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CICLO XXXI

EMERGING ROLE OF MONOCYTES AND OF THEIR INTRACELLULAR CALCIUM CONTENT IN SPONDYLOARTHRITIS

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INDEX

SUMMARY III

ABBREVIATIONS V

1. INTRODUCTION 1
 1.1 Spondyloarthritis 1
  1.1.1 Classification criteria 2
  1.1.2 Incidence and prevalence of Spondyloarthritis 4
  1.1.3 Measures of disease activity and outcome 5
  1.1.4 Diagnosis of Spondyloarthritis 6
 1.2 Treatment 8
  1.2.1 Non steroideal anti-inflammatory drugs 8
  1.2.2 Disease modyfing anti-rheumatic drugs 8
  1.2.3 Biological Agents 9
 1.3 Psoriatic Arthritis 12
 1.4 Ankylosing spondylitis 15
 1.5 Pathogenesis of Spondyloarthritis: Genetic factors 17
 1.6 Pathogenesis of Spondyloarthritis: Autoimmune or autoinflammatory 20
  1.6.1 IL-1β 21
  1.6.2 TNF-α 22
  1.6.3 TGF-β 23
 1.7 Pathogenesis of Spondyloarthritis: Calprotectin (S100A8/S100A9) 26
 1.8 Pathogenesis of Spondyloarthritis: Matrix Metalloproteinases 28
 1.9 Pathogenesis of Spondyloarthritis: Moncytes calcium-sensing cells 30

2. AIMS 33

3. MATERIALS AND METHODS 34
 3.1 Studied population 34
 3.2 Clinical Assessment 34
 3.3 Haematological and biochemical indices 35
 3.4 Isolation of human peripheral blood mononuclear cells 36
 3.5 Nucleic acids isolation 36
 3.6 Relative quantification analysis 37
 3.7 Genetic analysis 39
  3.7.1 TNFA gene analysis 39
  3.7.2 HLA-B27gene analysis 40
 3.8 Intracellular calcium fluxes analysis 40
 3.9 "In vitro" experiments in monocyte cells 41
 3.10 Statistical analysis 41

4. RESULTS 42
 4.1 Studied population 42
 4.2 Clinical characteristics of SpA patients 42
 4.3 Haematological and biochemical indices in SpA patients 45
 4.4 Cytokines and MMPs expression levels in PBMCs 46
 4.5 Calcium binding proteins S100A8 and S100A9 expression levels in PBMCs 49
 4.6 Intracellular calcium fluxes in Healty subjects and SpA patients 50
 4.7 Healthy subject serum induces intracelluar calcium oscillations in human monocytes 59
 4.8 Therapy effects 60

5. DISCUSSION and CONCLUSIONS 61

6. REFERENCES 66

SUMMARY

Background. The Spondyloarthritis (SpA) are a group of a multifactorial diseases characterised by a complex interplay between an inherited background and environmental factors that lead to immune response dysregulation and inflammation of the joints, mainly the sacro-ileal. Different from rheumatoid arthritis, there are no specific biomarkers for disease activity in the SpA that could be used in clinical practice. New biomarkers discovery could be helpful for early diagnosis, monitoring of disease activity, as well as for prognosis, outcome measures, and for assessing treatment efficacy. In SpA patients, macrophages infiltrating the inflamed joints, derive from circulating monocytes, express not only inflammatory cytokines, like TNF-α, IL-1β or TGF-β, but also enzymes causing tissue destruction and remodelling, like metalloproteinases. Metalloproteinases (MMPs), MMP3 in particular, have been reported to be highly expressed in synovial tissue and in peripheral blood of SpA patients. Recent studies have showed that MMP8 and MMP9, in particular, are produced by peripheral blood mononuclear cells (PBMCs) if they are stimulated by calprotectin (S100A8/S100A9 heterodimer). The SpA synovial tissue is characterized by an increased vascularization and an infiltrate composed of nucleated polymorphs, macrophages and lymphocytes. In these cells calcium signals are essential for various cellular functions, including proliferation, differentiation, apoptosis, and gene transcription. The aims of this work are to investigate whether the TNF-α, IL-1β, TGF-β, S100A8, S100A9, MMP3, MMP8 and MMP9 mRNA expression levels and intracellular calcium ($[Ca^{2+}]_i$) fluxes variations in PBMCs might be associated with SpA.

Methods. The study population comprised 64 patients with a diagnosis of SpA (39 males and 25 females; mean age±standard deviation: 39.5±13.2 years) and 100 healthy controls (58 males and 42 females; mean age±standard deviation: 46.6±8.5). Among patients, 26 (40.6%) had diagnosis of Ankylosing Spondylitis (AS), (modified New York criteria) and 38 (59.3%) had a diagnosis of Psoriatic Arthritis (PsA) (CASPAR criteria). Blood samples were collected and complete blood count, CRP, ESR, uric acid, ALT and glucose were evaluated. Relative quantification (Real Time PCR) of TNF-α, IL-1β, TGF-β, S100A8, S100A9, MMP3, MMP8 and MMP9 mRNA were performed. Intracellular calcium ($[Ca^{2+}]_i$) fluxes were studied in patients and controls monocyte cells by a fluorescent microscope.

Results. The mRNA expression levels in PBMCs of TNF-α, IL-1β, TGF-β were similar in AS and PsA patients when compared to controls. The variations of TNF-α, TGF-β and IL-1β were correlated each other. TNF-α mRNA expression levels also show a significant correlation if patient's relatives with SpA where found ($t=-2.5386$, $p=0.013$). MMP8 and MMP9 mRNA expression levels did not vary between controls and patients, nor they were related to disease clinical activity indices. S100A9 mRNA expression did not vary, the expression of S100A8 ($F=3.29$, $p=0.039$) was reduced in PsA patients. S100A8 and S100A9 expression levels were significantly correlated with circulating inflammatory cells and S100A8 was correlated with CRP and ESR. Monocytes from healthy controls had evident and frequent ($[Ca^{2+}]_i$) oscillations, while SpA patients monocytes did not. The percentage of cells exhibiting ($[Ca^{2+}]_i$) oscillations profile was significantly lower in AS with respect to controls ($F=6.15$, $p=0.003$). The percentage of monocytes with intracellular calcium oscillations and the studied molecules were not correlated with the type of therapy or of drug used.

Conclusions. SpA associates with a reduced expression of the inflammatory S100A8 calcium binding protein and with a decreased intracellular calcium fluxes in patients' cells compared to healthy subjects, suggesting that the presence of the disease affects the "on-off" mechanisms that regulate the concentration of intracellular calcium.

ABBREVIATIONS

ACPA: Anti-citrullinated protein/peptide antibodies

ADAMs: adamlysins

ADAMTSs: ADAMs with thrombospondin-like motifs

ALT: alanine transaminase

ANTXR2: anthrax toxin receptor 2

AP-1: activator protein 1

AS: ankylosing spondylitis

ASAS: Assessment of SpondyloArthritis international Society

ASDAS: Ankylosing Spondylitis Disease Activity Score

axSpA: axial spondyloarthritis

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index

BASFI: Bath Ankylosing Spondylitis Functional Index

BASMI: Bath Ankylosing Spondylitis Metrology Index

BMI: Body Mass Index

BMP: bone morphogenetic protein

bp: base pair

CARD9: caspase recruitment domain family, member 9

CaSR: Calcium sensing receptor

CASPAR: ClASsification criteria for Psoriatic Arthritis

CCP: cyclic citrullinated peptides

CI: Confidence Interval

CNV: copy number variation

CRP: C-reactive protein

CTLs: cytotoxic T lymphocytes

CVD: cardiovascular disease

DAS: Disease Activity Score

DAS-28: Disease Activity Score- 28

DMARDs: Disease-modifying antirheumatic drugs

dNTPs: deoxynucleotide triphosphates

ER: endoplasmic reticulum

ERAP1: endoplasmic reticulum aminopeptidase 1

ERK: extracellular signal-regulated kinases

ESR: erythrocyte sedimentation rate

ESSG: European Spondyloarhropathy Study Group

EULAR: European League Against Rheumatism

F: forward primer

GRAPPA: Group for Research and Assessment of Psoriasis and Psoriatic Arthritis

GDFs: growth differentiation factors

GWAS: genome-wide association studies

HAQ: Health Assessment Questionnaire

HRQoL: health related quality of life

IBD: Inflammatory Bowel Diseases

IBD- SpA: spondyloarthritis related to inflammatory bowel disease

IBP: Inflammatory back pain

IL: Interleukin

IL-1R2: interleukin-1 receptor 2

IL-23R: interleukin-23 receptor

IMIDs: Immune-mediated inflammatory diseases

LAP:latency-associated peptide

MASES: Maastricht Ankylosing Spondylitis Enthesitis Score

MAPK: mitogen-activated protein kinase

MEFV: Mediterranean fever

MDSC: myeloid-derived suppressor cell

MHC: Major Histocompatibility Complex

MMP: matrix metalloproteinase

MICA: major histocompatibility complex class I chain-related A

MRI: Magnetic Resonance Imaging

NF-κB: nuclear factor κappa-light-chain-enhancer of activated B cells

NK: natural killer cells

nr-axSpA: non-radiographic axial spondyloarthritis

NSAIDs: Non-steroidal anti-inflammatory drugs

OD: Odds Ratio

OP: osteoporosis

OPG: osteoprotegerin

PCR: polymerase chain reaction

PEG: polyethylene glicole

PMN: polymorphonuclear cells

PsA: Psoriatic Arthritis

PsC: cutaneous psoriasis

pSpA: peripheral spondyloarthritis

R: reverse primer

RA: rheumaotid arthritis

RANKL: Receptor activator of nuclear factor kappa-B ligand

RBCs: red blood cells

ReA: reactive arthritis

ROI: Regions of Interest

SD: Standard Deviation

SE: Standard Error

SNPs: single nucleotide polymorphisms

SpA: Spondyloarthritis

STAT3: signal transducer and activator of transcription 3

sTNF: TNF-α soluble form

sTNFR: TNF-α soluble receptors fragments

TACE: TNF-α converting enzyme

TASC: Australo-Anglo-American Spondyloarthritis Consortium

TβRI: type I receptor kinase

TβRII: type II receptor kinase

TGF: Transforming growth factor

Th: T helper

tmTNF: TNF-α transmembrane form

TNF-α: tumor necrosis factor α

TNFAIP3: tumor necrosis factor alpha-induced protein 3

TNFRI: tumor necrosis factor receptor type 1

TNFRII: tumor necrosis factor receptor type 2

TNFRSF1A: tumor necrosis factor (receptor) superfamily member 1A

TNFSF15: tumor necrosis factor (ligand) superfamily, member 15

TRADD: tumor necrosis factor receptor type 1-associated death domain protein

TRAPS: Tumor necrosis factor Receptor-Associated Periodic Syndrome

UPR: unfolded-protein response

uSpA: undifferentiated spondylarthropathy

VAS: visual analogue scale

VASg: visual analogue scale of global disease activity

JNK: c-Jun N-terminal kinase

WBC: White Blood Cells

1. INTRODUCTION

1.1 Spondyloarthritis

Spondyloarthritis (SpA) is a heterogeneous group of articular inflammatory disordes characterised by a broad spectrum of clinical manifestations, laboratory abnormalities, radiological features and genetic susceptibility in particular the association with the major histocompatibility complex class 1 antigen, HLA-B27. In 1974, Moll and colleagues established the concept of seronegative spondarthritides of inter-related disorders characterized by common clinical and imaging features different from other rheumatic diseases [1]. SpA, in particular, is a condition in wich both axial and peripheral joints could be affected; the inflammation of the axial skeleton involved sacroiliac joints and spine; the peripheral arthritis, commonly occur in a characteristic pattern, asymmetric, oligoarticular and preferentially in the lower extremities.

In SpA are also present extra-articular features, such as cutaneous (psoriasis, pustular lesions, erythemanodosum, pyoderma gangrenosum), ocular (uveitis) and mucosal (oral, intestinal, genital ulcers) manifestations as well as the inflammatory bowel disease (IBD) [2-4].

The absence of the rheumatoid factor and of anti-citrullinated protein/peptide antibodies (ACPA) in serum allowed to distinguish these disorders from rheumatoid arthritis and is referred to seronegative term.

The group of disease consist of psoriatic arthritis (PsA) or axial spondyloarthritis (ax-SpA), wich includes ankylosing spondylitis (AS) and non-radiographic axial SpA (nr-axSpA); the less common subgroups enteropatic SpA wich is associated with inflammatory bowel disease (Crohn's disease and ulcerative colitis), reactive arthritis and undifferentiated SpA (uSpA) [5].

Recent data showed that SpA tends to be associated with the development of some comorbidity like cardiovascular diseases (CVD), diabetes mellitus, osteoporosis (OP) and depressive disorders [6].

Signs and symptoms, in particular spinal symptoms, of SpA may be mistakenly attribuited to other causes of low back pain; this can lead to delays in diagnosis and treatment of the disease and related comorbidities. This delay results in a disease progression and disability that compromise work ability, work quality and health related quality of life (HRQoL) of these patients [7].

1.1.1 Classification criteria

Spa is carachterized by heterogeneous presenting features, diagnosis in fact depends on a combination of symptoms, physical examination, imaging, environmental factors and laboratory tests. In the Table 1 there is a list of the main features of the disease: each of these should be taken into account in making diagnosis [8].

Features of patients with SpA
Inflammatory back pain
Good response to NSAIDs
Positive family history of SpA
HLA-B27-positive
Increased CRP concentration
Enthesitis
Peripheral arthritis
Dactylitis
Prior urogenital or gastrointestinal infection
Inflammatory bowel disease (IBD)
Psoriasis
Acute anterior uveitis
Sacroiliitis detected by imaging (radiography or MRI)

SpA= Spondyloarthritis; NSAIDs = Non-steroidal anti-inflammatory; CRP: C-reactive protein; MRI= Magnetic Resonance Imaging

Table 1. Clinical, biochemical and genetic features of SpA [9].

The classification of SpA in the different subgroups of the SpA family can be difficult in particularly by the overlap of several symptoms making difficult an early diagnosis.

For these reasons, in the past decades, the classification criteria of SpA has been reviewed many times. In 1984 the modified New York (NY) criteria were the most classification criteria used for AS diagnosis; these criteria provided high specificity although they lack sensitivity, especially in the early phases of disease [10]. The diagnostic performance of modified New York criteria was based on clinical symptoms and signs and on the presence of radiographic sacroilitis. The modified New York criteria are only useful in the valuation of advanced disease and are strict limited to axial features of the disease [9]. In the early 1990s has been developed Amor criteria [11] and European Spondyloarthropathy Study Group (ESSG) criteria [12] due to the limitations of the modiefied NY criteria and to be applicable to early-stage and mild SpA.

In comparison with the modified NY criteria Amor and ESSG criteria cover the whole spectrum of SpA (including patients with undifferentiated SpA) and include a wider range of manifestations of the disease (Figure 1).

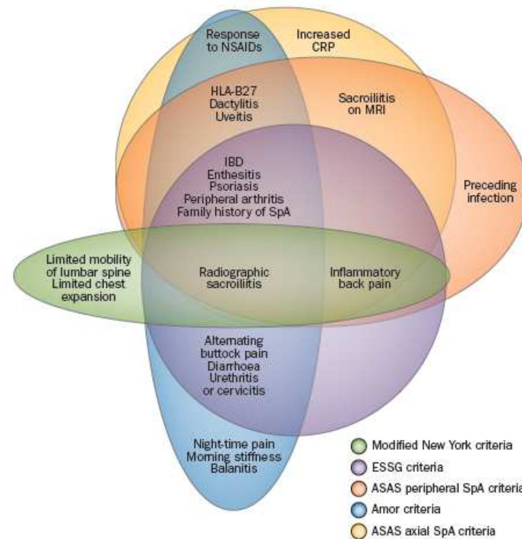


Figure 1. Venn diagram of features of the SpA criteria sets [9].

Amor and colleagues proposed a set of classification criteria for the whole group of SpA, allowing a patient to be classified as having SpA whatever the presenting symptoms. The Amor criteria do not have mandatory features for the classification but provide the score of 12 features covering 4 domains: symptoms, radiographic and genetic features, response to treatment. ESSG classification criteria required the presence of inflammatory back pain (IBP) and synovitis as a entry conditions. These criteria recognized five major subtypes of SpA: ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), spondyloarthritis related to inflammatory bowel disease (IBD-SpA) and uSpA.

The Amor criteria perform better than ESSG for early SpA showing higher sensitivity (85%) and specificity (90%) thanks to the inclusion of positivity for HLA-B27 and ocular manifestation [11]. Both sets of criteria cover the whole spectrum of SpA and were useful over the years in classification of this disease however they cannot differentiate axial from pheripheral disease, important distintion for providing good treatment strategies.

The Assessment of SpondyloArthritis international Society (ASAS) developed new criteria sets for axial SpA in 2009 [13] and for pheripheral SpA in 2011 [14] mainly do to the limitations of existing classification criteria.

The ASAS did a large cross-sectional study to propose new criteria on the basis of the two main clinical features identified in daily practice: axial symptoms and peripheral involvement [4].
ASAS classification criteria for axial and pheripheral SpA are shown in Figure 2.

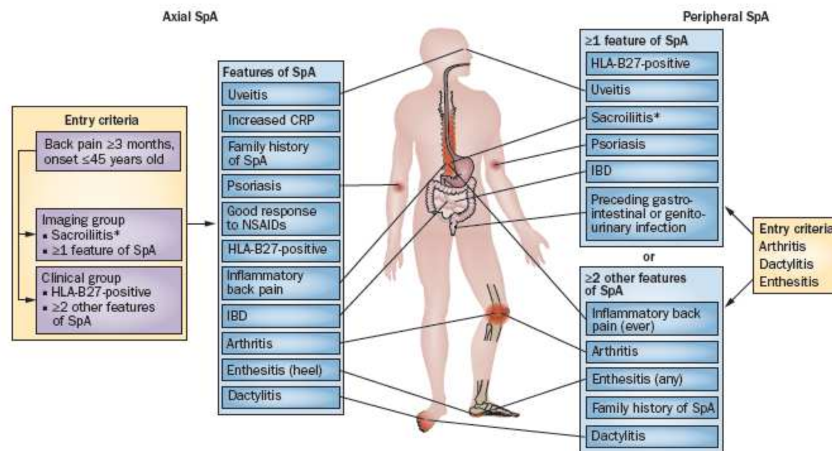


Figure 2. ASAS classification criteria for axial and pheripheral SpA [9].

