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CICLO XXIX

GENETICS IN TNF-TNFR PATHWAY: A COMPLEX NETWORK CAUSING SPONDYLOARTHRITIS AND CONDITIONING RESPONSE TO THERAPY

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SUMMARY

Background. The seronegative spondyloarthritis (SpA) are a group of chronic inflammatory diseases resulting from a complex interplay among genetic background (mainly represented by HLA-B27) and environmental factors, that leads to the activation of autoinflammation and the dysregulation of the immune-system.

In many cases, an early diagnosis and an appropriate monitoring of disease activity can be difficult because of the overlap of clinical features. Furthermore, because of the indices of inflammation, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), are in the normal range in at least half of SpA patients with a clear expression of disease activity, a delay in diagnosis and consequently in treatment in these patients has been documented. This imparts a tremendous symptomatic burden and loss of function in these patients during the productive years of life. For all these reasons, much attention is currently devoted to the identification of biochemical and genetic biomarkers to be used in the diagnosis as well as prognostic factors in evaluating the treatment effectiveness.

Among the genetic predisposing factors, a well-known role is that of HLA-B27, which contributes however to only 20–30% of the total heritability, whereas the whole major histocompatibility complex (MHC) accounts for about 40–50% of the genetic risk of developing SpA. This suggested that other genes are involved in pathogenetic mechanism. In fact, in addition to HLA-B27, a number of genetic factors in both, MHC and non-MHC locus, have been claimed to play a role in pathogenesis of SpA.

In this context, because of TNF-α is primarily involved in the propagation and perpetuation of inflammation in SpA, the study of TNF-α genetic is of great interest. Several polymorphisms (SNPs) in genes involved in TNF-α signalling, as TNFA, TNFSF15, TNFR1 and TRADD genes, have been identified as associated with SpA, even if results are controversial. Of great interest are also variants in MEFV gene, involved in the pathogenesis of the autoinflammatory disorder Familial Mediterranean Fever (FMF). Recent studies have shown that the SpA, and in particular the ankylosing spondylitis (AS), are very common among patients affected by FMF and that these patients can present with AS as a sole manifestation.

The present study, conducted in a cohort of 91 SpA patients and 223 controls, coming from a North-East Italian region, was aimed to identify biohumoral

(biochemical and haematological) and genetic factors to support the diagnostic and prognostic (response to therapy) work-up of SpA diseases. In particular, in addition to biochemical and haematological indices, we investigated whether SNPs in the promoter region of TNFA, or SNPs in the autoinflammatory TNFRSF1A and MEFV genes, might concur with HLA-B27 in enhancing the risk of developing SpA disease and/or in predicting the response to anti-TNFα drugs.

Methods. The study population comprised 91 patients with a diagnosis of SpA (mean age ± standard deviation: 52.1 ± 12.5 years; 57 males, 34 females) and 223 blood donors (mean age ± standard deviation: 46 ± 11 years; 146 males, 77 females) coming from Veneto Region, a North-East Italian region. Among patients, 36 had a diagnosis of AS and 55 patients of psoriatic arthritis (PsA), which were based on New York and CASPAR criteria respectively. The protocol of this study was approved by the Local Institutional Ethic Committee of University-Hospital of Padua, Italy (Prot.n. 3024P/13), and all participants gave written informed consent before entering the study.

Demographic and physiological data, medical and familial history data were collected for each participant. Blood samples were collected and complete blood count, CRP, ESR, uric acid, prealbumin, alanina aminotransferase (ALT) and glucose were evaluated.

Direct sequencing of MEFV (exons 2,3,5 and 10) and TNFRSF1A (exons 2,3,4 and 6) genes were performed. HLA-B27 and TNFA polymorphisms (-1031T>C;-857C>T;-376G>A;-308G>A;-238G>A) were assayed by Real Time-PCR. HLA-CW6 allele presence was analysed by molecular genetic testing using a commercially available CE-IVD microarray. Statistical analysis was performed using STATA software (version 13.1).

Results. An higher number of circulating polymorphonuclear cells and higher CRP levels could be detected in SpA patients with respect to controls, and in PsA higher levels of ALT could be observed with respect not only to controls but also to AS. Anyway these indices were not highly elevated and often comprised within the reference intervals.

As expected, HLA-B27 was associated with AS (χ^2=120.1; p<0.0001). Although a slightly higher frequency of HLA-CW6 carriers was observed among patients with AS (about 6%) or PsA (about 13%) with respect to controls (about 4%), the difference was not statistically significant.

Any single studied TNFA SNP was not associated with SpA diagnosis, nor with AS or PsA considered singly. The haplotypes deriving from the pairwise combinations of the five studied SNPs were also statistically inferred. The most frequent haplotypes in controls were selected as references, and only the haplotype -1031C/-308G was significantly associated with AS (p=0.015) exerting in this disease a protective role (Odds Ratio: 0.43; Confidence Interval 95%: 0.22-0.85).

Three SNPs were identified in TNFRSF1A gene and among them, only the R92Q (Minor Allele Frequency- MAF=0.034) and the c.625+10A>G (MAF=0.479) were selected for their potential functional implications. Both SNPs were not associated with the presence of SpA (χ^2=1.073 and p=0.300 for R92Q; χ^2=4.721 and p=0.094 for c.625+10A>G), but c.625+10A>G was associated with the response to anti-TNF therapy, assessed by BASDAI score lower /equal or higher than 4 at 10 months (p=0.031).

Twenty-one SNPs were identified in MEFV gene and among them, 10 with a known potential functional significance. Variant alleles were extremely rare in our population (MAF<0.025) except for R202Q (MAF=0.27). None was associated with SpA diagnosis (p>0.05).

Conclusions. In conclusion the results of this study indicate the relevant role of TNF-TNFR pathway genetics in the complex network causing SpA and conditioning response to therapy. TNFA was shown to be a predisposing factor for SpA, but mainly for AS, among Italian patients, while genetics of the autoinflammatory gene MEFV appears of no impact in this setting. The haplotype resulting from TNFA-1031C/-308G, potentially associated with lower TNF-α production, exerts a protective role in AS, while the TNFRSF1A c.625+10A>G polymorphism emerged as a potential predictor of response to anti- TNFα therapy.

RIASSUNTO

Introduzione. Le spondiloartriti sieronegative (SpA) sono un gruppo di malattie infiammatorie croniche risultanti da una complessa interazione tra fattori genetici (tra cui, HLA-B27 è il maggior predisponente) e ambientali. Ed è tale interazione ad indurre l'attivazione di processi autoinfiammatori e la disregolazione del sistema immunitario caratterizzanti la malattia.

In molti casi, una diagnosi precoce ed un adeguato monitoraggio dell' attività di malattia risultano difficili a causa della sovrapposizione delle caratteristiche cliniche tra le diverse forme. Il ritardo nella diagnosi e conseguentemente nel trattamento, è inoltre dovuto al fatto che, gli indici d'infiammazione comunemente utilizzati nella pratica clinica, la velocità di eritrosedimentazione (VES) ed la proteina C-reattiva (PCR), sono nella norma in almeno metà dei pazienti con chiara espressione dell'attività di malattia. Il ritardo nella diagnosi conferisce a questi pazienti un carico sintomatico importante ed una perdita di funzione durante gli anni di vita produttiva. Pertanto, forte attenzione è attualmente rivolta all'identificazione di marcatori biochimici e genetici utili alla diagnosi e di fattori prognostici necessari a valutare l'efficacia del trattamento.

Tra i fattori genetici predisponenti, è noto il ruolo di HLA-B27, che contribuisce però solo per il 20-30% all'ereditarietà totale, mentre il complesso maggiore di istocompatibilità (MHC) rappresenta circa il 40-50% del rischio genetico di sviluppare la patologia. Questo dato ha suggerito il probabile coinvolgimento di altri geni nel meccanismo patogenetico. Studi di associazione genetica hanno permesso di identificare un certo numero di altri geni, associati alla patologia, sia nel locus MHC che in altri loci.

In questo contesto, di grande interesse è lo studio della genetica di TNF-α, considerato il ruolo di tale citochina nel propagare e perdurare dell'infiammazione. Sebbene numerosi studi abbiano dimostrato l'associazione tra i polimorfismi di geni coinvolti nella via del segnale del TNF-α (es. TNFA, TNFSF15, TNFR1 e TRADD) e la patologia di SpA, i risultati sono discordanti. Di grande interesse sono anche le varianti del MEFV gene, coinvolto nella patogenesi della malattia autoinfiammatoria Febbre Mediterranea Familiare (FMF). Studi recenti hanno, infatti, dimostrato che le SpA, ed in particolare la spondilite anchilosante (AS), sono molto comuni tra i pazienti affetti da FMF e che questi pazienti possono presentarsi con AS come unica manifestazione.

Questo studio, condotto su 91 pazienti e 223 controlli, provenienti da una regione italiana del Nord-Est, si propone di identificare fattori bioumorali (biochimici ed ematologici) e genetici al fine di supportare i processi diagnostici e prognostici (risposta alla terapia). In particolare, oltre ai parametri biochimici ed ematologici, è stato valutato se polimorfismi nella regione del promotore del gene TNFA, o dei geni TNFRSF1A e MEFV, possano concorrere con l'allele HLA-B27 all'aumento del rischio di sviluppare la malattia e/o nel predire la risposta agli inibitori del TNF-α.

Metodi. La popolazione studiata comprendeva 91 pazienti con diagnosi di SpA (età media ± deviazione standard: 52.1 ± 12.5 anni; 57 maschi, 34 femmine) e 223 donatori di sangue (età media ± deviazione standard: 46 ± 11 anni; 146 maschi, 77 femmine) provenienti dalla Regione Veneto, una regione italiana del Nord-Est. Tra i pazienti, 36 presentavano AS e 55 artrite psoriasica (PsA), con diagnosi formulata sulla base dei criteri rispettivamente di New York e CASPAR. Il protocollo di questo studio è stato approvato dal Comitato Etico Istituzionale locale dell'Università-Azienda Ospedaliera di Padova, Italia (Prot.n. 3024P / 13), e tutti i soggetti arruolati hanno firmato un consenso informato prima di partecipare allo studio.

Per ciascun soggetto arruolato, sono stati raccolti i dati demografici e fisiologici, la storia clinica e familiare. Sono stati raccolti poi, campioni di sangue, al fine di valutare l'emocromo e la VES, e di determinare i livelli di PCR, acido urico, prealbumina, alanina aminotransferasi (ALT) e glucosio.

L'analisi molecolare dei geni MEFV (esoni 2,3,5 e 10) e TNFRSF1A (esoni 2,3,4 e 6) è avvenuta mediante sequenziamento diretto. La determinazione degli alleli HLA-B27 e dei polimorfismi del gene TNFA (-1031T>C;-857C>T;-376G>A;-308G>A;-238G>A) è stata condotta mediante PCR in Real-Time. La determinazione degli alleli HLA-CW6 è avvenuta mediante un test genetico molecolare CE-IVD, disponibile in commercio, che adotta la tecnologia microarray. L'analisi statistica è stata effettuata utilizzando il software STATA (versione 13.1).

Risultati. Un maggior numero di cellule polimorfonucleate circolanti e livelli di PCR più elevati sono stati rilevati nei pazienti affetti da SpA rispetto ai controlli. Inoltre, i pazienti affetti da PsA hanno mostrato livelli più elevati di ALT, non solo rispetto ai controlli, ma anche rispetto a pazienti affetti da AS. In ogni caso tali indici non erano molto elevati e spesso risultavano compresi entro gli intervalli di riferimento.

Come atteso, gli alleli HLA-B27 sono risultati associati all'AS (χ^2=120.1; p<0.0001). Sebbene una frequenza leggermente maggiore degli alleli HLA-CW6 sia

stata osservata tra i pazienti con AS (circa il 6%) o PsA (circa il 13%) rispetto ai controlli (circa 4%), la differenza non è risultata essere statisticamente significativa.

Nessuno dei polimorfismi del gene TNFA è risultato singolarmente associato alla diagnosi SpA, né a quella di AS o PsA, se valutate indipendentemente. Sono stati, poi, statisticamente dedotti gli aplotipi derivanti dalle coppie di combinazioni dei cinque polimorfismi studiati. Gli aplotipi più frequenti nei controlli sono stati selezionati come aplotipi di riferimento, e solo l'aplotipo -1031C/-308G è risultato significativamente associato con l'AS (p=0.015) esercitando in questa malattia un ruolo protettivo (odds ratio: 0.43; intervallo di confidenza al 95%: 0.22- 0.85).

Tre polimorfismi sono stati identificati nel gene TNFRSF1A e tra questi, solo i polimorfismi R92Q (Frequenza dell'allele minore- MAF = 0.034) e c.625 + 10A> G (MAF = 0.479) sono stati selezionati a causa del potenziale ruolo funzionale. Entrambi i polimorfismi non sono risultati associati con la diagnosi di SpA (χ^2 = 1.073 e p = 0.300 per R92Q; χ^2 = 4.721 e p = 0.094 per c.625 + 10A> G). Il polimorfismo c.625 + 10A> G è però, risultato essere associato con la risposta alla terapia con anti-TNF, valutato sulla base di un punteggio BASDAI inferiore / uguale o superiore a 4, a 10 mesi dall'inizio della terapia (p = 0.031).

Ventuno polimorfismi sono stati identificati nel gene MEFV e tra questi, 10 noti per il potenziale significato funzionale. Tali varianti alleliche sono risultate estremamente rare nella nostra popolazione (MAF <0.025) ad eccezione di R202Q (MAF = 0.27). Nessun polimorfismo è risultato essere associato con la diagnosi SpA (p> 0.05).

Conclusioni. In conclusione, i risultati di questo studio suggeriscono il ruolo rilevante della genetica della via del segnale TNF-TNFR nel complesso sistema che induce la patogenesi di SpA e condiziona la risposta alla terapia. Il gene TNFA, nella popolazione oggetto di studio, si è dimostrato un fattore predisponente per lo sviluppo di SpA, ma soprattutto di AS. Al contrario, la genetica del gene MEFV non sembra mostrare alcun impatto in questo gruppo di malattie. L'aplotipo TNFA-1031C/-308G, potenzialmente associato alla produzione di livelli più bassi di TNF-α, sembra esercitare un ruolo protettivo nella patogenesi di AS, mentre è emerso che il polimorfismo c.625 TNFRSF1A + 10A> G costituisce un potenziale fattore predittivo di risposta alla terapia con anti-TNFα.

ABBREVIATIONS

ALT: alanine transaminase

ANTXR2: anthrax toxin receptor 2

AP1: 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

β2m: β2-microglobulin

BMI: Body Mass Index

bp: base pair

CARD9: caspase recruitment domain family, member 9

CASPAR: ClASsification criteria for Psoriatic Arthritis

CCP: cyclic citrullinated peptides

CI: Confidence Interval

CRP: C-reactive protein

CTLs: cytotoxic T lymphocytes

DAS: Disease Activity Score

DAS-28: Disease Activity Score- 28

dbSNP: Single Nucleotide Polymorphism Database

DHPLC: denaturing high-performance liquid chromatography

DMARDs: Disease-modifying antirheumatic drugs

DMSO: dimethyl sulfoxide

dNTPs: deoxynucleotide triphosphates

ER: endoplasmic reticulum

ERAP1: endoplasmic reticulum aminopeptidase 1

ESR: erythrocyte sedimentation rate

ESSG: European Spondyloarhropathy Study Group

EULAR: European League Against Rheumatism

F: forward primer

FMF: Familiar Mediterranean Fever

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

GWAS: genome-wide association studies

HAQ: Health Assessment Questionnaire

IBD: Inflammatory Bowel Diseases

IBD- SpA: spondyloarthritis related to inflammatory bowel disease

IL: Interleukin

IL1R2: interleukin-1 receptor 2

IL23R: interleukin-23 receptor

mAbs: monoclonal antibodies

MAF: Minor Allele Frequency

MASES: Maastricht Ankylosing Spondylitis Enthesitis Score

MEFV: Mediterranean fever

MHC: Major Histocompatibility Complex

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

OCT1: Organic Cation Transporter 1

OD: Odds Ratio

PCR: polymerase chain reaction

PMN: polymorphonuclear cells

PsA: Psoriatic Arthritis:

pSpA: peripheral spondyloarthritis

R: reverse primer

ReA: reactive arthritis

SD: Standard Deviation

SNPs: single nucleotide polymorphisms

SpA: Spondyloarthritis

sTNF: TNF-α soluble form

sTNFR: soluble receptors fragments

TACE: TNF-α converting enzyme

TASC: Australo-Anglo-American Spondyloarthritis Consortium

T_H: T helper

tmTNF: TNF-α transmembrane form

TNF-α: tumor necrosis factor α

TNFR1: tumor necrosis factor receptor type 1

TNFR2: 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

U: Unit

UPR: unfolded-protein response

uSpA: undifferentiated spondylarthropathy

VAS: visual analogue scale

VASg: visual analogue scale of global disease activity

WBC: White Blood Cells

1. INTRODUCTION

1.1 Spondyloarthritis

In 1974, Moll and colleagues introduced the concept of *seronegative spondarthritides*, a group of chronic inflammatory diseases characterized by the sharing of genetic, clinical and radiological features, clearly different from the other rheumatic diseases.
The term '*seronegative*' referred to the absence of rheumatoid factor in serum, feature that allowed distinguishing these disorders from rheumatoid arthritis [1].
The term *'spondarthritides'* as well as '*spondyloenthesiticarthropathies'*, was used to highlight the three main aspects of these disorders: the inflammation of the axial skeleton (sacroiliac joints and spine); the inflammation at entheses (sites of attachment of tendon, ligament, fascia or joint capsule to bone); and less frequently, the peripheral arthritis, commonly occurring in a characteristic pattern, asymmetric, oligoarticular and preferentially in the lower extremities. Today, the term '***spondyloarthritis***' (SpA) is preferable to '*spondyloarthropathy'*, to better emphasize the inflammatory nature of these diseases [2,3].
Extra-articular features, such as cutaneous (psoriasis, pustular lesions, erythema nodosum, pyoderma gangrenosum), ocular (uveitis) and mucosal (oral, intestinal, genital ulcers) manifestations as well as the inflammatory bowel disease (IBD) are also present [2-4].
Furthermore, characteristic features of SpA family are the strong association with the human leukocyte antigen (HLA) B27 gene and the frequent familiar linkage.
The understanding of genetics, pathophysiology of inflammation (e.g., lesions on Magnetic Resonance Imaging- MRI), and structural damage (e.g., sacroiliitis on plain radiographs) affect clinical practice in the context of classification and diagnosis. Based on the specific pattern, patients are classified as belonging to a specific subgroup [4].

1.1.1 Classification criteria

In many cases the classification in subgroups can be difficult because of the overlap of clinical features. In fact, in addition to a common genetic background, there is often an overlap of several symptoms among SpA family diseases, making difficult an early diagnosis in a patient showing clinical signs attributable to a SpA.

For this reason, over the years it became necessary to identify a standardized and evidence-based approach to classify these diseases.

With the specific intention of classifying SpA including patients with undifferentiated spondylarthropathy (uSpA), in 1991, the European Spondyloarhropathy Study Group (ESSG), developed a set of classification criteria for the entire group of SpA (Figure 1) including inflammatory back pain and peripheral arthritis as major entry criteria.

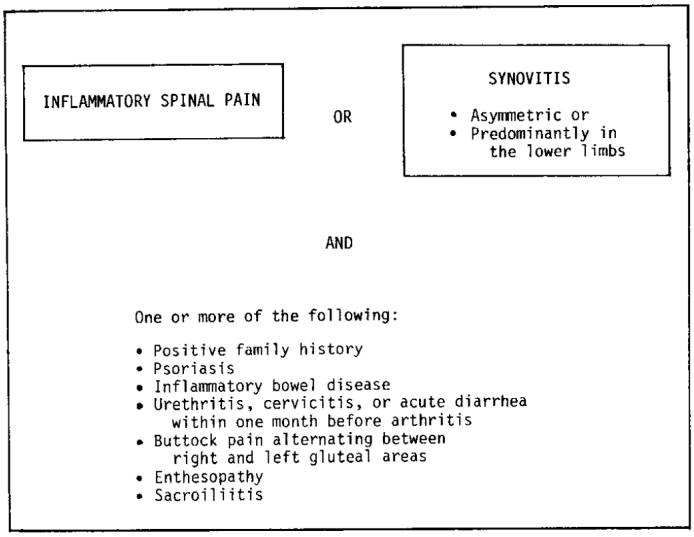

Figure 1. The ESSG criteria for the classification of SpA [5].

