper muscles may not be as accurate [Myoton website: https://www.myoton.com/technology/]. For this reason, in our study, we have excluded patients . Plus, even though a 10-minute resting period was required before all muscle measurements, no surface electromyography (sEMG) was of the muscle being measured.

Our study also has several strengths, such as the extensive muscle characterisation that includes, for thesfirt time, different body segments (trunk, UL and LL) for each participant. Despite being a small study, it already allowed us to identify important differences between patients with axSpA and HC, which warrants more in-depth research in future studies.

Overall, our study suggests that muscle physical properties were not different between axSpA patients and HC, not only at axial but also at appendicular levels. These results cannot be extrapolated for patients with longstanding disease (*e.g.* superior to 10 years of disease duration). Notwithstanding, a deterioration in physical performance and muscle strength, despite normal values of muscle mass and physical properties, seems to indicate a possible muscle dysfunction. Further robust studies are needed to determine its potential causes, and a genetic aetiology should also be pursued. Thesde fini ngs are of utmost importance, since physical performance is a strong predictor of adverse outcomes, including mortality (27). We also provide evidence for a potential new biomarker related to gait analysis with plausible interest for disease diagnosis and monitoring.

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Appendix 2: Supplemental Material

Table S1. List of patients with axSpA and matched healthy controls used for muscle characterization measurements.

Patient ID	Age	Gender	Healthy Control ID	Age	Gender
1.a.EA	45	M	19.b.s	48	M
5.a.EA	33	M	5.b.s	32	M
6.a.EA	34	M	6.b.s	35	M
7.a.EA	27	M	3.b.s	28	M
8.a.EA	40	M	230.b.s	41	M
9.a.EA	42	M	9.b.s	42	M
11.a.EA	44	F	2.b.s	45	F
12.a.EA	44	M	12.b.s	44	M
13.a.EA	44	M	13.b.s	47	M
14.a.EA	50	F	14.b.a	46	F
15.a.EA	44	M	15.b.s	44	M
16.a.EA	38	F	16.b.s	37	F
17.a.EA	39	M	17.b.s	36	M
18.a.EA	43	F	18.b.s	44	F
19.a.EA	37	M	1.b.s	38	M
20.a.EA	32	F	20.b.s	30	F
21.a.EA	34	F	21.b.s	31	F
22.a.EA	40	M	22.b.s	41	M
23.a.EA	31	M	8.b.s	30	M
24.a.EA	36	M	24.b.s	35	M
25.a.EA	35	M	25.b.s	35	M
26.a.EA	47	F	26.b.s	43	F
27.a.EA	34	F	27.b.s	33	F
28.a.EA	37	M	28.b.s	35	M
29.a.EA	29	F	29.b.s	27	F
30.a.EA	20	M	30.b.s	18	M
31.a.EA	21	M	31.b.s	22	M

Table S2. Differences in muscle strength and physical performance between patients with axSpA and healthy controls.

Predictors	Univariable analysis	Multivariable analysis	
	Regression coefficient (95% CI)	**Model 1** (without muscle physical properties)	**Model 2** (adjusted for muscle physical properties)
		Regression coefficient (95% CI)	**Regression coefficient** (95% CI)
	OUTCOME: UPPER LIMBS STRENGTH		
AxSpA vs controls	**-14.83 (-25.83; -3.84)**	**-14.85 (-25.05; -4.66)**	**-17.02 (-27.33; -6.70)**
Muscle mass of UL	14.71 (6.43; 22.99)	14.00 (6.32; 21.70)	13.13 (5.40; 20.86)
Stiffness of UL	0.07 (-0.08; 0.22)	-	0.07 (-0.11; 0.25)
Tonus of UL	1.89 (-3.11; 6.90)	-	0.07 (-6.02; 6.16)
Decrement of UL	-3.80 (-11.92; 4.33)	-	-6.03 (-12.87; 0.81)
	OUTCOME: LOWER LIMBS STRENGTH		
AxSpA vs controls	**-11.21 (-17.89; -4.54])**	**-11.83 (-18.67; -4.98)**	**-11.14 (-18.25; -4.04)**
Muscle mass of LL	0.87 (-1.45; 3.18)	0.01 (-2.12; 2.15)	0.23 (-2.04; 2.49)
Stiffness of LL	-0.03 (-0.10; 0.04)	-	-0.05 (-0.22; 0.13)
Tonus of LL	-0.64 (-2.36; 1.08)	-	0.33 (-4.11; 4.76)
Decrement of LL	3.31 (-7.98; 14.60)	-	2.44 (-8.56; 13.44)
	OUTCOME: TRUNK STRENGTH		
AxSpA vs controls	-4.26 (-12.04; 3.53)	-4.20 (-12.20; 3.81)	-6.05 (-14.18; 2.10)
Muscle mass of T	0.86 (-0.15; 1.87)	0.83 (-0.18; 1.84)	0.79 (-0.20; 1.79)
Stiffness of T	-0.01 (-0.07; 0.05)	-	-0.13 (-0.29; 0.03)
Tonus of T	-0.04 (-2.25; 2.17)	-	4.25 (-1.12; 9.62)
Decrement of T	4.98 (-8.29; 18.25)	-	17.28 (-0.50; 35.06)
	OUTCOME: TOTAL STRENGTH (5STS)		
AxSpA vs controls	**1.81 (0.53; 3.09)**	**2.00 (0.59; 3.42)**	**1.88 (0.43; 3.33)**
Muscle mass (total)	0.01 (-0.08; 0.08)	0.02 (-0.06; 0.09)	0.02 (-0.06; 0.10)
Total stiffness	0.01 (-0.01; 0.01)	-	0.01 (-0.00; 0.03)
Total tonus	0.05 (-0.16; 0.25)	-	-0.18 (-0.66; 0.30)
Total decrement	0.29 (-0.44; 1.02)	-	0.33 (-0.48; 1.13)
	OUTCOME: PHYSICAL PERFORMANCE		
AxSpA vs controls	-0.07 (-0.16; 0.03)	**-0.11 (-0.20; -0.14)**	**-0.11 (-0.21; -0.01)**
Muscle mass (total)	-0.00 (-0.01; 0.01)	-0.00 (-0.01; 0.01)	-0.00 (-0.01; 0.01)
Total Strength	-0.007 (-0.03; 0.02)	0.00 (-0.02; 0.02)	0.01 (-0.02; 0.03)
Total stiffness	0.00 (-0.01; 0.00)	-	0.00 (-0.01; 0.01)
Total tonus	-0.01 (-0.02; 0.01)	-	0.01 (-0.03; 0.03)
Total decrement	0.01 (-0.04; 0.06)	-	0.01 (-0.04; 0.05)

Model 1 (without muscle physical properties): adjusted for muscle mass and, in case of physical performance, also total strength.
Model 2 (with muscle physical properties): adjusted for the same covariates as model 1 plus stiffness, tonus, and decrement.
Independent variables (particularly, muscle mass, stiffness, tonus, decrement, and strength) refer to the 54 participants.
In the "axSpA *vs.* controls" variable, HC are the reference group.
p-values<0.05 are shown in bold.
UL: upper limbs. LL: lower limbs. T: trunk.