One important advance among the ASAS classification criteria is the inclusion of the MRI (Magnetic Resonance Imaging) allowed identifying the signs of axial inflammation in the early stages of the disease. The ASAS group, in fact, proposed the term "axial spondyloarthritis" (axSpA) for the first time to describe the full spectrum of axial disease and the "pheripheral spondyloarthritis" to describe the full spectrum of SpA diseases predominantly affecting the pheripheral skeleton [3]. The sensitivity and specificity of the entire set of criteria were 83% and 84% respectively [15].
The advantage of ASAS criteria has contributed to a better understanding of the spectrum of axial and pheripheral SpA, timely identification to avoid treatment options, unnecessary diagnostic testing and to acknowledge patient morbidity.

1.1.2 Incidence and prevalence of Spondyloarthritis

In the last years, data on the incidence and prevalence of SpA have become particularly important to improve the management of target therapies and consequently the health care costs.
Before the introduction of the ASAS criteria the global prevalence values of SpA were reported to be 1% [16,17] but range substantially, from 0.01% in Japan [18] to 2.5% in Northern Artic natives [19,20]. The incidence broadly vary from 0.48 cases per 100.000 person/year in Japan [18] to 62.5 per 100.000 person/year in Spain [21].

These differences are mainly due to the heterogeneity in the populations analysed, variation in the used criteria and the number of partecipants included in each study. Classification criteria are often developed for conducting clinical trials and are not always be available for epidemiological studies.

In different population and ethnic groups the incidence and prevalence of SpA widely vary and at least can be explained by differences in the prevalence of HLA-B27.

The overall prevalence of SpA using ASAS criteria has been evaluated in only one study in 2015. Costantino et al. in a large population-based cohort study (GAZEL cohort) consisting of 20625 French subjects (representative of French population) analysed the prevalence of SpA, in reference of HLA-B27, and founded that the estimated prevalence was 0.43%.

In healthy controls HL-B27 positivity rate was 6.9% and in SpA patients was 75% with an increased risk of developing the disease compared with HLA-B27 negative subjects [22].

Zlatkovic-Svenda and colleagues, estimated the prevalence of SpA in Serbian population; similar to that for rheumatoid arthritis (RA) the prevalence of SpA was 0.32% [23].

1.1.3 Measures of disease activity and outcome

In SpA, several tools for assessing disease activity and outcome are widely used in daily clinical practice. The most notably are: the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Bath Ankylosing Spondylitis Functional Index (BASFI) wich are self-administrated questionnaires. BASDAI consists of a questions related to the patients' self-assessment (e.g. fatigue, pain, swelling, axial and pheripheral symptoms, enthesopathy and duration and intensity of morning stiffness). To assess spinal mobility is used the Bath Ankylosing Spondylitis Metrology Index (BASMI) and to assess radiographic damage is used the modified Stroke Ankylosing Spondylitis Spinal Score (mSASSS) [24,25]. mSASS criteria defined the progression of radiographic changes focusing on the development of erosions and syndesmophytes in the lumbar and cervical spine.

Recently, to improve the BASDAI index, ASAS has developed the Ankylosing Spondilytis Disease Activity Score (ASDAS). This score is calculated on the basis of: the spinal pain, the duration of morning stiffness, the overall global assessment and peripheral arthritis plus laboratory assessment of either C-reactive protein (CRP) and

erythrocyte sedimentation rate (ESR) (www.asas-group.org/clinical-instruments/asdas_calculator/asdas.html).

Maastricht Ankylosing Sponylitis Enthesitis Score (MASES) [26] and Disease Activity Score-28 (DAS-28) [27] evaluate the peripheral joint commitment through the count of tender/swollen joints and the evaluation of dactylitis and enthesitis.

The spinal pain can be evaluated with a 10-cm visual analogue scale (VAS) and visual analogue scale of global disease activity (VASg) [28,29].

Other predictors include a history of smoking and elevated levels of inflammatory markers at the baseline [30].

The outcome indexes are important to support the clinicians in the choice of the treatements; recent data have suggested that long-term therapy with TNF-α inhibitors may reduce the rate of development of new lesions especially with early initiation of treatment and longer duration of follow-up [31].

1.1.4 Diagnosis of Spondyloarthritis

SpA diagnosis requires of the combination of assessment of symptoms, physical examination, imaging and laboratory analyses. In rheumatology available diagnostic criteria sets are usually designed and are also used in clinical practice. Diagnostic criteria are applied to individual patients and should be sensitive to the identification of patients at the early stage of the disease. The purpose of the diagnostic criteria is to distinguish "definite" patients who fulfill the criteria set from "probable" or "possible" patients who present only some features of the disease [32]. Early diagnosis and therapeutic intervention are important to modified disease progression and avoid unnecessary diagnostic and therapeutic procedures [33]. In SpA diseases, the presenting symptoms need to be distinguishd from those associated with other rheumatic conditions, moreover the absence of specific diagnostic biomarkers make it difficult to differentiate patients among inflammatory arthropaties group.

In SpA, the absence of rheumatoid factor in serum is a typical feature and distinguish these disorders from rheumatoid arthritis.

Levels of acute phase reactant as CRP or ESR can be higher in AS patients (40-50%), so a normal CRP or ESR does not rule out this condition. Levels of both of these acute phase reactants are higher in patients with AS than in those with nr-ax SpA.

The presence of HLA-B27 is a useful tool for SpA diagnosis and is an integral part of the ASAS criteria for ax-SpA. The prevalence of HLA-B27 vaires among different

ethnic groups, in fact 85-95% of AS white patients has HLA-B27 but only the 6% of HLA-B27 carriers in the general population develop the disease [34]. SpA diagnosis is also supported by imaging techniques to confirm the suspected diagnosis, to define the extent of disease and to monitor potential changes. The use of MRI to assess sacroiliac changes (inflammatory lesions of sacroiliac joints with definite bone marrow oedema and osteitis) is one of the important advance. Plain radiographs can detect only structural changes such as joint erosion and subchondral bone sclerosis seen at the late stage of the disease [4].

The development of early strategies to reduce the diagnostic delay of SpA remains to be investigated.

1.2 Treatment

The treatment goals for SpA include reducing symptoms, improving and maintaining spinal flexibility and normal posture, reducing functional limitation and decreasing the complication associated with the disease. Spa therapy is usually based on different treatment strategies based on main clinical manifestations; non-pharmacological methods (active exercise program) are often combined to drug therapies.

1.2.1 Non steroideal anti-inflammatory drugs

Non steroideal anti-inflammatory drugs (NSAIDs) agents is the first line therapy in patients with AS and PsA due to their good efficacy in reducing back pain and stiffness (48-72h after intake) [35]. There is no clear difference in the effectiveness of the different NSAIDs

In accordance with the severity of symptoms, continuous NSAID treatment is recommended for persistently active symptomatic disease. On-demand treatment is used when continuous treatment causes unacceptable side effects and is recommended for stable SpA patients [36].

Moreover, there is some evidence about the inhibitory effects of NSAIDs on radiographic progression in patients with AS. In a recent randomised multicentre study, Sieper and colleague enrolled and randomized 167 patients with AS for treatment with continuous or on demand diclofenac for two years [37] and demonstrated the efficacy of NSAID in the reduction of new bone formation in on demand group.

In 2016, ASAS-EULAR recommendations suggested the continuous use of NSAIDs if symptoms recur after stopping or reducing the dose therapy. The continuous use of NSAIDs is based on the symptoms of the patients instead of a possible protective effect on structural damage progression [6].

Recent studies demonstrated that there is no difference in the efficacy of different NSAIDs in AS (Indometacin, celecoxib, diclofenac, ketoprofen), the choice of the drugs should be based on patients history, risk factors for side effects and comoribities [38].

1.2.2 Disease modifyng anti rheumatic drugs

Conventional Disease modifyng anti rheumatic drugs (DMARDs) (methotrexate, sulfasalazine, leflunomide) have been shown to be effective for pheripheral arthritis and other extra-articular features such as psoriasis, uveitis, and inflammatory bowel disease

[39-41]. According to the EULAR recommendations, patients with peripheral PsA should be treated with conventional syntetic DMARDs, as methotrexate (MTX) as a first choice, when patients still have active disease despite NSAID therapy. EULAR/ASAS recommendations indicated that in AS patients with peripheral arthritis, sulfasalazine is the treatment option [6, 42]. It is well known that DMARDs are not useful for treating axial involvements, in fact biological drugs should be considered the best choice in patients with ax-SpA or in patients with PsA with persistently high disease activity [5].

1.2.3Biological agents

The use of TNF-α inhibitors is strongly recommended for SpA patients whose symptoms are not controlled by conventional therapy or for whom conventional therapy has unaccettable side effects [36].

Currently, five TNF-α inhibitors (infliximab, etanercept, adalimumab, golimumab and certolizumab) have been approved for clinical used for treatment SpA disease in the EC, USA and most other part of the world. Treatment with TNF-α inhibitors leads to a good improvement in all articular manifestations, CRP levels, MRI-detectable inflammation in sacroiliac joint or spine in patients when conventional treatment failed [43]. TNF-α inhibitors leads to an immediate reduction of disease activity (BASDAI, ASAS), improvement in function and spinal mobility (BASFI), pain reduction, reduction of pheripheral arthritis and enthesistis [44]. Partial or full remission of symptoms could be found in approximately 60% of patients who have an adequate response to these agents. The predictor of good response include: young age, short disease duration, high baseline levels of inflammatory markers and low baseline levels of functional disability, but patients at any disease stage may benefit [36].

TNF-α inhibitors comprise two types of agents: monoclonal antibodies (mAb) (infliximab, adalimumab, golimumab and certolizumab) and soluble receptors (etanercept). All TNF-α inhibitors have the same target but not all of them are equally efficacious in disease treatment (Figure 3).

Infliximab is a chimeric monoclonal IgG1 Ab composed by a human costant domain and murine variable regions. Golimumab and adalimumab are full human Abs that were produced by recombinant technology and certolizumab is a humanized Fab’ fragment coniugated to polyethylene glicole (PEG) to increase the serum half-life. Etanercept is a fusion protein of the extracellular domain of human TNFR2 receptor coupled to the Fc

region without CH1 domain of human IgG1, etanercept binds circulating soluble TNF-α and prevent TNF interaction with the cell surface receptors [45].

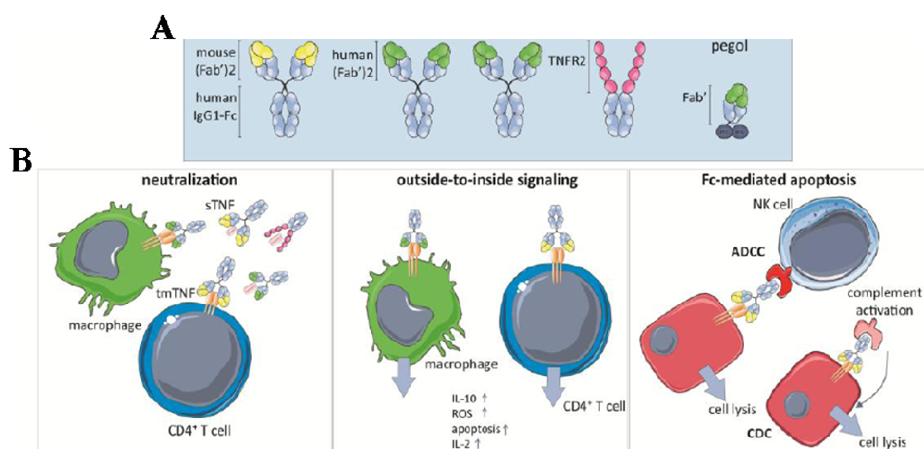


Figure 3. Structure (A) and mechanisms of action (B) of anti-TNF-α agents [45].

The various TNF-α inhibitors seem to be equally potent for the treatment of axial, peripheral and extra-articular features; etanercept is less effective than antibodies in treating anterior uveitis and inflammatory bowel disease and is not preferred in patients with these associated conditions.

Some studies demonstrated that the risk for tubercolosis and possibly herpes zoster might be lower with etanercept than with the monoclonal antibodies infliximab and adalimumab [46-48]. Before the initiaton of TNF-α inhibitors, patients should be tested for the presence of latent or active tubercolosis.

Generally, TNF-α inhibitors were found to be well tolerated and to improve healthy related quality of life but a high number of patients do not respond well to treatment in term to clinical, biological and imaging features that predict good response; primary non- responders patients (13-40%) fail to respond to initial TNF-α inhibitors and up to 50% of the patients lose responsiveness during therapy (secondary non responder) [49].

In case of failure of the first TNF-α inhibitor a second drug where used since many patients respond to different type of anti TNF-α tratments [50,51]. During the last year, new other biological targets have been studied, and in case of failure or toxicity of an TNF-α inhibitors, it is possible to choose other class of biological drugs.

Secukinumab, a monoclonal antibody that targets and neutralises Interleukin-17A (IL-17) showed good results in both PsA and AS patients. Interleukin-17A seems to have a

role in PsA pathogenesis; an increased number of IL-17 producing cells have been found in circulation, joints and psoriatic skin plaques [52].

Different studies have suggested the role of Interleukin-12 (IL-12) and Interleukin-23 (IL-23) in the pathophysiology of PsA. Ustekinumab, is a monoclonal antibody that target and inhibits the activity of both cytokines. Ustekinumab may be a therapeutic options in patients that presented skin and articular involvement [53].

1.3 Psoriatic Arthritis

Psoriatic arthritis (PsA) is a chronic, immune-mediated, inflammatory arthropathy associated with psoriasis and with inflammation of the joints and entheses. Psoriatic disease is characterized by varied clinical features which include enthesitis, dactylitis, nail dystrophy, uveitis and osteitis, in addition to associated comorbidities such as obesity, metabolic syndrome and cardiovascular disease [54]. PsA arises in about 30% of psoriasis patients and in 0.25% of the population [55]. The key feature of psoriatic arthritis is the inflammation of synovial membrane characterised by increased vascularisation and immune cell infiltration [56-58]. The immune cells release proinflammatory mediators that activate fibroblast-like synoviocytes, which then invade cartilage and bone. Further, osteoclast cells mediate bone resorption inducing joint deformity [59].

Traditionally, PsA is classified into five subtypes including: asymmetric oligoarticular pattern (involvement of five or fewer joints); symmetric polyarticular pattern (metacrpophalangeal and proximal interphalangeal joints involvement); distal interphalangeal joint disease pattern; spondyloarthropathy whose presentation is similar to AS and arthritis mutilans which is the most rare and severe form [60]. The prevalence of these specific pattern is variable, the most frequent is oligoarticular pattern even if patients with long term disease develop polyarticular pattern.

Specific features of PsA are dactylitis (inflammation of an entire digit) and enthesisits (inflammation of the connective tissue between tendon or ligament and bone). MRI studies suggest that enthesitis precedes clinical joint involvement although this notion remains controversial [61].

The primary forces driving the complex pathopysiology of PsA are mainly genetic, environmental and immunologic factors.

PsA is characterised by complex genotypes and have a strong genetic component. Based on twin and family studies in European populations the genetic contributions in PsA appear approximately 80-100% for heritability [62].

Compared to the most of the rheumatic diseases, heredity plays a particular important role in this form of SpA.

Initial genetic studies identified the HLA locus on chromosome 6p, in particular HLA-Cw6 locus, the strong determinant of psoriasis and psoriatic arthritis; recent study

published in 2016 indicated that this locus is primarly associated with cutaneous psoriasis [63], and HLA genes might define subphenotypes of PsA.
Results derived from cross-sectional cohrt analysis reported that HLA-B27:05:02 haplotype was positively associated with enthesis, dactylitis and symmetric sacroilitis, while HLA-B*08:01:01-HLA-C*07:01:01 haplotypes were associated with joint fusion, asymmetrical sacroilitis and dactylitis [63].
Several studies have reported an association with PsA and other gene in the HLA region as TNF-α and its promoter and in non-HLA region including IL-1, IL-23R and Tumor necrosis factor alpha-induced protein 3 TNFAIP3 gene [63]. Additionally, major histocompatibility complex class I chain-related A (MICA) alleles are also considered to be associated with PsA [64].
In genetically susceptible subjects, environmental factors, including infections, trauma, stress, obesity and smoking have been implicated in causing psoriatic arthritis. In 24.6 % of patients, the presence of local trauma precedes the development of PsA [65].
Furhermore, small data are available about the role of microbiota in this disease. There is substantial evidence about the role of the involvement of CD-8 positive T cells and the activation of the TNF-α and interleukin 23-Th17 pathways for immune-mediated inflammation in the pathogenesis of psoriatic arthritis.
The diverse range of PsA clinical features prevents its early detection and subsequent therapeutic intervention. Due to similarities to other inflammatory arthritides, the need for a diagnosis during pre-symptomatic and early clinical stages of PsA is well established.
PsA detections, is guided by the criteria outlined in the Classification Criteria for Psoriatic Arthritis (CASPAR) that include both clinical and radiological features [66].
To meet the CASPAR criteria the patients must have inflammatory articular disease (joint, spine or entheseal) and higher than/equal 3 points from the following categories: current psoriasis or personal or family history of psoriasis; psoriatic nail dystrophy on current examination; negative rheumatoid factor; dactylitis (current or on history as recorded by rheumatologist); radiographic evidence of iuxta-articular new bone formation. The sensitivity and the specificity of these criteria are 91.4% and 98.7% respectively.
Despite the advance in molecular technologies, PsA lacks of validated biomarkers for diagnosis, prediction of therapeutic response or remission of the disease. As demonstrated in a multicentre study conducted in a large cohort of Italian PsA patients,

CRP and ESR, the most popular laboratory markers of inflammation, are elevated in only half of the patients [67]. Serum levels of CRP is used as a measure of disease activity in PsA but also in several other inflammatory disease.

Hansson and colleagues, to search better prognostic markers of PsA, showed that serum S100 calcium binding protein S100A8/S100A9 (S-calprotectin) levels were significantly higher in PsA patients and were a better predictor for disease than CRP levels [68].

The investigation of PsA serum proteome showed that several pro-inflammatory mediators are upregulated, including cytokines and chemokines [69].