Five major subtypes of SpA are recognized on the basis of ESSG classification criteria: ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), spondyloarthritis related to inflammatory bowel disease (IBD- SpA) and uSpA. The ESSG classification criteria for SpA have been well studied and validated in population studies and have a good sensitivity of 75% and a specificity of 87% [5].

Therefore, these criteria were not sufficient to classify patients with isolated clinical manifestations, such as peripheral arthritis, dactylitis, enthesitis, inflammatory back pain or acute anterior uveitis [4].

In the same period, Amor and colleagues proposed an alternative set of classification criteria, allowing a patient to be classified as having SpA whatever the presenting symptoms. The Amor criteria scores out of 12 features covering 4 domains: symptoms, radiographic and genetic features, response to treatment (Table 1).

Table 1. The Amor classification Criteria for SpA (modified by *Amor et al*, 1990) [6].

Amor classification criteria		
Clinical symptoms or past history of		Score
1	Lumbar or dorsal pain at night or morning stiffness of lumbar or dorsal pain	1
2	Asymmetrical olygoarthritis	2
3	Buttock pain	1
		or
	If Alternate buttock pain	2
4	Sausage-like toe or digit	2
5	Heel pain or other well defined enthesitis	2
6	Iritis	1
7	Non-gonococcal urethritis or cervicitis within one month before the onset of arthritis	1
8	Acute diarrhea within one month before the onset arthritis	1
9	Psoriasis, balanitis, or inflammatory bowel disease (ulcerative colitis or Crohn's disease)	2
Radiological findings		
10	Sacroiliitis (bilateral grade 2 or Unilateral grade 3)	3
Genetic background		
11	Presence of HLA B27 and/or family history of ankylosing spondylitis, reactive arthritis, uveitis, psoriasis or IBD	2
Response to treatment		
12	Clear-cut improvement within 48 hours after NSAIDs intake or rapid relapse of the pain after their discontinuation	2
A patient is considered as suffering from a spondyloarthropathy if the sum is ≥ 6		

These criteria, showed higher sensitivity (85%) and specificity (90%) than the previous model, thanks to the inclusion of ocular manifestations of dactylitis and positivity for HLA-B27 [6].

Both these set of criteria, were useful over the years in classification of SpA but not in everyday clinical practice, where it is necessary to include patients in earlier stages of the disease. The main limitation of ESSG criteria consists in the low sensitivity and specificity, that have shown if applied in the early stages of the disease (i.e. within 12 months from onset of symptoms).

With this motivation and recognising of the drawbacks of criteria focused on a specific

subtype the Assessment of SpondyloArthritis international Society (ASAS) has decided to continue the work of ESSG group to improve the current classification criteria for SpA. 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 [7]. In fact, while some diseases within the SpA group affect the axial skeleton predominantly, some others involve the peripheral skeleton primarily. ASAS classification criteria for axial and peripheral SpA are shown in Figures 2 and 3, respectively.

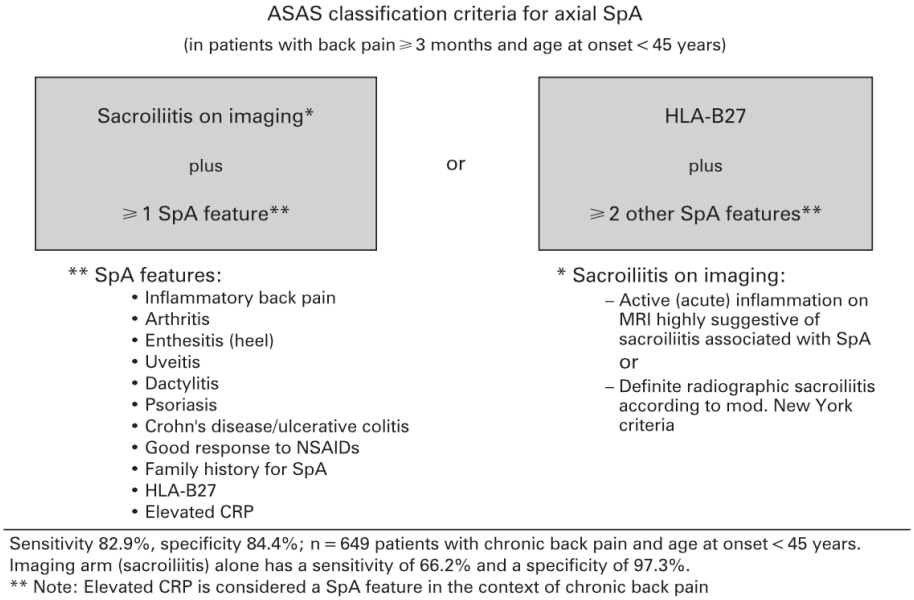

Figure 2. The ASAS classification Criteria for axial SpA [7].

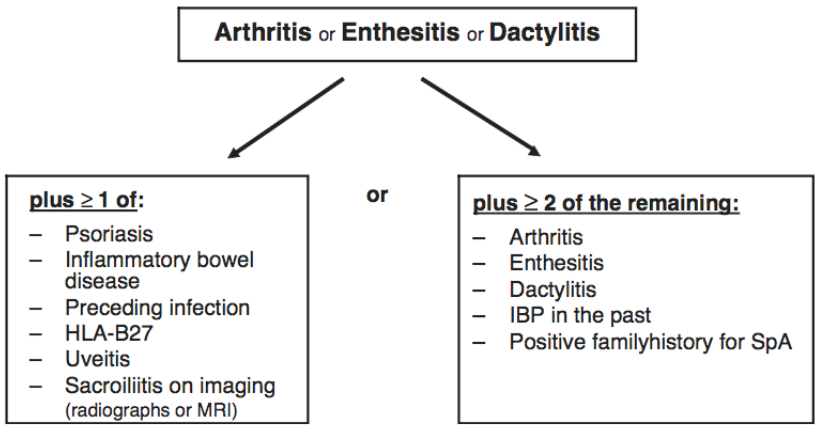

Figure 3. The ASAS classification Criteria for pheripheral SpA [7].

The inclusion of the MRI among the ASAS classification criteria allowed identifying the signs of axial inflammation in the early stages of the disease.

The advantage of this classification approaches is in a better representation of the disease at an early stage and improving the therapeutic choice, differing therapeutic strategies precisely according to the main axial or peripheral form.

According to ASAS criteria, today, SpA are classified into two main groups: the first one represented by **axial spondyloarthritis** (axSpA) includes non-radiographic axial spondyloarthritis (nr-axSpA) and AS; and the second one, the **peripheral spondyloarthritis** (pSpA) including PsA, ReA, and IBD- SpA (Figure 4) [3].

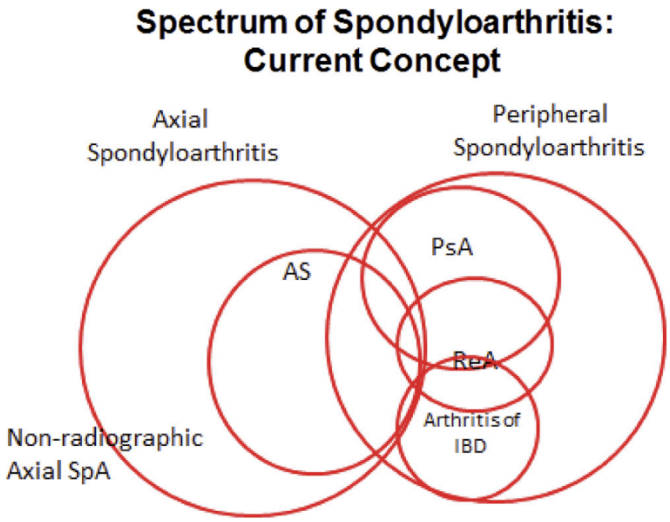

Figure 4. The Spectrum of SpA. The current concept [3].

1.1.2 Epidemiology of Spondyloarthritis

A wide variation in the prevalence of SpA and its specific subgroups has already been described in literature. The differences have been mainly related to geographic area and in particular to genetic characteristics, first of all the presence of HLA-B27. Moreover, demographical and methodological difference between studies (mean age of the patients, male:female ratio, year of data collection, sampling design), other than different criteria used to diagnose SpA and classify subtypes can explain heterogeneity in estimated prevalence of SpA.

Stolwijk and colleagues have recently published data concerning a meta-regression

analysis on the global prevalence of SpA. They reported a pooled prevalence of SpA ranging from 0.20% (95% Confidence Interval-CI: 0.00-0.66) in South-East Asia to 1.61% (95% CI: 1.27-2.00) in Northern Arctic communities (Figure 5).

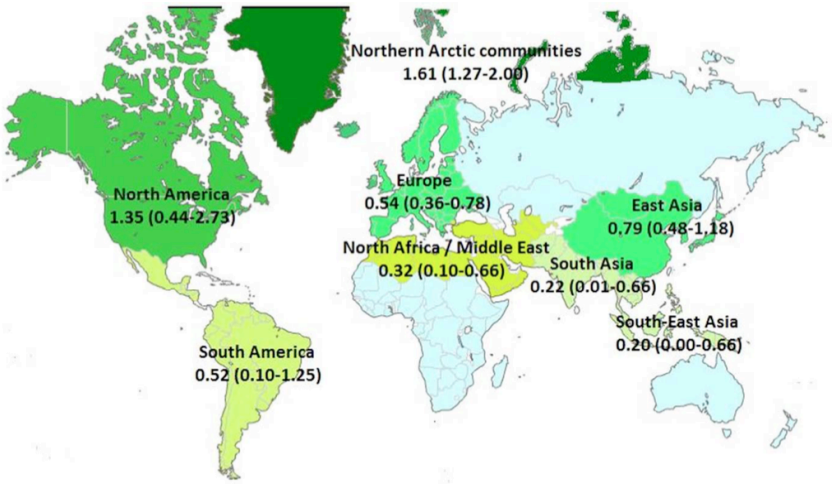

Figure 5. The Global prevalence of SpA [8].

The estimated prevalence of AS, stratified by groups, ranged from 0.02% (95% CI: 0.00-0.21) in Sub- Saharan Africa to 0.35% (95% CI: 0.24-0.48) in Northern Arctic communities; while the prevalence of PsA from 0.01% (95% CI: 0.00-0.17) in the Middle East to 0.19% (95% CI: 0.16-0.32) in Europe. Few data are available on the prevalence of other SpA subgroups, anyway generally low (ReA: 0.0-0.2%; IBD- SpA: 0.0-0.1%, uSpA: 0.0-0.7%) [8].

In literature, is available only one study on the prevalence of SpA in Italy, conducted on 2155 subjects coming from Marche, a region located in the centre of Italy. In this study, the most common SpA subsets were PsA, with a prevalence of 0.42% (95% CI: 0.31–0.61), and AS with 0.37% (95% CI: 0.23–0.49). Two cases with uSpA, two with ReA, and two with IBD-SpA were also observed (0.09%, 95% CI: 0.04–0.16) [9].

1.1.3 Pathogenesis of Spondyloarthritis

The onset of SpA typically occurs at a young age (<45 years, and usually between 20-40 years of age), but due to the lack of a pathognomonic clinical feature or laboratory test, early diagnosis is difficult. The average delay in diagnosis is estimated to be 8-11

years [10]. Without early diagnosis and with delayed treatment, SpA imparts a tremendous symptomatic burden and loss of function during the productive years of life.

The delay in diagnosis, the inflammation indices in the normal range in at least half of SpA patients with a clear disease activity and the lack of complete clinical response to treatment have raised interest in the pathogenetic mechanism involved in the genesis of this group of diseases.

Despite, the numerous studies available in literature, the pathogenesis of SpA is still not entirely clear. SpA are multifactorial diseases, that result of a complex interplay among an inherited genetic background (mainly represented by the HLA-B27 haplotype) and environmental factors (infections, mechanical stress, abnormal intestinal microbiota), that leads to the activation of autoinflammation and the dys-regulation of the immune-system [2-4].

1.1.4 Diagnosis

The wide variety of expression of these diseases is reflected in an equally variable spectrum of presentation, both from a clinical that biohumoral point of view.

Laboratory

As in rheumatoid arthritis and other inflammatory arthropathies, specific biomarkers of disease activity are not commonly used in clinical practice, because there is not a specific diagnostic biomarker.

The absence of rheumatoid factor in serum is a typical feature of SpA patients distinguishing these disorders from rheumatoid arthritis.

In the context of inflammatory spine symptoms, levels of acute phase reactants as C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) can be higher in severe AS (40–50% of patients) and acute exacerbations (notably ReA and PsA) than in those non-radiographic SpA, but at least half of patients with SpA presents indices of inflammation in the normal range, even in the presence of clear expression of disease activity with inflammatory synovial fluid, clinical pathologic expressions and radiographic signs, causing serious structural changes and damage involving the entheses, the peripheral joints, sacroiliac joints and column. Therefore, a normal ESR or CRP does not rule out this condition.

Where inflammatory disease is severe and prolonged, features of anaemia of chronic disease may be evident. Furthermore, persistently elevated serum immunoglobulin A (IgA) is a common, but non-specific finding.

The presence of HLA-B27 increases the chance of ultimately diagnosing a SpA, but is not sufficient to diagnose the condition. In fact, although 85-95% of white patients with AS has HLA-B27, only 6% of HLA-B27 carriers in the general population develop the condition. HLA-B27 is particularly useful for diagnosis of non-radiographic SpA [11].

Imaging

Although the diagnosis of SpA is based primarily on clinical manifestations, imaging techniques are fundamental to confirm the suspected diagnosis, to define the extent of disease and to monitor changes.

Radiographs may be normal, even if there is a long history of suspiciously relevant (previously undiagnosed). In fact, conventional radiology can usually detect late-alterations. In order to identify early-signs of enthesitis, it is necessary to resort to methods with higher sensitivity such as ultrasound combined with power Doppler and, above all, the MRI.

Therefore, in SpA the development of sensitive and specific imaging or biological markers for early diagnosis remains one of the major challenges [11].

1.1.5 Outcome Assessment

In the context of the patient management, five questions concerning the possible clinical presentations (axial, peripheral, enthesopathy, and extra-articular) must be continuously assessed: does the patient really have the disease, is the disease active, is the disease severe, is the disease potentially severe, and is the disease refractory?

Several outcome indexes are commonly used in daily practice and clinical trials to assess these domains [12].

Activity disease in SpA refers to the inflammation and is commonly assessed in daily practice with the bath ankylosing spondylitis disease activity index (BASDAI), a self-administered questionnaire. This index consists of questions related to the patient's self-assessment (e.g., fatigue, pain, swelling, axial and peripheral symptoms, enthesopathy, and duration and intensity of morning stiffness) [13,14].

To improve the objective properties of such an index has been developed another

activity disease index, the ankylosing spondylitis disease activity score (ASDAS) questionnaire. This index includes other than the four questions from the BASDAI, also the level of acute phase reactants evaluation [15,16].

Furthermore, the peripheral joint commitment can be assessed through the count of tender/swollen joints and the evaluation of dactylitis and enthesitis indices, as the Maastricht Ankylosing Spondylitis Enthesitis Score (MASES) [17] and Disease Activity Score- 28 (DAS-28) [18].

The spinal pain can be evaluated with a 10-cm visual analogue scale (VAS) and a visual analogue scale of global disease activity (VASg) [19, 20].

The severity of disease refers to structural damage, often due to tissue remodelling and its functional consequences. Because the structural damage and functional impairment in SpA are largely irreversible, it is necessary to predict the natural course of the disease at an early stage.

In this context, several outcomes domains have been proposed to show severity: death, job loss, functional impairment, range of motion, and hip involvement.

The Bath ankylosing spondylitis functional index (BASFI) [21] and the Health Assessment Questionnaire (HAQ) [22] assess the functional statement.

The spine mobility can be investigated through the Bath Ankylosing Spondylitis Metrology Index (BASMI) [23].

Furthermore, the outcome indexes are also important to lead the clinicians in the choice of treatments. In fact, whether the disease is refractory is important to move from a first-line to a second-line treatment [4].

1.1.6 Treatment

The objectives of treatment of SpA are to improve the condition of the patient (e.g., pain, functional disability) and to prevent subsequent clinical deterioration.

Considered the variable spectrum of presentation of SpA, the therapy is usually based on the use of different treatment strategies depending on the main clinical manifestations. In general, non-pharmacological methods (physical and occupational therapy) are combined to drug therapies.

Non-steroidal anti-inflammatory drugs (NSAIDs) represent the cornerstone of pharmacological therapy for SpA, especially for axSpA. In fact, NSAIDs rapidly (48–72 h after intake) reduce pain and stiffness (reducing the level of acute-phase reactants)

and are effective on outcome measures.

Despite the evidence of high rates of response with regard to the inflammatory spinal pain, approximately 50% of patients with early onset continue to have active disease. Furthermore, despite the benefits of the short-term therapy, the potential long-term gastrointestinal and cardiovascular toxic effects due to the daily intake of NSAIDs have to be taken in account in the choice of therapy [24].

Disease-modifying antirheumatic drugs (DMARDs) such as sulfasalazine, methotrexate, leflunomide and cyclosporine, effective in the treatment of peripheral arthritis and other extra- articular features such as psoriasis, uveitis, and inflammatory bowel disease, have no proven efficacy for either the axial or enthesopathic features of SpA.

Therefore, the pharmacological therapy of SpA is generally based on the association of NSAIDs with DMARDs.

The major clinical and therapeutic advance in SpA treatment is the successful use, in the last decade, of biological drugs and in particular of tumor necrosis factor α (TNF-α) inhibitors (etanercept, infliximab, adalimumab, golimumab, etc.) in active and refractory disease [25].

The rational use of TNF-α inhibitors (anti- TNFα) is based on the pro-inflammatory effects of this cytokine implicated in the pathogenesis of several diseases, such as SpA, psoriasis, rheumatoid arthritis and inflammatory bowel diseases. Anti-TNF-α agents act by inhibiting the binding of TNF-α to its receptors and therefore interfere with TNF-α signaling transduction pathways.

TNF-α inhibitors are highly effective in targeting the different disease features, not only axial disease but also peripheral arthritis, enthesitis, and extra-articular features such as psoriasis or uveitis [26]. Treatment with anti-TNFα drugs leads to a significant improvement in working life (employment, sick leave and productivity) and physical activity (participation rate, hours/week, and physical intensity) [27]. It also been proven that the effectiveness of TNF-α inhibitors is maintained for several years of treatment, and the different TNF-α inhibitors seem to show the same effect on different clinical manifestations [28].

Despite its major therapeutic effectiveness, a 20–40% of the patients do not respond well to treatment and the genetic background may play an important role in this context. In case of failure of a first TNF-α inhibitor, trying a second one is justified since many patients do still respond to a different anti-TNFα. Finally, although TNF-α inhibitors seem to halt joint destruction, they fail to slow new bone formation in SpA [29].

1.2 Ankylosing spondylitis

Ankylosing spondylitis (AS) is the most prevalent subtype of axSpA with an overall estimated prevalence ranging from 0.1 to 1.4%, in Middle-Europe. The estimated incidence ranges from 0.5 to 1.4 per 100000 people per year in studies from different countries. AS generally affects young people aged from 20 and 40 years and less than 5% of cases have an onset after 45 years. The male:female ratio is 3:1 and usually the clinical expression is more severe in males. AS is an inflammatory disease involving primarily the axial skeleton and sacroiliac joints, but peripheral arthritis and enthesitis can also occur. Characteristic symptoms of AS are spinal stiffness and loss of spinal mobility which originate from spinal inflammation, structural damage (syndesmophytes bridge between vertebrae that results in the iconic "bamboo spine"), or both. Extra-articular manifestations of the disease also include anterior uveitis, osteoporosis, cardiac disease with primarily valvular involvement, renal disease, lung disease, gastrointestinal disease, and skin disease [30-32].

Diagnosis of AS is based on the modified New York diagnostic criteria, initially proposed in 1984 [33].