Table S3. Differentially expressed genes between patients with axSpA and matched healthy controls.

Gene ID	Log FC	Ave Expression	*p*-value	adj *p*-value	Gene Name
ENSG00000115053	-0.206	8.256	0.0002238	0.046	*NCL*
ENSG00000183431	-0.224	6.166	0.0000180	0.019	*SF3A3*
ENSG00000102054	-0.227	5.885	0.0000286	0.019	*RBBP7*
ENSG00000168259	-0.241	5.656	0.0000507	0.022	*DNAJC7*
ENSG00000104129	-0.25	3.449	0.0001297	0.034	*DNAJC17*
ENSG00000145220	-0.254	4.584	0.0002138	0.046	*LYAR*
ENSG00000156697	-0.266	4.034	0.0000452	0.021	*UTP14A*
ENSG00000136950	-0.269	4.934	0.0000953	0.031	*ARPC5L*
ENSG00000100220	-0.276	5.249	0.0000055	0.009	*RTCB*
ENSG00000106443	-0.29	4.459	0.0000925	0.031	*PHF14*
ENSG00000173545	-0.297	4.633	0.0001186	0.034	*ZNF622*
ENSG00000117395	-0.317	3.857	0.0000644	0.025	*EBNA1BP2*
ENSG00000100911	-0.325	6.398	0.0000278	0.019	*PSME2*
ENSG00000168291	-0.338	4.829	0.0000121	0.016	*PDHB*
ENSG00000125445	-0.363	4.133	0.0000093	0.013	*MRPS7*
ENSG00000196262	-0.363	6.897	0.0001687	0.04	*PPIA*
ENSG00000204520	-0.379	4.068	0.0000520	0.022	*MICA*
ENSG00000069509	-0.384	2.226	0.0001253	0.034	*FUNDC1*
ENSG00000213551	-0.387	2.319	0.0000286	0.019	*DNAJC9*
ENSG00000134697	-0.392	4.367	0.0001681	0.04	*GNL2*
ENSG00000107949	-0.392	4.034	0.0000701	0.025	*BCCIP*
ENSG00000150456	-0.394	0.652	0.0002413	0.049	*EEF1AKMT1*
ENSG00000198034	-0.411	9.248	0.0000675	0.025	*RPS4X*
ENSG00000153774	-0.428	3.362	0.0000355	0.019	*CFDP1*
ENSG00000115539	-0.43	2.653	0.0000444	0.021	*PDCL3*
ENSG00000139168	-0.434	3.397	0.0000332	0.019	*ZCRB1*
ENSG00000152404	-0.439	4.843	0.0001271	0.034	*CWF19L2*
ENSG00000119421	-0.44	3.392	0.0000026	0.007	*NDUFA8*
ENSG00000114023	-0.444	3.201	0.0001188	0.034	*FAM162A*
ENSG00000143256	-0.463	3.105	0.0000181	0.019	*PFDN2*
ENSG00000171960	-0.463	3.05	0.0000239	0.019	*PPIH*
ENSG00000143222	-0.467	4.942	0.0000047	0.009	*UFC1*
ENSG00000126698	-0.47	5.464	0.0000313	0.019	*DNAJC8*
ENSG00000170627	-0.473	0.621	0.0001591	0.04	*GTSF1*
ENSG00000172115	-0.474	4.455	0.0000870	0.029	*CYCS*

ENSG00000132963	-0.478	4.096	0.0001406	0.036	*POMP*
ENSG00000150316	-0.478	3.84	0.0000304	0.019	*CWC15*
ENSG00000138495	-0.522	1.699	0.0000395	0.02	*COX17*
ENSG00000127922	-0.558	2.36	0.0000337	0.019	*SEM1*
ENSG00000228224	-0.611	0.662	0.0001254	0.034	*NACA4P*
ENSG00000227191	-0.611	4.599	0.0000670	0.025	*TRGC2*
ENSG00000260302	-0.639	0.239	0.0001909	0.044	*AP005482.1*
ENSG00000147400	-0.647	3.078	0.0000297	0.019	*CETN2*
ENSG00000196531	-0.649	8.043	0.0000018	0.006	*NACA*
ENSG00000118181	-0.671	5.913	0.0000681	0.025	*RPS25*
ENSG00000109536	-0.678	3.433	0.0000005	0.003	*FRG1*
ENSG00000102390	-0.686	2.906	0.0000003	0.002	*PBDC1*
ENSG00000235613	-0.709	1.229	0.0000992	0.031	*NSRP1P1*
ENSG00000181163	-0.717	6.498	0.0000201	0.019	*NPM1*
ENSG00000111716	-0.717	3.542	0.0001770	0.041	*LDHB*
ENSG00000168653	-0.748	2.756	0.0002120	0.046	*NDUFS5*
ENSG00000162398	-0.78	-0.33	0.0000602	0.024	*LEXM*
ENSG00000272221	-0.786	2.085	0.0001120	0.033	*AL645933.2*
ENSG00000145592	-0.817	7.271	0.0000031	0.007	*RPL37*
ENSG00000125356	-0.893	2.633	0.0000306	0.019	*NDUFA1*
ENSG00000111639	-0.897	3.499	0.0000019	0.006	*MRPL51*
ENSG00000239559	-0.982	2.013	0.0000051	0.009	*RPL37P2*
ENSG00000165502	-1.236	5.613	0.0000001	0.001	*RPL36AL*
ENSG00000230629	-1.306	-0.04	0.0000464	0.021	*RPS23P8*
ENSG00000115561	0.245	5.859	0.0002418	0.049	*CHMP3*
ENSG00000215769	0.328	2.622	0.0002240	0.047	*ARHGAP27P1-BPTFP1-KPNA2P3*
ENSG00000278918	0.373	3.004	0.0001101	0.033	*AC080112.3*
ENSG00000145428	0.42	3.341	0.0001677	0.04	*RNF175*
ENSG00000134152	0.423	5.346	0.0002106	0.046	*KATNBL1*
ENSG00000155744	0.449	6.74	0.0000794	0.027	*FAM126B*
ENSG00000241886	0.553	1.468	0.0000343	0.019	*AC112496.1*
ENSG00000042832	0.573	0.886	0.0001738	0.041	*TG*
ENSG00000187554	0.582	4.679	0.0001103	0.033	*TLR5*
ENSG00000243055	0.595	1.507	0.0000601	0.024	*GK-AS1*
ENSG00000100505	0.67	0.399	0.0001336	0.035	*TRIM9*
ENSG00000151012	0.706	-0.26	0.0002127	0.046	*SLC7A11*
ENSG00000128918	0.717	-0.453	0.0001031	0.032	*ALDH1A2*

ENSG00000138722	0.726	1.271	0.0000406	0.02	*MMRN1*
ENSG00000184557	0.74	5.464	0.0000518	0.022	*SOCS3*
ENSG00000206341	1.239	7.142	0.0000255	0.019	*HLA-H*
ENSG00000109272	1.438	0.688	0.0000311	0.019	*PF4V1*

Table S4. List of genes and their primer sequences utilized in RT-qPCR experiment.