Significantly higher levels of interleukin-6 (IL-6) were detected in PsA patents respect to patients with cutaneous psoriasis (PsC) [70]. IL-6 correlated with CRP and ESR among PsA patients with joint manifestations and their levels seems to be associated with the disease activity.

Chandran and colleagues discovered more potential diagnostic biomarkers in the serum of PsA patients, they found increased levels of highly sensitive CRP (hs CRP), osteoprotegerin (OPG) and matrix metalloproteinase 3 (MMP-3) [71].

Predictive biomarkers are important for distinguishing patients responders and non responders to target therapy and to avoid unnecessary expenses and risk of adverse events. The recommendations for the management of PsA have been developed by several expert groups and focused the attention at the pharmacological treatments although an optimal management of patients with PsA should also include non pharmacological strategies with patients education and regular exercise. At the international levels, Group for Research and Assessment of Psoriasis and Psoriatic Arthritis (GRAPPA) and EULAR developed the recomandations in 2009 and in 2012 respectively and updates of these recommendations are currently ongoing [72,42].

Both of these sets of recommendations propose non-steroideal anti-inflammatory drugs as first treatment for joint inflammation then, if necessary, introduction of DMARDs such as methotrexate, sulfasalzine and lefunomide, and finally, if inflammation persists introduction of TNF-α inhibitors [72,42].

1.4 Ankylosing Spondylitis

Ankylosing spondylitis (AS), a prototype of the spondyloarthritis family, is a chronic, progressive, inflammatory disease of the axial skeleton that mainly involves the axial spine and sacroiliac joints [73] and can manifest with various clinical signs and symptoms. The characteristic symptoms of AS are spinal stiffness and loss of spinal mobility which originate from spinal inflammation, structural damage or both. Extra-articular manifestations of the disease of AS include acute uveitis, pheripheral arthritis, enthesitis, psoriasis, aortic root and gut inflammation [74]. AS is also associated with an increased risk of cardiovascular disease principally due to the presence of systemic inflammation. AS is typically diagnosed in people aged from 20 to 40 years, about 80% of patients develop first symptoms when they are younger than 30 years. Less than 5% of cases have an onset after 45 years. AS is more common among male patients than female patients and the ratio is 3:1. Due to the insidiously progressive nature of AS, delay between onset of symptoms and diagnosis is up to 8-10 years [75].

Modified New York criteria are used for AS diagnosis, these criteria based their diagnostic performance on clinical symptoms and signs and on the presence of radiographic sacroilitis [10].

Different studies have demonstrated that the delay in the definition of structural damage is mainly due to the late appearance of definite sacroilitis using conventional plain radiographs.

ASAS classification criteria take into account MRI techniques, able to show the presence of active inflammation. ASAS criteria permitted the classification of patients in an early phase of the disease when structural damage is not yet present [15].

The discovery of biomarkers has been growing interest to facilitate early diagnosis of AS and appropriate selection of patients for treatment. Laboratory indices, as CRP and ESR, are generally non specific tool in diagnosis AS. About 50 to 70% of patients with active AS have elevated levels of both indices but normal CRP and ESR, however, not exclude the disease.

In AS patients, the assessment of genetic profile is mandatory for diagnosis, there is a strong and well-established association of HLA-B27 with AS, more than 90% of patients had this haplotype [8].

HLA-B27 is recentely proposed as a part of diagnostic algorithm in the diagnostic process of AS.

Novel molecular pathways that improve the understanding about AS are been discovered. In 2007, a genetic study identified variants among non-HLA genes as a risk factor of AS. Multiple genes are involved in the development and activity of a recently identified population of T helper (Th) cells known Th17 that produced IL-17, in the IL-23/IL-23R pathways, in the influence the activity of the pro-inflammatory transcription factor nuclear factor kappa-light-chain-ehancer of activated B cells (NF-κB) and in the production of other inflammatory mediators [76].

Furthermore, other gene are involved in AS pathogenesis including endoplasmic reticulum aminopeptidase 1 (ERAP 1) that palys central role in peptide trimming, a step required for the generation of HLA peptide [36].

1.5 Pathogenesis of Spondyloarthritis: Genetic factors

SpA is a multifactorial disease and the pathogenesis are not yet fully understood. Genetic factors (HLA-B27 and non HLA-B27 related genes), inflammatory cytokines (TNF-α, IL-1, IL-6, IL-7, IL-17 and IL-23) and environmental factors (infections, mechanical stress, abnormal intestinal microbiota) play an important role [52].The complex interaction of these different factors can cause the activation of autoinflammation and autoimmunity. The onset of SpA typically occurs at young age (usually <45 years), early diagnosis is difficult mainly due to the lack of pathognomonic clinical feature or laboratory test.

The average delay in diagnosis is estimated to be 8-11 years [75].

It is crucial to identify SpA patients and instituite treatments as early as possible after the onset of symptoms and in order to retard the radiological progression of the disease.

Numerous studies in the literature tried to determine the mechanisms of SpA pathogenesis but it is still completely understood. Genetic, immunological and environmental factors play an important role in this disease.

Genetic factors have long been recognized to play an important role in SpA pathogenesis; some familial aggregation studies have estimated that genetic risk factors contribute to 80-90% of the susceptibility to AS [77].

The most studied genetic markers involved in AS and in PsA are located in the major histocompatibility complex (MHC) region but genome-wide association studies (GWAS) identified the genetic contribution of genes located in non MHC region [52].

HLA-B27, is an HLA class I surface protein encoded at the locus B of the MHC, on the short arm of chromosome 6. In 1969 was discovered its serological specificity [78] and four years later its association with SpA and related forms [79]. It was later observed that HLA-B27 is the major genetic risk factor in SpA. This association is present in many genetically diverse populations and across all major HLA-B27 subtypes [4]. HLA-B27 haplotype is present in 80-90% of AS patients and in 50-75% of patients with other SpA subtypes, the mechanisms about this association are partially understood [2].

The HLA-B27 gene consist at least of 132 different alleles coding for 105 protein subtypes (HLA-B*27:01 to HLA-B*27:105). In Mediterranean populations, the most common subtypes associated with AS is HLA-B*27:02, in Chinese population is HLA-B*27:04, in Caucasian is HLA-B*27:05 and in South Asian and Middle Eastern

population is HLA-B*27:07. Two subtypes, HLA-B27*06 and HLA-B27*09, seem to have no disease association [80].

The difference between the proteins encoded by the HLA-B27 alleles associated with SpA and those not associated with the disease included: structural conformation, the protein binding, the thermodynamic stability and the cell surface expression [81].

The main function of HLA-B27 is to present peptides derived from degradation of endogenous proteins to the surface of nucleated cells for recognition by CD8+ T-lymphocytes. The basis of this hypothesis is the presence of the molecular mimicry between intracellular pathogens and self-antigens that may break self-tolerance leading to autoimmunity [82].

Three main theories have been proposed to explain the pathogenic role of HLA-B27 in SpA: the presentation of arthritogenic peptides to autoreactive T lympochytes, the misfolding of HLA-B27 during its biosynetsis in the endoplasmic reticulum (ER) leading to an unfolded-protein response (UPR) and the cell surface HLA-B27 homodimers hypoteses. The arthritogenic-peptide theory, which was formulated in 1990 [83], is the most accepted pathophysiological framework for SpA. It is based on the premise of a molecular mimicry between pathogenic antigens (e.g., intracellular bacteria or an ubiquitous virus) and cartilage/bone-derived self-peptides that activate cytotoxic T lymphocytes (CTLs) after HLA-B27 antigen presentation [80]. This hypothesis has been supported by the finding of autoreactive HLA-B27-restricted CTLs that recognize peptides derived from intracellular bacteria as well as uninfected healthy cells in the synovial fluid of AS patients [84], and by the observation that gastrointestinal or urogenital infections can trigger SpA. The theory has not, however, been entirely corroborated by the identification of an autoimmune arthritogenic peptide [85] or in animal models [86,87]. The other two hypotheses argue in favour of the theory that HLA-B27 plays an autoinflammatory role in triggering the innate immune responses [52]. The misfolded HLA-B27 heavy chains tend to accumulate in the endoplasmic reticulum (ER), triggering ER stress, which leads to the activation of the unfolded protein response and the NF-κB pathway which, in turn, leads to the release of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, mainly by monocytes/macrophages, thus favouring the inflammatory process [88]. Animal disease models argue both in favour and against this hypothesis [89,90]. The cell surface HLA-B27 homodimers hypothesis is, instead, based on the observation that HLA-B27 homodimers produced at the cell surface bind to specific receptors expressed on NK cells, T-lymphocytes, and

myelomonotic cells producing an immunomodulatory effect [91]. This hypothesis has been supported by the finding that the number of NK and $CD4^+$ T-cells expressing a receptor that recognizes HLA-B27 homodimers but not heterodimers is higher in HLA-B27 positive patients [92].

HLA-B27 plays a critical role in the disease pathogenesis, recent studies suggest that it only accounts for 20-25% of the total heritability and 40% of the genetic risk. Fewer that 5% of HLA-B27 carriers in the general population develop SpA disease [93,94]. Other than HLA-B27 other MHC genes are involved in SpA: HLA-Cw6 is the most frequent allele associated to psoriasis, it is associated with more severe and early onset of both psoriasis and PsA [95]; HLA-B (B*40:01) appear to be associated with AS [96]. GWAS association studies have identified several SNPs in non-MHC genes strongly associated with SpA: endoplasmic reticulum aminopeptidase 1 (ERAP1), interleukin 23 receptor (IL-23R), tumor necrosis factor (TNF) receptor 1 (TNFSF1A), the signalling molecule TNF receptor 1 associated death domain preotein (TRADD), the TNF superfamily cytokine (TNFSF15), interleukin 1 alpha (IL-1A), interleukin 1 receptor 2 (IL-1R2), the vascular morphogenesis protein gene anthrax toxin receptor 2 (ANTXR2) and the innate immune receptor caspase recruitment domain family member 9 (CARD9) [97]. Other genes such as Familial Mediterranean Fever gene (MEFV) and signal transducer and activator of transcription 3 (STAT3) need additional confirmation.

1.6 Phatogenesis of Spondyloarthritis: autoimmune or autoinflammatory

Immune-mediated inflammatory diseases (IMIDs) are characterized by a continuum ranging from autoimmunity to autoinflammation where innate immune response is activated by specific tissue triggers, such as micro-organisms or microtrauma [98].

Spa are considered to be polygenic IMID characterized by inflammation of the spine and peripheral joints, extra-articular manifestations and is not associated with typical autoimmune genes but displays polymorphisms in genes involved in innate immune recognition (CARD9) and cytokine signalling pathways (TNF-α, IL-1 and IL-23/IL-17 axis) [99-102].

Spa disease is not associated with disease specific autoantibodies and does not show a female predominance, thus unmetting the classical features of autoimmune diseases.

In the past, some studies have considered the SpA pathogenesis (AS in particular) with an adaptive immune response involvements; however in a recent view SpA is classified as a autoimmune disease where innate immune system play major role[103].

Clinical trials with B and T cell targeted therapies (abatacept, alefacept, efalizumab and rituximab) showed very modest therapeutic efficacy in SpA pointing out that the lack of classical autoimmune features explain the hypothesis that adaptive immune response are not primary in SpA [104].

Due to the autoinflammatory origin of SpA, some studies have pointed the role of bacterial and local mechanical stress as triggers in the pathogenesis of the disease. Both factors are able to activate the innate immune responses. Genome-wide association studies and copy number variation (CNV) analyses identified SpA susceptibility genes that encode the components of innate immune system [94,105].

This evidence and the good clinical efficacy of the innate cytokine blockers suggest the contribution of innate immune system in SpA pathogenesis [106].

The activation and the regulation of the immune response are mediated by the cells of the innate immune system through the release of effector proteins know as cytokine such as IL-1β, TNF-α and other related members of this family. The IL-1 family contains nine genes with high sequence homology and the most studied members are IL-1α and IL-1β which are potent pro-inflammatory cytokines. IL-1β and TNF-α are produced by various types of immune cells; IL-1β can be secreted by all nucleated cells

especially macrophages that recruit other immune cells to produce inflammation while TNF-α are produced by macrophages and T cells [104].

An important cytokine with a broad regulatory role in the immune system is transforming growth factor-β (TGF-β). TGF-β is produced by and has effects on many different cells of the immune system, and plays fundamental roles in the regulation of immune responses during homeostasis, infection and disease.

1.6.1 IL-1β

Among the IL-1 family members, IL-1β seems to be the primary therapeutic target for a large number of inflammatory conditions. IL-1β is secreted as inactive precursor that is cleaved by Caspase-1 via inflammasome into an active cytokine [107]. IL-1β binds type I (IL-1RI) and type II (IL-1RII) specific receptor. IL-1RI is the main functional receptor for IL-1 signalling and after IL-1 binding undergoes a conformational change required for the recruitment of downstream signalling molecules that lead to activation of many transcription factors, such as NF-κB, AP-1, c-jun N-terminal kinase (JNK) and p38 MAPK (Figure 4) [107,108].

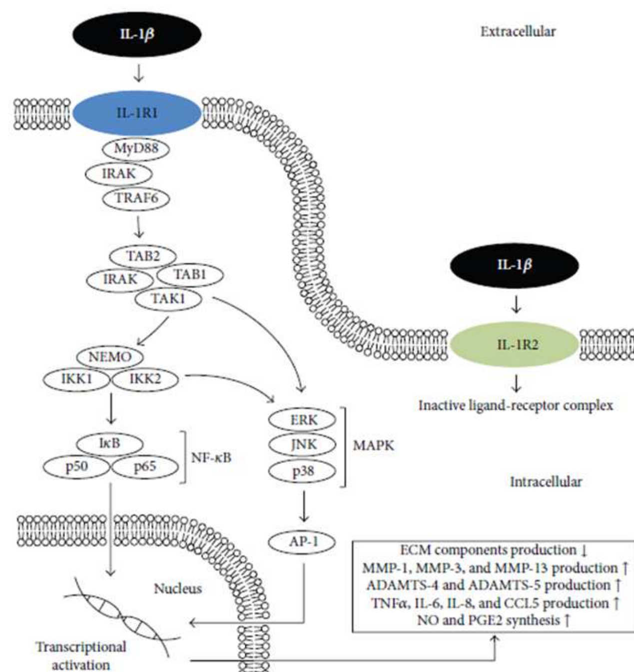
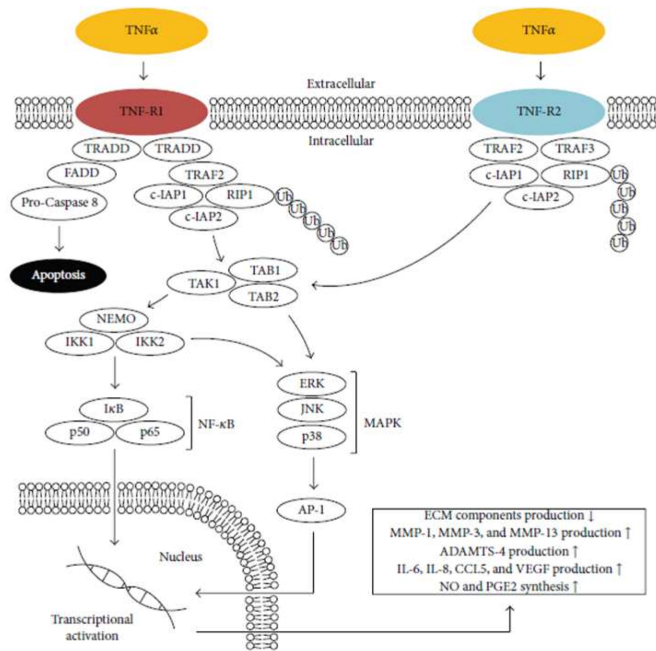


Figure 4. IL-1β associated intracellular signaling pathways and downstream cellular targets and effects [109]

In animals model of arthritis both IL-1α and IL-1β can activate bone resorption osteoclast cells and induce other enzymes involved in joint destruction [110]. In vitro and in vivo models demonstrated that IL-1β is a strong stimulator of bone resorption because it upregulates the production of Receptor activator of nuclear factor kappa-B ligand (RANKL) enhancing its activity and stimulating osteoclastogenesis [111].
RANKL is the main cytokine involved in osteoclastogenesis and is a member of the membrane associated TNF ligand family, The RANKL-RANK receptor interaction stimulates several transcription factors that modulate osteoclast differentiation, activation, and survival thus leading the bone resorption [112]. In the inflammatory osteoclastogenesis IL-1β and TNF-α have an intimate relationship where many effects of TNF-α are upregulated by IL-1β. Experimental studies showed that blocking both IL-1β and TNF-α cause a total arrest of bone resorption [112-114]. AS patients are characterized by an association of new bone formation and an increased bone resorption; these patients have an increased osteoporosis and vertebral fractures compared to healthy controls [115,116]. The inflammatory process and cytokine production are the main cause of osteoporosis in AS patients. Only few data are available regarding the efficacy of anti IL-1β in the treatment of AS; Anakira (IL-1R antagonist) was tested in a small prospective open-label trials in AS patients and did not have a better effect than an expected placebo response. In this study, anakira was not able to induce any improvement of sign and symptoms of AS and this study did not evaluate the bone loss or the bone new formation [117].

1.6.2 TNF-α

TNF-α is a pleiotropic pro-inflammatory cytokine that play an essential role during the inflammatory response inducing the upregulation of adhesive molecules, lymphocyte activation, fibroblast proliferation and the production of other pro-inflammatory cytokines [118]. TNF-α is primarly produced as a transmembrane form (tmTNF) that can be cleaved by the TNF-α converting enzyme (TACE) into a soluble homotrimeric cytokine (sTNF). TNF-α exert its biological activities by binding two types of receptor: TNF receptor type I (TNFRI) and type II (TNFRII) (Figure 5).



Figue 5. TNF-α associated intracellular signaling pathways and downstream cellular targets and effects [109].

These receptors bind with different affinities the sTNF-α and tmTNF-α; TNFRI (encoded by *TNFRSF1A* gene) binds both forms equally well and is the main receptor that mediates the proinflammatory response of TNF while TNFRII has a higher affinity for the tmTNF.

The expression of multiple cytokine and immunoregulatory molecules are induced by the activation of both receptors [45].

TNF-α is implicated in the pathogenesis of SpA, the analysis of sacroiliac biopsies have indicated the increases number of TNF expressing macrophages in AS patients [119].