These criteria required the evidence of advanced sacroiliitis (unilateral grade 3 or 4 sacroilitis or bilateral grade 2 to 4 sacroilitis) on radiographic imaging together with any one of three clinical criteria: inflammatory back pain, limitation of the motion of the lumbar spine, and chest expansion. Although these criteria are quite specific, they do not allow an early diagnosis of the disease, for this reason recently, have been developed other criteria that take into account MRI images, able to highlight the early lesions [7]. Therefore, considering that sacroiliitis is the hallmark of AS, conventional radiography is sufficiently sensitive in establishing more than 95% of patients having structural changes in the sacroiliac joints, while the detection of typical syndesmophytes could be useful for diagnosis in individual patients. Furthermore, MRI could provide an additional diagnostic benefit in visualising the active inflammation in early stages of the disease. Laboratory indices, as CRP and ESR, are only a support tool in diagnosis of AS, in fact only half of AS patients have raised CRP concentrations. In addition the correlation of disease activity with laboratory indices of inflammation is restricted. AS is strongly associated with HLA-B27, but as stated above HLA-B27 is not sufficient to diagnose the disease. However, associations with AS are firmly established for subtypes B*27:02 (Mediterranean populations), B*27:04 (Far Eastern populations), B*27:05

(white and worldwide populations), and B*27:07 (South Asian and Middle Eastern populations), while the subtypes B*27:06 (Southeast Asian populations) and B*27:09 (southern Italian and Sardinian populations) are not associated with AS [34]. In addition to HLA-B27, definite associations have been identified with the non-MHC genes, IL23R (interleukin-23 receptor) and ERAP1 (endoplasmic reticulum aminopeptidase 1), and with the gene deserts 2p15 and 21q22. Furthermore, there is strongly suggestive evidence implicating other genes including IL1R2 (interleukin-1 receptor 2), ANTXR2 (anthrax toxin receptor 2), TNFSF15 (tumor necrosis factor (ligand) superfamily, member 15), TNFR1 (tumor necrosis factor receptor 1) and TRADD (Tumor necrosis factor receptor type 1-associated death domain protein) [35].

Treatment goals for AS include reducing symptoms, improving and maintaining spinal flexibility and normal posture, reducing functional limitations, maintaining the ability to work, and decreasing the complications associated with the disease.

According to recommendations for the management of AS proposed by ASAS working group and European League Against Rheumatism (EULAR), the best treatment of the disease is a combination of non-pharmacological (spa treatment, education and physical therapy) and pharmacological methods. The standard treatment of spinal pain and stiffness for patients with AS consists of NSAIDs including selective inhibitors of cyclo-oxygenase 2, and structured exercise programmes. Continuous NSAIDs treatment is recommended for persistently active, symptomatic disease, with doses adjusted in accordance with the severity of symptoms. DMARDs, such as methotrexate and sulfasalazine, have not been shown to be effective in AS and more in general in axial SpA. Anti-TNF therapy should be given according to ASAS recommendations in patients who have had inadequate response to at least two NSAIDs used for 4 weeks or more, or in patients who develop unacceptable side effects (cardiovascular, gastrointestinal, and renal effects) [36-38].

1.3 Psoriatic arthritis

Psoriatic arthritis (PsA) is a chronic inflammatory arthritis associated with psoriasis and characterized by: asymmetric distribution, distal interphalangeal joint involvement, dactylitis (inflammation of the whole digit), enthesitis, spinal involvement, and association with HLA-B27. On the basis of these characteristics, PsA has been classified as a HLA- B27-associated SpA [39]. As stated above, the prevalence of PsA ranges from 0.01% (95% CI: 0.00-0.17) in the Middle East to 0.19% (95% CI: 0.16-0.32) in Europe [8], and is equal to 0.42% (95% CI 0.31–0.61) in Italy according to an only study conducted on subjects coming from the centre of Italy [9]. PsA affects man and women equally with onset typically between the ages of 30-50 years. Skin disease usually predates the onset of joint disease by an average of 10 years.

PsA is a heterogeneous disorder traditionally classified into five subtypes including: asymmetric oligoarticular pattern, which involves five or fewer joints (usually medium-large joints, e.g, wrist and knee); symmetric polyarticular pattern, which predilects the metacarpophalangeal and proximal interphalangeal joints; distal interphalangeal joint disease pattern; spondyloarthropathy, affecting the sacroiliac joints and the apophyseal joints of the spine, whose presentation is similar to AS but it can usually differentiated from the later age of onset and presence of psoriasis; and arthritis mutilans, which is the most severe form, in which extensive bone destruction and remodelling result in extreme deformities and loss of function [40]. Specific features of PsA are dactilitys (swelling of a finger or toe, also called as ‘sausage finger/toe’) and enthesitis, usually affecting the Achilles tendon, but the most important element is the personal or family history of psoriasis. In reality, patients rarely fit closely into one subtype and the pattern of joint involvement changes over time, therefore the pattern at presentation is not particularly useful for classification.

Therefore, in 2006, the Classification for Psoriatic Arthrithis group proposed the CASPAR (ClASsification criteria for Psoriatic ARthritis) criteria, that include both clinical and radiological features, resulting in a sensitivity of 98.7% and a specificity of 91.4%. To meet the CASPAR criteria, a patient must have inflammatory articular disease (joint, spine, or entheseal) accompanied by a score higher than/equal to 3 resulting from the sum of the following 5 criteria: 1) evidence of current psoriasis, or a personal/family history of psoriasis; 2) typical psoriatic nail dystrophy including onycholysis, pitting, and hyperkeratosis observed on current physical examination; 3) a

negative test result for the presence of rheumatoid factor; 4) current dactylitis; 5) radiographic evidence of juxtaarticular new bone formation, appearing as ill-defined ossification near joint margins (but excluding osteophyte formation) on plain radiographs of the hand or foot [41]. These criteria are also considered the standard inclusion criteria in clinical trials involving patients with PsA.

Conventional radiology shows the classical features of the PsA comprising the combination of erosive change with bone proliferation, in a predominantly distal distribution (specially interphalangeal joints). In the case of enthesitis and tenosynovitis (e.g. dactylitis) of particular importance are the echo-power Doppler and MRI.

Moreover, PsA also lacks of laboratory diagnostic markers. The most popular laboratory markers of inflammation, such as ESR and CRP, are elevated in only half of the patients, as demonstrated in a multicentre study conducted on a large cohort of Italian PsA patients [42]. Interestingly, these indices are significantly elevated in elderly onset PsA, probably reflecting more increased levels of proinflammatory cytokines in comparison with younger onset PsA and a more severe outcome [43]. Furthermore, elevated levels of ESR have been proposed, as one of the best predictors of damage progression and, while low levels of ESR seems to be protective. Moreover an ESR >15 mm/h is one of the factors associated with an increased mortality in PsA [44]. The synovial fluid effusion is much higher in PsA than in other arthropathies. When available, synovial fluid analysis may offer additive information useful for the diagnosis, such as the increased number of leukocytes, which underlines the inflammatory nature of the effusion even in a patient with normal serum levels of acute phase response. Some biomarkers are utilised to differentiate PsA from other diseases more than to characterize PsA. For example in polyarticular PsA, which may be in some cases indistinguishable from rheumatoid arthritis, the rheumatoid factor or antibodies to cyclic citrullinated peptides (anti-CCP), may be useful to better identify rheumatoid arthritis. However, rheumatoid factor was found in 5- 13% of patients with PsA, and anti-CCP may be observed in almost similar percentage [45].

Among the genetic markers most studied, alleles at the HLA locus on chromosome 6p have been most informative. HLA associations with PsA have been demonstrated particularly for class I alleles, at the B and C loci. Compared to most of the rheumatic diseases, heredity plays a particularly important role in this form of SpA. About 15% of the relatives of a patient with psoriasis manifest the PsA, and a further 30-45% have psoriasis. The most involved allele for psoriasis is HLA-CW6 and in particular, the

presence of HLA-CW*0602 is associated with more severe and early onset in both psoriasis and PsA. Moreover, HLA-CW*0602 alleles confer a phenotype characterized by a longer interval (> 10 years) between the onset of skin psoriasis and arthritis [46]. HLA-B16, HLA-B38 and HLA-B39 are have been associated with peripheral arthritis in PsA, while HLA-B27 with spondyloarthropathy subset, as in AS. In subjects B27 and B39 positive, musculoskeletal manifestations seems to occur simultaneously with the skin manifestations [47]. There is also evidence that other genes in the HLA region on chromosome 6 may be important in PsA, including TNF-α and its promoter. Other genes, as Interleukin (IL)-1 gene cluster on chromosome 2q12-13 and IL-23R gene on chromosome 1p31 were found to be associated with PsA [39, 48].

The management of PsA requires attention to both skin and joint manifestations. Several groups of expert have developed various sets of recommendations, either at the national level or at the international level. At the international level, two recommendations sets are available: these have been developed by the Group for Research and Assessment of Psoriasis and Psoriatic Arthritis (GRAPPA) and by the EULAR, published in 2009 and 2012 respectively [49, 50]. These recommendations deal mainly with pharmacological treatments, although an optimal management of patients with PsA should also include non- pharmacological strategies with patient education and regular exercise.

Both recommendations sets propose a graduated, overlapping approach to the treatment of PsA, in particular propose NSAIDs drugs as first treatment for joint inflammation then, if necessary, introduction of conventional synthetic DMARDs (such as methotrexate, sulfasalazine and leflunomide), and finally, if inflammation persists, introduction of anti- TNFα. The main pillar of pharmacological treatment is represented by DMARDs [51].

1.4 Genetics of Spondyloarthritis

The pathogenesis of SpA encompasses a complex array of genetic, immunological and environmental factors. Genetic factors have long been recognized to play an important role in pathogenesis of SpA. The most consistent and dominant genetic effect of AS and PsA is located within the major histocompatibility complex (MHC) region, but several genome-wide association studies (GWAS) identified a number of genes associated with SpA and located in non MHC regions.

1.4.1 MHC-related genetics

1.4.1.1 HLA-B27 genetics

The HLA-B27 expression is closely related to the pathogenesis of SpA, and in particular to the susceptibility of developing the axial location. However, the prevalence of this factor varies depending on the subtype (80- 90% in patients with AS, 50-75% in patients with other SpA subtypes) and geographic area. Although the presence of HLA-B27 in 80–90% of patients with AS suggests a direct and dominant effect of the gene encoding the molecule, the mechanisms underlying the striking association of SpA with the HLA-B27 remain poorly understood [2].

The HLA-B27 gene consists of at least 132 different alleles coding for 105 different protein subtypes (named HLA-B*27:01 to HLA-B*27:106). The most common subtypes associated with AS are HLA- B*27:02 in Mediterranean populations, HLA-B*27:04 in Chinese population, HLA- B*27:05 among Caucasians, and B*27:07 in South Asian and Middle Eastern populations.

Two subtypes, HLA- B*27:06 (Southeast Asian populations) and B*27:09 (southern Italian and Sardinian populations) do not seem to be associated with the AS [34].

The researches carried out show subtle differences (one or more amino acid substitutions) between proteins encoded by the alleles associated with SpA, such as B*27:05, and those not associated with the disease, such as, for example, the B*27:09. They include the structural conformation, the protein binding, the thermodynamic stability, the cell surface expression and are able to influence the type of link between the various ligands with the translated protein [52].

The role of HLA–B27 molecule is to bind to peptides derived from both self-antigens (arising from degradation of endogenous proteins) and intracellular pathogens

(mimicking self-proteins) and present them at the cell surface for recognition by T lymphocytes. Unresponsiveness to self-peptides presented by HLA–B27 molecules is ensured by tolerance, which is largely acquired through thymic selection of the T-cell repertoire. To the contrary, antigenic challenge breaks self-tolerance, leading to autoimmunity. Multiple theories based on either the structural or functional properties of HLA–B27 have been proposed over time to explain its pathogenic role in SpA [53].
The three main accredited hypotheses include: the presentation of arthritogenic peptides to autoreactive T lymphocytes, the misfolding of HLA-B27 during its biosynthesis in the endoplasmic reticulum (ER) leading to an unfolded-protein response (UPR) and the formation of heavy-chain homodimers activating natural killer cells (NK).
While the first theory supports the autoimmune mechanism, the other two hypotheses argue for an autoinflammatory role of HLA-B27 in triggering innate immune responses [2].

Arthritogenic peptide hypothesis

The arthritogenic-peptide theory, formulated in 1990 [54], is the most accepted pathophysiological framework for SpA. It was assumed that HLA–B27 molecules binding to pathogenic antigens (e.g. intracellular bacteria or an ubiquitous virus) activates cytotoxic T lymphocytes (CTLs) response. If this antigen show molecular or anti- genic mimicry with a constitutive self-ligand of HLA-B27, activated CTLs overcome self-tolerance against that peptide, leading to autoimmunity, tissue injury and inflammation. There are several self-peptides revealed as possible ligands of HLA-B27 (many derived from cartilage/bone-related proteins) that show high homology to peptides derived from pathogenic bacterial proteins [52].
The arthritogenic-peptide theory has been supported by the identification, in the synovial fluid of AS patients, of autoreactive HLA–B27–restricted CTLs that recognize peptides derived from intracellular bacteria as well as uninfected healthy cells [55]. Other evidence for this hypothesis is provided by the triggering of SpA by gastrointestinal or urogenital infections, and the presence of HLA-B27-restricted CTLs that are reactive against bacterial antigens as well as against self-proteins from cartilage in the inflamed joint. The observation that the onset of disease is often preceded by infection with enteric bacteria has contributed to the continued popularity of this theory. However, there are several points leading to think that additional hypotheses could explain HLA-B27 association with the SpAs' pathogenesis. First of all the arthritogenic

peptide has not been demonstrated [56], moreover, two indipendent groups proved that transgenic rats developed inflammatory phenotype independently to CD8+ T-cells [57, 58]. Therefore two additional hypotheses have emerged to explain the role of HLA-B27.

Misfolding hypothesis

The proper folding of a protein is closely related to its function. Therefore cellular quality control processes, generally, degrade partially folded or misfolded proteins to avoid the totally or partially loss of function and the activation of abnormal processes.
Class I molecules associate in ER with β2-microglobulin (β2m) and antigenic peptides for cell surface expression and presentation to T-cells. However, HLA-B27 can form covalent homodimers and polymers though a cysteine-67 residue in the α1 domain. Misfolded HLA-B27 heavy chains tend to accumulate in ER, triggering ER stress, which leads to activation of UPR [59] resulting in activation of nuclear factor κappa-light-chain-enhancer of activated B cells (NF-κB). In certain cell types, especially in monocytes/macrophages, the activation of NF-κB induces the release of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, favouring inflammatory processes [60]. This hypothesis was confirmed by the finding that misfolded HLA-B27 molecules and UPR activation were observed in transgenic rats with inflammatory disease [61]. On the contrary, the study of a transgenic rat model, in which arthritis, spondylitis, and enthesitis were developed even though the accumulation of HLA-B27 misfolded heavy chains was reversed by increasing the expression of β2m protein, challenged this theory [62].

Cell surface HLA-B27 homodimers hypothesis

HLA-B27 heavy chains can form homodimers and polymers independently in the ER and at the cell surface. Generally, the cellular quality control processes ensure that only the correctly folded MHC class I-peptide complexes are exported to the cell surface, on the contrary misfolded HLA-B27 molecules accumulate in ER, as previously described, and activate the UPR. The HLA-B27 heavy chain homodimers detected at the cell surface, are thought to be produced locally during endosomal recycling [63].
HLA-B27 homodimers produced at cell surface bind to specific receptors expressed on NK cells, T-lymphocytes, and myelomonotic cells playing an immunomodulatory effect in the pathogenesis of autoimmune disorders HLA-B27-associated [64].

This hypothesis was supported by the finding that the number of NK and CD4+ T-cells expressing a receptor which recognizes HLA-B27 homodimers but not heterodimers increase in HLA-B27 positive patients [65].
Other findings challenged this hypothesis. For example, the residue Cys67, critical in homodimers formation, exists in HLA-B27 subtypes related to SpA as well as in subtypes not SpA related [53].

HLA-B27 is the most important gene predisposing to AS, but it not seems to be the only one. In fact, studies in twins estimate that HLA-B27 contributes only 20–30% of the total heritability, whereas the whole MHC accounts for about 40–50% of the genetic risk. In addition, fewer than 5% of HLA-B27 carriers in the general population develop disease. Beside HLA-B27, other MHC genes such as HLA-B60 and HLA-DR1 seem to be associated with AS. These considerations suggest the contribution of additional genes in the pathogenesis of SpA [2].

1.4.1.2 TNFA genetics

Moreover, non-HLA genes within the MHC region have been implicated in SpA but the existence of long-range linkage disequilibrium at 6p21 does not allow a stringent distinction to be made between true susceptibility alleles and markers that are simply linked to the disease. From a biologic point of view, the association between TNFA promoter single nucleotide polymorphisms (SNPs) and SpA is of particular interest, because increased levels of various cytokines (TNF, IL-1, IL-6, and IL-18), derived primarily from monocyte/macrophages, have been observed in the psoriatic skin, synovial fluid, and synovial membrane of patients with PsA [66], as well as in blood of AS patients. Moreover its expression by peripheral T cells correlated well with AS activity [67, 68]. In this context, it is assumed that the levels of TNF-α in vivo may be affected by polymorphisms of the TNFA gene. The study of the TNFA promoter polymorphisms is of particular interest not only because of their potential functional role in pathogenesis of SpA, but also because the most biologic drugs used to treat the disease targets the TNF-α protein.
TNFA, the gene encoding TNF-α is located in the class III region of the major histocompatibility complex on chromosome 6 between the HLA-B and HLA-DR genes. Several TNFA polymorphisms have been identified inside the TNFA promoter at the positions (relative to the transcription start site): −1031 (T>C), −863 (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) (Figure 6). Some of these have also been shown to influence the rate of transcription and protein production of TNF-α and TNF-β associations with diseases [69, 70].

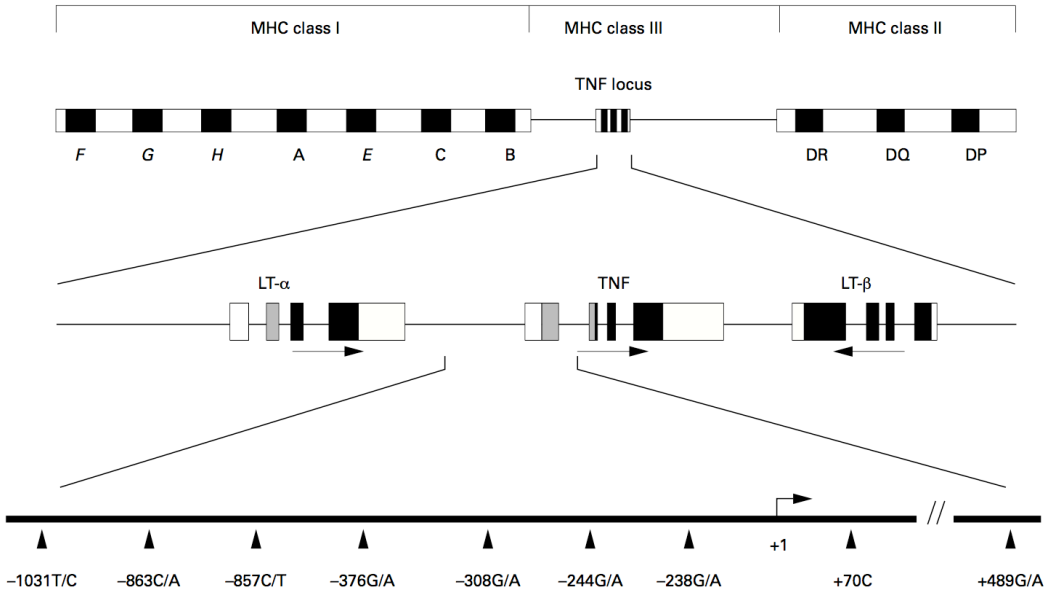

Figure 6. Location of TNF gene within the MHC region. The arrow indicate the transcriptional orientation of TNF gene. The position of the SNPs within TNF gene is indicated [69].

Although the association between polymorphisms in the TNFA promoter region and SpA diseases has been reported in various studies and different populations, the role of these polymorphisms in SpA patients is still unclear.