Ensemble ID	Gene Name	Primer Sequences	PCR Product Length (bp)
ENSG00000111640	*GAPDH*	F: ACAACTTTGGTATCGTGGAAGG R: GCCATCACGCCACAGTTTC	101
ENSG00000184557	*SOCS3*	F: GGGGAGTACCACCTGAGTCT R: CGAAGTGTCCCCTGTTTGGA	128
ENSG00000136950	*ARPC5L*	F: CGTCTTGTCAGTCCCGTGAT R: CACTTAGTACGAGGAGGCCG	98
ENSG00000109536	*FRG1*	F: GGGAAAATGGCTTTGTTGGC R: TTGCTTCTATGTCCCCTGCT	70
ENSG00000196531	*NACA*	F: ATCCCAGCAAGCACAACTAG R: CAGTTGGAGTCTGTGTGTTTTCT	92
ENSG00000275485	*N6AMT2*	F: TGATGAGACACCCCAGCTTT R: CTCGCCTGGCTCAATTTGTT	88
ENSG00000227191	*TRGC2*	F: CAGGAGGGGAACACCATGAA R: GTGACTCTTCTGGCACCGTT	73
ENSG00000196262	*PPIA*	F: GGTATAAAAGGGGCGGGAGG R: AAGAACACGGTGGGGTTGA	100
ENSG00000100393	*EP300*	F: CCTATTGTACCCCGGCAAAC R: GGTTGAGAGCTCCAGGTTGA	70
ENSG00000107742	*SPOCK2*	F: GATTCTCTCTCCCTCTCCGC R: GGCTGAAATGTGACCTGGTT	77
ENSG00000142541	*RPL13A*	F: GCTTACGCTGCACCATCTAC R: CCGCAGACCATCGTGAGATAA	100
ENSG00000166333	*ILK*	F: AACGGTTGGTGGATGAGAGG R: TTACACTGGCTAGCAAAGGC	98
ENSG00000033327	*GAB2*	F: ACAAGAAGGAGCTGCAGGAT R: TCTTCTGTCTCAGCCACCAG	82
ENSG00000124831	*LRRFIP1*	F: TGGTTTCCAATGCTCAGCTAG R: AGCCAGCTGTTCTTCAAGC	98
ENSG00000188342	*GTF2F2*	F: GTGGAAAACCAGCTTCAGTCA R: GTCTGTCCTCCAACACTTTGC	70
ENSG00000114503	*NCBP2*	F: CATAAATGGGACGCGTCTGG R: CGTATTGCCTGCCCTCCTTA	77

Table S5. Relative normalized genes expression of patients with axSpA and healthy controls by RT-qPCR.

Target Gene	Biological Group	Expression Level	Expression SD	Mean Cq	Cq SD	*p*-value	Biological Function
SOCS3	Healthy Controls	1.00	0.10	25.75	0.10	N/A	Suppressor Of Cytokine Signalling 3 that inhibits the activity of JAK2 kinase.
SOCS3	Patients	1.64	0.17	24.88	0.13	0.010	
FRG1	Healthy Controls	1.00	0.09	22.1	0.08	N/A	May play a role in regulation of pre-mRNA splicing or in the assembly of rRNA into ribosomal subunits.
FRG1	Patients	0.72	0.07	22.4	0.11	0.574	
ARPC5L	Healthy Controls	1.00	0.13	28.68	0.10	N/A	Component of the Arp2/3 complex which is involved in regulation of actin polymerization.
ARPC5L	Patients	0.97	0.11	28.56	0.13	0.477	
NACA	Healthy Controls	1.00	0.09	20.98	0.08	N/A	Its muscle-specific isoform contributes in myofibril organization.
NACA	Patients	0.51	0.06	21.8	0.14	0.003	
ILK	Healthy Controls	1.00	0.12	27.11	0.06	N/A	Regulates integrin-mediated signal transduction.
ILK	Patients	0.90	0.11	27.09	0.14	0.151	
RPL13A	Healthy Controls	1.00	0.12	27.53	0.05	N/A	Encodes a member of the L13P family of ribosomal proteins.
RPL13A	Patients	0.91	0.10	27.5	0.11	0.101	
N6AMT2	Healthy Controls	1.00	0.09	27.87	0.09	N/A	Encodes proteins are expected to have molecular functions (methyltransferase activity, nucleic acid binding) and to localize in cytoplasm.
N6AMT2	Patients	0.72	0.06	28.19	0.10	0.048	
TRGC2	Healthy Controls	1.00	0.10	23.75	0.09	N/A	Participates in the antigen recognition.
TRGC2	Patients	0.63	0.05	24.26	0.09	0.110	
PPIA	Healthy Controls	1.00	0.10	28.77	0.11	N/A	Catalyses the cis-trans isomerization of proline imidic peptide bonds in oligopeptides.
PPIA	Patients	0.72	0.06	29.08	0.10	0.012	
EP300	Healthy Controls	1.00	0.09	23.75	0.07	N/A	It functions as histone acetyltransferase that regulates transcription via chromatin remodelling and is important in the processes of cell proliferation and differentiation.
EP300	Patients	0.98	0.07	23.61	0.07	0.913	
SPOCK2	Healthy Controls	1.00	0.09	23.85	0.09	N/A	Encodes a protein which binds with glycosaminoglycans to form part of the extracellular matrix.
SPOCK2	Patients	1.02	0.07	23.66	0.07	0.851	
GAB2	Healthy Controls	1.00	0.08	23.47	0.05	N/A	Acts as adapters for transmitting various signals in response to stimuli through cytokine and growth factor receptors, and T- and B-cell antigen receptors.
GAB2	Patients	1.14	0.09	23.12	0.09	0.108	
LRRFIP1	Healthy Controls	1.00	0.09	21.85	0.07	N/A	Transcriptional repressor which preferentially binds to the GC-rich consensus sequence and may regulate expression of TNF, EGFR and PDGFA
LRRFIP1	Patients	0.91	0.06	21.81	0.06	0.935	
GTF2F2	Healthy Controls	1.00	0.09	26.11	0.09	N/A	transcription initiation factor that binds to RNA polymerase II and helps to recruit it to the initiation complex in collaboration with TFIIB.
GTF2F2	Patients	0.79	0.06	26.28	0.08	0.133	
NCBP2	Healthy Controls	1.00	0.10	26.23	0.10	N/A	The product of this gene is a component of the nuclear cap-binding protein complex, which binds to the monomethylated 5' cap of nascent pre-mRNA in the nucleoplasm.
NCBP2	Patients	0.82	0.07	26.35	0.10	0.205	

Expression SD: Expression Standard Deviation
Mean Cq: Mean for Quantitation Cycle (Cq for short). Lower Cq values mean higher initial copy numbers of the target.
Cq SD: Quantitation Cycle Standard Deviation
p-value indicates if gene expression in patients with axSpA is significantly different from healthy controls.