Some studies have shown increased levels of TNF-α in blood of AS patients compared to subject with non-inflammatory back-pain or to healthy controls [120,121].

TNF-α is encoded by *TNFA* gene which is located within the class III region of the MHC on chromosome 6 between the HLA-B and HLA-DR genes [122].

Among the mechanisms that regulate TNF-α expression, several polymorphisms have been identified within the TNF promoter region relative to the transcription start site: -1031 (T>C), 836 (C>A), -857 (C>T), -851 (C>T), -419(G>C), -376 (G>A), -308 (G>A), -238 (G>A), -162 (G>A), and -49 (G>A). These variable regions have also been

shown to influence the differential TNF transcription and protein production between individuals [123,124].

The association between polymorphisms in the *TNFA* promoter region and SpA disease have been reported in different studies and different populations but the role of these polymorphisms in SpA patients is still unclear. Most studies have focused on Adenine (A) to Guanosine (G) transition at position -308 [125] and -238 [126].

The decrease frequency of -238A and -308A alleles (high TNF-α secreting polymorphisms) found in some population have not been confirmed in all population-based studies and meta-analyses. Two meta-analyses have detected no association between polymorphisms and AS susceptibility except for the AA genotype at -238 position [127,128]. Other studies demonstrated an association of -308 G>A polymorphism with AS susceptibility [129,130]. Moreover, -308A allele has a protective role against AS and was associated with lower disease activity and severity of the disease [131].

1.6.3 TGF-β

TGF-β is a potent cytokine and growth factor belonging to TGF-β superfamily together with bone morphogenetic proteins (BMPs), activins, nodal, and growth differentiation factors (GDFs) [132]. TGF-β exerts its pleiotropic action in multiple biological processes such as embryonic development, immune responses, inflammation and repair, cell proliferation and differentiation [133,134]. TGF-β is transcribed and translated as a small latent complex composed of active TGF-β and latency-associated peptide (LAP) which prevents the interaction of TGF-β with its specific receptor on the cell surface [135]. The TGF-β receptor is a tetrameric cell surface complex consisting of two transmembrane serine-threonine kinase pairs, type I (TβRI) and type II (TβRII) receptor kinase. Interaction of TGF-β with this receptor on target cells induces a intracellular signalling cascade by the phosphorylation of cytosolic Smad 2 and Smad 3 proteins [136,137]. Smad 2/3 forms a trimeric complex with Smad4 which then translocates into the nucleus where it binds SMAD-binding elements and recruits co-transcriptional factors to transactivate or repress target genes [138]. Smad 7, an inhibitory Smad, is induced by Smad2/3 complex as a feedback regulatory mechanism. In addition, TGF-β can activate numerous Smad independent signalling pathways such as the ERK, p38 and JNK mitogen-activated protein kinases, Rho-like GTPase and phosphatidylinositol-3-

kinase/AKT pathways [139]. In the autoimmune inflammatory diseases the TGF-β effects are complex and difficult to predict also due to the clinical application of TGF-β inhibitors [140]. Several studies have demonstrated the presence of TGF-β in synovial tissue and synovial fluid of RA patients and higher expression of TβRII in rheumatoid synovial fibroblast [141,142].

Other study detected, in rheumatoid synovial fibroblast, an increased expression of TGF-β and TβRII. This expression correlates positively with clinical markers of disease activity depicted a strong correlation with TGF-β and inflammation [143].

1.7 Phatogenesis of Spondyloarthritis:Calprotectin (S100A8/S100A9)

Calprotectin is a member of the S100 protein family and is secreted primarly by neutrophilic granulocytes, monocytes and macrophages. Calprotectin is a calcium and zinc binding protein and is a heterocomplex composed by two different proteins S100A8 and S100A9 (MRP14/MRP8 or calgranulin A/B) [144], encoded by the S100A8/S100A9 gene located on chromosome 1q21 [145]. The presence of calcium induces conformational changes in the heterodimer, thereby allowing the binding of other proteins. Furthermore in the presence of calcium, MRP8/14 heterodimeric complexes may tetramerize into heterotetramers [146].

The zinc-binding domains are involved in the antibacterial activity of calprotectin [147]. The concentration of calprotectin in neutrophils is abundant and constitutes about half (30-60 %) of total cytosolic protein [148,149]; conversely calprotectin is not usually present in lymphocytes [148].

Calprotectin is also an important mediator of many regulatory functions such as chemotactic activity, deactivation of macrophages and inhibition of immunoglobulin synthesis. Elevated levels of calprotectin have been identified at sites of inflammation and in the extracellular fluid in patients with RA, cystic fibrosis, Sjogren's syndrome and abscesses [149]. In experimental antigen-induced arthritis, MRP8 and MRP14 significantly contribute to joint inflammation and leucocyte infiltration [150-153].

The concentration of calprotectin is known to be increased in plasma of patients with RA respect to those found in healthy individuals. Furthermore calprotectin was reported to be alevated in serum [154] and feces [155] and is an independent marker for radiographic spinal progression in axSpA [156].

Contradictory results have been found on the expression of calprotectin in SpA. Calprotectin was reported to be elevated in SpA as compared to the healthy controls and decrease rapidly and consistently upon effective treatment [154] while in another work, no differences in serum calprotectin between AS patients and healthy controls were found [155].

Huang and colleagues, demonstrated that calprotectin was to be elevated in both the nr-axSpA and AS patients compared to healthy controls and correlated with CRP, ESR BASDAI and ASDAS indicating that it is a marker for inflammation signal. There is no difference between the nr-axSpA and AS patients in calprotectin levels [157]. Baseline calprotectin was higher in patients with more radiographic spinal progression, was

decrease upon tratement with TNF-α inhibitors and may represent a biomarker for clinical response [158,159].
High levels of calprotectin have been found in synovial fluid from patients with PsA and RA respect to healthy controls [160].
One of the most extra articular manifestations in SpA patients is IBD and fecal calprotectin was frequently used in the assessment of disease activity, response to treatment prediction of disease relapse or postoperative recurrence in IBD [161]. Elevated levels of fecal calprotectin at baseline in AS patients was the strongest predictor of the development of IBD, then calprotectin could be a potential biomarker to identified patients with AS at risk of developing IBD.

1.8 Phatogenesis of Spondyloarthritis:Matrix Metalloproteinases

Metalloproteinases belong to a superfamily of zinc-dependent proteases known as metzincins. The superfamily of zinc-dependent proteases includes also: adamlysins (ADAMs), ADAMs with thrombospondin-like motifs (ADAMTSs), astacins, serralysins and pappalysins [162]. The term “matrix metalloproteinase” (MMP) defines these enzymes emphasizing the dependence of their activity on metal ions and their ability to degrade the structural proteins of the extracellular matrix. The regulated turnover of the molecules that compose the ECM is crucial for the interaction of individual cells with the surrounding environment, for proper physiological function, and for the development of the multicellular organisms [163]. The ECM present in the synovial membrane and cartilage is a key component of the joints. Far from being an inert structure, it undergoes continuous re-modeling which, in a homeostatic equilibrium, guarantees joints’ integrity. The modulation of cartilage re-modeling in SpA patients is due to inflammatory conditions where monocytes and macrophages release inflammatory molecules TNF-α, IL-1 and IL-6. The inflammatory mediators also induce the production of MMPs, the main enzymes involved in extra-cellular matrix degradation (Figure 6).

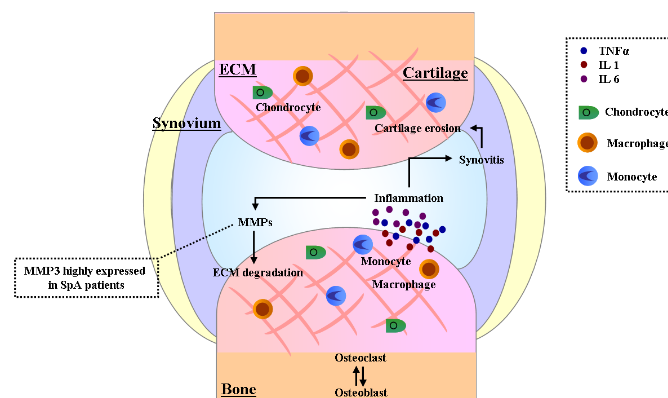


Figure 6. Cartilage re-modeling in SpA pathogenesis [164].

We have recently demonstrated that metalloproteinases, MMP8 and MMP9 in particular, are produced by peripheral blood mononuclear cells if they are stimulated by calprotectin, the S100A8/S100A9 heterodimer [165]. This finding seems potentially relevant to the pathophysiology of arthritis since peripheral blood mononuclear cells represent a dynamic cellular population that can infiltrate the inflamed tissues where

they differentiate into inflammatory macrophages and thereby contribute to sustaining the inflammatory process. It has been reported that MMPs, and MMP3 in particular, are produced in response to cytokines in the joints, being more highly expressed in the synovial tissues of SpA patients than in peripheral blood mononuclear cells [166]. In view of these findings, studies have been performed in order to verify if serum MMPs level assessment can be used as a biomarker for diagnosing SpA, assessing disease severity, and predicting response to therapy with TNF-α inhibitors. The majority of studies focusing on MMP3 are in agreement with the finding of higher serum levels in SpA, in both AS and PsA subtypes, with respect to those in healthy controls, and these results are confirmed by a recent meta-analysis which demonstrated that increased serum MMP3 levels are associated to higher AS risk. However, the serum MMP3 levels related to the development and progression of AS were found to be conditioned by different geographical and genetic factors. In fact, at the ethnicity-stratified analysis, the MMP3 levels were higher in Asians and Caucasians than in African AS patients [167]. Although higher MMP3 baseline values appear to be correlated with the severity of SpA assessed biochemically (CRP) or using the BASDAI or BASFI severity indices, and seem to suggest peripheral joint involvement, the correlation between MMP3 serum levels and disease activity indexes is sometimes contrastive [164]. MMP3 together with CTX-II (C-terminal cross-linking telopeptide of type II collagen) seem to be particularly useful to predict radiographic progression of AS patients treated conventionally, outperforming over baseline CRP levels and BASDAI [168]. Besides MM3, MMP1, MMP2, MMP8 and MMP9 seem to be correlated with CRP in AS patients and MMP8 and MMP9 seem to have a stronger association with BASDAI [169].

1.9 Pathogeneisis of Spondyloarthritis: Moncytes calcium-sensing cells

SpA presents with multiple manifestations of inflammation that are due to complex causes. Many different cell components are involved in the development of inflammation, including neutrophils, mastocytes, T and B lymphocytes, and monocytes/macrophages. Activation of these cells leads to the production of cytokines and mediators responsible for inflammation. TNF-α has been shown to be the master element of inflammation in SpA.

Monocyte cells are considered as a precursor for macrophages and dendrtic cells, moreover, monocytes are made of numerous subpopulations with distinct functions according to molecules expressed at their cell curface. Furthermore monocyte cells are able to differentiate in different cell type depending upon the stimuli they recived [170,171].

Considering the role that monocytes have in chronic inflammatory disease, recent studies emphasized that monocytes could be interesting therapeutic targets [172,173].

As well as in SpA, monocyte recruitment contributes to the pathogenesis of various other inflammatory disease such as chronic inflammatory rheumatism and RA in wich circulating monocytes recruitment in the joint could trigger inflammatory cascade [174].

The physiological functions of monocyte cells depend on calcium (Ca^{2+}) signals; monocyte can detect variations of extracellular calcium through the calcium-sensing receptor (CaSR) activation. This process could induce the chemotactic response of monocytes to calcium concentrations in different sites of bone resorption or inflammatory sites [174]. At the intracellular levels Ca is a highly versatile signal that can regulate different cellular functions

The Ca^{2+} signaling system operate in many different ways to regulate cellular processes that function over a wide dynamic range. Ca^{2+} play an important role in signal transduction pathways, where they act as a second messenger, in neurotransmitter release from neurons, in contraction of all muscle cell types, and in fertilization. Many enzymes require Ca^{2+} ions as a cofactor, those of the blood-clotting cascade being notable examples. Extracellular Ca^{2+} is also important for maintaining the potential difference across excitable cell membranes, as well as proper bone formation [175]. At any moment in time, the level of intracellular Ca^{2+} is determined by a balance between the “on” and “off” reactions (Figure 7) and cell survival is dependent on Ca^{2+} homeostasis, whereby the Ca^{2+} fluxes during the “off” reactions exactly match those

during the "on" reactions. Ca^{2+} that enters the cell from the outside is a principal source of signal Ca^{2+} during the "on" reaction. Cells use this external source of signal Ca^{2+} by activating various entry channels with widely different properties

During the "off" reactions Ca^{2+} leaves the effectors and buffers and is removed from the cells by various exchangers and pumps.

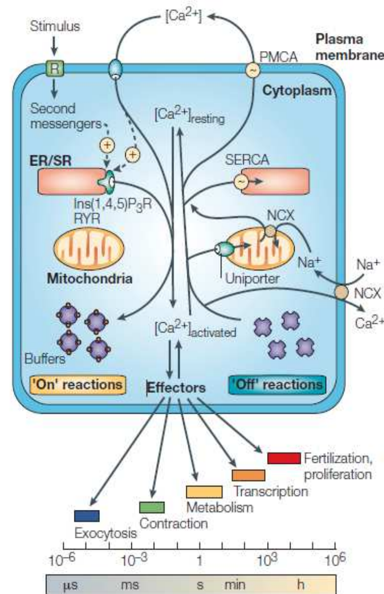


Figure 7. Calcium-signaling dynamics and homeostasis [175].

Ca^{2+} signals are highly organized in time and space in the form of transients, oscillations and cell-wide waves [176]. This array of Ca^{2+} signal modalities is thought to underlie regulation of physiological processes through effector specificity. These findings underscore the physiological importance of the spatial and temporal diversity of Ca^{2+} signaling and reveal the need for statistical analysis tools that encompass this diversity.

Relative cellular Ca^{2+} levels are commonly measured as time lapse image sequences using fluorescent Ca^{2+} indicators, such as Fluo-4 that responds to Ca^{2+} binding with an increase of fluorescence intensity but not spectral shift.

Fluo-4 is a fluorescent dye for quantifying cellular Ca^{2+} concentrations in the 100nM to 1µM range. It was synthesized by substituting two fluorines for the two chlorine substituents in the fluorophore. It is easy to use and it can be loaded into cells either in membrane-soluble AM (acetoxymethyl) ester form or in salt form. Fluo-4 exhibits high Ca^{2+}-dependent fluorescence enhancement, and a dissociation costant for Ca^{2+} ($Kd(Ca^{2+})$) of 345 nM, making it well suited for measuring physiologically important

Ca^{2+} changes in a wide range of cells. The excitation and emission wavelengths for Fluo-4 are 488 nm and 510nm respectively, making it suitable for use in flow cytometry, confocal laser scanning microscopy, microplate screening assays, or light microscopy when used in conjunction with standard fluorescein filter sets [177].

The time lapse image sequences which are obtained by Fluo-4 dye are often analyzed with user-defined Regions of Interest (ROIs) at sites determined to have fluctuations in fluorescence intensity, and the mean intensity within each ROI is measured as a function of time.

2. AIMS

Spondiloarthritis is a family of chronic rheumatic disease predominantly affecting the axial skeleton, but also charachterized by the involvement of peripheral joints and tissues/organs outside the skeleton. The symptoms at the onset of disease, are not specific and this often determine a delay in diagnosis. The diagnostic process is often laborious because of the absence of pathognomonic clinical and/or laboratory findings. Diagnosis is supported by imaging and unfortunately not enough by biochemistry. The first aim of this study was to verify whether biochemical markers commonly used in clinical practice and the mRNA expression levels in mononuclear cells of inflammatory cytokines, calcium binding proteins and matrix metalloproteinases could be associated with the presence of SpA (AS or PsA), and/or could be predicting anti-TNF-α treatment failure.

The second aim is to investigate the role of circulating monocytes and of their intracellular calcium oscillations and if monocytes maintain or not their characteristic pattern of intracellular calcium behaviour when SpA is present.

Specific aims were to investigate whether diagnosis of SpA (AS or PsA) and failure of response to anti-TNF-α drugs are associated with the followings markers:

- Specific haematological and biochemical pattern of inflammation considering ESR and complete blood count, CRP, prealbumin, ALT, glucose, creatinine and uric acid;
- mRNA expression levels in mononuclear cells of inflammatory cytokine (TNF-α, IL-1β, TGF-β), S100 calcium binding protein (S100A8 and S100A9) and matrix metalloproteinases (MMP3, MMP8,MMP9);
- Five SNPs (−1031 T>C, −857 C>T, −376 G>A, −308 G>A, −238 G>A), in the promoter region of TNFA, evaluated singely or as haplotypes;
- Variations of intracellular calcium oscillations in circulating monocyte cells.

3. MATERIALS AND METHODS

3.1 Sudied population

In this study, was enrolled a cohort of 164 subjects belonging to the Veneto Region, a North-East Italian region, from January 2016 to December 2017.

The cohort comprised 100 healthy controls (58 males and 42 females; mean age±standard deviation: 46.6±8.5) attending the Department of Transfusion Medicine University-Hospital of Padova (Italy) and 64 patients (39 males and 25 females; mean age±standard deviation: 39.5±13.2 years) affected by SpA and attending the Rheumatology Unit of the Department of Medicine-University-Hospital of Padova (Italy).

Among patients, 26 (40.6%) had a definite diagnosis of AS, according to the modified New York criteria [10] and 38 (59.3%) had a diagnosis of PsA according to the CASPAR criteria [67]. The mean disease duration was 14 years (range 2–38 years).

At enrollment, 57 patients (89%) were under therapy with anti- TNF-α agents (8 with Infliximab, 21 with Adalimumab, 20 with Etanercept, 4 with Golimumab, 3 with Ustekinumab and 1 with Secukinumab), of which 8 (14%) experienced in their clinical history, at least one or more switch of TNF-α inhibitor before entering the study. Among patients treated with anti- TNF-α agents, two patients had combined therapy with DMARDs and 7 patients were treated with DMARDs only. A subset of 8 patients started their therapy with anti-TNF-α agent at enrollment; for these patients a second sample was obtained after six months from the start of therapy.

Patients were followed for at least 12 months after the beginning the anti-TNF-α agent. At least 10 months of treatment were considered to define a delayed response to TNF-α inhibitors.