Controversial results emerged studying the relation between TNFA SNPs and SpA. TNFA polymorphisms seems to have no independent effect on AS susceptibility [71, 72] but their modulating effect on TNF-α expression were well relevant to the phenotypic diversity in AS [73, 74]. In contrast, other studies demonstrated an association of −308G>A polymorphism with susceptibility to AS [75, 76]. Moreover, the A allele was thought to have a protective role against AS [71, 77], and was associated with: a lower risk of developing AS, with the age at disease onset, disease severity and response to anti-TNFα treatment [78]. Höhler and co-workers demonstrated an increased frequency of the TNF-308G allele among patients with PsA suggesting an independent predisposition to the development of arthritic complications [79]. Interestingly, an association of this promoter allele has been reported in German

[80, 81] and Scottish [82] patients with AS, although this association was not found in Spanish [83], and British AS cohorts [81].

Kaijzel et al., observed a significantly decrease of TNFA-238A allele in HLA-B27 positive AS patients. Assessment of association showed that the TNF-238G allele is in linkage disequilibrium with the HLA-B27 allele, suggesting that the association between TNFA-238G and AS is secondary to the HLA-B27 gene [84]. On the contrary, Höhler and co-workers observed a highly significant association among TNFA-238A polymorphism and psoriasis and PsA, which was independent of HLA-B27. The authors also demonstrated that this variant decreased transcriptional activity of the TNFA promoter and consequently reduced production of TNF-α by peripheral blood mononuclear cell [79]. Higher prevalence of both wild-type alleles at positions -238 (GG) and -1031 (TT) were found with moderate to severe psoriasis compared to healthy control group [85].

A large genetics study, conducted on 909 PsA patients and 1315 healthy controls, robustly demonstrated that TNFA-857T represents a risk allele for PsA independent of the HLA-CW6 [86]. Although the functional role of TNFA-857T remains to be determined, it has been shown that allele T increases the transcription of TNF-α [87].

In summary, the data from the studies on TNFA genetic polymorphisms seem to vary from one study to another. This variation could be related to the differences in the ethnic origin or the number of the individuals under study, other than to the low statistical power and clinical heterogeneity in the available studies. Although these polymorphisms do affect the expression level of TNF-α, the activation and abundance of other molecules that interact directly or indirectly with the promoter sequence must affect the expression of TNF-α.

1.4.2 Non MHC-related genetics

Recently, GWASs have identified several SNPs in non-MHC genes (involved in innate and adaptive immunity) significantly associated with SpA, such as: ERAP1, IL23R, IL1R2, ANTXR2, CARD9 (caspase recruitment domain family, member 9), TNFSF15, TNFR1 and TRADD [88, 89].

A strong association has been identified with the combination of three polymorphisms (rs17482078, rs10050860 and rs30187) in the ERAP1, a gene that interacts with HLA-

B27 and is involved in the intracellular processing of the antigen. The mechanism by which ERAP1 predisposes to SpA remains unknown, but one hypothesis is that ERAP1 potentially contributes to the pathogenesis of AS, altering HLA-B27 peptide presentation [90].

A GWAS, conducted by Australo-Anglo-American Spondyloarthritis Consortium (TASC) reported 14 SNPs in and around IL1R2 with at least nominal association with AS. IL-1R2 binds IL-1A and IL-1B with high affinity, and the IL-1 antagonist IL-1RA with lower affinity. IL-1R2 is cleaved from cell membranes, possibly by ERAP1, and acts as a receptor, interfering with the binding of IL-1 to IL-1R1. One possible explanation for the associations of ERAP1 and IL1R2 with AS is that the disease-associated genetic variants affect cleavage of IL-1R2 from the cell surface [88].

IL-23R is another gene strongly associated with SpA and especially with AS, PsA and IBD. Several studies confirmed the role of polymorphisms in that gene in predisposition to the development of AS and PsA, but also on disease severity [91]. Polymorphisms of IL-23R seem to be involved in T helper (T_H) 17 lymphocyte activation/differentiation [89].

Strong evidences support the association of ANTXR2 variants with AS. Two GWAS identified eight SNPs associated with AS, the strongest being with rs12504282. Seven of these SNPs were associated with HLA-B27-positive subgroup, but none was associated with HLA-B27-negative AS [92]. ANTXR2 encodes the protein capillary morphogenesis protein-2 (CMP2), a transmembrane protein expressed during capillary morphogenesis to bind laminin and collagen IV. It is known that recessive mutations of ANTXR2 cause juvenile hyaline fibromatosis and infantile systemic hyalinosis, but the functional mechanism in AS is still unclear [89].

SNPs in CARD9 and CARD14 genes, encoding member of the family of caspase recruitment domain-containing scaffold proteins and involving in the recruitment and activation of the NF-kB pathway, have been also shown in association with AS [93] and PsA [94], respectively.

A strongly suggestive evidence of the association of a region on chromosome 9q31-4, near TNFSF15, with SpA has been reported [95]. The SNPs of TNFSF15 gene, are also associated with Crohn's disease, but it is not clear whether TNFSF15 is the true disease-associated gene in this region, either it is a candidate [96]. Following binding to another TNF superfamily member, DR3 (death receptor 3), TNFSF15 has been shown to stimulate the proliferation of T_H17 lymphocytes, and in an inflammatory colitis mouse

model, to upregulate T_H1 and T_H17 lymphocyte activity [97].

Two SNPs (rs9033, rs868213) of TRADD gene, have been also shown as strongly, but not definitly, associated with AS. In addition to being a key component of the TNFR1-signaling cascade, TRADD is involved in signalling from the innate immunity receptors Toll-like receptors TLR3 and TLR4, and in DR3 signalling [98].

1.4.2.1 TNFRSF1A genetics

TNF-α, is one of the cytokines of particular interest in the propagation and perpetuation of inflammation in SpA. The key role of TNF has been also shown through the effectiveness of TNF blockers, but the mechanism driven by TNF remains unclear. Furthermore, remains poorly understood which form of TNF (soluble or transmembrane), which receptor (TNFR1 or TNFR2) and which target cells are predominantly involved in SpA.

TNF-α is primarily produced as a transmembrane form (tmTNF) that can be cleaved by the TNF-α converting enzyme (TACE) into a soluble 51-kDa homotrimeric cytokine (sTNF). The two TNF-a receptors, TNFR1 and TNFR2, bind with different affinities the sTNF-α and the tmTNF-α: TNFR1 binds both forms equally well; TNFR2 has a higher affinity for the tmTNF.

TNFR1 is constitutively expressed in most tissues and is considered the key mediator of TNF-α signalling, whereas TNFR2 is highly regulated and generally expressed in cells of the immune system. Both receptors contain four cysteine-rich domains in their extracellular domains and the pre-ligand assembly domain. The extracellular domain can be cleaved by proteases, releasing soluble receptors fragments (sTNFR1 and sTNFR2). The soluble receptors by binding their ligand in the circulation neutralize TNF-α signalling. Based on this, the anti-TNF agents are compounds that have been engineered as IgG dimeric fusion proteins, which mimick the soluble receptors fragments [99]. The differences between TNFR1 and TNFR2 intracellular domains, explain the different signalling events evoked by TNF-α stimulation as depicted in Figure 7.

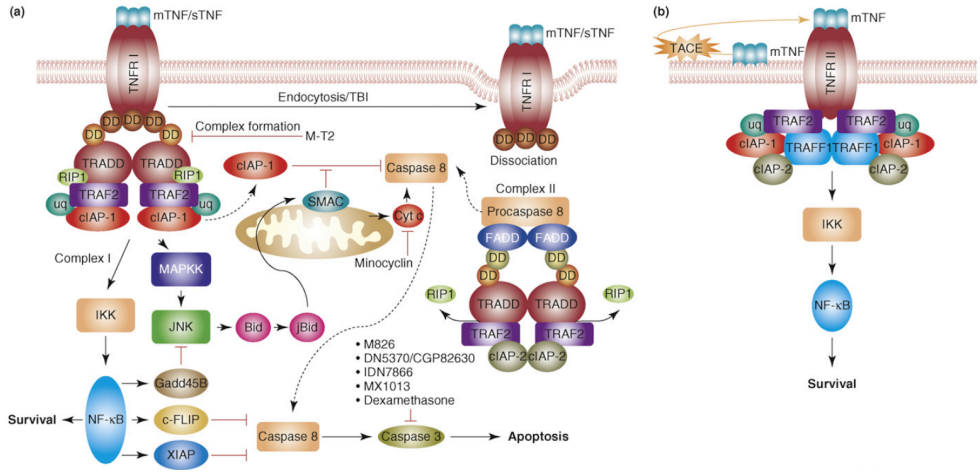

Figure 7. TNF-TNFR signalling mechanisms are mediated via intracellular protein complexes. The binding of TNF-α to TNFR1 (a) and TNFR2 (b) activates different pathways [99].

TNFR1 and TNFR2 are encoded by the human TNFR genes, which are located on the short arm of chromosome 12 (12p13) and cromosome 1 (1p36) respectively. Polymorphisms of the TNFR genes have been described and associated with the susceptibilty for development of different diseases, e.g. diabetes type 1 and sepsis [100, 101].

In addition to polymorphisms in TNFA promoter and in TRADD gene, several SNPs in TNFRSF1A (tumor necrosis factor (receptor) superfamily, member 1A) gene, the gene encoding TNFR1, were also found in association with AS. The TASC study, demonstrated that the strongest TNFRSF1A gene SNP associated with AS, was c.625+10A>G (rs1800693), with respect to other SNPs moderately associated [88]. Karaderi et al., also demonstrated the association between AS and TNFRSF1A in Caucasians and Chinese population [102]. Davidson et al., found a significant association between another TNFRSF1A gene SNP (rs4149577) and AS in Chinese population [103].

The particular interest for TNFRSF1A gene derives also from the fact that it is also known to be the genetic cause of the autosomal dominant autoinflammatory disorder TRAPS (Tumor necrosis factor Receptor-Associated Periodic Syndrome). To date, more than 100 different TNFRSF1A mutations have been reported among TRAPS patients and listed on the INFEVERS website. All the mutations related to TRAPS are located on extracellular domains of TNFR1, CRD1 and CRD2, rispectively involved in the homotrimeric formation and in the ligand binding. Mutations in the gene result in

different aberrant functions of the protein that involve modification in the shedding or in protein folding or in intracellular signalling of TNFR1. The shedding hypotesis suggests that defective TACE-induced shedding of cell surface TNFR1 leads to decreased sTNFR1 available to buffer the effect of TNF, and then increased TNFR1 activation. Mutations can also result in a protein with structural defects that is incapable to reach the cell surface and it accumulates in the ER. Oligomers reteined in the ER can form trimers and constitutively activate, in the cytoplasm, the NF-κB signalling. Furthermore the TNFRSF1A mutations can increase mitochondrial respiration and reactive oxygen species production, potentiating MAPK (Mitogen-activated protein kinase) signalling by inhibiting MAPK phosphatases [104-111].

The most frequent mutations are the Arg92Gln (R92Q) and Pro46Leu (P46L) substitutions, which are associated with a mild TRAPS phenotype with low penetrance [112]. In the last years it has been compared a high frequency of R92Q variant in other inflammatory disorders as rheumatoid arthritis, multiple sclerosis [113- 115]. Recently Amigues and colleagues have presented an atypical case of AS in a patient carrier of R92Q. The Authors conclude that, because of arthralgias and flares of sacroiliitis, typical manifestations of AS, were always associated with other symptoms of TRAPS, the presence of R92Q mutation probably confers particularly inflammatory presentations to AS [116].

1.4.2.2 MEFV genetics

In the context of inflammation and in particular of autoinflammation, an interesting potential candidate is MEFV (Mediterranean fever) gene. This gene, located on chromosome 16p13.3, encodes a 781-aminoacid protein, namely pyrin or marenostrin, involved in the control of inflammation through regulation of leukocyte apoptosis and activation of IL-1β and NF-κB. Missense variations of the gene have been identified as causes of Familiar Mediterranean Fever (FMF), an autosomal recessive autoinflammatory disease [117, 118]. To date, 199 sequence variants have been identified in MEFV gene, mainly located in exons 2 and 10. The most common identified missense variations among FMF patients, also considered disease-causing mutations, are: M680I, M694V, M694I, and V726A in exon 10; R202Q and E148Q in exon 2 [119, 120]. Two different models have been proposed to explain why mutations of pyrin lead to the development of FMF: in the first pro-inflammatory model, the

mutated protein promote a process of hyper- or auto-activation of the inflammosome, while in the second anti -inflammatory model the mutated protein would not be able to inhibit the activation of caspase–1. In both models, however, the abnormal activation of the inflammosome is central in the initiation of the disease process. The inflammosome is a multiprotein complex that mediates intracellular activation of inflammatory caspases (caspase-1 and caspase-11). The main consequence of the activation of caspase-1 is the secretion of inflammatory cytokines IL- 1β, IL- 18 and IL- 33 [121].
Considering that muskoloskeletal involvement, mostly in the form of arthritis, is the second common manifestation of FMF and shows some similarity to the SpA [122, 123] and the AS, are very common among FMF patients and among their first-degree relatives in the Turkish population, the MEFV variations have been proposed as a contributing genetic factor to AS. It has been suspected that the upregulation of the inflammatory response may predisposes MEFV to certain types of inflammatory conditions. Furthermore febrile episodes, acute rheumatic fever and rheumatoid arthritis are reported to be more frequent in MEFV carriers than in controls [124]. Moreover, considering that FMF is mainly prevalent in Jews, Turkish, Armeninans and Arabs, and the carrier frequency of MEFV variations in these populations has been reported to be as high 39%, several association studies have been conducted in Turkish population [125-130]. Controversial results were found. In particular, Durmus et al., found a similar MEFV mutation frequency in AS patients compared to controls, though MEFV mutations exacerbated the severity of disease [129]. On the contrary Cinar et al, demonstrated a high frequency of MEFV mutations (M694V, M680I, V726A, F479L, M694I, P369S) among AS patients, but no significant differences were found between MEFV carriers and non-carriers with respect to clinical and demographic characteristics, as well as in NSAIDs response rate [130]. Anyhow, higher frequency was observed for M694V mutation in the AS patients than in controls [124, 127, 130]. It has been concluded that MEFV gene variations can be considered a potential geographic-specific pathogenetic factor [124].

Genetic studies strongly suggested the presence of non-MHC genes involved in SpA. While GWASs have yielded great insights into the genes that contribute to the pathogenesis of SpA, replication in large cohorts, fine mapping, together with functional studies of genetic variants identified, are warranted to better understand susceptibility, pathogenesis, and to develop more effective therapy.

1.5 Response to TNF-α inhibitors and genetics

The inflammatory nature and the relevant role of TNF-α in the pathogenesis of SpA pose the basis of treatment with drugs targeting the TNF-α pathway.

TNF-α inhibitors comprise two main types of agents: monoclonal antibodies (mAbs) (infliximab, adalimumab, golimumab and certolizumab) and soluble receptors (etanercept). Infliximab is a chimeric mouse-human anti-TNFα mAb composed of a murine variable region (25%) and a human IgG1 constant region (75%). Adalimumab and golimumab are fully humanized anti-TNFα mAbs, which are indistinguishable from the normal human IgG1. Infliximab, adalimumab and golimumab differ in their IgG1 isotypes, the Fc regions of which govern effector functions: complement fixation and Fc receptor-mediated biological activities. Certolizumab is a Fab'fragment portion of humanized anti-TNFα mAbs lacking of the Fc portion, therefore lacking of effector funtions. Its hinge region is linked to two cross-linked chains of polyethylene glycol (PEG) to enhance solubility and hal-life in vivo. Etanercept is a genetically engineered fusion protein composed of a dimer of the extracellular portions of human TNFR2 linked to the Fc portion (CH2 and CH3 domains) of human IgG1 [131,132]. The structures of TNF-α inhibitors are schematically represented in Figure 8.

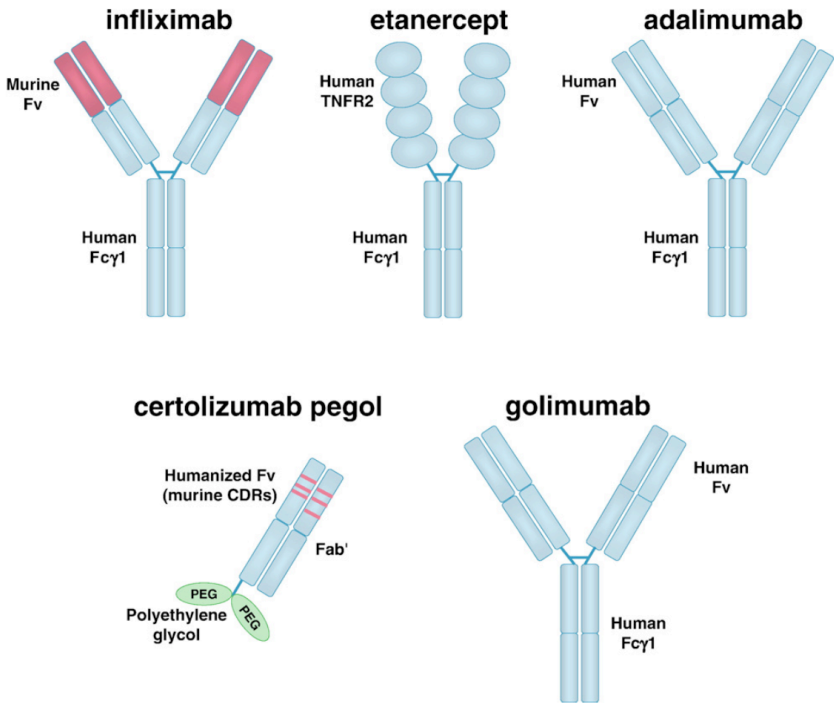

Figure 8. TNF-α inhibitors: the molecular structures [131].

TNF-α inhibitors, other than molecular structures, differ also for their binding specificities and the manner in which they neutralize TNF-α. Their mechanism of action is then quite different. Infliximab binds to both monomer and trimer forms of sTNF, whereas etanercept bind only to the trimer form, suggesting that they probably bind to different epitopes on sTNF (Figure 9A). As bivalent mAbs, infliximab and adalimumab are capable of binding to two TNF-α simultaneously and up to three infliximab molecules can bind to each TNF-α homotrimer blocking all receptor-binding sites on TNF-α. In this way stable complexes mAbs-sTNF are generated. In contrast, etanercept is supposed to form 1:1 complex with the TNF-α trimer in which two of three receptor-binding sites on TNF are occupied by etanercept, and the third receptor-binding sites is open, resulting in relatively unstable complexes (Figure 9B). In addition, TNFR2 is known to have fast rates of association and dissociation to TNF-α, suggesting that etanercept may only transiently neutralizes the activity of TNF-α molecules [131, 133]. Infliximab, adalimumab, etanercept and certolizumab bind also to tmTNF with similar affinities that are lower than for sTNF. As in the case of sTNF, up to three molecules of infliximab can bind one tmTNF, one etanercept can bind one molecule of tmTNF [132].

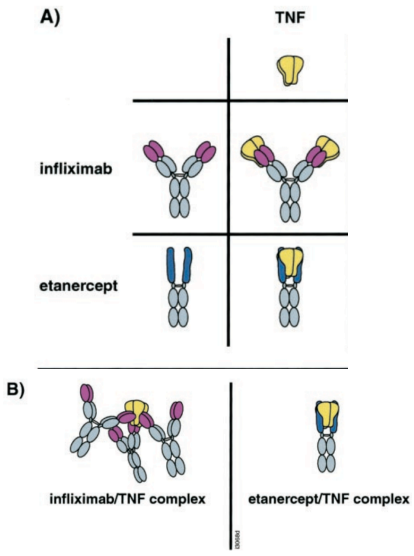

Figure 9 A-B. Infliximab versus etanercept: schematic illustration summarizing differences in binding specificities [133].

Although the binding and neutralizing activities against TNF-α are common mechanisms of action of these drugs, pharmacokinetic effects may influence the

functions of TNF-α inhibitors. Infliximab, for example, may reach higher concentrations in circulation following intravenous infusion and in tissue microenvironments than etanercept or adalimumab and, therefore, have a grater opportunity to bind to tmTNF on cells and induce signalling or Fc receptor-mediated biological activities. The elimination half- lives for infliximab and etanercept are 210 hours and 115 hours, respectively. These differences may account for the somewhat different clinical efficacy of these compounds [131].