Table S6. Differentially regulated proteins between patients with axSpA and matched healthy controls.

Peak Name	Group	*p*-value	Log (Fold Change)	Protein
sp|P02741|CRP_HUMAN	C-reactive protein OS=Homo sapiens OX=9606 GN=*CRP* PE=1 SV=1	2.800E-07	0.7515	CRP
sp|P03951|FA11_HUMAN	Coagulation factor XI OS=Homo sapiens OX=9606 GN=*F11* PE=1 SV=1	5.680E-14	0.7434	FA11
sp|P0DOY3|IGLC3_HUMAN	Immunoglobulin lambda constant 3 OS=Homo sapiens OX=9606 GN=*IGLC3* PE=1 SV=1	4.180E-03	0.7166	IGLC3
sp|P02768|ALBU_HUMAN	Serum albumin OS=Homo sapiens OX=9606 GN=*ALB* PE=1 SV=2	4.479E-02	0.6064	ALBU
sp|Q99832|TCPH_HUMAN	T-complex protein 1 subunit eta OS=Homo sapiens OX=9606 GN=*CCT7* PE=1 SV=2	1.800E-04	0.5212	TCPH
sp|P10809|CH60_HUMAN	60 kDa heat shock protein, mitochondrial OS=Homo sapiens OX=9606 GN=*HSPD1* PE=1 SV=2	2.610E-03	0.5005	CH60
sp|P55060|XPO2_HUMAN	Exportin-2 OS=Homo sapiens OX=9606 GN=*CSE1L* PE=1 SV=3	1.410E-06	0.4989	XPO2
sp|Q9Y6R4|M3K4_HUMAN	Mitogen-activated protein kinase kinase kinase 4 OS=Homo sapiens OX=9606 GN=*MAP3K4* PE=1 SV=2	8.060E-08	0.4454	M3K4
sp|P13639|EF2_HUMAN	Elongation factor 2 OS=Homo sapiens OX=9606 GN=*EEF2* PE=1 SV=4	2.090E-03	0.4415	EF2
sp|Q96SN8|CK5P2_HUMAN	CDK5 regulatory subunit-associated protein 2 OS=Homo sapiens OX=9606 GN=*CDK5RAP2* PE=1 SV=5	4.200E-04	0.4178	CK5P2
sp|P26599|PTBP1_HUMAN	Polypyrimidine tract-binding protein 1 OS=Homo sapiens OX=9606 GN=*PTBP1* PE=1 SV=1	8.580E-03	0.3692	PTBP1
sp|P0C0L4|CO4A_HUMAN	Complement C4-A OS=Homo sapiens OX=9606 GN=*C4A* PE=1 SV=2	7.370E-11	0.3606	CO4A
sp|P68104|EF1A1_HUMAN	Elongation factor 1-alpha 1 OS=Homo sapiens OX=9606 GN=*EEF1A1* PE=1 SV=1	9.008E-02	0.3421	EF1A1
sp|P04278|SHBG_HUMAN	Sex hormone-binding globulin OS=Homo sapiens OX=9606 GN=*SHBG* PE=1 SV=2	1.430E-03	0.2877	SHBG
sp|P02748|CO9_HUMAN	Complement component C9 OS=Homo sapiens OX=9606 GN=*C9* PE=1 SV=2	1.030E-12	0.2720	CO9
sp|Q9NPH3|IL1AP_HUMAN	Interleukin-1 receptor accessory protein OS=Homo sapiens OX=9606 GN=*IL1RAP* PE=1 SV=2	2.440E-03	0.2576	IL1AP
sp|P06733|ENOA_HUMAN	Alpha-enolase OS=Homo sapiens OX=9606 GN=*ENO1* PE=1 SV=2	2.734E-01	0.2576	ENOA
sp|Q8TDX9|PK1L1_HUMAN	Polycystic kidney disease protein 1-like 1 OS=Homo sapiens OX=9606 GN=*PKD1L1* PE=1 SV=1	2.680E-01	0.2540	PK1L1
sp|P00734|THRB_HUMAN	Prothrombin OS=Homo sapiens OX=9606 GN=*F2* PE=1 SV=2	1.250E-05	0.2516	THRB
sp|P26927|HGFL_HUMAN	Hepatocyte growth factor-like protein OS=Homo sapiens OX=9606 GN=*MST1* PE=1 SV=2	7.840E-07	0.2352	HGFL
sp|Q03591|FHR1_HUMAN	Complement factor H-related protein 1 OS=Homo sapiens OX=9606 GN=*CFHR1* PE=1 SV=2	9.630E-07	0.2105	FHR1
sp|P15169|CBPN_HUMAN	Carboxypeptidase N catalytic chain OS=Homo sapiens OX=9606 GN=*CPN1* PE=1 SV=1	2.700E-06	0.1905	CBPN
sp|P01859|IGHG2_HUMAN	Immunoglobulin heavy constant gamma 2 OS=Homo sapiens OX=9606 GN=*IGHG2* PE=1 SV=2	2.076E-01	0.1837	IGHG2
sp|P02774|VTDB_HUMAN	Vitamin D-binding protein OS=Homo sapiens OX=9606 GN=*GC* PE=1 SV=2	1.300E-04	0.1761	VTDB
sp|Q92954|PRG4_HUMAN	Proteoglycan 4 OS=Homo sapiens OX=9606 GN=*PRG4* PE=1 SV=3	1.517E-02	0.1671	PRG4
sp|P18428|LBP_HUMAN	Lipopolysaccharide-binding protein OS=Homo sapiens OX=9606 GN=*LBP* PE=1 SV=3	1.200E-04	0.1668	LBP
sp|P02765|FETUA_HUMAN	Alpha-2-HS-glycoprotein OS=Homo sapiens OX=9606 GN=*AHSG* PE=1 SV=2	2.900E-07	0.1664	FETUA
sp|P01042|KNG1_HUMAN	Kininogen-1 OS=Homo sapiens OX=9606 GN=*KNG1* PE=1 SV=2	9.250E-07	0.1643	KNG1
sp|P19652|A1AG2_HUMAN	Alpha-1-acid glycoprotein 2 OS=Homo sapiens OX=9606 GN=*ORM2* PE=1 SV=2	2.081E-01	0.1512	A1AG2