All patients patients gave fully informed written consent for the study, which was approved by the Local Institutional Ethic Committee ("Comitato Etico per la Sperimentazione, Azienda Ospedaliera di Padova", protocol number: 3024P/13).

3.2 Clinical Assessment

All subjects attending the study were questioned about demographic (gender, age, place of birth, weight, height) and physiological (smoking, alcohol use) data.

Medical (age at diagnosis, laboratory tests at diagnosis, disease duration, axial/peripheral involvement, enthesitis, inflammatory back pain, articular and extra-articular manifestations, pre-existing/current drug therapy) and family history (arthritis, psoriasis, other rheumatic diseases) data were also collected in SpA patients.The disease activity and physical functioning of patients were assessed with self-reported questionnaires: BASDAI, BASFI and ASDAS indexes. BASDAI includes six questions useful to investigate the intensity of pain; BASFI includes ten questions which explore the degree of difficulty encountered in carrying out specific activities of daily life and ASDAS is a composite index comprising subjective measures and laboratory parameters. The functional statement was also investigated with HAQ questionnaire which comprises 20 queastions about daily living activities. The axial involvement was investigated through the BASMI that indicates a condition that ranges from normal to the severely restricted spinal mobility (from 0 o 10 score). DAS index was used for the evaluation of the peripheral joint commitment through the count of tender/swollen joints and the evaluation of dactylitis and enthesistis.

3.3 Haematological and biochemical indices

For SpA patients and healthy donors, six blood samples were collected: two EDTA-K_2 tubes (6 ml blood each),one for mRNA expression analyses and the other one for monocyte cells isolations, two EDTA-K_2 tubes (3 ml blood each), one of them for the determination of ESR and the complete blood count, the other one for genetic analyses; one lithium-heparin tube (3 ml blood) for the determination in plasma of glucose, uric acid, creatinine and alanine transaminase (ALT); one whole blood sample (4 ml) for the determination in serum of CRP and prealbumin.

Complete blood count was performed using Sysmex XE-2100 analyser (Dasit, Milan, Italy) based on fluorescence flow cytometry analysis technology.

TEST1 analyser (Alifax, SIRE Analytical System, Udine, Italy) which adopts a method based on capillary photometry-kinetic technology was used to ESR determination.

Glucose, uric acid, creatinine and ALT levels were determined on Cobas 8000 modular analyser (Roche Diagnostics, USA) through the enzymatic- colorimetric assays widely used in routine testing and in particular: glucose: hexokinase method; creatinine: the Jaffé method; uric acid: uricase-peroxidase method; ALT: pyridoxal- 5-phosphate

method, according to procedure released by International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).
CRP and prealbumin were determined by immunometric assays on Dimension Vista 1500 System (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA).

3.4 Isolation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-K_2 peripheral blood from each enrolled subject by differential density gradient centrifugation (Histopaque®-1077, Sigma-Aldrich, Milano, Italy, F/H). This method is based on the different density of mononuclear cells compared to the other blood elements: PBMCs and platelets are concentrated over the layer of F/H while red blood cells (RBC) and granulocytes, characterized by an increased density compared to F/H, are collected on the bottom of the tube. After being the cells were washed twice with saline solution 1% phosphate buffered saline (PBS) (Euroclone, MI, Italy) supplemented with 2% fetal calf serum (FCS) (Thermo Fisher Scientific, Waltham, MA, USA) to remove contaminating platelets and centrifuged at 1,200 rpm for 10 minutes. PBMCs were then treated with a hemolysis solution (NH4Cl, KHCO3, EDTA Na4) for 10 minutes in order to remove RBCs cells, centrifuged at 1,200 rpm for 10 minutes and used for the RNA extraction.

3.5 Nucleic acids isolation

Total RNA was extracted from PBMCs using High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The extraction method generate high-purity template and is based on the use of affinity spin columns. RNA was eluted in 60 µl of elution provided buffer. RNA concentration and purity were assessed for each sample by spectrophotometric measurement of the absorbance at the wavelenght of 260 nm and 280 nm (Nanodrop spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). RNA concentration was 116.43±68.92 ng/µl (mean±standard deviation) with a purity, calculated as 260/280 nm ratio, of 2.31±2.06 (mean±standard deviation).
Five hundred ng of total RNA were reverse transcribed into cDNA (Random primers and Superscript TM II RNasiH-Reverse Trascriptase, Waltham, MA, USA).

Genomic DNA, was extracted from 200 µl EDTA-K_2 peripheral blood using the MagNA Pure96 System (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer instructions. The extraction method is based on the binding affinity of the DNA to the glass (silica) surface of Magnetic Glass Particles. DNA was eluted in 100 µl of elution provided buffer. DNA concentration and purity were assessed for each sample by spectrophotometric measurement of the absorbance at the wavelenght of 260 nm and 280 nm (Nanodrop spectrophotometer, Thermo Fisher Scientific, Waltham, MA USA). DNA concentration was 72.7±17.8 ng/µl (mean±standard deviation) with a purity, calculated as 260/280 nm ratio, of 1.86±0.13 (mean±standard deviation).

3.6 Relative quantification analysis

Relative quantification of S100A8, S100A9, MMP3, MMP8, MMP9, TNF-α, IL-1β and TGF-β1 genes were undertaken by Realt-Time PCR (qPCR) with ABI Prism 7900 HT (Thermo Fisher Scientific, Waltham, MA USA). Primers and fluorogenic probes for S100A8 (exon boundary: exon 1/exon 2), S100A9 (exon boundary: exon 1/exon 2), MMP8 (exon boundary: exon 5/exon 6) and MMP9 (exon boundary: exon 2/exon 3) analyses are reported in Table 2, 200 nM probe and 900 nM (S100A8 and S100A9) or 500 nM (MMP8 and MMP9) each primer were used. Relative quantification of TNF-α, IL-1β and TGF-β1 and MMP3 was undertaken using the primers and probe set supplied by Thermo Fisher Scientific (Waltham, MA,USA, catalogue number 4331182, assay ID Hs00174128_m1 (TNF-α), Hs01555410_m1 (IL-1β), Hs00998133_m1 (TGF-β1), Hs00968305_m1(MMP3)). Relative quantifications were performed in a final volume of 20 uL with 33 ng cDNA. The reference gene, HPRT1 (Hypoxanthine-guanine phosphoribosyltransferase), was selected according to the method commonly used for internal control for quantitative gene expression analyses, and its expression was determined by commercially available HPRT1 primers and probe sets (Part Number 4326321E, Thermo Fisher Scientific, Waltham, MA USA). S100A8, S100A9, MMP3, MMP8, MMP9, TNF-α, IL-1β and TGF-β1 and HPRT1 were analyzed in duplicate for each sample. PCR was run at 2 minutes at 50°C, 10 min at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 min at 60 °C. To determine the relative mRNA expression levels of all selected genes we used the comparative Ct method, a mathematical model that calculates changes in gene expression as a relative fold difference between an

experimental and a pool derived from 20 healthy blood donors used as calibrator sample.

Gene	Primers [Final concentration for S100s: 900nM] [Final concentration for MMPs: 500nM]	Probe [Final concentration:200nM]
S100A8	F: 5'CTCTTGTCAGCTGTCTTTCAGAAGA3', R:5'TTTCTCCAGCTCGGTCAACA3',	5'-FAM-AAGTCCGTGGGCATC-MGB3'
S100A9	F: 5'CTCTTGTCAGCTGTCTTTCAGAAGA3', R: 5'TTTCTCCAGCTCGGTCAACA3'	5'-FAM-AAGTCCGTGGGCATC-MGB3'
MMP8	F: 5'CACTCCCTCAAGATGACATCGA3' R:5'ACGGAGTGTGGTGATAGCATCA3'	5'FAM-CAAGCAACCCTATCCAACCTACTGGACCAA-TAMRA3'
MMP9	F: 5'CCTGGGCAGATTCCAAACCT3' R: 5'GCAAGTCTTCCGAGTAGTTTTGGAT3'	5'FAM-CTCAAGTGGCACCACCACAACATCACC-MGB3'

Table 2. Primers and fluorogenic probes for S100A8, S100A9, MMP8 and MMP9 analysis.

3.7 Genetic analysis

3.7.1 TNFA gene analysis

The genotype discrimination of five TNFA SNPs (-1031T>C, rs1799964; -857C>T, rs1799724; -376G>A, rs1800750; -308G>A, rs1800629; -238G>A, rs361525) was performed by a TaqMan dual probes allelic discrimination assay. Amplification was carried out with Realt-Time PCR instrument ABI Prism 7900 HT (Thermo Fisher Scientific, Waltham, MA USA).

50 ng of genomic DNA were amplified in a reaction mix containing primers and probes at a final concentration specified in Table 2 and 1X TaqMan Universal Master Mix (Thermo Fisher Scientific, Waltham, MA USA), in a final volume of 20 µl.

Primers and probes sequences and thermocycling conditions were shown in Table 3.

TNFA SNPs	**Primers, [Final concentration, nM]**	**Probes, [Final concentration, nM]**	**Thermocycling Conditions**
-1031T>C rs1799964	F:5'-AGGTGAGGCCGCCAGACT-3', [900] R:5'-TCCTCAGAGCCGCTACATGTG-3', [900]	FAM:AGCTGAGAAGACGAAG (C), [200] VIC:AGCTGAGAAGATGAAG (T), [150]	50 °C 2 min,□ 95 °C 10 min,□ 40 cycles 95°C 15s, 62 °C 1 min
-857C>T rs1799724	F:5'-GGTCCTGGAGGCTCTTTCACT-3', [50] R:5'-AGAATGTCCAGGGCTATGAAAGTC-3', [900]	FAM:CCCTGTCTTCGTTAAG (C), [200] VIC:CCCTGTCTTCATTAAG (T), [100]	50 °C 2 min,□ 95 °C 10 min,□ 40 cycles 95°C 15s, 60 °C 1 min
-376G>A rs1800750	F:5'-CCCCTCCCAGTTCTAGTTCTATCTT-3', [300] R:5'-CCTATTGCCTCCATTTCTTTTGG-3', [900]	FAM:CTGTCTGGAAATTAGAAG(A), [100] VIC:CTGTCTGGAAG TTAGAAG (G), [150]	50 °C 2 min,□ 95 °C 10 min,□ 40 cycles 95 °C15s, 60 °C 1 min
-308G>A rs1800629	F:5'-CAAAAGAAATGGAGGCAATAGGTT-3', [900] R:5'-GGCCACTGACTGATTTGTGTGT-3', [900]	FAM:AACCCCGTCCTCATG (A), [200] VIC:AACCCCGTCCCCATG (G), [150]	50 °C 2 min,□ 95 °C 10 min,□ 40 cycles 95 °C15s, 56 °C 1 min
-238G>A rs361525	F:5'-AAATCAGTCAGTGGCCCAGAA-3', [300] R:5'-GCATCAAGGATACCCCTCACA-3', [900]	FAM:CTCGGAATCAGAGCAG (A), [200] VIC:CTCGGAATCG GAGCAG (G), [200]	50 °C 2 min,□ 95 °C 10 min,□ 40 cycles 95 °C15s, 60 °C 1 min

TNFA: Tumor necrosis factor α, F: forward primer, R: reverse primer.

Table 3. Primers, probes sequences and amplification profiles designed for five TNFA SNPs genotype discrimination.

3.7.2 HLA-B27 haplotypes detection

HLA-B27 presence was determined in all subjects studied with an ABI Prism 7900 HT (Thermo Fisher Scientific, Waltham, MA USA).

50 ng of genomic DNA were amplified in a reaction mix containing primers and probes at a final concentration specified in Table 4 and 1X TaqMan Universal Master Mix (Thermo Fisher Scientific, Waltham, MA USA), in a final volume of 20 µl.

HLA-B27	Primers, [Final concentration, 900nM]	Probes, [Final concentration, 250nM]	Thermocycling Conditions
Exon 2	F:5'-CTACGTGGACGACACGCT-3', R:5'-GCAAGGCCAAGGCACAGACT -3',	5' FAM-CGTGAGGTTCGACAGC-MGB 3'	50 °C 2 min, 94 °C 7 min, 50 cycles 92°C 20s, 60 °C 1,10 min

Table 4. Primers, probes sequences and amplification profiles designed for HLA-B27 analysis.

3.8 Intracellular calcium fluxes analysis

Intracellular calcium $[Ca^{2+}]_i$ fluxes analysis were carried out using an inverted epifluorescence microscope Nikon Eclipse Ti (Nikon Instruments, Amsterdam, Netherlands) using Fluo-4 AM (Thermo Fisher Scientific, Waltham, MA USA) high affinity Ca^{2+} indicators. As an illumination source, we used a mercury arc discharge lamp and selected excitation and emission wavelengths with an FITC filter cube (EX 460-500 DM505 EM 510–560 nm). We used 40X1.30 oil objective (Nikon Instruments, Amsterdam, Netherlands) and, in order to minimize phototoxicity and photobleaching, a back-thinned electron multiplied CCD camera DS-U3 Digital sight (Nikon Instruments, Amsterdam, Netherlands). After the first minute, required to achieve optimal culture conditions, intracellular fluorescence was continuously monitored (5 frames/sec) for 12 minutes.

The fluorescence signal was quantified by measuring the mean pixel value of a manually selected cellular area for each frame of the image stack using the NIS-Elements software (Nikon Instruments, Amsterdam, Netherlands).

Monocyte cells were isolated from EDTA-K_2 peripheral blood from 51 Spa patients and 48 healthy controls using Human Monocyte Enrichment Cocktail (RosetteSep kit, StemCell Technologies, Vancouver, BC, Canada). Unwanted cells are targeted for removal with Tetrameric Antibody Complexes (TAC) recognizing non-monocyte cells and RBCs. Whole blood was incubated for twenty minutes with Human Monocyte

Enrichment Cocktail and monocyte cells were isolated by differential density gradient centrifugation as described above.

10^6 monocytes were seeded on coverslips that had been inserted in six well culture plates and cultured for 24 hours in 2 mL of complete medium (RPMI, 10% FCS) (Thermo Fisher Scientific, Waltham, MA USA).

Coverslips were incubated for twenty minutes at 37°C with 5µM Fluo-4AM in imaging buffer (IB) containing 137 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 5 mM glucose, 20 mM HEPES pH 7.4. The cells were carefully washed two times and then incubated with IB at 37°C for ten minutes in order to allow Fluo-4 to stay inside the cells.

The coverslip was placed in an appropriate chamber, added 1 mL of IB supplemented with 1mM $CaCl_2$ and $[Ca^{2+}]_i$ fluxes were analysed.

3.9 "In vitro" experiments in Monocyte cells

Monocyte cells were isolated from the buffy coat of healthy donors as described above and were seeded (3×10^6 cells per well) in six well culture plates and kept in continuous culture at 37 °C in a humidified atmosphere (5% CO_2) for 24 hours. The cells were cultured in five different types of conditioned media: 1. RPMI 1640, 0.1% Gentamycin, 1% L-Glutamine, 0% FCS, 2. RPMI 1640, 0.1% Gentamycin, 1% L-Glutamine 10% FCS, 3. RPMI 1640, 0.1% Gentamycin, 1% L-Glutamine, 10% of AS patients serum, 4. RPMI 1640, 0.1% Gentamycin, 1% L-Glutamine, 10% of PsA patients serum, 5. RPMI 1640, 0.1% Gentamycin, 1% L-Glutamine, 10% of heathy subjects serum. Each experiment was performed at least in triplicate. Intracellular calcium fluxes were analysed as described above.

3.10 Statistical analysis

Mean, standard error (SE), standard deviation (SD) and 95% confidence interval (CI) were used as descriptive statistics for continuous data, while χ^2 and Fisher's exact tests were used for categorical data. The χ^2 test, Fisher's exact test, binary logistic regression analysis, Student's t test for unpaired data, analysis of variance (ANOVA) and Bonferroni's adjustement of p value for multiple testing were performed using Stata software, version 13.1 (StataCorp, Lakeway Drive, TX, USA).

4. RESULTS

4.1 Studied population

In this study, a total of 164 subjects from the Veneto Region (a North-east Italian region) were enrolled. These included 100 controls and a cohort of 64 patients with an established SpA diagnosis comprising 26 AS and 38 PsA. In statistical analyses, patients were evaluated as a single group (SpA) in comparison to controls, or AS and PsA groups were considered singly. Table 5 reports the demographic characteristics of controls and patients with results from the statistical analyses. Among PsA the percentage of females and mean age were higher with respect to AS and controls (Table 5). Height did not differ between the studied groups while Body Mass Index (BMI) and weight were lower in AS patients.

	Controls (n=100)	**AS (n=26)**	**PsA (n=38)**	**Statistics**
Gender M/F (% of F)	58/42 (42%)	19/7 (26%)	20/18 (47%)	χ^2=2.80,p=0.245
Age mean±SD (years)	46±8	48±15	54±10**	F=7.36,**p=0.0009**
Weight mean±SD (Kg)	78.8±15.89	68.76±9.35*	75.38±11.11	F=5.40,**p=0.0054**
Height mean±SD (cm)	171.75±7.72	171.76±7.40	169.26±8.34	F=1.48,p=0.2305
BMI mean±SD (Kg/m^2)	26.61±4.63	23.38± 2.89*	26.28±3.57	F=6.28,**p=0.0024**

Bonferroni's test for pairwise comparisons: *p<0.05 with respect to controls, **=p=0.001 with respect to controls. Significant p values are reported in bold face. AS: ankylosing spondylitis, PsA:psoriatic arthritis, M: male, F: female, SD: standard deviation, BMI: Body Mass Index.

Table 5. Demographic characteristics in Controls, AS and PsA patients.

4.2 Clinical characteristic of SpA patients

The clinical characteristics of the two groups of patients are shown in Table 6. Family history of SpA, age at diagnosis and clinical symptoms were evaluated. Back pain and enthesitis were more frequent among AS, while peripheral arthritis, dactylitis and psoriasis were more frequent among PsA patients as expected. Extra-articular clinical manifestations, such as uveitis, IBD or urethritis/cervicitis were rare in both groups of patients.