The efficacy and safety of these drugs has been supported by clinical trials. Placebo-controlled randomized trials revealed similar efficacy of TNF-α inhibitors in controlling active disease, however, not all patients (20-40%) respond to these therapies and, furthermore they are not exempt from serious adverse events.

TNF-α inhibitors have been associated with increased risk of infections, including reactivation of tuberculosis and sepis, probably as result of interference with innate immunity, but they have not been found to be broadly immunosuppressive. Less clear are the effects of anti-TNFα agents on host defence against malignancies, particularly lymphomas. Other adverse events associated with TNF-α inhibitors were: haematologic disorders such as anemia and pancytopenia, demyelinating disorders and neuropathy, worsening of congestive heart failure, occurrence of autoantibodies and autoimmunity, and hypersensitivity reactions [134].

In the past few years new therapies have been approved for the treatment of SpA, increasing the therapeutic options for these patients. How best to use these drugs remain unclear. The development of predictors of response might identify responders and thus help with making therapeutic decision in clinical practice. Recently, Maneiro and colleagues performed a systematic review and meta-analysis of clinical trials and observational study to identify predictors of response to TNF-α inhibitors in patients affected by AS and PsA. Male gender, HLA-B27, younger age, higher CRP level, higher BASDAI and lower BASFI at baseline predict better response to TNF-α inhibitors in AS but not in PsA [135].

TNF-α genetics has been hypothesized as one of the determinants causing failure of response in terms of both efficacy and toxicity. The majority of studies in this field were performed in patients with rheumatoid arthritis, while only few studies have analysed the role of genetic markers in the response to anti- TNFα agents in SpA patients, considering mainly AS [136- 138]. Studies in this field, summarized in two recent meta-analyses [139, 140], demonstrated that a better response to TNF-α antagonists occurs

among Caucasian patients carrying the common TNFA-308G, -238G and -857C alleles [139]. These genetic variants, however, did not uniformly predict response to treatment, their effect being dependent on the type of drug and on the SpA subtype.

In particular, G alleles of TNFA-308 and -238 could predict the response to etanercept (OR = 4.02 [2.24-7.23]; 5.17 [2.29-11.67]) but not to infliximab/adalimumab (OR = 1.68 [1.02-2.78]; 1.28 [0.57-2.86]). Moreover the TNFA-308 (G>A) polymorphism could predict the response to TNF blockers in PsA and IBD- SpA patients, but not in AS patients [140].

The relation of TNFRSF1A mutations and response to infliximab was investigated in patients affected by inflammatory bowel disorders. Pierik et al., demonstrated that the TNFRSF1A 36G mutation was associated with lower response to infliximab [141]. Other Authors observed a significant association between TNFRSF1A, rs767455 and the efficacy of infliximab in eighty Crohn's disease patients. In particular the minor allele carrier of rs767455 showed a significant association with a lack of efficacy compared to major genotype [142].

Schiotis et al., investigated also the role of SNPs, located in other genes and reported as associated with pathogenesis of SpA, in the response to anti-TNFα agents. 384 SNPs, distributed in 190 genes, were tested in 121 AS patients. Among these, five indipendent predictors of nonresponse to the anti-TNFα therapy were identified: rs917997 in the IL18RAP (interleukin 18 receptor accessory protein) gene (OR 3.35, 95 % CI 1.38–8.15), rs755622 (OR 3.14, 95 % CI 1.19–8.22) in the MIF (macrophage migration inhibitory factor) gene, rs1800896 in the IL10 gene (OR 3.09, 95 % CI 1.04–9.15), rs3740691 (OR 2.90, 95 % CI 1.12–7.51) in the ARFGAP2 (ADP-ribosylation factor GTPase-activating protein 2) gene, rs1061622 (OR: 2.46, 95 % CI 1.00–6.04) in the TNFRSF1B (tumor necrosis factor receptor superfamily, member 1B) gene [143].

Overall, these data further support the relevant role of inflammatory cytokines genetics and of the TNF pathway not only in disease pathogenesis, but also in response to treatment with anti-TNF-a agents.

Pharmacogenomic studies focusing on genes involved in SpA etiology and pathogenesis, in order to analyse the role of allelic polymorphisms in the individual difference in treatment response to TNF-α inhibitors, are limited in SpA. Taking account the cost and the potential severe side effects of these agents, identification of genetic biomarkers of treatment inefficacy would be of major use for prospectively selecting patients that will most likely respond to such treatment.

2. AIMS

The main aim of this retrospective study was to verify whether biochemical markers commonly used in clinical practice and genetic variants of HLA locus, TNF-TNFR pathway and MEFV play a part in increasing the risk of SpA (AS or PsA), and/or in predicting anti-TNFα treatment failure in subjects from the Veneto Region (North-East of Italy).

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

- Specific haematological and biochemical pattern of inflammation considering ESR and complete blood count, CRP, prealbumin, ALT, glucose, creatinine and uric acid;
- HLA-B27 and HLA-CW6 haplotypes;
- 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;
- known or unknown variants of TNFRSF1A by the analysis of exons 2,3,4 and 6;
- known or unknown variants of the autoinflammatory gene MEFV, by the analysis of exons 2,3,5 and 10.

3. MATERIALS AND METHODS

3.1 Studied population

A cohort of 314 subjects belonging to the Veneto Region, a North-East Italian region, was enrolled in this study from January to December 2014.

The cohort comprised 223 controls (146 males and 77 females; mean age ± standard deviation: 46.1 years ± 10.5) and 91 patients (57 males and 34 females; mean age ± standard deviation: 52.2 ± 12.5 years) affected by SpA and attending the Rheumatology Unit of the Department of Medicine- University-Hospital of Padova (Italy).

Among patients, 36 (39.6%) had a definite diagnosis of AS, according to the modified New York criteria [33] and 55 (60.4%) had a diagnosis of PsA according to the CASPAR criteria [41]. The mean disease duration was 15 ± 11.7 years (range 2–56 years).

At enrollment, 58 patients (63.7%) were under therapy with anti- TNFα agents (25 with Infliximab, 21 with Adalimumab, 11 with Etanercept and 1 with Golimumab), of which 11 (19%) experienced in their clinical history, at least one switch of TNF-α inhibitor before entering the study. Patients were followed for at least 12 months after the beginning the anti-TNFα agent. At least 10 months of treatment were considered to define a delayed response to TNF-α inhibitors.

The study protocol was approved by the Local Research Ethics Committee of the University-Hospital of Padova (Comitato Etico per la Sperimentazione, protocol number: 3024P/13) and all subjects gave their fully informed written consent before entering the study.

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 familiarly history (arthritis, psoriasis, other rheumatic diseases) data were also collected in patients.

Outcome Assessment

All patients underwent clinical, clinimetric and functional examinations.

The peripheral joint commitment was assessed through the count of tender/swollen joints and the evaluation of dactylitis and enthesitis indices, as the Disease Activity Score (DAS) [18].

The axial involvement was investigated through the BASMI [23]. A BASMI score from 0 to 10 indicates a condition that ranges from normal spine mobility to the severely restricted spinal mobility.

To evaluate the functional statement was asked patients to fill out two validated questionnaires: BASFI [21] and HAQ [22]. In particular, BASFI includes 10 questions presented in the form of numerical scale, which explores the degree of difficulty encountered in carrying out specific activities of daily life and whose total score is calculated as an average of 10 score questions. HAQ comprises 20 questions about daily living activities divided into 8 categories, the sum of the scores ranging between 0-24 and divided by 8 provides the final score on a scale 0 to 3. The highest values reflect the maximum impairment.

The disease activity was evaluated by using BASDAI [13, 14] and ASDAS [15, 16]. BASDAI includes six questions useful to investigate the intensity of pain in the neck, back, hips and peripheral joints, the pain caused by contact / pressure, as well as the intensity and the duration of morning stiffness. ASDAS is a composite index comprising both, subjective measures and laboratory parameters (spine and peripheral joint pain, morning stiffness duration, disease activity). Values are expressed, for both, on a scale ranging from 0 to 10, where a score >4 identifies active disease.

3.3 Haematological and biochemical indices

For each enrolled subject, four blood samples were collected: 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.

ESR was performed within 4 hours of collection on a Sysmex XE-2100 analyser (Dasit, Milan, Italy), which adopts a method based on light scatter, impedance, and

fluorescence. TEST1 analyser (Alifax, SIRE Analytical System, Udine, Italy), based on capillary photometry-kinetic technology, was used to perform complete blood count.
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 Genetics analyses

Genomic DNA, was extracted from 200 μl EDTA-K_2 peripheral blood using the MagNA Pure96 System (Roche S.p.A., Monza, Italy) 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, Wilmington, DE, 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).

3.4.1 HLA-B27 and HLA-CW6 haplotypes detection

HLA-B27 and HLA-CW6 alleles presence was determined by two molecular genetic testing using commercially available CE-IVD microarray: EUROArray HLA-B27–Direct and EUROArray HLA-CW6–Direct, respectively (Euroimmun AG, Luebeck, Germany).
EUROArray technology is capable to detect all together, 130 different HLA-B27 subtypes (B*27:01–B*27:105) or 51 HLA-CW6 subtypes (C*06:02:01:01–C*06:55), which differ only in some bases. 200 ng of genomic DNA, previously isolated from the enrolled subjects (patients and controls), is mixed with the ready for use polymerase chain reaction (PCR) reagents (DNA polymerase and validated specific primers)

supplied by the EUROArray kits.

In the first reaction step, HLA gene fragments (two sequences of the HLA-B or one of HLA-C gene) and a b-globin gene fragment as positive control were amplified from the genomic DNA sample using a PCR protocol, according to the manufacturer instructions. The HLA-B gene sections are only amplified if the sample contains an HLA- B27 allele, while the HLA-C gene fragment only when the sample contains HLA-C06. All PCR products were labelled with a fluorescence dye during PCR reaction, so that they could be analysed in the second reaction step. The hybridisation of the fluorescing PCR product to the corresponding microarray (containing immobilised probes complementary to the amplified DNA) was detected using the EUROIMMUN Microarray Scanner. A fluorescence signal on the HLA (HLA-B27 or HLA-C06)-specific spots indicates the presence of an HLA-allele in the analysed DNA. The EUROArrayScan software automatically evaluated all spot signals and, on the basis of these data, gave out a test result.

3.4.2 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 an ABI Prism 7900 HT (Applied Biosystem, CA, 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 (Applied Biosystem, CA, USA), in a final volume of 20 μl.

Primers and probes sequences and thermocycling conditions were shown in Table 2.

Table 2. Primers, probes sequences and amplification profiles designed for five TNFA SNPs genotype discrimination.

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.

3.4.3 TNFRSF1A gene analysis

Exons 2, 3, 4 and 6 of the TNFARSF1A gene were analysed by means of denaturing high-performance liquid chromatography (DHPLC; Wave® 2100 Fragment Analysis, Transgenomic, Omaha, NE, USA) and gene sequencing (ABI PRISM 3130 Genetic Analyzer, Applied Biosystem, CA, USA) of identified heteroduplexes.

Exons 2, 3, 4 and 6 of TNFRSF1A gene were at first amplified by PCR using 100 ng of genomic DNA, 480 nM for each primer (forward and reverse primer), 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 2.5 U of Taq polymerase (AmpliTaq Gold, Applied Biosystems, CA, USA) and 1X manufacturer-provided buffer (Applied Biosystems, USA) in a final volume of 50 µl. PCRs were run with an initial denaturation at 95 °C for 7 minutes, followed by 35 cycles at 94 °C for 30 seconds, 55 °C for 45 seconds and 72 °C for 45 seconds, with a final extension at 72 °C for 20 minutes. Water was used as negative control in each PCR run. After amplification of DNA, to verify the presence of amplification products and to exclude the presence of contamination in mixes, gel electrophoresis of 10 µl of the amplification products was performed using 2% agarose

gels (E-Gel, Invitrogen, Life Technologies, Monza, Italy) with ethidium bromide as staining.

DHPLC analysis was then performed using the Wave® 2100 Fragment Analysis (Transgenomic, Omaha, NE, USA). DHPLC is based on a reversed phase chromatography in which the hydrophobic stationary phase was 2 μm of alkylated nonporous poly (styrene- divinylbenzene) particles packed into a 4.6x50 mm separation column (Transgenomic). The polar mobile phase is acetonitrile. Triethylammonium acetate (TEAA) 0.1 M buffer (pH 7.0) is used as an ion-pairing reagent.

This first step is the denaturation of the PCR amplicons at 95 °C for 10 minutes and a slow renaturation to create hetero- and homoduplexes molecules. The presence of a mutation can be established by the differential retention of hetero- and homoduplex DNA on the stationary phase under partial thermal denaturation condition. Heteroduplex molecules are primarily eluted from the column in an increasing gradient of acetonitrile (0.5% for minute).

Homozygous polymorphism was identified in the same way by premixing 1:1 each amplicon with one obtained from a wild type subject to mimic a heteroduplex after the denaturation and renaturation step. Navigator 3.1.0 software was used to predict the melting temperature of each PCR fragment and the appropriate linear acetonitrile gradient necessary to distinguish hetero- and homoduplexes. Primers for PCR amplifications, amplicon lengths, DHPLC temperature and gradient conditions are listed in Table 3.

Table 3. Primer sequences designed for the amplification of exons 2, 3, 4 and 6 of the TNFRSF1A gene, and denaturing high-performance liquid chromatography (DHPLC) temperature conditions.

Exon	Forward and reverse primer sequences	Amplicon bp	Denaturing HPLC temperature, °C	Timeshift	Acetonitrile gradient % (Start-end)
2	F-5'-TCCCTCTTTGATGGTGTCTCC-3' R-5'-CAGACCTGAGGGCATTCACC-3'	234	59.3/59.8	0/0	13.1-15.3
3	F-5'-TTCCTTGTGTTCTCACCCGCAG-3' R-5'-TCAAGACCCGCCTGACTCTC-3'	214	62.7/63.2	0/0	12.9-15.1
4	F-5'-AGGATGCAGGACTCATACCC-3' R-5'-AAAGGAAGTGCCACCGCATGG-3'	268	63.5	1	12.9-15.1
6	F-5'-GTGTTCCTCCAATGGTAGGG-3' R-5'-AAGCAGGTGTTGGTCAGAGG-3'	258	60.5/61	0/0	13.3-15.5

F: forward primer, R: reverse primer, bp: base pair, HPLC: high-performance liquid chromatography.

Exons with altered elution profiles in DHPLC assay were directly sequenced.

TNFRSF1A gene sequencing was performed on the automatic sequencer 3130ABI PRISM Genetic Analyzer (Applied Biosystem, CA, USA), using 1 µL of previously purified (ExoSAP, GE Healthcare, Fairfield, CT, USA) amplification product mixed with 300 nM of each forward and reverse PCR primer, 4 µl of Big Dye terminator Mix (Applied Biosystem, CA, USA) in a final volume of 20 µL. Chromatograms were analysed with Chromas Lite 2.6.1 software (Technelysium Pty Ltd., South Brisbane, QLD, Australia).

3.4.4 MEFV gene analysis

The analysis of polymorphisms of the MEFV gene was performed by direct sequencing of exons 2, 3, 5 and 10.

Exons were amplified by PCR (2720 Thermal Cycler, Applied Biosystem, CA, USA), using primers sequences shown in Table 4.

Table 4. Primer sequences for the amplification of exons 2, 3, 5 and 10 of the MEFV gene.

Exon	Forward and reverse primer sequences	Amplicon (bp)
2	F-5'-GGGGA TTCTCTCTCCTCTGC-3' R-5'-CTCAAAGTCTTGGCCTCCAG-3'	903
3	F-5'-AACTTGGGTTTGCCATTCAG-3' R-5'-AAGTGCCTGGCAGAGAAGAG-3'	438
5	F-5'-CCACCTCTTATCCACCTCCA-3' R-5'-CTAGGCCTTAGGGGCTTCAC-3'	449
10	F-5'-CAGGTGGGGAGAACCCTGTAG-3' R-5'-CCGTGGGCACAGTAACTATT-3'	757

F: forward primer, R: reverse primer, bp: base pair.

For each exon, 100 ng of genomic DNA were amplified in a reaction mix composed by 500 nM of each forward and reverse primer, 200 µM of each deoxynucleotide triphosphates (dNTPs), 2.5 mM of $MgCl_2$, 1 Unit (U) of Taq polymerase (Super Taq, AB Analitica, Padova, Italy), 1X manufacturer-provided buffer, 10% dimethyl sulfoxide (DMSO) (only for exon 2) in a final volume of 25 µl. Therefore, four different reactions mix was prepared for a single enrolled subject. PCR reactions were run with an initial denaturation at 94 °C for 5 minutes, followed by a touchdown step (94 °C for 30 seconds, 68°C in the first cycle for 30 seconds and -0.5 °C/cycle for 9 cycles, 72°C for 45 seconds) and more 26 cycles at 94 °C for 30 seconds, 62 °C for 30 seconds, 72 °C for 45 seconds, with a final extension at 72 °C for 7 minutes. Water was used as

negative control in each PCR run. After amplification of DNA, to verify the presence of amplification products and to exclude the presence of contamination in mixes, gel electrophoresis of 10 μl of the amplification products was performed using 2% agarose gels (E-Gel, Invitrogen, Life Technologies, Monza, Italy) with ethidium bromide as staining.

MEFV gene sequencing was performed on the automatic sequencer 3130ABI PRISM Genetic Analyzer (Applied Biosystem, CA, USA), using 1 μL of previously purified (ExoSAP, GE Healthcare, Fairfield, CT, USA) amplification product mixed with 300 nM of each forward and reverse PCR primer, 4 μl of Big Dye terminator Mix (Applied Biosystem, CA, USA) and 5% DMSO (only for exon 2) in a final volume of 20 μL. Two additional internal primers were also used to perform the sequence of the exon 2 (Fint: 5'-CCCTGAGCAAACGCAGAG-3' and Rint: 5'-GTACACTTCGAAGGGCCTGC-3'). Chromatograms were analysed with Chromas Lite 2.6.1 software (Technelysium Pty Ltd., South Brisbane, QLD, Australia).

MEFV gene: R202Q SNP genotyping

The genotype discrimination of the R202Q SNP (rs224222) in MEFV gene (exon 2) was performed by a TaqMan dual probes allelic discrimination assay. Probes and primers mix was purchased as manufacturer-provided assay from Applied Biosystem (Taqman SNP genotyping, catalog number C_2394721_10). 50 ng of genomic DNA was amplified in a reaction mix containing 1X of primers and probes mix and 1X TaqMan Universal Master Mix (Applied Biosystem, CA, USA) in a final volume of 20 μl. Amplification was carried out with an ABI Prism 7900 HT (Applied Biosystem, CA, USA) with a step at 50 °C for 2 minutes and 95 °C for 10 minutes, followed by a 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute.

3.5 Statistical analysis

Mean, standard deviation (SD) or median and interquartile range (IQR) 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).

Hardy-Weinberg calculation

Genotype frequencies were tested for Hardy-Weinberg equilibrium proportions using χ^2 test. For each SNP, the allele frequencies were first calculated on the basis of the observed genotypes. The expected genotypes frequencies were then calculated on the basis of the following formula:

$$p^2 + 2pq + q^2 = 1$$

p and q being the observed alleles frequencies.

Haplotype Estimation

Haplotypes phases and frequencies were estimated by the retrospective profile-likelihood approach using the Stata haplologit package. Odds ratio (OR) was calculated by logistic regression analysis using the additive model [144].

4. RESULTS

4.1 Studied population

In this study, a total of 314 subjects from the Veneto Region (a North-east Italian region) were enrolled. These included 223 controls and a cohort of 91 patients with an established SpA diagnosis comprising 36 AS and 55 PsA. In statistical analyses, patients were evaluated as a single group (SpA) in comparison to controls, or else AS and PsA groups were considered singly. Table 5 reports the demographic characteristics of controls and patients with results from the statistical analyses. The percentage of females was higher among PsA (49%) than AS (19%) or controls (35%), and mean age was lower among controls than in SpA patients (Table 5). Weight, Height and body mass index (BMI) did not differ between the studied groups. Gender and age were therefore considered as potential confounding factor in all subsequent statistical analyses.