sp|P35542|SAA4_HUMAN	Serum amyloid A-4 protein OS=Homo sapiens OX=9606 GN=SAA4 PE=1 SV=2	1.200E-02	0.1433	SAA4
sp|P0C0L5|CO4B_HUMAN	Complement C4-B OS=Homo sapiens OX=9606 GN=*C4B* PE=1 SV=2	7.400E-04	0.1402	CO4B
sp|P09172|DOPO_HUMAN	Dopamine beta-hydroxylase OS=Homo sapiens OX=9606 GN=*DBH* PE=1 SV=3	6.440E-03	0.1283	DOPO
sp|Q9NZP8|C1RL_HUMAN	Complement C1r subcomponent-like protein OS=Homo sapiens OX=9606 GN=*C1RL* PE=1 SV=2	3.900E-04	0.1247	C1RL
sp|P36980|FHR2_HUMAN	Complement factor H-related protein 2 OS=Homo sapiens OX=9606 GN=*CFHR2* PE=1 SV=1	2.870E-03	0.1187	FHR2
sp|Q9NQ79|CRAC1_HUMAN	Cartilage acidic protein 1 OS=Homo sapiens OX=9606 GN=*CRTAC1* PE=1 SV=2	1.612E-02	0.1182	CRAC1
sp|P02750|A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein OS=Homo sapiens OX=9606 GN=*LRG1* PE=1 SV=2	1.100E-04	0.1182	A2GL
sp|P02652|APOA2_HUMAN	Apolipoprotein A-II OS=Homo sapiens OX=9606 GN=*APOA2* PE=1 SV=1	1.539E-01	0.1145	APOA2
sp|P01011|AACT_HUMAN	Alpha-1-antichymotrypsin OS=Homo sapiens OX=9606 GN=*SERPINA3* PE=1 SV=2	1.503E-02	0.1100	AACT
sp|P05160|F13B_HUMAN	Coagulation factor XIII B chain OS=Homo sapiens OX=9606 GN=*F13B* PE=1 SV=3	1.800E-04	0.1094	F13B
sp|P27918|PROP_HUMAN	Properdin OS=Homo sapiens OX=9606 GN=*CFP* PE=1 SV=2	6.910E-03	0.1075	PROP
sp|P05543|THBG_HUMAN	Thyroxine-binding globulin OS=Homo sapiens OX=9606 GN=*SERPINA7* PE=1 SV=2	6.500E-04	0.0996	THBG
sp|Q8WZ42|TITIN_HUMAN	Titin OS=Homo sapiens OX=9606 GN=*TTN* PE=1 SV=4	4.520E-01	0.0978	TITIN
sp|O95497|VNN1_HUMAN	Pantetheinase OS=Homo sapiens OX=9606 GN=*VNN1* PE=1 SV=2	3.352E-01	0.0960	VNN1
sp|P04070|PROC_HUMAN	Vitamin K-dependent protein C OS=Homo sapiens OX=9606 GN=*PROC* PE=1 SV=1	3.100E-04	0.0957	PROC
sp|P00751|CFAB_HUMAN	Complement factor B OS=Homo sapiens OX=9606 GN=*CFB* PE=1 SV=2	2.910E-03	0.0954	CFAB
sp|P00736|C1R_HUMAN	Complement C1r subcomponent OS=Homo sapiens OX=9606 GN=*C1R* PE=1 SV=2	3.300E-05	0.0905	C1R
sp|P13671|CO6_HUMAN	Complement component C6 OS=Homo sapiens OX=9606 GN=*C6* PE=1 SV=3	6.100E-04	0.0901	CO6
sp|Q15848|ADIPO_HUMAN	Adiponectin OS=Homo sapiens OX=9606 GN=*ADIPOQ* PE=1 SV=1	2.105E-01	0.0887	ADIPO
sp|Q9UGM5|FETUB_HUMAN	Fetuin-B OS=Homo sapiens OX=9606 GN=*FETUB* PE=1 SV=2	2.024E-02	0.0861	FETUB
sp|P25311|ZA2G_HUMAN	Zinc-alpha-2-glycoprotein OS=Homo sapiens OX=9606 GN=*AZGP1* PE=1 SV=2	2.944E-02	0.0832	ZA2G
sp|P02749|APOH_HUMAN	Beta-2-glycoprotein 1 OS=Homo sapiens OX=9606 GN=*APOH* PE=1 SV=3	5.230E-03	0.0816	APOH
sp|P0DOX5|IGG1_HUMAN	Immunoglobulin gamma-1 heavy chain OS=Homo sapiens OX=9606 PE=1 SV=2	4.045E-01	0.0791	IGG1
sp|P08603|CFAH_HUMAN	Complement factor H OS=Homo sapiens OX=9606 GN=*CFH* PE=1 SV=4	1.199E-02	0.0727	CFAH
sp|P00450|CERU_HUMAN	Ceruloplasmin OS=Homo sapiens OX=9606 GN=*CP* PE=1 SV=1	1.396E-02	0.0698	CERU
sp|P02746|C1QB_HUMAN	Complement C1q subcomponent subunit B OS=Homo sapiens OX=9606 GN=*C1QB* PE=1 SV=3	7.810E-03	0.0691	C1QB
sp|P05156|CFAI_HUMAN	Complement factor I OS=Homo sapiens OX=9606 GN=*CFI* PE=1 SV=2	3.264E-02	0.0623	CFAI
sp|P36955|PEDF_HUMAN	Pigment epithelium-derived factor OS=Homo sapiens OX=9606 GN=*SERPINF1* PE=1 SV=4	8.348E-02	0.0613	PEDF
sp|P09871|C1S_HUMAN	Complement C1s subcomponent OS=Homo sapiens OX=9606 GN=*C1S* PE=1 SV=1	7.100E-04	0.0596	C1S
sp|P08571|CD14_HUMAN	Monocyte differentiation antigen CD14 OS=Homo sapiens OX=9606 GN=*CD14* PE=1 SV=2	1.019E-02	0.0594	CD14
sp|P10643|CO7_HUMAN	Complement component C7 OS=Homo sapiens OX=9606 GN=*C7* PE=1 SV=2	3.743E-02	0.0550	CO7
sp|P48740|MASP1_HUMAN	Mannan-binding lectin serine protease 1 OS=Homo sapiens OX=9606 GN=*MASP1* PE=1 SV=3	7.025E-02	0.0520	MASP1