	AS (n=26)	PsA (n=38)	Statistics
Family history of SpA, n (%)	9 (35%)	25(66%)	χ^2=6.02,**p=0.014**
Age at diagnosis, mean ± SD (years)	36.5±13.96	41.65±12.48	F=2.39,p=0.1269
Inflammatory back pain, n (%)	25(96%)	22(58%)	χ^2=11.58,**p=0.001**
Peripheral arthritis, n (%)	9(35%)	38(100%)	χ^2=33.83,**p=0.000**
Enthesitis, n (%)	13(50%)	29(76%)	χ^2=4.74,**p=0.029**
Buttock pain, n (%)	22(84%)	10(26%)	χ^2=20.98,**p=0.000**
Dactylitis, n (%)	3(11%)	8(21%)	χ^2=0.98,p=0.322
Uveitis, n (%)	1(4%)	0(0%)	χ^2=1.48,p=0.223
Psoriasis, n (%)	5(19%)	35(92%)	χ^2=34.98,**p=0.000**
IBD, n (%)	3(11%)	0(0%)	χ^2=4.60,**p=0.032**
Urethritis/Cervicitis/Diarrhoea, n (%)	2(7%)	7(18%)	χ^2=1.47,p=0.225

Significant p values are reported in bold face. SpA: spondyloarthritis, AS: ankylosing spondylitis, PsA: psoriatic arthritis, SD: standard deviation, IBD: Inflammatory Bowel Diseases.

Table 6. Clinical characteristics in AS and PsA patients at diagnosis.

Outcome measures at diagnosis were also evaluated and reported in Table 7. Only the BASMI index significantly differed between groups, being higher among AS than PsA.

	AS (n=26)	PsA (n=38)	Statistics
ESR, mean ± SD (mm/hr)	27.23±19.72	31±11.62	F=0.92,p=0.3402
CRP, mean ± SD (mg/L)	8.62±5.31	9.99±5.92	F=0.90,p=0.3465
DAS, mean ± SD	3.22±0.96	3.74±0.99	F=4.29,**p=0.0424**
BASMI, mean ± SD	3.27±1.806	1.31±1.58	F=21.07,**p=0.0000**
BASFI, mean ± SD	5.52±1.90	4.62±1.90	F=3.45,p=0.0681
HAQ, mean ± SD	0.96±0.52	0.91±0.53	F=0.16,p=0.6918
BASDAI, mean ± SD	6.18±1.55	6.39±1.43	F=0.33,p=0.5649
ASDAS-PCR, mean ± SD	3.12±0.64	2.98±0.48	F=1.02,p=0.3174

Significant p values are reported in bold face. AS: ankylosing spondylitis, PsA: psoriatic arthritis, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, DAS: Disease Activity Score, BASMI: Bath Ankylosing Spondylitis Metrology Index, BASFI: Bath Ankylosing Spondylitis Functional Index, HAQ: Health Assessment Questionnaire, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, ASDAS: Ankylosing Spondylitis Disease Activity Score, SD: Standard Deviation.

Table 7. Laboratory indices and outcome measures in AS and PsA patients at diagnosis.

Patients enrolled in this study attended the Rheumatology Unit of the Department of Medicine- University-Hospital of Padova (Italy). All patients received NSAIDs or DMARDs therapy soon after diagnosis for variable time periods. In non-responders, anti-TNF-α was replaced to NSAIDs or DMARDs. Therefore at enrollment 7 PsA (not

AS) patients were on NSAIDs or DMARDs only, 2 patients (1 PsA and 1 AS) were on DMARDs and anti-TNF-α and 55 were on anti-TNF-α only. Among the 57 patients on anti-TNF-α, 8 cases switched anti-TNF-α agent twice (n=4), three (n=3) or four times (n=1) before the enrollment. A subset of 8 patients started their therapy with anti-TNF-α agent at enrollment; for these patients a second sample was obtained after six months from the start of therapy. Table 8 shows the number (and percentage) of patients treated with an anti-TNF-α agent, and the type of administered drug.

The number of AS patients treated with a TNF-α inhibitor was higher with respect to the treated PsA patients.

	AS (n=26)	**PsA (n=38)**	**Fisher's exact**
Therapy with anti-TNF-α agents, n (%)	26(100%)	31(82%)	**p=0.035**
Adalimumab, n (%)	13(50%)	8(21%)	**p=0.002**
Etanercept, n (%)	7(27%)	13(34%)	
Golimumab, n (%)	0(0%)	4(10%)	
Infliximab, n (%)	6(23%)	2(5%)	
Ustekinumab, n (%)	0(0%)	3(8%)	
Secukinumab, n (%)	0(0%)	1(3%)	
More than one anti-TNF-α n(%), (range)	3(11%),(1-3)	5(16%), (1-4)	p=0.091

Significant p values are reported in bold face. AS: ankylosing spondylitis, PsA: psoriatic arthritis, TNF-α = Tumor Necrosis factor alpha

Table 8. Treatment with anti-TNF-α agents: patients affected by AS and PsA at study enrollement.

4.3 Haematological and biochemical indices in SpA patients

Table 9 reports the mean and standard deviation of haematological and biochemical parameters evaluated in controls and in SpA patients at enrollment. The number of white blood cells (WBC) and polymorphonuclear cells (PMN) were higher in SpA patients with respect to controls, being the highest values recorded among AS, which also had significantly higher levels of haemoglobin with respect to controls. Moreover, in SpA patients, the number of lymphocytes, the glucose and CRP levels were higher with respect to controls, although there is no differences between the two SpA groups.

	Controls (n=100)	**AS (n=26)**	**PsA (n=38)**	**Statistics**
WBC (x10^3/μL)	5.46±1.25	7.47±2.72**	6.42±1.83*	F=15.91,**p=0.0000**
Haemoglobin (g/L)	137.46±17.07	147.38±14.11**	142±13.95	F=4.34,p=**0.0145**
Platelets (x10^3/μL)	239.81±55.38	256.42±76.43	260.79±57.60	F=2.07,p=0.1301
PMN (x10^3/μL)	3.03±1.06	4.73±2.18**	3.75±1.39*	F=17.14,**p=0.0000**
Monocyte (x10^3/μL)	0.47±0.16	0.47±0.21	0.48±0.18	F=0.11,p=0.8973
Lymphocyte(x10^3/μL)	1.83±0.49	2.13±0.75	2.06±0.65	F=3.97,p=**0.0208**
Glucose (mmol/L)	4.93±0.86	5.30±1.13	5.44±1.20*	F=4.21,p=**0.0165**
Creatinine (μmol/L)	80±13.77	73.42±17.47	75.42±12.17	F=3.01,p=0.0519
Uric acid (mmol/L)	0.30±0.07	0.29±0.08	0.28±0.07	F=0.55,p=0.5806
ALT (U/L)	23.91±9.29	27.77±18.15	30.10±20.42	F=2.89,p=0.0583
Prealbumin (mg/L)	284.34±39.72	295.28±57.58	286.86±47.90	F=0.60,p=0.5509
CRP (mg/L)	3.07±2.88	4.79±4.39	4.18±4.35	F=3.09,p=**0.0480**

Haematological and biochemical parameters are reported in terms of mean ± standard deviation. Bonferroni's test for pairwise comparisons: *=p<0.05 with respect to controls; **=p<0.005 with respect to controls. Significant p values are reported in bold face. AS: ankylosing spondylitis, PsA: psoriatic arthritis, WBC: white blood cells, PMN: polymorphonuclear cells, ALT: alanine transaminase, CRP: C-reactive protein.

Table 9. Haematological and biochemical parameters in controls, AS and PsA patients at study enrollement

4.4 Cytokines and MMPs expression levels in PBMCs

The expression levels of TNF-α did not differ between groups (Figure 8; F= 0.92, p=0.400).

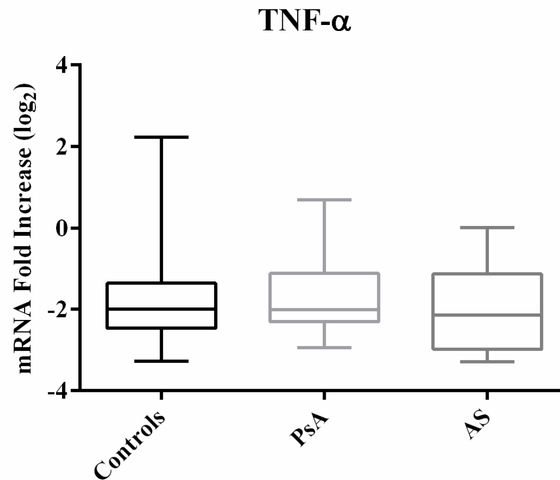


Figure 8. TNF-α mRNA expression (log_2 fold increase) in PBMCs of Healthy controls (Controls), Psoriatic Arthritis patients (PsA) and Ankylosing Spondyilits (AS) patients.

Among patients, the TNF-α expression levels were not associated with treatment type (DMARDs vs anti-TNF-α) (t=-0.5291; p=0.598), nor with any of the biochemical or clinical indexes considered. Interestingly TNF-α expression levels among patients were directly correlated with family history of SpA (Figure 9, t= -2.5386, p= 0.013).

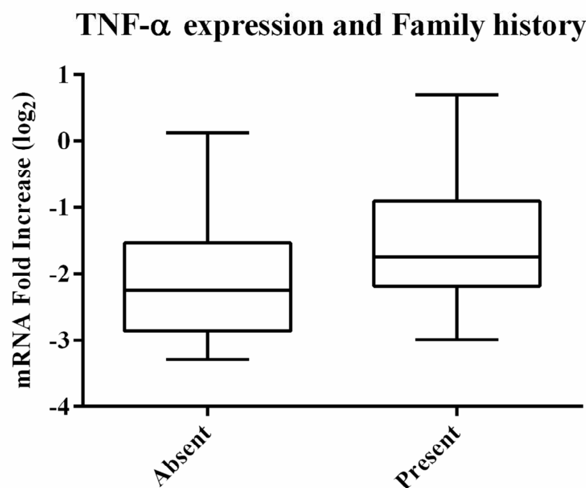


Figure 9. TNF-α mRNA expression (log_2 fold increase) in PBMCs of SpA patients without or with Family history of SpA disease.

This finding prompted us to hypothesize a role for genetics in influencing TNF-α expression. For this reason five single nucleotide polymorphisms in the promoter region of TNFA gene were analysed, (-1031T>C, rs1799964; -857C>T, rs1799724; -376G>A, rs1800750; -308G>A, rs1800629; -238G>A, rs361525). None of the studied polymorphisms considered singly was correlated with TNF-α mRNA expression levels (F=1.15, p=0.331 for -1031T>C; F=0.40, p=0.672 for -857C>T; F=0.39, p=0.535 for -376G>A; F=0.33, p=0.717 for -308G>A and F=0.00, p=0.977 for -238G>A). In a previous study [178] we demonstrated an association between the haplotype resulting from the combination of the rare *TNFA* -1031 C allele with the common *TNFA* -308 G allele with SpA. We verified whether the genotypes resulting from SNPs -1031T>C and -308G>A of the *TNFA* gene haplotypes combinations exerted any influence on TNF-α expression levels. Six genotypes were inferred (TG/TG, TG/TA, TG/CG, TA/TA, CG/TA, CG/CG) and grouped on the basis of our previous observation that the most frequent *TNFA* haplotype was -1031T/-308G while the protective *TNFA* haplotype was -1031C/-308G. Genotypes were grouped as follows: common homozygous (TG/TG), carriers of the protective haplotype (CG/TG, CG/TA and CG/CG), and others (TG/TA and TA/TA). TNF-α expression in PBMCs obtained from a series of 95 controls was found to be significantly reduced among carriers of the protective *TNFA* -1031C/-308G haplotype (Figure 10; F=3.276, p=0.042). This association between *TNFA* genetics and TNF-α expression was not confirmed in SpA patients (F=0.380, p=0.684).

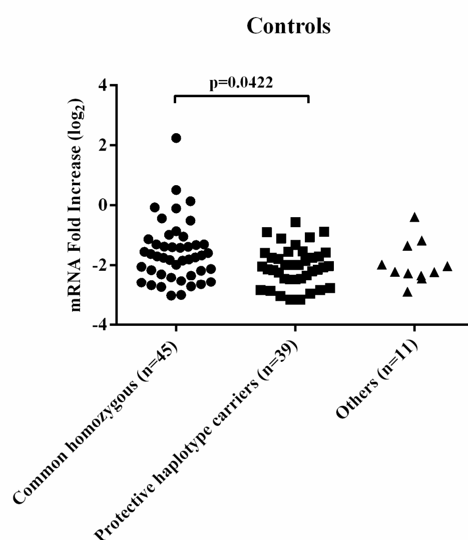


Figure 10.TNF-α mRNA expression (log_2 fold increase) in PBMCs obtained from 95 controls grouped on the basis of genotypes derived from the TNFA -1031/-308 haplotypes combinations [178].

Both MMP8 (F=0.19, p=0.825) and MMP9 (F=0.86, p=0.425) expression levels were not associated with diagnosis as depicted in Figure 11

Similarly TGF-β1 (F=0.42, p=0.655) and IL-1β (F=0.85, p=0.427) varied independently from diagnosis.

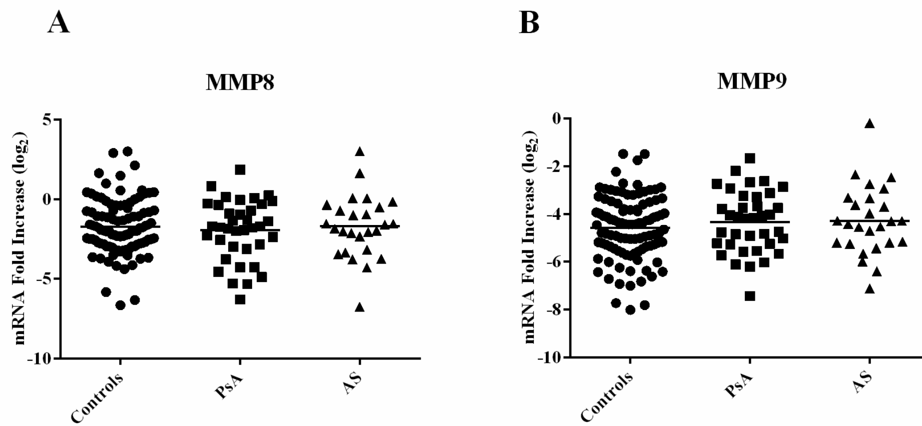


Figure 11. MMP8 (Panel A) and MMP9 (Panel B) mRNA expression (log_2 fold increase) in PBMCs of Healthy controls (Controls), Psoriatic Arthritis patients (PsA) and Ankylosing Spondyilits (AS) patients.

4.5 Calcium binding proteins S100A8 and S100A9 expression levels in PBMCs

The expression levels of S100A8 were found to be significantly lower in PsA patients with respect to controls (Figure 12; F=3.29, p=0.039), while S100A9 were not (F=1.94, p=0.147)

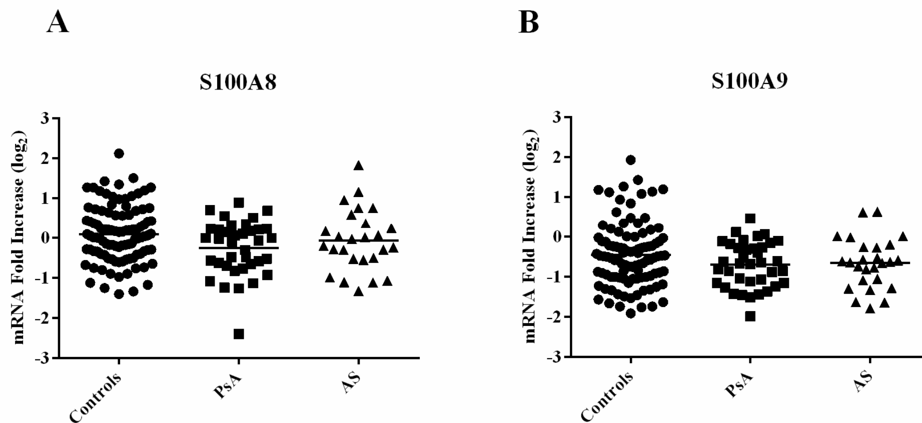


Figure 12. S100A8 (Panel A) and S100A9 (Panel B) mRNA expression (log_2 fold increase) in PBMCs of Healthy controls (Controls), Psoriatic Arthritis patients (PsA) and Ankylosing Spondyilits (AS) patients.

Both S100A8 and S100A9 were not correlated with the severity indexes of disease BADAI, BASFI, BASMI, ASDAS-PCR, DAS and HAQ nor with metabolic and organ function indices. By contrast both S100A8 and S100A9 were significantly correlated with circulating inflammatory cells and S100A8, not S100A9, with the inflammatory indices ESR and CRP (Table 10).

	S100A8	S100A9
WBC (x10^3/µL)	R=0.2121,p=0.095	R=0.1518, p=0.234
Haemoglobin (g/L)	R=-0.1466, p=0.251	R=-0.0387, p=0.763
Platelets (x10^3/µL)	R=0.0557, p=0.664	R=-0.0047, p=0.970
PMN (x10^3/µL)	R= 0.3150, **p=0.011**	R=0.2913, **p=0.020**
Lymphocyte (x10^3/µL)	R=-0.1646, p=0.197	R=-0.2800, **p=0.026**
Monocyte (x10^3/µL)	R=0.2935, **p=0.026**	R=0.3368, **p=0.010**
ESR (mm/hr)	R=0.3202, **p=0.010**	R=0.1631, p=0.201
CRP (mg/L)	R=0.2679, **p=0.033**	R=0.1699, p=0.183

Significant p values are reported in bold face. WBC: white blood cells, PMN: polymorphonuclear cells, ALT: alanine transaminase, ESR: Eritrocyte sedimentation rate, CRP: C-reactive protein.

Table 10. Correlations between the expression levels of calcium binding proteins S100A8 and S100A9 (log_2 fold increase) and inflammatory cells and inflammatory indices in PBMCs of patients with SpA (n=64)

4.6 Intracellular calcium fluxes in Healty subjects and SpA patients

Monocytes recruitment at joint inflammatory sites plays a key role in the inflammatory cascade and in joint destruction. It has been recently pointed out that calcium signalling in circulating monocytes regulates their chemotaxis and cytokines production. With this in mind, in this study we verified whether the pattern of intracellular calcium oscillations differs between healthy subjects (n=48) and patients with SpA (n=51). To do this, blood monocytes were first isolated by negative selection and then used to ascertain intracellular calcium oscillations by epifluorescence microscope analysis. Monocytes obtained from healthy blood donors had evident and frequent intracellular calcium oscillations, while monocytes obtained from SpA patients did not. Figure 13 shows individual cellular data from representative experiments from 4 controls and 4 SpA patients.