Table 5. Demographic characteristics in three groups: controls, AS and PsA patients.

	Controls (n=223)	**AS (n=36)**	**PsA (n=55)**	**Statistics**
Gender (M/F)	146/77	29/7*	28/27*	$\chi^2 = 8.59$, **p = 0.014**
Age mean±SD (years)	46 ± 11	52±13*	53±12***	F = 10.22, **p=0.0001**
Weight mean±SD (Kg)	76.52 ± 15.33	76.17 ± 9.85	77.54 ± 14.24	F = 0.13, p = 0.8782
Height mean±SD (cm)	172.77 ± 10.94	175.11 ± 6.21	170.80 ± 8.75	F = 2.01, p = 0.1354
BMI mean±SD (Kg/m^2)	25.91 ± 8.27	24.81 ± 2.73	26.50 ± 4.13	F = 0.62, p = 0.5410

Bonferroni's test for pairwise comparisons: *=p<0.05 with respect to controls; ***=p<0.0001 with respect to controls. Significant p values are reported in bold. AS: ankylosing spondylitis, PsA: psoriatic arthritis, M: male, F: female, SD: standard deviation, BMI: Body Mass Index.

4.2 Clinical characteristics 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.

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

	AS (n=36)	**PsA (n=55)**	**Statistics**
Family history of SpA, n (%)	10 (27.78)	45 (81.82)	$\chi^2 = 26.57$, **p < 0.0001**
Age at diagnosis, mean ± SD (years)	40.53 ±12.43	40.58 ±13.16	t = 0.000, p = 0.984
Inflammatory back pain, n (%)	35 (100.00)	36 (65.45)	$\chi^2 = 15.33$, **p < 0.0001**
Peripheral arthritis, n (%)	4 (11.11)	50 (90.91)	$\chi^2 = 57.42$, **p < 0.0001**
Enthesitis, n (%)	34 (94.44)	35 (63.64)	$\chi^2 = 11.27$, **p < 0.001**
Buttock pain, n (%)	33 (91.67)	15 (27.27)	$\chi^2 = 36.20$, **p < 0.0001**
Dactylitis, n (%)	0 (0.00)	33 (60.00)	$\chi^2 = 33.89$, **p < 0.0001**
Uveitis, n (%)	2 (5.56)	1 (1.82)	$\chi^2 = 0.95$, p = 0.329
Psoriasis, n (%)	1 (2.78)	45 (81.82)	$\chi^2 = 54.38$, **p < 0.0001**
IBD, n (%)	2 (5.56)	2 (3.64)	$\chi^2 = 0.19$, p = 0.662
Urethritis/Cervicitis/Diarrhoea, n (%)	4 (11.11)	14 (25.45)	$\chi^2 = 2.82$, p = 0.093

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

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.

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

	AS (n=36)	**PsA (n=55)**	**Statistics**
ESR, mean ± SD (mm/hr)	22.97 ± 13.91	28.62 ± 15.07	t = 3.24, p =0.0751
CRP, mean ± SD (mg/L)	5.85 ± 5.81	8.022 ± 6.52	t = 2.63, p =0.1082
DAS, mean ± SD	3.50 ± 1.33	3.49 ± 0.81	t = 0.00, p =0.9677
BASMI, mean ± SD	3.67 ± 1.29	2.53 ± 1.33	t = 16.36, **p <0.0001**
BASFI, mean ± SD	4.9 ± 2.6	4.2 ± 2.4	t = 1.35, p =0.2476
HAQ, mean ± SD	0.79 ± 0.68	0.79 ± 0.56	t = 0.00, p =0.9790
BASDAI, mean ± SD	6.1 ± 1.9	5. 6 ± 2.1	t = 1.22, p = 0.2720
ASDAS-PCR, mean ± SD	3.00 ± 0.59	2.94 ± 0.50	t = 0.29, p =0.5933

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.

Patients enrolled in this study attended the Rheumatology Unit of the Department of Medicine- University-Hospital of Padova (Italy). Patients who not responded to

NSAIDs or DMARDS were treated with anti- TNFα agents. In those patients who did not respond to the first anti- TNFα drug, another one, or in certain cases, more than one anti- TNFα agent was switched. Table 8 shows the number (and percentage) of patients treated with an anti- TNFα drug, and the type of administered drug. At enrollment, 58 patients (63.7%) were under therapy with anti- TNFα agents; of these 11 (19%) experienced in their clinical history, before enrollment, at least one switch of TNF-α drug inhibitor. The number of AS patients treated with a TNF-α inhibitor was higher with respect to the treated PsA patients. Most of patients affected by AS were under treatment with infliximab, while patients affected by PsA were treated with adalimumab.

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

	AS (n=36)	**PsA (n=55)**
Therapy with anti- TNFα agents, n (%)	30 (83.3)	28 (50.9)
Adalimumab, n (%)	6 (20)	15 (53.6)
Etanercept, n (%)	4 (13.3)	7 (25)
Golimumab, n (%)	0 (0)	1 (3.6)
Infliximab, n (%)	20 (66.7)	5 (17.8)

AS: ankylosing spondylitis, PsA: psoriatic arthritis, n: number.

4.3 Haematological and biochemical indices in SpA patients: differences in polymorphonuclear cells number and ALT levels in patients affected by PsA

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 polymorphonuclear cells (PMN) were higher in SpA patients with respect to controls, being the highest values recorded among PsA, which also had significantly higher levels of ALT with respect to controls. Serum levels of prealbumin were lower among SpA patients than in controls, although the differences were not powerful enough to reach the statistical significance.

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

	Controls (n=223)	**AS (n=36)**	**PsA (n=55)**	**Statistics**
WBC ($x10^3/\mu L$)	6.11 ± 1.42	6.9475 ± 1.85	7.09 ± 2.02	F= 2.68, p=0.0728
Haemoglobin (g/L)	144.60 ± 12.8	144.03 ± 12.68	143.87 ± 13.62	F= 0.09, p=0.9162
Platelets ($x10^3/\mu L$)	236.5 ± 46.0	243.22 ± 63.89	252.21 ± 60.32	F= 2.17, p=0.1162
PMN ($x10^3/\mu L$)	3.32 ± 1.05	3.90 ± 1.33*	3.98 ± 1.55 **	F= 9.02, **p=0.0002**
Monocyte ($x10^3/\mu L$)	0.43 ± 0.35	0.51 ± 0.17	1.01 ± 4.26	F= 2.28, p= 0.1036
Lymphocyte($x10^3/\mu L$)	1.90 ± 1.90	2.15 ± 0.84	2.24 ± 0.75	F= 1.12, p= 0.3289
Glucose (mmol/L)	4.71 ± 0.92	4.88 ± 0.66	4.92 ± 0.79	F= 1.61, p= 0.2014
Creatinine (μmol/L)	76.97 ± 12.39	76.97 ± 15.50	74.27 ± 17.78	F= 0.86, p= 0.4228
Uric acid (mmol/L)	0.3 ± 0.058	0.31 ± 0.08	0.30 ± 0.08	F= 0.40, p=0.6685
ALT (U/L)	22.14 ± 8.99	24.46 ± 10.77	29.22 ± 21.49**	F=7.35, **p= 0.0008**
Prealbumin (mg/L)	287.1 ± 46.1	273.63 ± 50.63	273.25 ± 44.55	F=2.79, p= 0.0630
CRP (mg/L)	3.49 ± 4.04	6.23 ± 6.65**	5.03 ± 4.54	F=7.20, **p=0.0009**

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.

4.4 Genetics associations in Spondyloarthritis

The genetic basis of SpA is complex and includes, in addition to the well established HLA-B27 haplotype for AS, a number of variants in genes encoding for inflammatory and immunomodulatory cytokines and for their receptors, in genes of the HLA region and in genes, like CARD9, involved in the inflammasome. These data come mainly from GWASs and requires validation before the potential use in the clinical setting. In this study we investigated the presence of association between SpA and any of the following genes: two HLA haplotypes, namely HLA-B27 and HLA-CW6; genes involved in the TNF pathway, namely TNFA and TNFRSF1A; a gene involved in the inflammasome pathway, MEFV. The HLA-B27, HLA-CW6, and five SNPs in the promoter region of TNFA, involved in transcription regulation were specifically studied. Differently from these common variants, TNFRSF1A and MEFV may affect protein function by the presence of uncommon variants. For this reason with the aim to identify any potentially relevant sequence variation, the complete sequence analysis of exons 2, 3, 4 and 6 of TNFRSF1A gene and of exons 2, 3, 5 and 10 of MEFV gene were performed. We selected the above reported exons because they are those described to carry hotspot mutations more frequently. All above described genes were analysed in all SpA patients. HLA-B27 and TNFA promoter polymorphisms were analysed in all controls. HLA-CW6, MEFV and TNFRSF1A sequence analysis were performed in an exploratory cohort of controls (n=27). After interim analysis, those variants found to be of potential significance were subsequently analysed in all control series.

4.4.1 HLA-B27, not HLA-CW6 haplotype, associates with Ankylosing Spondylitis

The results of HLA-B27 haplotype in patients and controls are shown in Table 10. The frequency of patients carrying the HLA-B27 haplotype was significantly higher than in controls (χ^2= 120.12, p< 0.0001), being this haplotype mainly associated with AS than with PsA.

Table 10. HLA-B27 haplotype in controls and in AS and PsA patients.

Controls (n=214)		**AS (n=36)**			**PsA (n=55)**		
Number (frequency, %)		**Number (frequency, %)**		**χ^2, p**	**Number (frequency, %)**		**χ^2, p**
Carriers 9 (4.21)	***Non carriers*** 205 (95.79)	***Carriers*** 26 (72.22)	***Non carriers*** 10 (27.78)	χ^2= 118.41 ***p<0.0001****	***Carriers*** 7 (12.73)	***Non carriers*** 48 (87.27	χ^2= 5.68 **p=*0.051****

Bonferroni's test for pairwise comparisons: **Bonferroni's adjusted p value with respect to controls. Significant p values are reported in bold face. The determination of HLA-B27 haplotype was not possible in 9 subjects for depletion of biological material. AS: ankylosing spondylitis, PsA: psoriatic arthritis.

The results of HLA-CW6 haplotype in patients and controls are shown in Table 11. Although a slightly higher frequency of HLA-CW6 carriers was observed among patients with AS (about 6%) or PsA (about 13%) with respect to controls (about 4%), the difference was not statistically significant (χ^2= 2.57, p= 0.277) and for this reason the analysis of HLA-CW6 was not extended to the remaining controls.

Table 11. HLA-CW6 haplotype in controls and in AS and PsA patients. Results from the exploratory study.

Controls (n=27)		**AS (n=36)**		**PsA (n=55)**	
Number (frequency, %)		**Number (frequency, %)**		**Number (frequency, %)**	
Carriers 1 (3.70)	***Non carriers*** 26 (96.30)	***Carriers*** 2 (5.56)	***Non carriers*** 34 (94.44)	***Carriers*** 7 (12.96)	***Non carriers*** 48 (87.04)

AS: ankylosing spondylitis, PsA: psoriatic arthritis.

4.4.2 TNFA genetic: a risk factor for Spondyloarthritis

4.4.2.1 TNFA -1031C/-308G haplotype reduces AS risk

Table 12 reports the results of the genotype discrimination of five SNPs (TNFA -1031 T>C, -857C>T -376G>A -308G>A -238G>A), in the promoter region of TNFA gene. Considering each studied SNP, the Minor Allele Frequency (MAF), the number and frequency of genotypes in patients (divided on the basis of diagnosis) and controls are shown. The polymorphisms were all in Hardy-Weinberg equilibrium (p >0.01). None of the studied TNFA SNPs was singly associated with AS or PsA diagnosis.

Table 12. TNFA gene polymorphisms in controls and in AS and PsA patients.

dbSNP (TNFA gene)	MAF	Controls (n=223)			AS (n=36)			PsA (n=55)			χ^2, p
		GENOTYPES Number (frequency)			GENOTYPES Number (frequency)			GENOTYPES Number (frequency)			
-1031T>C rs1799964	C (0.263)	C/C 8 (0.09)	T/C 62 (0.41)	T/T 63 (0.50)	C/C 1 (0.03)	T/C 10 (0.28)	T/T 25 (0.69)	C/C 4 (0.06)	T/C 20 (0.38)	T/T 31 (0.56)	χ^2= 6.37 p= 0.17
-857C>T rs1799724	T (0.208)	T/T 10 (0.04)	C/T 66 (0.32)	C/C 142 (0.64)	T/T 3 (0.05)	C/T 10 (0.35)	C/C 23 (0.60)	T/T 2 (0.06)	C/T 22 (0.37)	C/C 29 (0.57)	χ^2= 3.69 p= 0.45
-376G>A rs1800750	A (0.021)	A/A 0 (-)	G/A 9 (0.06)	G/G 149 (0.94)	A/A 0 (-)	G/A 0 (-)	G/G 36 (1)	A/A 0 (-)	G/A 1 (0.02)	G/G 47 (0.98)	χ^2= 3.04 p= 0.22
-308G>A rs1800629	A (0.107)	A/A 2 (0.01)	G/A 41 (0.19)	G/G 172 (0.80)	A/A 0 (-)	G/A 6 (0.16)	G/G 30 (0.84)	A/A 0 (-)	G/A 14 (0.25)	G/G 39 (0.75)	χ^2= 2.51 p= 0.64
-238G>A rs361525	A (0.044)	A/A 0 (-)	G/A 18 (0.09)	G/G 188 (0.91)	A/A 0 (-)	G/A 1 (0.03)	G/G 35 (0.97)	A/A 1 (0.00)	G/A 5 (0.12)	G/G 49 (0.88)	χ^2= 5.10 p= 0.20

The analysis was not performed, because of biological material depletion, in: 133 controls for -1031 T>C SNP, 5 controls for -857C>T SNP, 65 controls for -376G>A SNP, 8 controls for -308G>A SNP and 17 controls for -238G>A SNP. AS: ankylosing spondylitis, PsA: psoriatic arthritis, dbSNP: Single Nucleotide Polymorphism Database, TNFA: Tumor Necrosis Factor α, MAF: minor allele frequency.

The studied polymorphisms of the TNFA gene, all closely located in the promoter region, are carried as different haplotypes. Therefore the haplotypes resulting from the

pairwise combination of TNFA-1031T>C, -857C>T, -376G>A, -308G>A and -238G>A polymorphisms were inferred by statistical analysis. Table 13 reports the haplotypes combinations with their respective frequencies inferred from control subjects.

Table 13. TNFA haplotypes combinations: frequencies inferred from controls.

SNPs	SNPs	TNFA haplotypes	Frequencies (%)
-1031T>C	-857C>T	-1031T/-857C	49.19
		-1031C/-857C	30.21
		-1031T/-857T	20.60
		-1031C/-857T	<0.6
-1031T>C	-376G>A	-1031T/-376G	69.55
		-1031C/-376G	27.59
		-1031C/-376A	2.86
		-1031T/-376A	<0.6
-1031T>C	-308G>A	-1031T/-308G	59.19
		-1031C/-308G	30.77
		-1031T/-308A	10.04
		-1031C/-308A	<0.6
-1031T>C	-238G>A	-1031T/-238G	70.98
		-1031C/-238G	22.14
		-1031C/-238A	6.88
		-1031T/-238A	<0.6
-857C>T	-376G>A	-857C/-376G	76.52
		-857C/-376A	2.88
		-857T/-376G	2.06
		-857T/-376A	<0.6
-857C>T	-308G>A	-857C/-308G	69.38
		-857T/-308G	20.60
		-857C/-308A	10.01
		-857T/-308A	<0.6
-857C>T	-238G>A	-857C/-238G	72.56
		-857T/-238G	20.61
		-857C/-238A	6.84
		-857T/-238A	<0.6
-308G>A	-376G>A	-308G/-376G	86.98
		-308A/-376G	10.04
		-308G/-376A	2.98
		-308A/-376A	<0.6
-308G>A	-238G>A	-308G/-238G	83.10
		-308A/-238G	10.05
		-308G/-238A	6.85
		-308A/-238A	<0.6
-238G>A	-376G>A	-238G/-376G	93.18
		-238A/-376G	4.30
		-238A/-376A	2.52
		-238G/-376A	<0.6

SNP: Single Nucleotide Polymorphism, TNFA: Tumor Necrosis Factor α.

To verify whether any TNFA haplotype exerts any independent role over SpA diagnosis, logistic regression analyses were performed, on considering SpA diagnosis as the outcome variable and TNFA haplotype combinations as predictors. Table 14 reports the results of the analyses. For any combination the reference haplotype was the most frequent among controls (Table 13). The possible pairwise combinations between TNFA SNPs were ten and the overall seventeen haplotypes were inferred. The OR with 95% confidence intervals (CI) with respect to the reference haplotype are shown.

Only the TNFA -1031C/-308G haplotype was significantly associated with SpA ($p<0.05$), with an OR lower than 1, this suggesting its protective role.

Table 14. Logistic regression analysis considering SpA diagnosis as the outcome variable and TNFA haplotype combinations as predictors.

SNP	SNP	Reference TNFA haplotype	Risk TNFA haplotype	OR (95% CI)	p
-1031T>C	-857C>T	-1031T/-857C	-1031C/-857C	0.66 (0.41 - 1.04)	0.073
			-1031T/-857T	1.02 (0.67 - 1.58)	0.916
			-1031C/-857T	-	-
-1031T>C	-376G>A	-1031T/-376G	-1031C/-376G	0.71 (0.45 - 1.11)	0.133
			-1031C/-376A	-	-
			-1031T/-376A	-	-
-1031T>C	-308G>A	-1031T/-308G	-1031C/-308G	**0.63 (0.40 - 0.99)**	**0.047**
			-1031T/-308A	1.00 (0.57 - 1.75)	0.998
			-1031C/-308A	-	-
-1031T>C	-238G>A	-1031T/-238G	-1031C/-238G	0.80 (0.50 - 1.29)	0.362
			-1031C/-238A	-	-
			-1031T/-238A	-	-
-857C>T	-376G>A	-857C/-376G	-857C/-376A	0.21 (0.03 - 1.68)	0.141
			-857T/-376G	1.16 (0.77 - 1.74)	0.483
			-857T/-376A	-	-
-857C>T	-308G>A	-857C/-308G	-857T/-308G	1.21 (0.80 - 1.83)	0.357
			-857C/-308A	1.19 (0.68 - 2.08)	0.536
			-857T/-308A	-	-
-857C>T	-238G>A	-857C/-238G	-857T/-238G	1.16 (0.77 - 1.74)	0.487
			-857C/-238A	0.65 (0.29 - 1.43)	0.284
			-857T/-238A	-	-
-308G>A	-376G>A	-308G/-376G	-308A/-376G	1.10 (0.64 - 1.91)	0.722
			-308G/-376A	0.20 (0.03 - 1.57)	0.125
			-308A/-376A	-	-
-308G>A	-238G>A	-308G/-238G	-308A/-238G	1.04 (0.60 - 1.81)	0.888
			-308G/-238A	0.47 (0.19 - 1.14)	0.095
			-308A/-238A	-	-
-238G>A	-376G>A	-238G/-376G	-238A/-376G	0.82 (0.33 - 2.02)	0.661
			-238A/-376A	-	-
			-238G/-376A	-	-

Significant p values are reported in bold face. SNP: Single Nucleotide Polymorphism, TNFA: Tumor Necrosis Factor α, OR: Odds Ratio, CI: Confidence Interval.

The identified TNFA -1031C/-308G haplotype was confirmed to be a protective factor for AS, not for PsA, at logistic regression analysis performed comparing controls to these two diseases separately (Table 15).

Table 15. Logistic regression analysis considering AS or PsA diagnosis as the outcome variable and the 1031T>C/308G>A SNPs combination as predictor.

				AS		PsA	
Reference haplotype	**Risk haplotype**	**Controls**		**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
-1031T/-308G	-1031C/-308G	Ref.	-	**0.43 (0.22 - 0.85)**	**0.015**	0.80 (0.48 - 1.35)	0.400
	-1031T/-308A	Ref.	-	0.66 (0.27 - 1.60)	0.354	1.29 (0.67 - 2.47)	0.444

Significant p values are reported in bold face. AS: ankylosing spondylitis, PsA: psoriatic arthritis, OR: Odds Ratio, CI: Confidence Interval.