sp|P01860|IGHG3_HUMAN	Immunoglobulin heavy constant gamma 3 OS=Homo sapiens OX=9606 GN=*IGHG3* PE=1 SV=2	6.823E-01	0.0511	IGHG3
sp|P02790|HEMO_HUMAN	Hemopexin OS=Homo sapiens OX=9606 GN=*HPX* PE=1 SV=2	2.841E-02	0.0486	HEMO
sp|P05090|APOD_HUMAN	Apolipoprotein D OS=Homo sapiens OX=9606 GN=*APOD* PE=1 SV=1	5.346E-01	0.0475	APOD
sp|Q08380|LG3BP_HUMAN	Galectin-3-binding protein OS=Homo sapiens OX=9606 GN=*LGALS3BP* PE=1 SV=1	7.215E-01	0.0468	LG3BP
sp|P02743|SAMP_HUMAN	Serum amyloid P-component OS=Homo sapiens OX=9606 GN=*APCS* PE=1 SV=2	4.172E-01	0.0407	SAMP
sp|P0DP01|HV108_HUMAN	Immunoglobulin heavy variable 1-8 OS=Homo sapiens OX=9606 GN=*IGHV1-8* PE=3 SV=1	6.992E-01	0.0386	HV108
sp|P07358|CO8B_HUMAN	Complement component C8 beta chain OS=Homo sapiens OX=9606 GN=*C8B* PE=1 SV=3	1.201E-01	0.0379	CO8B
sp|P04004|VTNC_HUMAN	Vitronectin OS=Homo sapiens OX=9606 GN=*VTN* PE=1 SV=1	1.486E-01	0.0358	VTNC
sp|P0DOX3|IGD_HUMAN	Immunoglobulin delta heavy chain OS=Homo sapiens OX=9606 PE=1 SV=1	7.441E-01	0.0349	IGD
sp|P04217|A1BG_HUMAN	Alpha-1B-glycoprotein OS=Homo sapiens OX=9606 GN=*A1BG* PE=1 SV=4	8.249E-02	0.0347	A1BG
sp|Q9UK55|ZPI_HUMAN	Protein Z-dependent protease inhibitor OS=Homo sapiens OX=9606 GN=*SERPINA10* PE=1 SV=1	1.387E-01	0.0324	ZPI
sp|P05546|HEP2_HUMAN	Heparin cofactor 2 OS=Homo sapiens OX=9606 GN=*SERPIND1* PE=1 SV=3	2.303E-01	0.0321	HEP2
sp|Q6EMK4|VASN_HUMAN	Vasorin OS=Homo sapiens OX=9606 GN=*VASN* PE=1 SV=1	3.044E-01	0.0319	VASN
sp|P08519|APOA_HUMAN	Apolipoprotein(a) OS=Homo sapiens OX=9606 GN=LPA PE=1 SV=1	7.493E-01	0.0291	APOA
sp|P10909|CLUS_HUMAN	Clusterin OS=Homo sapiens OX=9606 GN=*CLU* PE=1 SV=1	2.255E-01	0.0238	CLUS
sp|P14151|LYAM1_HUMAN	L-selectin OS=Homo sapiens OX=9606 GN=*SELL* PE=1 SV=2	7.644E-01	0.0195	LYAM1
sp|P01024|CO3_HUMAN	Complement C3 OS=Homo sapiens OX=9606 GN=*C3* PE=1 SV=2	7.728E-01	0.0172	CO3
sp|O00391|QSOX1_HUMAN	Sulfhydryl oxidase 1 OS=Homo sapiens OX=9606 GN=*QSOX1* PE=1 SV=3	5.482E-01	0.0160	QSOX1
sp|P23142|FBLN1_HUMAN	Fibulin-1 OS=Homo sapiens OX=9606 GN=*FBLN1* PE=1 SV=4	5.253E-01	0.0158	FBLN1
sp|P0DOX7|IGK_HUMAN	Immunoglobulin kappa light chain OS=Homo sapiens OX=9606 PE=1 SV=1	8.926E-01	0.0125	IGK
sp|Q06033|ITIH3_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H3 OS=Homo sapiens OX=9606 GN=*ITIH3* PE=1 SV=2	7.539E-01	0.0111	ITIH3
sp|P00747|PLMN_HUMAN	Plasminogen OS=Homo sapiens OX=9606 GN=*PLG* PE=1 SV=2	7.817E-01	0.0082	PLMN
sp|P04196|HRG_HUMAN	Histidine-rich glycoprotein OS=Homo sapiens OX=9606 GN=*HRG* PE=1 SV=1	7.986E-01	0.0081	HRG
sp|P00740|FA9_HUMAN	Coagulation factor IX OS=Homo sapiens OX=9606 GN=*F9* PE=1 SV=2	7.391E-01	0.0078	FA9
sp|P63261|ACTG_HUMAN	Actin, cytoplasmic 2 OS=Homo sapiens OX=9606 GN=*ACTG1* PE=1 SV=1	8.531E-01	0.0063	ACTG
sp|P06276|CHLE_HUMAN	Cholinesterase OS=Homo sapiens OX=9606 GN=*BCHE* PE=1 SV=1	8.468E-01	0.0052	CHLE
sp|P06681|CO2_HUMAN	Complement C2 OS=Homo sapiens OX=9606 GN=*C2* PE=1 SV=2	9.274E-01	0.0033	CO2
sp|P04003|C4BPA_HUMAN	C4b-binding protein alpha chain OS=Homo sapiens OX=9606 GN=*C4BPA* PE=1 SV=2	9.737E-01	0.0014	C4BPA
sp|P19827|ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1 OS=Homo sapiens OX=9606 GN=*ITIH1* PE=1 SV=3	9.914E-01	-0.0003	ITIH1
sp|P0DOX2|IGA2_HUMAN	Immunoglobulin alpha-2 heavy chain OS=Homo sapiens OX=9606 PE=1 SV=2	9.866E-01	-0.0014	IGA2
sp|P05452|TETN_HUMAN	Tetranectin OS=Homo sapiens OX=9606 GN=*CLEC3B* PE=1 SV=3	9.365E-01	-0.0023	TETN
sp|P22105|TENX_HUMAN	Tenascin-X OS=Homo sapiens OX=9606 GN=*TNXB* PE=1 SV=5	9.402E-01	-0.0025	TENX

