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

1. CEDOC, NOVA Medical School, Universidade NOVA de Lisboa, Lisbon, Portugal
2. Institute of Hygiene and Tropical Medicine, Universidade NOVA de Lisboa and Global Health and Tropical Diseases Research Centre Lisbon, Portugal
3. Centro Hospitalar de Lisboa Ocidental, Hospital de Egas Moniz, Lisboa, Rheumatology Department, Portugal
4. Instituto Politécnico de Setúbal, Escola Superior de Saúde de Setúbal, Setúbal, Portugal
5. MSK imaging Unit (UIME), Imaging Center, Hospital da Luz, Lisbon, Portugal
6. Champalimaud Centre for the Unknown, Lisbon, Portugal
7. Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa
8. Leiden University Medical Center, Leiden, The Netherlands
9. University of Illinois, College of Medicine at Peoria, USA
10. Bradley University, Mechanical Engineering department, USA
11. ITQB-NOVA, Instituto de Tecnologia Quimica e Biologica Antonio Xavier, Oeiras, Portugal
12. iBET - Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal
13. UCIBIO, DCV, FCT-NOVA, Caparica, Portugal

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

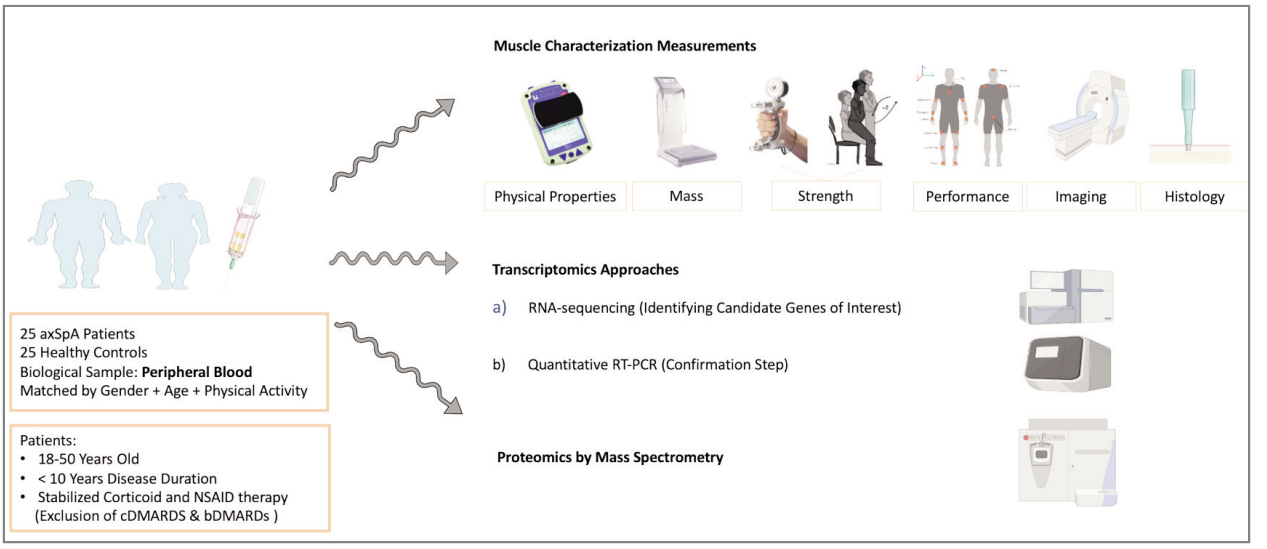

FIGURE 1. Experimental Study Approach; 1. Muscle Characterization Measurements (Physical Properties, Mass, Strength, Physical Performance, Imaging and Histology). 2. Transcriptomics Approach (a) RNA-sequencing, b) Quantitative RT-PCR). 3. Proteomics by Mass Spectrometry.
cDMARD: conventional disease-modifying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

FUNDING

This study was supported by iNOVA4Health (consortia to create a multidisdiplinary/translational network at the NOVA University, Lisbon, Portugal) and Portuguese Society of Rheumatology grants.

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.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the individuals who will share their clinical data and biological samples with us.