The estimated frequencies of the -1031/-308 TNFA haplotypes in controls and in patients affected by PsA and AS are also shown in Figure 10. The frequency of the TNFA -1031C/-308A haplotype was very low (< 0.6), therefore it was not considered.
In agreement with findings from logistic regression analyses, the frequency of the TNFA -1031C/-308G haplotype was significantly lower in AS patients (17%) than in PsA patients (25%) and controls (31%) (χ^2= 14.50 and p = 0.0059).

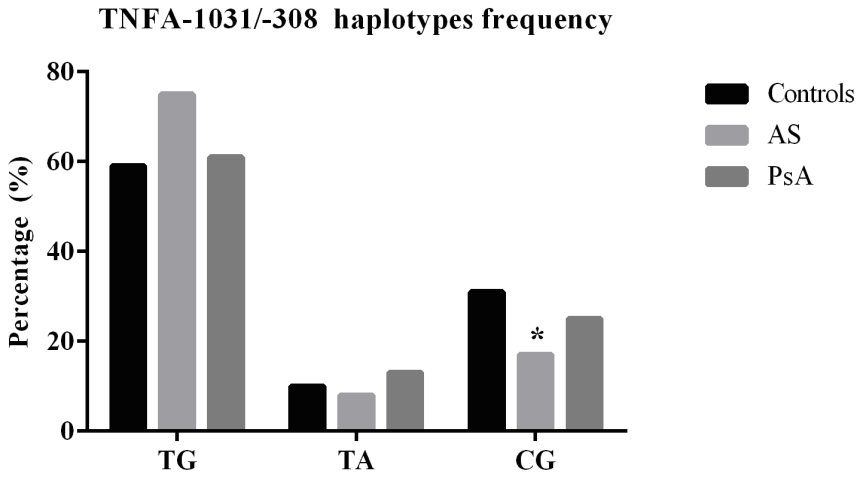

Figure 10. Frequency of haplotypes derived from the TNFA-1031T>C/-308G>A SNPs combination. *p < 0.05

By combining the two SNPs -1031T>C and -308G>A of the TNFA gene, the six following genotypes were also inferred: TG/TG, TG/TA, TG/CG, TA/TA, CG/TA, CG/CG. On the basis of the most frequent TNFA haplotype (-1031T/-308G), genotypes were grouped in three categories as follows: homozygous genotype (TG/TG), heterozygous genotypes (TG/other), and genotypes identified by rare haplotypes (rare haplotypes).

The frequency of these three categories, in controls and in patients affected by AS and PsA are shown in Figure 11.

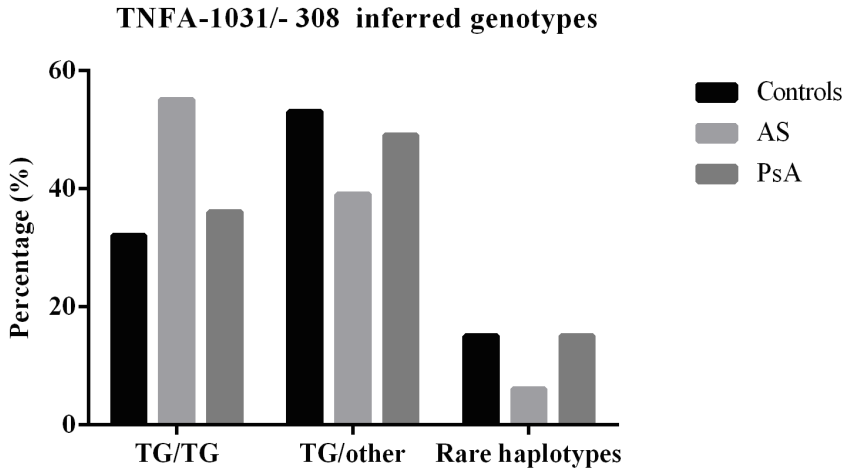

Figure 11. Frequency of the genotypes derived from the TNFA -1031/-308 haplotypes combination in controls and in patients.

The rare haplotypes were less frequent in patients affected by AS (about 6%) than in controls (about 16%) (χ^2= 7.47 and p = 0.024). No significant difference was found between PsA patients and controls (χ^2= 0.29 and p = 0.865).

Intriguingly, the TNFA -1031/-308 inferred genotypes were associated with HLA-B27. Figure 12 shows the frequencies of the three inferred genotypes in HLA-B27 positive and negative subjects.

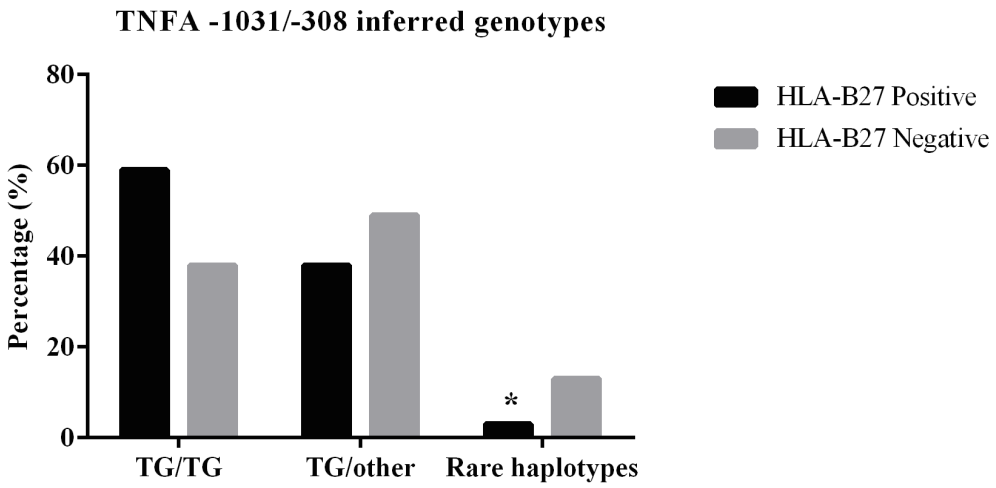

Figure 12. Frequency of the genotypes derived from the TNFA -1031/-308 haplotypes combination in HLA-B27 positive and negative subjects. *p < 0.05

Among HLA-B27 carriers, the rare inferred genotypes were less frequently found than in HLA-B27 negative subjects (χ^2= 6.24 and p = 0.044).

4.4.2.2 TNFA and HLA-B27 haplotypes are closely linked to the risk of developing SpA

Since TNFA is located in the MHC region of chromosome 6, we verified whether haplotypes resulting from the combination of each TNFA SNP and HLA-B27 might affect SpA risk. Table 16 shows the HLA-B27/TNFA haplotypes with their frequencies among controls.

Table 16. HLA-B27/TNFA haplotypes combinations: frequencies inferred from controls.

HLA-B27/ TNFA SNP	TNFA haplotypes	Frequencies (%)
HLA-B27/ -1031T>C	HLA-B27 neg/ -1031T	69.04
	HLA-B27 neg/ -1031C	28.65
	HLA-B27 pos/ -1031C	2.31
	HLA-B27 pos/ -1031T	<0.6
HLA-B27/ -857C>T	HLA-B27 neg/ -857C	77.87
	HLA-B27 neg/ -857T	19.96
	HLA-B27 pos/ -857C	1.53
	HLA-B27 pos/ -857T	0.64
HLA-B27/ -376G>A	HLA-B27 neg/ -376G	94.95
	HLA-B27 neg/ -376A	2.90
	HLA-B27 pos/ -376G	2.15
	HLA-B27 pos/ -376A	<0.6
HLA-B27/ -308G>A	HLA-B27 neg/ -308G	88.10
	HLA-B27 neg/ -308A	9.74
	HLA-B27 pos/ -308G	1.86
	HLA-B27 pos/ -308A	<0.6
HLA-B27/ -238G>A	HLA-B27 neg /-238G	9.10
	HLA-B27 neg /-238A	6.87
	HLA-B27 pos /-238G	2.10
	HLA-B27 pos /-238A	<0.6

SNP: Single Nucleotide Polymorphism, TNFA: Tumor Necrosis Factor α, HLA: human leukocyte antigen.

A logistic regression analysis was then performed considering SpA diagnosis as the outcome variable, and HLA-B27/TNFA haplotypes as predictor variables. The obtained results, reported in terms of OR with 95% CI with respect to the reference haplotype (that most frequent among controls), are shown in Table 17.

Table 17. Logistic regression analysis considering SpA diagnosis as the outcome variable, and the HLA-B27/TNFA haplotypes as predictors.

HLA-B27/ TNFA SNP	Reference haplotype	Risk haplotype	OR (95% CI)	p
	HLA-B27 neg	**HLA-B27 pos**	**12.96 (5.87 – 28.63)**	**<0.0001**
HLA-B27/ -1031T>C	HLA-B27 neg/ -1031T	HLA-B27 neg/ -1031C	0.80 (0.49 - 1.31)	0.377
		HLA-B27 pos/ -1031C	-	-
		HLA-B27 pos/ -1031T	-	-
HLA-B27/ -857C>T	HLA-B27 neg/ -857C	HLA-B27 neg/ -857T	1.14 (0.70 - 1.84)	0.608
		HLA-B27 pos/ -857C	**10.42 (3.75 -28.93)**	**<0.0001**
		HLA-B27 pos/ -857T	**9.88 (1.53 - 63.65)**	**0.016**
HLA-B27/ -376G>A	HLA-B27 neg/ -376G	HLA-B27 neg/ -376A	0.24 (0.03 - 1.95)	0.183
		HLA-B27 pos/ -376G	**9.86 (4.61 - 21.10)**	**<0.0001**
		HLA-B27 pos/ -376A	-	-
HLA-B27/ -308G>A	HLA-B27 neg/ -308G	HLA-B27 neg/ -308A	1.40 (0.81- 2.43)	0.232
		HLA-B27 pos/ -308G	**10.53 (4.90 - 22.60)**	**<0.0001**
		HLA-B27 pos/ -308A	-	-
HLA-B27/ -238G>A	HLA-B27 neg /-238G	HLA-B27 neg /-238A	0.75 (0.34 - 1.66)	0.480
		HLA-B27 pos /-238G	10.13 (4.73-21.70)	**<0.0001**
		HLA-B27 pos /-238A	-	-

Significant p values are reported in bold face. SNP: Single Nucleotide Polymorphism, TNFA: Tumor Necrosis Factor α, HLA: human leukocyte antigen, AS: ankylosing spondylitis, PsA: psoriatic arthritis.

HLA-B27 appeared to have a dominant effect on SpA risk, independently from its combined haplotype with any single TNFA SNP.

The logistic regression analysis was performed also considering the differential diagnosis, AS and PsA. Results are reported in Table 18.

Among the studied combinations, three haplotypes were significantly associated with the risk of AS as well as of PsA: HLA-B27 positive/-376G, HLA-B27 positive/-308G and HLA-B27 positive/-238G. The TNFA -376G, -308G, -238G alleles, not only singly (Table 18), but also when inherited all together with HLA-B27 haplotype, significantly enhance the risk of developing PsA (OR: 3.298; CI: 1.193-9.115; p=0.021) and AS (OR: 25.895; CI: 11.324-59.213; p<0.0001). Differently from the PsA group, in the AS group in addition to the three already mentioned haplotypes, the HLA-B27 positive/-857C haplotype was also significantly associated to the risk of developing the disease.

Table 18. Logistic regression analysis considering AS or PsA diagnosis as the outcome variable, and HLA-B27 alone or combined with TNFA SNPs in haplotypes as predictors.

			AS		PsA	
HLA-B27/ TNFA SNP	**Reference haplotype**	**Risk haplotype**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
	HLA-B27 neg	**HLA-B27 pos**	**57.78 (21.49 - 155.32)**	**<0.0001**	**3.24 (1.15- 9.14)**	**0.026**
HLA-B27/ -1031T>C	HLA-B27 neg/ -1031T	HLA-B27 neg/ -1031C	0.58 (0.23 -1.47)	0.252	0.87 (0.52 -1.48)	0.616
		HLA-B27 pos/ -1031C	-	-	-	-
		HLA-B27 pos/ -1031T	-	-	-	-
HLA-B27/ -857C>T	HLA-B27 neg/ -857C	HLA-B27 neg/ -857T	1.14 (0.54 - 2.38)	0.733	1.20 (0.70 - 2.06)	0.500
		HLA-B27 pos/ -857C	**25.48 (10.89 - 59.59)**	**<0.0001**	2.80 (0.63 - 12.43)	0.176
		HLA-B27 pos/ -857T	-	-	4.13 (0.40 - 42.31)	0.232
HLA-B27/ -376G>A	HLA-B27 neg/ -376G	HLA-B27 neg/ -376A	7.11e-07 (-)	0.989	0.37 (0.05 - 2.98)	0.351
		HLA-B27 pos/ -376G	**24.97 (11.03 - 56.54)**	**<0.0001**	**3.04 (1.10 - 8.35)**	**0.031**
		HLA-B27 pos/ -376A	-	-	-	-
HLA-B27/ -308G>A	HLA-B27 neg/ -308G	HLA-B27 neg/ -308A	1.31 (0.53 - 3.24)	0.556	1.44 (0.77 - 2.71)	0.258
		HLA-B27 pos/ -308G	**26.63 (11.67 - 60.79)**	**<0.0001**	**3.24 (1.18 - 8.95)**	**0.023**
		HLA-B27 pos/ -308A	-	-	-	-
HLA-B27/ -238G>A	HLA-B27 neg / -238G	HLA-B27 neg / -238A	0.29 (0.04 - 2.20)	0.233	0.97 (0.42 - 2.24)	0.936
		HLA-B27 pos / -238G	**25.00 (11.03 - 56.68)**	**<0.0001**	**3.16 (1.15-8.69)**	**0.026**
		HLA-B27 pos / -238A	-	-	-	-

Significant p values are reported in bold face. AS: ankylosing spondylitis, PsA: psoriatic arthritis SNP: Single Nucleotide Polymorphism, TNFA: Tumor Necrosis Factor α, HLA: human leukocyte antigen.

With respect to the reference haplotype, only two further haplotypes were inferred from the combination of HLA-B27 and TNFA SNP -376G>A, -308G>A and -238G>A, being the frequent TNFA allele associated with positive HLA-B27, while the rare alleles with negative HLA-B27. By contrast HLA-B27 resulted in more haplotypes with

TNFA -857C>T SNP. The haplotype HLA-B27 positive/TNFA -857C only being correlated with AS diagnosis and this was confirmed by the analysis of HLA-B27/TNFA-857 haplotypes frequencies found in the different diseases in comparison with controls (Figure 13) (χ^2= 52.82 and p < 0.0001).

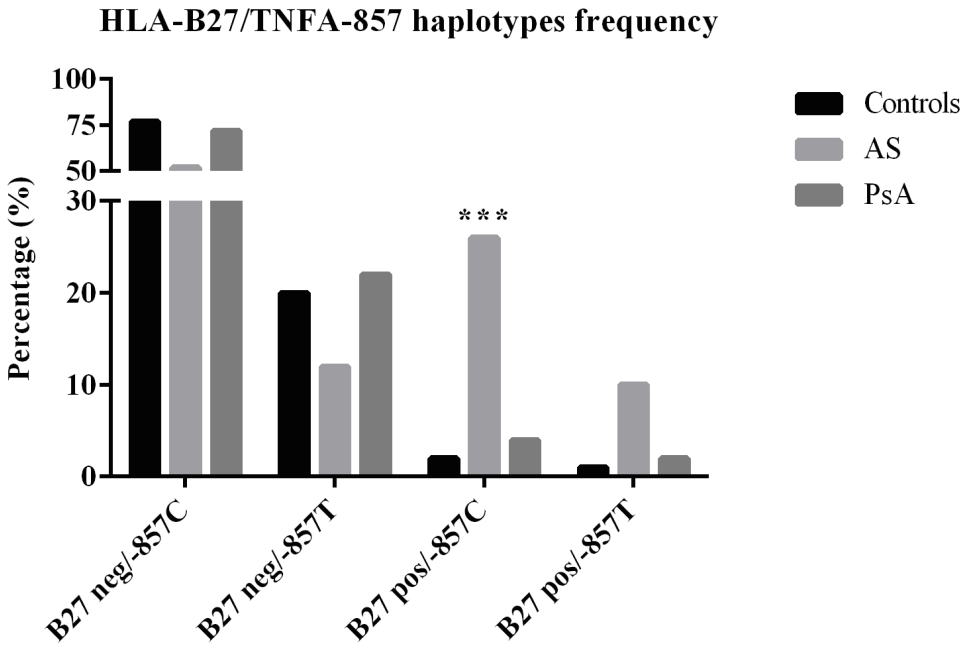

Figure 13. Frequency of the HLA-B27/TNFA-857 haplotypes in controls and in patients.

***p < 0.0001

4.4.3 TNFRSF1A gene variants do not associate with Spondyloarthritis

By the DHPLC screening of exons 2, 3, 4 and 6 of the TNFRSF1A gene followed by direct sequence analysis of positive samples, a total of three polymorphisms were identified during the explorative study and, among them, only the R92Q and the c.625+10A>G were selected for their potential functional implications (Table 19). MAF were 0.034 and 0.483 respectively. Both SNPs were not correlated with the presence of SpA (χ^2 = 1.073 and p = 0.300 for R92Q; χ^2= 4.721 and p= 0.094 for c.625+10A>G). Therefore the study of these gene variations was not extended to the entire number of controls.

Table 19. TNFRSF1A gene polymorphisms in controls and patients (AS and PsA). Results from the exploratory study.

dbSNP TNFRSF1A gene	MAF	Controls (n=27)			AS (n=36)			PsA (n=55)			χ^2, p
		GENOTYPES Number (frequency)			GENOTYPES Number (frequency)			GENOTYPES Number (frequency)			
R92Q c.362G>A rs4149584	A (0.034)	A/A 0 (-)	G/A 3 (0.11)	G/G 24 (0.89)	A/A 0 (-)	G/A 2 (0.05)	G/G 34 (0.95)	A/A 0 (-)	G/A 3 (0.05)	G/G 52 (0.95)	χ^2= 1.04 p= 0.60
c.625+10A>G rs1800693	G (0.483)	G/G 4 (0.25)	G/A 19 (0.50)	A/A 4 (0.25)	G/G 8 (0.20)	G/A 16 (0.49)	A/A 12 (0.31)	G/G 14 (0.25)	G/A 27 (0.50)	A/A 14 (0.25)	χ^2= 5.22 p= 0.27

AS: ankylosing spondylitis, PsA: psoriatic arthritis, dbSNP: Single Nucleotide Polymorphism Database, TNFRSF1A: Tumor Necrosis Factor Receptor Superfamily Member 1A, MAF: minor allele frequency.

4.4.4 MEFV gene variants do not associate with Spondyloarthritis

In the exploratory study, direct sequencing of exons 2, 3, 5 and 10 of MEFV gene allowed the identification of a total of twenty-one SNPs. Among them, eleven were synonymous base substitutions (exon 2: D102D, A165A, G138G; exon 3: R314R; exon 5: E474E, D510D, Q476Q, R501R, Q489Q; exon 10: F721F, P706P), and therefore they were not considered in the subsequent statistical analyses. The remaining ten variants (exon 2: E148Q, S179N, R202Q; exon 3: P369S, R408Q, R348H; exon 5: A457V; exon 10: A744S; K695M, M680IGA) were missense SNPs of potential functional significance and their genotypes with minor alleles frequencies are reported in Table 20.

The variant alleles of almost all the identified missense SNPs were extremely rare in our population, being their MAF lower or equal to 0.025 for nine out of ten SNPs. The R202Q SNP had a MAF of 0.336 and the rare homozygous AA genotype was found only among AS or PsA patients, not in controls, although the difference was not statistically significant. Since this might be dependent on the low number of AS and PsA cases with the rare genotype, we performed the statistical analysis by comparing SpA overall with controls, and this was significant ($\chi^2 = 7.4837$; $p = 0.006$).

Table 20. MEFV gene polymorphisms in controls and in patients (AS and PsA).