sp|P03952|KLKB1_HUMAN	Plasma kallikrein OS=Homo sapiens OX=9606 GN=*KLKB1* PE=1 SV=1	9.100E-01	-0.0031	KLKB1
sp|P80108|PHLD_HUMAN	Phosphatidylinositol-glycan-specific phospholipase D OS=Homo sapiens OX=9606 GN=*GPLD1* PE=1 SV=3	8.816E-01	-0.0064	PHLD
sp|P02763|A1AG1_HUMAN	Alpha-1-acid glycoprotein 1 OS=Homo sapiens OX=9606 GN=*ORM1* PE=1 SV=1	9.446E-01	-0.0064	A1AG1
sp|P01876|IGHA1_HUMAN	Immunoglobulin heavy constant alpha 1 OS=Homo sapiens OX=9606 GN=*IGHA1* PE=1 SV=2	9.427E-01	-0.0095	IGHA1
sp|Q04756|HGFA_HUMAN	Hepatocyte growth factor activator OS=Homo sapiens OX=9606 GN=*HGFAC* PE=1 SV=1	7.017E-01	-0.0102	HGFA
sp|P43251|BTD_HUMAN	Biotinidase OS=Homo sapiens OX=9606 GN=*BTD* PE=1 SV=2	5.852E-01	-0.0146	BTD
sp|Q16610|ECM1_HUMAN	Extracellular matrix protein 1 OS=Homo sapiens OX=9606 GN=*ECM1* PE=1 SV=2	7.267E-01	-0.0154	ECM1
sp|Q15582|BGH3_HUMAN	Transforming growth factor-beta-induced protein ig-h3 OS=Homo sapiens OX=9606 GN=*TGFBI* PE=1 SV=1	5.807E-01	-0.0159	BGH3
sp|P06396|GELS_HUMAN	Gelsolin OS=Homo sapiens OX=9606 GN=*GSN* PE=1 SV=1	4.241E-01	-0.0197	GELS
sp|O43866|CD5L_HUMAN	CD5 antigen-like OS=Homo sapiens OX=9606 GN=*CD5L* PE=1 SV=1	5.919E-01	-0.0206	CD5L
sp|P08697|A2AP_HUMAN	Alpha-2-antiplasmin OS=Homo sapiens OX=9606 GN=*SERPINF2* PE=1 SV=3	3.645E-01	-0.0211	A2AP
sp|P07360|CO8G_HUMAN	Complement component C8 gamma chain OS=Homo sapiens OX=9606 GN=*C8G* PE=1 SV=3	4.789E-01	-0.0226	CO8G
sp|P00742|FA10_HUMAN	Coagulation factor X OS=Homo sapiens OX=9606 GN=*F10* PE=1 SV=2	1.987E-01	-0.0265	FA10
sp|P02760|AMBP_HUMAN	Protein AMBP OS=Homo sapiens OX=9606 GN=*AMBP* PE=1 SV=1	1.332E-01	-0.0311	AMBP
sp|P01031|CO5_HUMAN	Complement C5 OS=Homo sapiens OX=9606 GN=*C5* PE=1 SV=4	5.859E-01	-0.0316	CO5
sp|Q96IY4|CBPB2_HUMAN	Carboxypeptidase B2 OS=Homo sapiens OX=9606 GN=*CPB2* PE=1 SV=2	1.225E-01	-0.0350	CBPB2
sp|Q14624|ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4 OS=Homo sapiens OX=9606 GN=*ITIH4* PE=1 SV=4	6.461E-02	-0.0352	ITIH4
sp|P02775|CXCL7_HUMAN	Platelet basic protein OS=Homo sapiens OX=9606 GN=*PPBP* PE=1 SV=3	7.307E-01	-0.0384	CXCL7
sp|P00915|CAH1_HUMAN	Carbonic anhydrase 1 OS=Homo sapiens OX=9606 GN=*CA1* PE=1 SV=2	7.082E-01	-0.0386	CAH1
sp|B9A064|IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5 OS=Homo sapiens OX=9606 GN=*IGLL5* PE=2 SV=2	7.353E-01	-0.0387	IGLL5
sp|P49747|COMP_HUMAN	Cartilage oligomeric matrix protein OS=Homo sapiens OX=9606 GN=*COMP* PE=1 SV=2	3.455E-01	-0.0388	COMP
sp|P49908|SEPP1_HUMAN	Selenoprotein P OS=Homo sapiens OX=9606 GN=*SELENOP* PE=1 SV=3	4.819E-01	-0.0395	SEPP1
sp|P22792|CPN2_HUMAN	Carboxypeptidase N subunit 2 OS=Homo sapiens OX=9606 GN=*CPN2* PE=1 SV=3	2.382E-02	-0.0429	CPN2
sp|O00533|NCHL1_HUMAN	Neural cell adhesion molecule L1-like protein OS=Homo sapiens OX=9606 GN=*CHL1* PE=1 SV=4	1.162E-01	-0.0460	NCHL1
sp|Q00839|HNRPU_HUMAN	Heterogeneous nuclear ribonucleoprotein U OS=Homo sapiens OX=9606 GN=*HNRNPU* PE=1 SV=6	9.077E-01	-0.0469	HNRPU
sp|O75636|FCN3_HUMAN	Ficolin-3 OS=Homo sapiens OX=9606 GN=*FCN3* PE=1 SV=2	7.877E-02	-0.0482	FCN3
sp|P01619|KV320_HUMAN	Immunoglobulin kappa variable 3-20 OS=Homo sapiens OX=9606 GN=*IGKV3-20* PE=1 SV=2	5.325E-01	-0.0496	KV320
sp|P01008|ANT3_HUMAN	Antithrombin-III OS=Homo sapiens OX=9606 GN=*SERPINC1* PE=1 SV=1	8.353E-02	-0.0509	ANT3
sp|P00748|FA12_HUMAN	Coagulation factor XII OS=Homo sapiens OX=9606 GN=*F12* PE=1 SV=3	4.849E-02	-0.0510	FA12
sp|P01019|ANGT_HUMAN	Angiotensinogen OS=Homo sapiens OX=9606 GN=*AGT* PE=1 SV=1	4.201E-01	-0.0520	ANGT
sp|P04275|VWF_HUMAN	von Willebrand factor OS=Homo sapiens OX=9606 GN=*VWF* PE=1 SV=4	5.874E-01	-0.0538	VWF
sp|P19823|ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2 OS=Homo sapiens OX=9606 GN=*ITIH2* PE=1 SV=2	6.064E-02	-0.0541	ITIH2