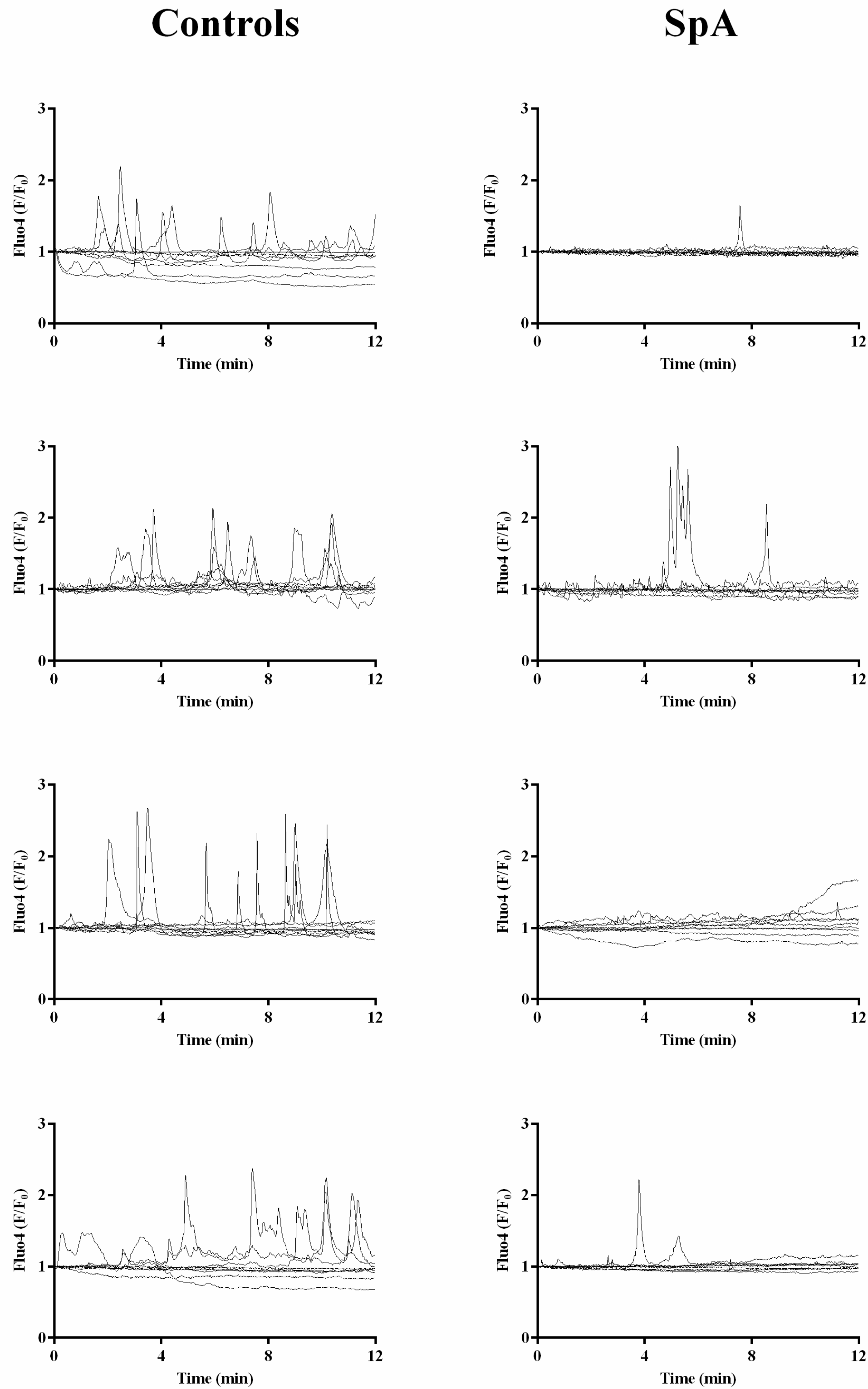
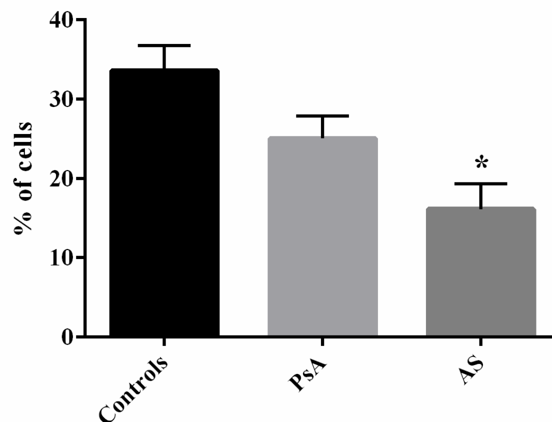


Figure 13. Intracellular calcium fluxes ($[Ca^{2+}]_i$) of monocyte cells from Healthy controls (Controls) and SpA patients (SpA).

For any individual experiment, each referred to any individual case and control, the number of monocytes exhibiting intracellular calcium oscillations profile with respect to

the whole number of cells examinated was calculated and expressed as percentage (% of cells). Figure 14 shows the results obtained in healthy controls (Controls), patients with PsA or with AS. AS patients had a significant lower percentage of active monocytes with respect to controls (F=6.15, p=0.003).



Bonferroni's test for pairwise comparisons: *=p<0.005 with respect to controls;

Figure 14. Percentage of monocyte cells (% of cells) with intracellular calcium oscillations in Healty controlos (Controls) in Psoriatic Arthritis patients (PsA) and Ankylosing Spondyilits (AS) patients.

We then evaluated considering only the cells exhibitng intracellular calcium oscillations, the frequency of the oscillations which was expressed as oscillations/minute. No difference was observed between patients and controls (F=0.03, p=0.973).

The percentage of cells with intracellular calcium oscillations in SpA patients (n=51) was not correlated with the severity clinical indices BASDAI, BASFI, BASMI, ASDAS-CRP, DAS and HAQ as detailed in Table 11, nor with the biochemical indices of inflammation, namely circulating PMN, lymphocytes, monocytes, CRP and ESR, as detailed in Table 12.

	SpA patients			
	Coefficent	**SE**	**95% CI**	**p-Value**
BASDAI	-0.094	0.178	-0.454 to 0.266	0.601
BASFI	0.107	0.132	-0.159 to 0.373	0.421
BASMI	-2.128	1.336	-4.823 to 0.565	0.118
ASDAS-CRP	-5.615	4.444	-14.577 to 3.346	0.213
DAS	3.214	2.592	-2.013 to 8.441	0.222
HAQ	-0.756	4.546	-9.925 to 8.412	0.869

Significant p values are reported in bold face. BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, BASFI: Bath Ankylosing Spondylitis Functional Index, BASMI: Bath Ankylosing Spondylitis Metrology Index, DAS: Disease Activity Score, ASDAS: Ankylosing Spondylitis Disease Activity Score, CRP: C-reactive protein, HAQ: Health Assessment Questionnaire, SE: Standard Error, CI= Confidence Interval.

Table 11. Multiple regression analysis considerenig the percentage of cells with intracelluar calcium oscillations as dependent variable and the clinical indices as predictor variables.

	SpA patients			
	Coefficent	**SE**	**95% CI**	**p-Value**
Age	0.364	0.238	-0.120 to 0.849	0.135
Gender	7.459	6.579	-5.912 to 20.829	0.265
BMI	1.513	0.839	-0.192 to 3.218	0.080
WBC(x10^3/μL)	-9.747	20.115	-50.627 to 31.133	0.631
PMN (x10^3/μL)	10.380	20.427	-31.134 to 51.894	0.615
Lymphocyte (x10^3/μL)	8.155	20.615	-33.740 to 50.051	0.695
Monocyte (x10^3/μL)	3.925	24.545	-45.958 to 53.808	0.874
CRP (mg/L)	-0.425	0.923	-2.302 to 1.450	0.648
ESR (mm/hr)	-0.175	0.260	-0.705 to 0.354	0.506
Constant	-33.840	20.448	-75.397 to 7.716	0.107

Significant p values are reported in bold face.WBC: white blood cells, PMN: polymorphonuclear cells, CRP: C-reactive protein, ESR= Eritrocyte sedimentation rate. SE: Standard Error, CI= Confidence Interval.

Table 12. Multiple regression analysis considering the percentage of of cells with calcium oscillations as dependent variable and biochemical indices of inflammation as a predictor variables.

The percentage of cells exhibiting a calcium oscillation was then analysed in the light of peripheral joint involvement (peripheral arthritis, enthesitis, dactylitis), psoriasis and presence or absence of buttock pain or back pain. Table 13 reports mean values with standard errors and a statistical analysis of data (student’s t test). Uveitis and IBD were

not evaluated due to the low number of patients with any of these conditions. The proportion of cells exhibiting an intracellular calcium oscillations were significantly reduced among patients without dactylitis or psoriasis, and slightly reduced among patients with buttock pain.

	SpA patients				
Clinical condition	**Absent**	**Mean±SE**	**Present**	**Mean±SE**	**Statisitcs**
Peripheral arthritis	10	14.8±3.9	40	23.6±2.5	t=-1.61, p=0.114
Enthesitis	15	19.61±3.52	35	22.82±2.81	t=-0.66, p=0.514
Dactylitis	41	19.59±2.29	9	32.16±5.69	t=-2.26, **p=0.028**
Psoriasis	16	14.18±3.09	34	25.47±2.74	t=-2.49, **p=0.016**
Buttock pain	27	25.52±3.06	23	17.55±3.06	t=1.83, p=0.074
Back pain	14	21.38±4.61	36	22.04±2.55	t=-0.13, p=0.895

Significant p values are reported in bold face. SE: Standard Error

Table 13. Student's t test for unpaired data considering the absence and the presence of peripheral joint involvement psoriasis, buttock pain and back pain and the percentage of monocyte cells with intracellular calcium oscillations.

Since the reduced proportion of cells with calcium oscillations was correlated with features that characterize AS with respect to PsA, binary logistic regression analysis was performed in order to verify whether cells with calcium oscillations were independently correlated with diagnosis. In the analysis, AS diagnosis was considered the outcome variable, PsA diagnosis was the reference, buttock pain, dactylitis, psoriasis, HLA-B27 and the percentage of cells with calcium oscillations were independent variables (Table 14). Absence of psoriasis and presence of buttock pain were independently correlated with AS diagnosis, while cells with calcium oscillations were not. This indicates that this latter variable is not per sè and index of AS, but this does not exclude its potential pathophysiological role.

	Coefficent	SE	95% CI	p-Value
Age	0.060	0.059	-0.057 to 0.178	0.315
Gender	-1.267	1.176	-3.572 to 0.037	0.281
BMI	-0.474	0.267	-0.997 to 0.049	0.076
Buttock pain	2.355	1.111	0.177 to 4.534	**0.034**
Dactylitis	2.355	1.573	-0.727 to 5.438	0.134
Psoriasis	-2.542	1.077	-4.653 to -0.430	**0.018**
% of cells	-0.041	0.044	-0.127 to 0.045	0.348
HLA-B27	0.085	1.096517	-2.064 to 2.235	0.938
Constant	10.387	5.946946	-1.268 to 2.043	0.081

Significant p values are reported in bold face. BMI: Body Mass Index; SE: Standard Error; CI= Confidence Interval.

Table 14. Logistic regression analysis considering AS diagnosis as the outcome variable buttock pain, dactylitis, psoriasis, percentage of cells with calcium oscillations (% of cells), HLA-B27 age and gender as predictors.

Since all 51 patients SpA were under treatment, we verified whether variations in cells with calcium oscillations were correlated with the drug type used. Values found in patients under DMARDs treatment (n=6, Mean±SE: 22.928±6.462) were not different form values of patients under anti-TNF-α (n=44, Mean±SE: 21.709±2.394), (student's t test for paired data: t= 0.17, p= 0.861). This result was further confirmed in the subset of 8 patients who had a replicated analysis before and after 6 months from the beginning of anti-TNF-α therapy (student's t test for paired data: t = 0.21, p=0.833).

The percentage of cells with calcium oscillations was then correlated with the metabolic and organ function biochemical indices, namely glucose, creatinine, ALT, uric acid and with age and BMI. No significant colleration was found when cases and control were considered overall, while significant correlation were recorded among patients between the percentage of cells with calcium oscillations and age (R=0.3808, p= 0.006), BMI (R= 0.3714, p=0.007), creatinine (R=-0.3029, p=0.032) and with uric acid (R= -0.3415, p=0.015). Multiple linear regression analysis performed considering SpA patients confirmed the significant and independent association between cells with calcium oscillations and BMI and uric acid (Table 14)

	SpA patients			
	Coefficent	**SE**	**95% CI**	**p-Value**
Age	0.206	0.180	-0.157-0.569	0.259
BMI	1.455	0.590	0.266-2.644	**0.018**
Creatinine (µmol/L)	-0.272	0.155	-0.585-0.040	0.086
Uric acid (mmol/L)	-70.071	34.350	-139.255--0.886	**0.047**

Significant p values are reported in bold face. BMI: Body Mass Index; SE: Standard Error; CI= Confidence Interval.

Table 14. Multiple regression analysis considering the percentage of percentage of cells with calcium oscillations (% of cells), as dependent variable and Age, BMI, Creatinine and Uric Acid as a predictor variables.

We then evaluated whether the variations found in cell with calcium oscillations among patients were correlated with the expression levels of inflammatory cytokines, S100 proteins and MMPs. While in control subjects no correlation was found (Figures 15 and Figure 16, left panels), the percentage of cell with calcium oscillations was significantly correlated with the expression levels of TNF-α and IL-1β among patients (Figure 15, righ panels), but not with S100 proteins, MMPs or TGF-β (Figure16, right panels).

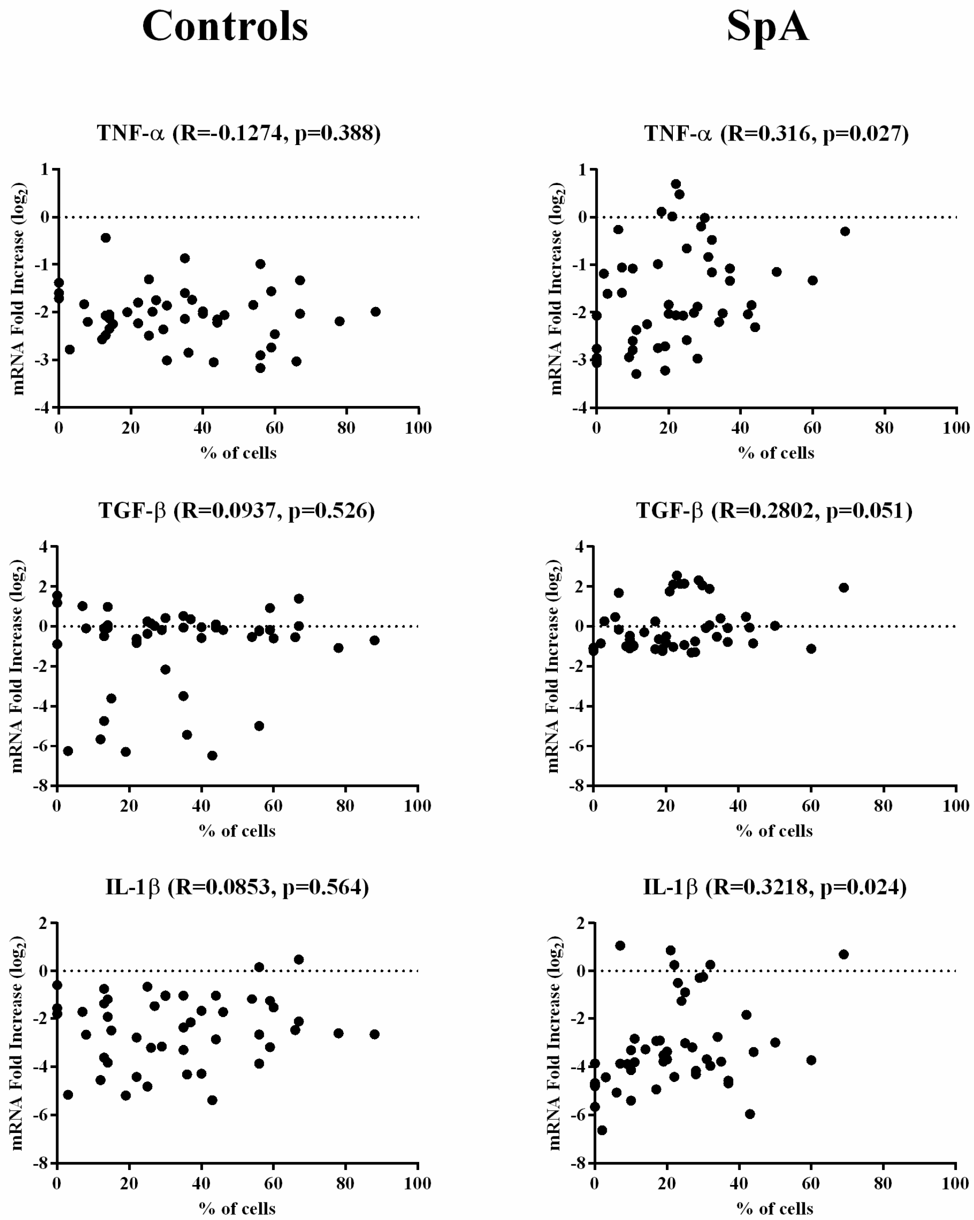


Figure 15. Correlation among monocyte cells with intracellular calcium oscillations (% of cells) and TNF-α, TGF-β, IL-1β mRNA expression levels (log_2 fold increase) in PBMCs of Healthy Controls (Controls) and SpA patients (SpA).