CORRESPONDENCE TO

Atlas Mashayekhi Sardoo
Rua Câmara Pestana 6, 1150-082 Lisboa
Portugal
E-mail: atlas.sardoo@nms.unl.pt

REFERENCES

1. Sieper J, Poddubnyy D. Axial spondyloarthritis. The Lancet. 2017.
2. Rudwaleit M, Van Der Heijde D, Landewé R, Listing J, Akkoc N, Brandt J, et al. The development of Assessment of SpondyloArthritis international Society classification criteria for axial spondyloarthritis (part II): Validation and final selection. Ann Rheum Dis. 2009;
3. Branco JC, Rodrigues AM, Gouveia N, Eusébio M, Ramiro S, Machado PM, et al. Prevalence of rheumatic and musculoskeletal diseases and their impact on health-related quality of life, physical function and mental health in Portugal: Results from EpiReumaPt- a national health survey. RMD Open. 2016;
4. van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. Arthritis Rheum. 1984;
5. Peng J, Gong Y, Zhang Y, Wang D, Xiao Z. Immunohistological analysis of active sacroiliitis in patients with axial spondyloarthritis. Med (United States). 2017;
6. Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DCO, Sturrock RD. ANKYLOSING SPONDYLITIS AND HL-A 27. Lancet. 1973;
7. Brown MA, Kennedy LG, MacGregor AJ, Darke C, Duncan E, Shatford JL, et al. Susceptibility to ankylosing spondylitis in twins: The role of genes, HLA, and the environment. Arthritis Rheum. 1997;
8. Schlosstein L, Terasaki PI, Bluestone R, Pearson CM. High Association of an HL-A Antigen, W27, with Ankylosing Spondylitis. N Engl J Med. 1973;
9. Yang L, Wang L, Wang X, Xian CJ, Lu H. A possible role of intestinal microbiota in the pathogenesis of ankylosing spondylitis. International Journal of Molecular Sciences. 2016.
10. Burton PR, Clayton DG, Cardon LR, Craddock N, Deloukas P, Duncanson A, et al. Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. Nat Genet. 2007;
11. Reveille JD, Sims AM, Danoy P, Evans DM, Leo P, Pointon JJ, et al. Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. Nat Genet. 2010;
12. de Winter JJ, van Mens LJ, van der Heijde D, Landewé R, Baeten DL. Prevalence of peripheral and extra-articular disease in ankylosing spondylitis versus non-radiographic axial spondyloarthritis: A meta-analysis. Arthritis Res Ther. 2016;
13. Masi AT, Nair K, Andonian BJ, Prus KM, Kelly J, Sanchez JR, et al. Integrative Structural Biomechanical Concepts of Ankylosing Spondylitis. Arthritis. 2011;
14. Nair K, Masi AT, Andonian BJ, Barry AJ, Coates BA, Dougherty J, et al. Stiffness of resting lumbar myofascia in healthy young subjects quantified using a handheld myotonometer and concurrently with surface electromyography monitoring. J Bodyw Mov Ther. 2016;
15. Cortes A, Hadler J, Pointon JP, Robinson PC, Karaderi T, Leo P, et al. Identification of multiple risk variants for ankylosing spondylitis through high-density genotyping of immune-related loci. Nat Genet. 2013;