sp|P02655|APOC2_HUMAN	Apolipoprotein C-II OS=Homo sapiens OX=9606 GN=*APOC2* PE=1 SV=1	3.652E-01	-0.0554	APOC2
sp|P29622|KAIN_HUMAN	Kallistatin OS=Homo sapiens OX=9606 GN=*SERPINA4* PE=1 SV=3	4.930E-03	-0.0554	KAIN
sp|Q96PD5|PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase OS=Homo sapiens OX=9606 GN=*PGLYRP2* PE=1 SV=1	5.567E-02	-0.0557	PGRP2
sp|P04114|APOB_HUMAN	Apolipoprotein B-100 OS=Homo sapiens OX=9606 GN=*APOB* PE=1 SV=2	2.185E-01	-0.0563	APOB
sp|P17936|IBP3_HUMAN	Insulin-like growth factor-binding protein 3 OS=Homo sapiens OX=9606 GN=*IGFBP3* PE=1 SV=2	9.551E-02	-0.0655	IBP3
sp|P22352|GPX3_HUMAN	Glutathione peroxidase 3 OS=Homo sapiens OX=9606 GN=*GPX3* PE=1 SV=2	6.906E-02	-0.0668	GPX3
sp|P00738|HPT_HUMAN	Haptoglobin OS=Homo sapiens OX=9606 GN=*HP* PE=1 SV=1	3.336E-01	-0.0871	HPT
sp|O75882|ATRN_HUMAN	Attractin OS=Homo sapiens OX=9606 GN=*ATRN* PE=1 SV=2	8.930E-03	-0.0915	ATRN
sp|P51884|LUM_HUMAN	Lumican OS=Homo sapiens OX=9606 GN=*LUM* PE=1 SV=2	1.010E-03	-0.0928	LUM
sp|P07225|PROS_HUMAN	Vitamin K-dependent protein S OS=Homo sapiens OX=9606 GN=*PROS1* PE=1 SV=1	3.410E-05	-0.0933	PROS
sp|P27169|PON1_HUMAN	Serum paraoxonase/arylesterase 1 OS=Homo sapiens OX=9606 GN=*PON1* PE=1 SV=3	7.650E-03	-0.0990	PON1
sp|P13647|K2C5_HUMAN	Keratin, type II cytoskeletal 5 OS=Homo sapiens OX=9606 GN=*KRT5* PE=1 SV=3	1.002E-01	-0.1008	K2C5
sp|P05154|IPSP_HUMAN	Plasma serine protease inhibitor OS=Homo sapiens OX=9606 GN=*SERPINA5* PE=1 SV=3	4.162E-02	-0.1011	IPSP
sp|P02649|APOE_HUMAN	Apolipoprotein E OS=Homo sapiens OX=9606 GN=*APOE* PE=1 SV=1	5.601E-02	-0.1053	APOE
sp|P07359|GP1BA_HUMAN	Platelet glycoprotein Ib alpha chain OS=Homo sapiens OX=9606 GN=*GP1BA* PE=1 SV=2	8.885E-02	-0.1091	GP1BA
sp|P43652|AFAM_HUMAN	Afamin OS=Homo sapiens OX=9606 GN=*AFM* PE=1 SV=1	7.300E-04	-0.1108	AFAM
sp|P02745|C1QA_HUMAN	Complement C1q subcomponent subunit A OS=Homo sapiens OX=9606 GN=*C1QA* PE=1 SV=2	1.200E-04	-0.1182	C1QA
sp|P01023|A2MG_HUMAN	Alpha-2-macroglobulin OS=Homo sapiens OX=9606 GN=*A2M* PE=1 SV=3	4.498E-02	-0.1243	A2MG
sp|P07357|CO8A_HUMAN	Complement component C8 alpha chain OS=Homo sapiens OX=9606 GN=*C8A* PE=1 SV=2	1.350E-06	-0.1266	CO8A
sp|P04264|K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens OX=9606 GN=*KRT1* PE=1 SV=6	1.348E-02	-0.1462	K2C1
sp|Q96KN2|CNDP1_HUMAN	Beta-Ala-His dipeptidase OS=Homo sapiens OX=9606 GN=*CNDP1* PE=1 SV=4	8.270E-06	-0.1464	CNDP1
sp|P02747|C1QC_HUMAN	Complement C1q subcomponent subunit C OS=Homo sapiens OX=9606 GN=*C1QC* PE=1 SV=3	2.800E-04	-0.1471	C1QC
sp|O14791|APOL1_HUMAN	Apolipoprotein L1 OS=Homo sapiens OX=9606 GN=*APOL1* PE=1 SV=5	2.550E-03	-0.1561	APOL1
sp|P05155|IC1_HUMAN	Plasma protease C1 inhibitor OS=Homo sapiens OX=9606 GN=*SERPING1* PE=1 SV=2	3.530E-03	-0.1562	IC1
sp|O95445|APOM_HUMAN	Apolipoprotein M OS=Homo sapiens OX=9606 GN=*APOM* PE=1 SV=2	3.570E-05	-0.1578	APOM
sp|P20742|PZP_HUMAN	Pregnancy zone protein OS=Homo sapiens OX=9606 GN=*PZP* PE=1 SV=4	3.705E-01	-0.1681	PZP
sp|P02753|RET4_HUMAN	Retinol-binding protein 4 OS=Homo sapiens OX=9606 GN=*RBP4* PE=1 SV=3	5.230E-10	-0.1685	RET4
sp|P35858|ALS_HUMAN	Insulin-like growth factor-binding protein complex acid labile subunit OS=Homo sapiens OX=9606 GN=*IGFALS* PE=1 SV=1	1.480E-12	-0.1761	ALS
sp|P01591|IGJ_HUMAN	Immunoglobulin J chain OS=Homo sapiens OX=9606 GN=*JCHAIN* PE=1 SV=4	4.457E-02	-0.1782	IGJ
sp|P06727|APOA4_HUMAN	Apolipoprotein A-IV OS=Homo sapiens OX=9606 GN=*APOA4* PE=1 SV=3	9.090E-03	-0.1803	APOA4
sp|P35908|K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens OX=9606 GN=*KRT2* PE=1 SV=2	2.495E-02	-0.1859	K22E

sp|P07996|TSP1_HUMAN	Thrombospondin-1 OS=Homo sapiens OX=9606 GN=*THBS1* PE=1 SV=2	1.370E-07	-0.1886	TSP1
sp|P68871|HBB_HUMAN	Hemoglobin subunit beta OS=Homo sapiens OX=9606 GN=*HBB* PE=1 SV=2	9.260E-03	-0.1972	HBB
sp|P01871|IGHM_HUMAN	Immunoglobulin heavy constant mu OS=Homo sapiens OX=9606 GN=*IGHM* PE=1 SV=4	8.900E-04	-0.2137	IGHM
sp|P35527|K1C9_HUMAN	Keratin, type I cytoskeletal 9 OS=Homo sapiens OX=9606 GN=*KRT9* PE=1 SV=3	1.490E-03	-0.2193	K1C9
sp|P69905|HBA_HUMAN	Hemoglobin subunit alpha OS=Homo sapiens OX=9606 GN=*HBA1* PE=1 SV=2	2.840E-03	-0.2290	HBA
sp|P02533|K1C14_HUMAN	Keratin, type I cytoskeletal 14 OS=Homo sapiens OX=9606 GN=*KRT14* PE=1 SV=4	5.780E-03	-0.2371	K1C14
sp|P13645|K1C10_HUMAN	Keratin, type I cytoskeletal 10 OS=Homo sapiens OX=9606 GN=*KRT10* PE=1 SV=6	1.030E-03	-0.2446	K1C10
sp|O00187|MASP2_HUMAN	Mannan-binding lectin serine protease 2 OS=Homo sapiens OX=9606 GN=*MASP2* PE=1 SV=4	2.491E-02	-0.2792	MASP2
sp|P08185|CBG_HUMAN	Corticosteroid-binding globulin OS=Homo sapiens OX=9606 GN=*SERPINA6* PE=1 SV=1	1.090E-03	-0.2841	CBG
sp|P02647|APOA1_HUMAN	Apolipoprotein A-I OS=Homo sapiens OX=9606 GN=*APOA1* PE=1 SV=1	3.140E-03	-0.2963	APOA1
sp|P24821|TENA_HUMAN	Tenascin OS=Homo sapiens OX=9606 GN=*TNC* PE=1 SV=3	3.100E-06	-0.3229	TENA
sp|P02751|FINC_HUMAN	Fibronectin OS=Homo sapiens OX=9606 GN=*FN1* PE=1 SV=5	2.270E-05	-0.3343	FINC
sp|P01009|A1AT_HUMAN	Alpha-1-antitrypsin OS=Homo sapiens OX=9606 GN=*SERPINA1* PE=1 SV=3	5.430E-08	-0.3865	A1AT
sp|P02787|TRFE_HUMAN	Serotransferrin OS=Homo sapiens OX=9606 GN=*TF* PE=1 SV=3	2.570E-08	-0.4734	TRFE