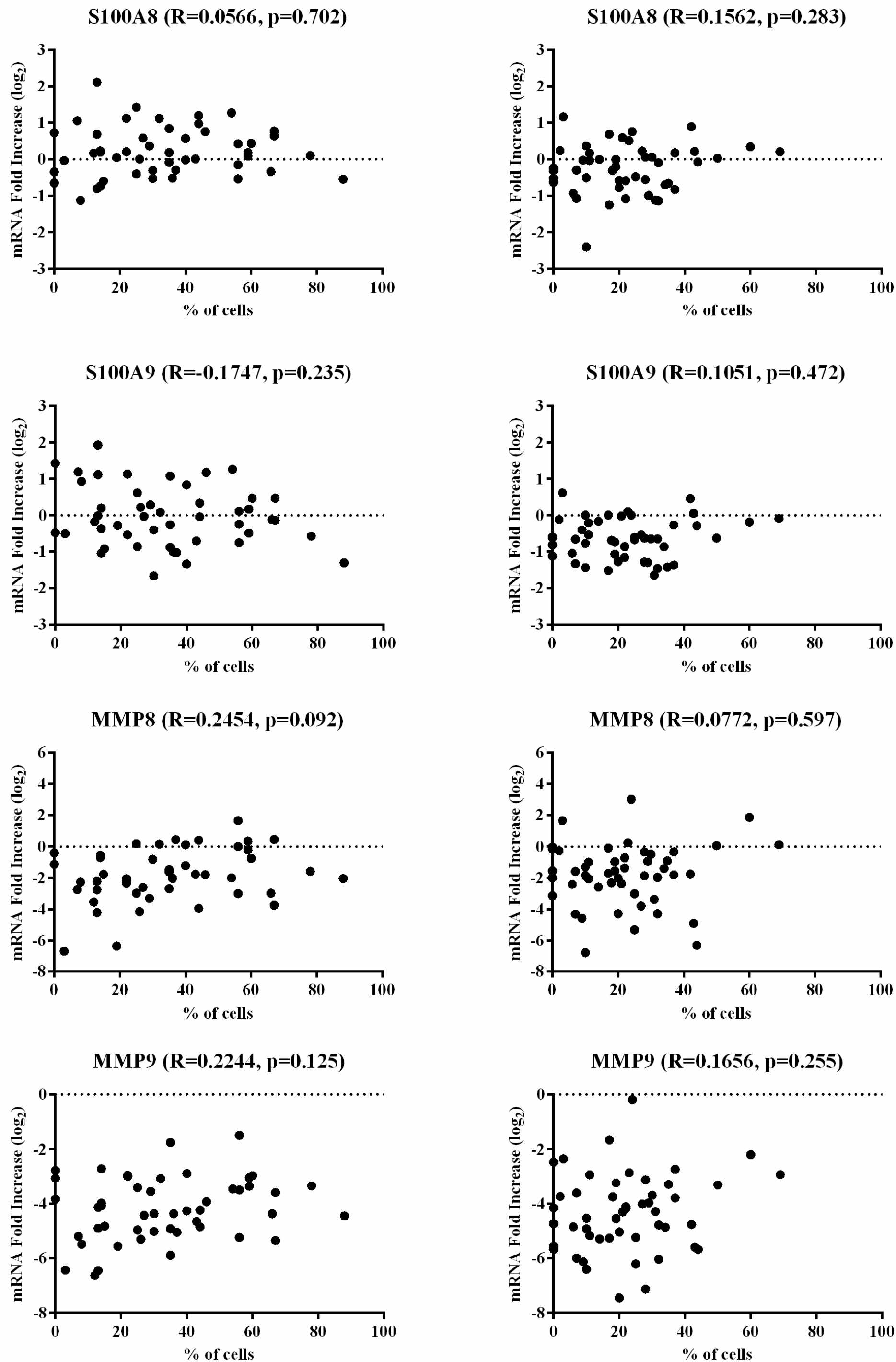


Figure 16. Correlation among percentage of monocyte cells with intracellular calcium oscillations (% of cells) and S100A8, S100A9, MMP8, MMP9 mRNA expression levels ($\log_2$ fold increase) in PBMCs of Healthy Controls (Controls) and SpA patients (SpA).

4.7 Healthy subject serum induces intracelluar calcium oscillations in human monocytes

The observed disease-related pattern of cell with calcium oscillations might be consequent to intrinsic monocytes alterations and/or to soluble blood mediators. To verify this latter hypothesys we performed in vitro experiments using three healthy donors blood monocytes which were conditioned for 24 hours with FCS or with human serum pools obtained from healthy subjects (n=3), from patients with AS (n=3) and from patients with PsA (n=3). At the end of the conditioning period, intracellular calcium oscillations of monocytes were analysed and the percentages of cells with intracellular calcium were calculated as described above. Figure 17 shows the obtained results. Intringuinly the percentage of cell with calcium oscillations significantly increased in the presence of healthy human sera, but not in the presence of disease sera (Kruskal Wallis test: p=0.038), supporting the hypothesis that in SpA patients soluble mediators are released into the bloodstream that dampen monocytes intracellular calcium oscillations. This finding poses the basis for the potential identification of new blood biomarkers of disease.

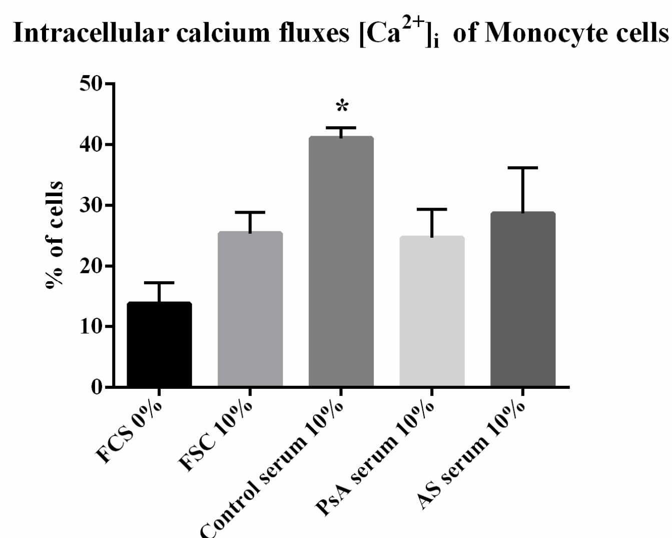


Figure 17. Percentage of monocyte cells (% of cells) with intracellular calcium oscillations in healty blood donors cultured in the absence or presence of 10% FCS, 10% of Healthly controls (Control), 10% of Psoriatic Arthritis patients (PsA) and 10 % of Ankylosing Spondyilits (AS) patients serum.

4.8 Therapy effects

The expression levels of the studied molecules were evaluated taking into account the type of drug used for treatment. Patients were then subdivided according to treatment with DMARDs only (n=7), Adalimumab (n=21) or Etanercept (n=20); the remaining patients under treatment with Golimumab, Infliximab, Ustekimumab and Sekukimumab were grouped all together due to their low number (n=15).

Table 15 reports the results of statistical analysis (one way ANOVA). Type of treatment did not influence any of the studied molecules.

	Statistics
IL-1β	F=1.21, p=0.313
TGF-β	F=0.60,p=0.616
TNF-α	F=0.20,p=0.896
MMP8	F=0.23,p=0.877
MMP9	F=0.93,p=0.430
S100A8	F=0.53,p=0.663
S100A9	F=0.39,p=0.760
% of cells	F=0.27,p=0.850

Table 15. IL-1β, TGF-β, TNF-α, MMP8, MMP9, S100A8, S100A9 mRNA expression levels (log_2 Fold increase) and percentage of monocyte cells with intracellular calcium fluxes (% of cells) in the response to treatment with DMARDs and anti- TNF-α therapy.

Moreover, none of the studied molecules expression levels were modified by therapy also considering the subset of 8 patients for whom results were available before and after six months of anti TNF-α therapy (Table 16 shows Student's t test results for paired data).

	Student's t test	p-Value
IL-1β	t=0.6085	0.562
TGF-β	t=0.2326	0.822
TNF-α	t=-0.7228	0.493
MMP8	t=0.4725	0.650
MMP9	t=1.1111	0.309
S100A8	t=0.4329	0.678
S100A9	t=1.2433	0.253

Table 16. IL-1β, TGF-β, TNF-α, MMP8, MMP9 S100A8, S100A9 mRNA expression levels (log_2 Fold increase) in the absence or presence of anti-TNF-α therapy.

5. DISCUSSION and CONCLUSIONS

Spondyloarthritis comprises two main diseases, AS and PsA, that primarily involve the spine, but they can also affect peripheral joints and tissues/organs outside the skeleton, like eyes, skin and intestine. The symptoms that patients refer at the onset of disease, mainly back pain, are not specific and this often determine a delay in diagnosis on the one hand and repeated cycles of therapy with anti-inflammatory agents. The delayed diagnosis with a consequent delay in appropriate treatments might associate with disease progression that cause irreversible joint damage. Diagnosis is supported by imaging and unfortunately not enough by biochemistry. In fact no specific blood biomarkers are available for these diseases. Moreover, despite the intensive inflammatory process involving sacro-ileal joints, the common blood inflammatory indexes, e.g. CRP and ESR, remains within the reference ranges or are only modestly altered. These considerations have prompted the research of new potential biomarkers that may help and support diagnosis and monitoring. Among these a special attention has been posed on the circulating levels of metalloproteinases, mainly on MMP3, and on emerging inflammatory proteins, like the calcium binding proteins S100A8 and S100A9 and their derived complex calprotectin. In this study we focused on circulating mononuclear cells as key players of the inflammatory process of SpA. In fact macrophages infiltrating the inflamed joints derive from circulating monocytes and contribute to the process of tissue destruction be expressing not only inflammatory cytokines, like TNF-α or IL-1β, but also enzymes causing tissue destruction and remodelling, like metalloproteinases. With this in mind we first evaluated whether circulating mononuclear cells have different mRNA transcription levels of the inflammatory cytokines TNF-α, IL-1β and TGF-β. When compared to controls, AS and PsA patients had similar cytokines expression levels in their circulating mononuclear cells. This finding was in part unexpected, since increased protein levels have been reported in these patients and the role of these cytokines, mainly TNF-α and IL-1β, in disease pathogenesis is well established and supported by the dramatic efficacy of anti-TNF-α therapy. Our unexpected findings might be explained considering that very small variations in the expression levels of mRNA, not traceable with the actual quantitative assay sytems, can result in wider variations of the encoded proteins; moreover mononuclear cells might enhance the expression levels of inflammatory cytokines only when homed at the joint tissue sites.

Despite the lack of association between cytokines expression levels and disease diagnosis, the variations of TNF-α, TGF-β and IL-1β were correlated each other in controls and in SpA patients, suggesting that these inflammatory pathways share common drivers and probably influence each other. For the relevance of TNF-α in SpA, we analysed in more depth this cytokine, by evaluating in our series of patients whether its expression was associated with the clinical indixeses of disease activity, the extra-spine lesions, the biochemical inflammatory and metabolic indices, but none of those analysed was found to influence TNF-α results. By contrast a significant correlation was detected between TNF-α and family history of SpA, being higher expression levels recorded among patients with a positive than in those with a negative history. This intringuing finding further supports the potential role of TNF-α in disease risk and suggests a genetic search. TNF-α is encoded by TNFA gene which is located in close proximity of the MHC locus in chromosome 6 between the HLA-B and HLA-DR genes, which carries well known predisposing alleles for AS, the HLA-B27, and for PsA, the HLA-Cw6. Some TNFA variants, which might be in linkage with HLA predisposing alleles, might be implicated in disease pathogenesis. We verified five single nucleotide polymorphsims of the TNFA promoter, that result in haplotypes that might influence TNF-α expression. As hypothesized the haplotype resulting from the combination of the rare TNFA -1031C and TNFA -308G alleles was significantly associated with reduced TNF-α expression levels in mononuclear cells of healthy controls. This finding supports our previous observation that this haplotype has a protective role for SpA [178]. However in patients TNFA genetics did not associate with TNF-α expression, suggesting that the role of genetics in the regulation of mRNA expression can be masked by other disease related factors, like other cytokines or other triggers of the TNF-α pathway.

On the basis of the above results, TNF-α, TGF-β and IL-1β appear of no clinical utility in SpA diagnosis, nor in the stratification of disease severity. We therefore moved our attention to other potential inflammatory biomarkers, choosing the metalloproteinases MMP8 and MMP9 for their potential role in SpA. In this approach we did not forget another metalloproteinase, MMP3, which was reported as the most promising diagnostic tool for SpA, both at serum protein level and as mRNA expression level. MMP3 mRNA expression was never measurable in both patients and controls (data not shown). The discrepancy of our results with previous data in the literature might be explained considering that MMP3 protein was described as increased in serum, while

increased transcripts have been described in synovial fluid or tissues. Although MMP3 and mononuclear cells are both potentially involved in disease pathogenesis, this does not necessarily imply that this metalloproteinase would be produced directly by mononuclear cells. Other MMPs could be implicated, and we focused on MMP8 and MMP9 for which very few data are present in the literature. However, although both expressed at measurable levels in circulating mononuclear cells, they did not vary between controls and patients, nor they were related to disease clinical activity indices.

An emerging concept in inflammatory joint diseases is the role played by intracellular calcium as a trigger for the release of inflammatory molecules and of the related calcium binding inflammatory S100 proteins. Two members of this protein family appear of particular relevance: S100A8 and S100A9, which mRNA and protein expression levels was shown to be increased in the synovial fluid and in blood of patients with rheumatoid arthritis. They appear involved in joint destruction, probably also because they are inducers of metalloproteinases, including MMP8 and MMP9. S100A8 and S100A9 proteins are both expressed by mononuclear cells and, based on this premise, we verified whether their expression by circulating mononuclear cells is altered in SpA. While S100A9 expression did not vary, the expression of S100A8 was reduced in PsA patients as compared to controls. This finding is hard to be explained, taking also into account that among patients S100A8 expression levels were correlated with the inflammatory indices CRP and ESR, and with the number of blood inflammatory cells, namely PMN and monocytes. However, these correlations might spurious and consequent to the observation that PMN, CRP and ESR values were higher in AS than in PsA, as S100A8. The main question is why S100A8 is reduced. One of the mechanisms underlying S100A8 expression might be variations in intracellular calcium, which regulates several biological processes of the cells. Intracellular calcium balance is tightly regulated by transporters expressed both at cell membrane and mitochondrial membrane [179]. Alterations in intracellular calcium might be characterized by increased and constant accumulation of the ion, increased frequency in its oscillations, but also the opposite might also occur, i.e. decreased intracellular levels and/or frequency of oscillations. These alterations may evoke different biological effects, which depend on cell type and cell context. It has been recently reported that monocytes from patients with arthritis might have alterations in intracellular calcium due to an altered expression of the calcium sensing receptor (CaSR) [174]. On the other hand the ORAI Ca^{2+} channels, also expressed by monocytes and found to be reduced by

bacterial products, have been suggested to play a role in arthritis. We verified whether circulating monocytes maintain or not their characteristic pattern of intracellular calcium behaviour when SpA is present. Our findings clearly demonstrate that monocytes from patients with SpA, but mainly with AS, loose their typical intracellular calcium pattern. In other words, while the majority of monocytes from controls maintained in culture for 24 hours show regular calcium oscillations, only a minority of monocytes from AS patients maintain this pattern, while the majority of them do not show regular calcium oscillations. This finding requires some comments: 1. the reduced number of monocytes with intracellular calcium oscillations might be the expression of a differentiation shift of monocytes towards the dendritic cell or the mMDSC phenotypes. In fact, as previously demonstrated by us, these cellular subsets of the monocyte population present reduced or even absent intracellular calcium fluxes [180]. The role of MDSC as contributors in arthritis and psoriasis is actually an emerging concept: Cao et al. described in psoriasis increased monocytic MDSC that produce increased amounts of IL23, IL-1β and the Chemokine (C-C motif) ligand 4 (CCL4), and a de-regulated immunosuppressive loop in this disease has been suggested [181], while Zhang et al. supported the potential pathophysiological role of these immunosuppressive cells demonstrating in the animal model their role in bone erosion [182]. In our series of patients, not in controls, an association between the percentage of monocytes with intracellular calcium oscillations and the expression levels of TNF-α and IL-1β was effectively recorded, but this association was inverse, a progressive reduction of cytokines expression and the percentage of monocytes with intracellular calcium oscillations were reduced; 2. in patients with SpA structural alterations of the CaRS might determine the observed pattern and these alterations might be due to genetic variants. However another hypothesis should also be taken into account, i.e. the expression of calcium sensing receptors might vary in inflammatory diseases of the joints as demonstrated by Séjourné et al. who found reduced CaSR in monocytes isolated from synovial fluid and peripheral blood from patients with reumathoid arthritis [174]. We support this latter hypothesis. In fact in our experiments serum from PsA patients were demonstrated to cause a reduction of intracellular calcium oscillations in peripheral blood mononuclear cells of healthy blood donors. This suggests that in serum of patients soluble factors might act on monocytes and confer them new phenotypes. Although these soluble factors remain unknown, the role of cytokines may be hypothesized, but small metabolites may also be involved. We observed an inverse

significant correlation between the percentage of monocytes with intracellular calcium oscillations and uric acid. This finding poses the attention on a relevant metabolite well known to be implicated in another joint disease, gout. However in plasma of patients with gout, uric acid is usually above the upper reference limit, while in our series of SpA patients it was not. Therefore its potential pathogeneic role in these diseases is probably different from that described in gout, i.e. crystals deposits in joints, and a complex interplay between small uric acid variations and expansion of mMDSC appears a possible hypothesis.

The interpretation of our findings might be limited by the fact that SpA patients were under treatment at enrollment. Therefore one might argue that the observed variations might be due to therapy not to disease. The study of a population of patients free of any medication is extremely difficult for the reasons already described above, i.e. diagnosis if often delayed but from symptom onset to diagnosis patients received usually symptom-guided therapy. At enrollment our patients, however, were on different therapies, the main being DMARDs and anti-TNF-α and a subset of 8 patients were enrolled before starting anti-TNF-α therapy and for them a second evaluation 6 months later was available. The percentage of monocytes with intracellular calcium oscillations was not correlated with the type of therapy or of drug used, and it did not vary before and after six months of anti-TNF-α therapy. Similarly therapy was not found to affect the expression levels of the studied cytokines, calprotectin and MMPs. Therefore our findings appear to be mainly disease- not drug- related.

In conclusion the results of this study indicate that the analysis of the expression levels of inflammatory cytokines, calprotectin and MMPs in blood circulating mononuclear cells appear of no clinical utility in SpA diagnosis nor in the stratification of disease groups. SpA patients shown a reduced number of monocytes with intracellular calcium oscillations probably due to shift toward mMDSC phenotype which could involved in SpA inflammatory process.

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