16. Haynes KR, Pettit AR, Duan R, Tseng HW, Glant TT, Brown MA, et al. Excessive bone formation in a mouse model of ankylosing spondylitis is associated with decreases in Wnt pathway inhibitors. Arthritis Res Ther. 2012;
17. Kehl AS, Corr M, Weisman MH. Review: Enthesitis: New Insights into Pathogenesis, Diagnostic Modalities, and Treatment. Arthritis and Rheumatology. 2016.
18. McGonagle D, Stockwin L, Isaacs J, Emery P. An enthesitis based model for the pathogenesis of spondyloarthropathy. Additive effects of microbial adjuvant and biomechanical factors at disease sites. Journal of Rheumatology. 2001.
19. Andonian BJ, Masi AT, Aldag JC, Barry AJ, Coates BA, Emrich K, et al. Greater Resting Lumbar Extensor Myofascial Stiffness in Younger Ankylosing Spondylitis Patients Than Age-Comparable Healthy Volunteers Quantified by Myotonometry. Arch Phys Med Rehabil. 2015;
20. Yeremenko N, Noordenbos T, Cantaert T, Van Tok M, Van De Sande M, Cañete JD, et al. Disease-specific and inflammation-independent stromal alterations in spondylarthritis synovitis. Arthritis Rheum. 2013;
21. Micallef L, Vedrenne N, Billet F, Coulomb B, Darby IA, Desmoulière A. The myofibroblast, multiple origins for major roles in normal and pathological tissue repair. Fibrogenes Tissue Repair. 2012;
22. Layh-Schmitt G, Lu S, Navid F, Brooks SR, Lazowick E, Davis KM, et al. Generation and differentiation of induced pluripotent stem cells reveal ankylosing spondylitis risk gene expression in bone progenitors. Clin Rheumatol. 2017;
23. ICH harmonised guideline [Internet]. Available from: https://ichgcp.net/
24. WMA DECLARATION OF HELSINKI [Internet]. 2018 [cited 2018 Jul 9]. Available from: https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/
25. Pimentel-Santos FM, Mourão AF, Ribeiro C, Costa J, Santos H, Barcelos A, et al. Spectrum of ankylosing spondylitis in Portugal. Development of BASDAI, BASFI, BASMI and mSASSS reference centile charts. Clin Rheumatol. 2012;
26. Reuma.pt [Internet]. Available from: http://reuma.pt/pt_PT/Default.aspx
27. Craig CL, Marshall AL, Sjöström M, Bauman AE, Booth ML, Ainsworth BE, et al. International physical activity questionnaire: 12-Country reliability and validity. Med Sci Sports Exerc. 2003;
28. Myoton AS. Patented technology [Internet]. Available from: https://www.myoton.com/technology/
29. Cruz-Jentoft AJ, Bahat G, Bauer J, Boirie Y, Bruyère O, Cederholm T, et al. Sarcopenia: Revised European consensus on definition and diagnosis. Age and Ageing. 2019.
30. Creemers MCW, Franssen MJAM, Van't Hof MA, Gribnau FWJ, Van De Putte LBA, Van Riel PLCM. Assessment of outcome in ankylosing spondylitis: An extended radiographic scoring system. Ann Rheum Dis. 2005;
31. Braun J, Sieper J, Bollow M. Imaging of sacroiliitis. Clinical Rheumatology. 2000.
32. Rudwaleit M, Jurik AG, Hermann KGA, Landewé R, Van Der Heijde D, Baraliakos X, et al. Defining active sacroiliitis on magnetic resonance imaging (MRI) for classification of axial spondyloarthritis: A consensual approach by the ASAS/OMERACT MRI group. Ann Rheum Dis. 2009;
33. Lin Z, Bei JX, Shen M, Li Q, Liao Z, Zhang Y, et al. A genome-wide association study in Han Chinese identifies new susceptibility loci for ankylosing spondylitis. Nat Genet. 2012;
34. Hollingsworth KG, de Sousa PL, Straub V, Carlier PG. Towards harmonization of protocols for MRI outcome measures in skeletal muscle studies: Consensus recommendations from two TREAT-NMD NMR workshops, 2 May 2010, Stockholm, Sweden, 1-2 October 2009, Paris, France. Neuromuscul Disord. 2012;
35. Dixon WT. Simple proton spectroscopic imaging. Radiology. 1984;
36. Mercuri E, Jungbluth H, Muntoni F. Muscle imaging in clinical practice: Diagnostic value of muscle magnetic resonance imaging in inherited neuromuscular disorders. Current Opinion in Neurology. 2005.
37. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics. 2013;
38. Liao Y, Smyth GK, Shi W. FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics. 2014;
39. Robinson MD, McCarthy DJ, Smyth GK. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2009;
40. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma. Nucleic Acids Res. 2015;
41. CFX Maestro Software [Internet]. Available from: https://www.bio-rad.com/en-pt/product/cfx-maestro-software-for-cfx-real-time-pcr-instruments?ID=OKZP7E15
42. Palma AS, De Carvalho M, Grammel N, Pinto S, Barata N, Conradt HS, et al. Proteomic analysis of plasma from Portuguese patients with familial amyotrophic lateral sclerosis. Amyotroph Lateral Scler. 2008;
43. Charro N, Hood BL, Faria D, Pacheco P, Azevedo P, Lopes C, et al. Serum proteomics signature of Cystic Fibrosis patients: A complementary 2-DE and LC-MS/MS approach. J Proteomics. 2011;
44. Cunha B, Aguiar T, Carvalho SB, Silva MM, Gomes RA, Carrondo MJT, et al. Bioprocess integration for human mesenchymal stem cells: From up to downstream processing scale-up to cell proteome characterization. J Biotechnol. 2017;