Results from the exploratory study.

dbSNP MEFV gene	MAF	Controls (n=27)			AS (n=36)			PsA (n=55)			χ^2, p
		GENOTYPES Number (frequency)			GENOTYPES Number (frequency)			GENOTYPES Number (frequency)			
R202Q c.605G>A rs224222	A (0.288)	A/A 0 (-)	G/A 13 (0.42)	G/G 14 (0.58)	A/A 2 (0.09)	G/A 17 (0.41)	G/G 17 (0.50)	A/A 6 (0.10)	G/A 22 (0.43)	G/G 27 (0.48)	χ^2= 2.37 p= 0.67
S179N c.536G>A	A (0.004)	A/A 0 (-)	G/A 0 (-)	G/G 27 (1.00)	A/A 0 (-)	G/A 1 (0.03)	G/G 35 (0.97)	A/A 0 (-)	G/A 0 (-)	G/G 55 (1.00)	χ^2= 2.30 p= 0.32
E148Q c.442G>C rs3743930	C (0.013)	C/C 0 (-)	G/A 0 (-)	G/G 27 (1.00)	C/C 0 (-)	G/C 1 (0.03)	G/G 35 (0.97)	C/C 0 (-)	G/C 2 (0.04)	G/G 53 (0.96)	χ^2= 0.98 p= 0.61
P369S c.1105C>T rs11466023	T (0.025)	T/T 1 (0.01)	C/T 1 (0.10)	C/C 25 (0.89)	T/T 0 (-)	C/T 0 (-)	C/C 36 (1.00)	T/T 0 (-)	C/T 3 (0.05)	C/C 52 (0.95)	χ^2= 5.39 p= 0.25
R408Q c.1223G>A rs11466024	A (0.017)	A/A 0 (-)	G/A 1 (0.04)	G/G 26 (0.96)	A/A 0 (-)	G/A 0 (-)	G/G 36 (1.00)	A/A 0 (-)	G/A 3 (0.05)	G/G 52 (0.95)	χ^2= 1.99 p= 0.37
R348H c.1043G>A rs104895198	A (0.004)	A/A 0 (-)	G/A 0 (-)	G/G 27 (1.00)	A/A 0 (-)	G/A 0 (-)	G/G 36 (1.00)	A/A 0 (-)	G/A 1 (0.02)	G/G 54 (0.98)	χ^2= 1.16 p= 0.56
A457V c.1370C>T rs104895151	T (0.004)	T/T 0 (-)	C/T 0 (-)	C/C 27 (1.00)	T/T 0 (-)	C/T 0 (-)	C/C 36 (1.00)	T/T 0 (-)	C/T 1 (0.02)	C/C 54 (0.98)	χ^2= 1.15 p= 0.56
A744S c.2230G>T rs61732874	T (0.004)	T/T 0 (-)	G/T 0 (-)	G/G 27 (1)	T/T 0 (-)	G/T 0 (-)	G/G 36 (1.00)	T/T 0 (-)	G/T 1 (0.02)	G/G 54 (0.98)	χ^2= 1.15 p= 0.56
K695M c.2084A>T rs104895129	T (0.008)	T/T 0 (-)	A/T 1 (0.04)	A/A 26 (0.96)	T/T 0 (-)	A/T 1 (0.03)	A/A 35 (0.97)	T/T 0 (-)	A/T 0 (-)	A/A 55 (1.00)	χ^2= 1.86 p= 0.40
M680IGA c.2040G>A rs28940580	A (0.004)	A/A 0 (-)	G/A 0 (-)	G/G 27 (1.00)	A/A 0 (-)	G/A 0 (-)	G/G 36 (1.00)	A/A 0 (-)	G/A 1 (0.02)	G/G 54 (0.98)	χ^2= 1.15 p= 0.56

AS: ankylosing spondylitis, PsA: psoriatic arthritis, dbSNP: Single Nucleotide Polymorphism Database, MEFV: Mediterranean fever, MAF: minor allele frequency.

To confirm this result, i.e. the association between R202Q SNP and SpA, the study of this SNP was extended to the entire controls population (Table 21). The frequency of the rare homozygous genotype AA, was very similar in patients (0.09) and in controls (0.07) and the difference was not statistically significant. Therefore the study extended to a higher number of controls does not confirm the association between SpA diagnosis and R202Q SNP.

Table 21. R202Q polymorphism in the entire studied population.

Gene	**dbSNP**	**MAF**	**SpA (n=91)**			**Controls (n=218)**			**χ^2, p**
			GENOTYPES Number (frequency)			**GENOTYPES Number (frequency)**			
MEFV	R202Q c.605G>A rs224222	A (0.272)	A/A 8 (0.09)	G/A 39 (0.42)	G/G 44 (0.49)	A/A 15 (0.07)	G/A 83 (0.38)	G/G 120 (0.55)	χ^2= 1.05 p= 0.59

The determination of R202Q SNP was not possible in 5 subjects for depletion of biological material. SpA: Spondyloarthritis, dbSNP: Single Nucleotide Polymorphism Database, MEFV: Mediterranean fever, MAF: minor allele frequency.

4.4.5 TNFRSF1A c.625+10A>G SNP associates with effective anti-TNFα treatment in SpA

On the basis of the previously reported results, we verified whether HLA-B27 and TNFA genetics correlate with response to anti-inflammatory treatment.

Patients were classified in two groups, on the basis of the time to reach a BASDAI score lower than or equal to 4 (response time). Patients with a BASDAI score lower than/equal to 4 were regarded as having no active disease. To evaluate the response to treatment the selected target was 10 months from the starting of therapy. Patients were subdivided in two groups on the basis of their response to therapy at 10 months after starting the last therapy: Group 1= BASDAI score lower than/equal to 4; Group 2= BASDAI score higher than 4. Table 22 reports descriptive statistics of Groups 1 and 2. Thirty-six of the 65 patients reached a BASDAI score lower than/equal to 4 in a time ranging from 1 to 10 months, while the remaining 29 in a timeframe spanning from 12 and 36 months.

Table 22. Months to reach a BASDAI score lower than/equal to 4 in groups 1 and 2.

	BASDAI ≤ 4						
Anti-TNFα therapy	**N of SpA patients**	**Mean**	**Max**	**Min**	**50th percentile**	**IQR**	**SD**
Group 1 (≤ 10 months)	36	5.94	10	1	6	0	1.19
Group 2 (>10 months)	29	16.55	36	12	12	5	7.16

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, TNFα: Tumor Necrosis Factor α, SpA: Spondyloarthritis, N: number, Max: maximum value (months), Min: minimum value (months), IQR: interquartile range, SD: standard deviation.

Table 23 reports the number and percentage of patients belonging to group 1 or 2 in relation to the identified genotypes in HLA-B27, TNFA and TNFRSF1A genes.

The TNFRSF1A c.625+10A/G SNP was significantly associated ($p = 0.031$) with a slower response to therapy. No association was found between the response to treatments and the other investigated polymorphisms.

Table 23. HLA-B27, TNFA and TNFRSF1A genetics in the response to treatment.

	BASDAI ≤ 4		
	≤ 10 months n (%)	**> 10 months** n (%)	
HLA-B27			
Negative	22 (33.8%)	20 (30.8%)	χ^2 = 0.4334 p = 0.510
Positive	14 (21.5%)	9 (13.8%)	
TNFA			
-1031T>C			
T/T	23 (35.4%)	17 (26.1%)	χ^2 = 0.1960 p = 0.907
T/C	11 (16.9%)	10 (15.4%)	
C/C	2 (3.1%)	2 (3.1%)	
-857C>T			
C/C	22 (34.9%)	16 (25.4%)	χ^2 = 4.3876 p = 0.111
T/C	14 (22.2%)	8 (12.7%)	
T/T	0 (0.0%)	3 (4.8%)	
-376G>A			
G/G	34 (55.7%)	26 (42.6%)	χ^2 = 0.7552 p = 0.385
G/A	1 (1.6%)	0 (0.0%)	
A/A	-	-	
-308G>A			
G/G	30 (47.6%)	20 (31.7%)	χ^2 = 0.8077 p = 0.369
G/A	6 (9.5%)	7 (11.1%)	
A/A	-	-	
-238G>A			
G/G	33 (50.8%)	27 (41.5%)	χ^2 = 0.0467 p = 0.829
G/A	3 (4.6%)	2 (3.1%)	
A/A	-	-	
TNFRSF1A			
R92Q (c.362G>A)			
G/G	35 (53.8%)	26 (40%)	χ^2 = 1.5925 p = 0.207
G/A	1 (1.5%)	3 (4.6%)	
A/A	-	-	
c.625+10A>G			
A/A	13 (20%)	5 (7.7%)	χ^2 = 6.9648 p = **0.031**
G/A	18 (27.7%)	12 (18.5%)	
G/G	5 (7.7%)	12 (18.5%)	

Significant p values are reported in bold face. The analysis was not performed, because of biological material depletion, in: 2 patients for -857C>T SNP, 2 patients for -376G>A SNP and 2 patients for -308G>A SNP. BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, TNFA: Tumor Necrosis Factor α, TNFRSF1A: Tumor Necrosis Factor Receptor Superfamily Member 1A.

The association between c.625+10A>G polymorphism and response time was verified among patients treated with anti-TNFα drugs and confirmed among those treated with infliximab but not in those treated with adalimumab (Table 24). The number of patients

treated with etanercept or golimumab was too low to support statistical analysis and these drugs were therefore not considered in separate analysis.

Table 24. c.625 +10 A>G polymorphism in the response to treatment with anti-TNFα therapy.

c.625+10A>G	**Infliximab**			**Adalimumab**		
	BASDAI ≤ 4		Fisher's exact	**BASDAI ≤ 4**		Fisher's exact
	≤ 10 months n (%)	**> 10 months** n (%)		**≤ 10 months** n (%)	**> 10 months** n (%)	
A/A	5 (38.5%)	1 (20.0%)	**0.026**	3 (23.1%)	0 (0%)	1.000
G/A	9 (50.0%)	2 (16.7%)		5 (27.8%)	0 (0%)	
G/G	1 (20.0%)	5 (41.7%)		4 (80%)	1 (8.3%)	

Significant p values are reported in bold face. BASDAI: Bath Ankylosing Spondylitis Disease Activity Index

5. DISCUSSION and CONCLUSIONS

The SpA are a group of chronic inflammatory diseases characterized by the sharing of distinctive pathological, clinical and radiographic aspects and a strong genetic predisposition, that leads to the activation of autoinflammation and the dys-regulation of the immune-system [3]. The two main distinct and frequent SpA subgroups are AS and PsA.

In many cases, an early diagnosis and an appropriate monitoring of disease activity can be difficult because of the overlap of clinical features. In addition, at least half of patients with SpA present indices of inflammation (ESR and CRP) in the normal range, even in the presence of clear expression of disease activity. Therefore, unlike rheumatoid arthritis and other inflammatory arthropathies, specific biomarkers of disease activity are not commonly used in clinical practice both in AS and PsA [11]. Among the genetic predisposing factors, a well-known role is that of HLA-B27, which contributes however only 20–30% of the total heritability, whereas the whole MHC accounts for about 40–50% of the genetic risk of developing SpA. Even the presence of HLA-B27, which increases the chance of ultimately diagnosing a SpA especially in the context of non-radiographic SpA, cannot be considered sufficient to diagnose the condition. Moreover, several SNPs in non-HLA-B genes have been identified as associated with AS, and more in general with SpA [2, 4].

For all these reasons, much attention is currently devoted to the identification of biochemical and genetic biomarkers to be used in the diagnosis as well as prognostic factors for evaluating the effectiveness of treatment. Must be remembered that, in these patients, a delay in diagnosis (generally estimates in 8-11 years by the symptoms onset) and consequently in treatment, imparts a tremendous symptomatic burden and loss of function during the productive years of life [10].

The present study, conducted in a cohort of 91 SpA patients coming from a North-East Italian region, was aimed to identify biohumoral and genetic factors to support the diagnostic and prognostic (response to therapy) work-up of SpA diseases. In particular, in addition to biochemical and haematological indices, we investigated whether SNPs in the promoter region of TNFA, or SNPs of the autoinflammatory genes TNFRSF1A and MEFV might concur with HLA-B27 in enhancing the risk of developing SpA disease and/or in predicting the response to treatment, including anti-TNFα drugs.

Considering the wide variety of expression of these diseases, both from a clinical that biohumoral point of view, only the two most prevalent subgroups of SpA patients, AS and PsA patients, were recruited in the study. Therefore, patients affected by non-radiographic axial SpA, ReA, IBD- SpA and uSpA were not enrolled because of their very low prevalence among worldwide patients [8] as well as among Italian patients [9]. Furthermore, while the AS belongs to the group of axial involvement SpA, PsA is representative of the peripheral SpA. The two groups of patients, as expected, were well-distinguished by characteristic clinical manifestations. Family history of disease, personal history of psoriasis, dactilitys and pheripheral arthritis were more frequently found among PsA, and this fit well with the Classification Criteria for Psoriatic Arthritis (CASPAR) [41]. Back pain and buttock pain, although commonly shared by the two disease groups, were more frequently recorded among patients affected by AS than PsA. Some extra-articular clinical manifestations were extremely rare in both groups, which, at diagnosis, had very similar scores of outcome, and similar demographic and anthropometric characteristics, such as age and BMI. The differences and similarities between AS and PsA support the notion that these diseases probably share a common background on the one hand, but specific genetic and/or environmental factors dictate AS or PsA evolution.

We first evaluated whether these two diseases could be distinguished by routinely used biochemical and haematological parameters and found that in both diseases a higher number of circulating PMN and higher CRP levels could be detected with respect to controls, and in PsA higher levels of ALT could be observed with respect not only to controls but also to AS. Aspartate aminotransferase is an index of liver injury of any cause and in the present series it might indicate the presence of mild liver damage due to non-alcoholic fatty liver disease, which is observed mainly in PsA than in AS [145, 146]. The mild degree of liver damage was hypothesized because the observed ALT levels were never above twice the cut-off. The finding of higher circulating PMN and CRP in both PsA and AS deserves some attention. Although both indices were not highly elevated and often comprised within the reference intervals, their increased levels in SpA suggest that the systemic inflammatory process underlying these diseases cannot be completely switch off despite treatment that means, in other words, a persisting smouldering inflammatory fire. This conclusion is supported by fact that biochemical data were collected at enrollment of patients, when all were under treatment with anti-TNFα drugs or NSAIDs/DMARDS. Although the differences in

WBC count and CRP levels between patients and controls were statistically significant, they cannot be proposed for diagnosis especially in patients under therapy because their variations were too light.

We focused our efforts in evaluating whether genetics could be of some help in this setting, taking into care consideration the fact that it is not affected by therapy. We first evaluated the association between disease diagnosis and HLA-B27, a genetic paradigm for AS [2, 35, 56]. In agreement a strong association between AS diagnosis and HLA-B27 positivity was found. This genetic predisposing factor was also slightly associated with PsA diagnosis, further supporting the notion that HLA-B27 is generally a risk factor for different subgroups of SpA [56]. The different degree of association, strong for AS and mild for PsA, might be interpreted in the light of the hypothesized roles of this leukocyte antigen in SpA pathogenesis. This antigen appears to play a relevant role in autoimmunity by the selective presentation of self antigens to immune competent T cells, being selectivity potentially dependent on allelic variants of HLA-B27, expression of the surface cells of homo- or hetero- HLA-B27 dimers and on a various grade of protein misfolding [53, 56-64]. The molecular analysis of HLA-B27 allowed us to analyse the presence of the most frequent alleles, but not to distinguish them one from another, nor was it able to give us indications about misfolding or dimerization. Therefore we might only hypothesize that AS and PsA share in part the common HLA-B27 genetic background, but their full clinical manifestations depend on more complex interplay with other genetic and/or environmental factors.

HLA-B27 gene belongs to the family of the major histocompatibility complex located in the short arm of chromosome 6. The HLA region includes the major genetic risk factors for SpA [2, 34, 47] and, in addition to HLA-B27, HLA-CW6 was demonstrated to be associated with PsA risk [46- 48]. In our series of patients this variant was more frequently found among PsA, than AS or controls, but the difference was not statistically significant. This is not surprising since HLA-CW6 was described in association with vulgaris psoriasis and our patients had only few cutaneous manifestations and onychopathy, and HLA-CW6 was described to account with more than other 30 genes only about 20% genetic predisposition to PsA [46, 48].

Within the HLA region is located also the TNFA gene, encoding TNF [69, 70]. Due to the pathophysiological role of TNF in inflammation [66, 67], a basic phenomenon found in many diseases including SpA, and taking into account that anti-TNF therapy reaches often a great success in these patients [25-28], we asked for any potential

impact of TNFA genetics in SpA. A number of polymorphisms in the promoter of TNFA gene have been studied in different inflammatory and autoimmune diseases, including SpA [70-71, 83]. These polymorphisms might have a potential functional significance since they, by binding with different affinities transcription factors like OCT1 (Organic Cation Transporter 1) or NF-kB, up- or down-regulate mRNA transcription levels [70]. Five main TNFA promoter polymorphisms have been described in Caucasians, namely -1031T>C (rs1799964), -857C>T (rs1799724), -376G>A (rs1800750), -308G>A (rs1800629), and -238G>A (rs361525) and among them association data with SpA have been reported with the 857C>T, -308G>A, and -238G>A with contrastive results [71-83]. The opposite findings regarding these SNPs in SpA might be dependent on differences in ethnicity of the studied populations, in number of studied patients and on differences in selection criteria of patients and controls. Due to these uncertainty, in this study we verified whether these SNPs in addition to other two SNPs not previously investigated in depth, play a part in SpA alone or combined in haplotypes among them or with HLA-B27. Any single TNFA SNP was not associated with SpA diagnosis, nor with PsA or AS considered singly, and this is in agreement with previous data collected from a English AS population [81] as well as with data reported by Lee an co-workers in a meta-analysis [73]. This might mean that TNFA promoter polymorphisms do not play any role in predisposing to SpA disease or, otherwise, they may act only when combined in haplotypes. It is known, in fact, that the regulation of TNFA expression by transcription factors involve more than one single SNP, being the gene regulated by several transcription factors such as OCT1, NF-kB and AP1 (activator protein 1). The transcription factor OCT1 can strongly bind with the allele -857T (but not the -857C) thus blocking the interaction of NF-κB to the nearby region -873 to -863 leading to inhibition of TNF-α transcription. The transcription factor AP1 binds a region close to the -1031T>C SNP [70]. Based on these assumptions we evaluated by inferred statistical analysis the haplotypes deriving from the pairwise combinations of the five studied SNPs. Due to the very low frequency of the -376A, -308A and -238A minor alleles (<0.1), the expected haplotypes including these alleles were almost completely absent in our population. Similarly the haplotype resulting from the combination of the minor alleles -1031C and -857T was very rare in our population despite a minor allele frequency for any single SNP higher than 0.2. We selected as a reference the most frequent haplotypes in controls and found that only the haplotype -1031C/-308G was significantly associated with SpA exerting in these

diseases a protective role, confirmed mainly in AS when any disease was considered singly. This finding might be explained taking into account that any single allele concurring in determining the studied haplotype was demonstrated to be associated with reduced TNF release. The -308G common allele was described to be associated with a lower spontaneous or stimulated TNF release both in vitro and in vivo with respect to the rare -308A allele [68, 147]. It is expected that the rare -1031C allele is also associated with low TNF production since it is in complete linkage disequilibrium with the -863A allele, which was demonstrated to be associated with reduced TNF release [148]. Therefore it may be assumed that low TNF producing alleles might exert effects on disease only when inherited together. To further verify the role of TNFA -1031/-308 haplotype association with AS risk, we defined the resulting genotypes when possible and inferred them from haplotype frequencies in those cases that were heterozygotes for both SNPs. In agreement with the above reported observations, a higher frequency of TNFA -1031T/-308G homozygotes was found among AS cases than in controls or PsA, while the opposite was found considering subjects carrying the genotype combination of rare haplotypes.

The above finding, however, should be evaluated taking into account also HLA-B27 status, because of the nearby location of these genes. TNFA -1031T/-308G homozygotes were most frequent, while rare haplotypes were less frequently found among HLA-B27 carriers, suggesting the existence of an association between these two inherited conditions.

We assumed that the risk effects on SpA exerted by TNFA and HLA-B27 might derive from their combination and to verify this assumption haplotypes were inferred. The frequency of haplotypes including HLA-B27 and the rare TNFA -376A, -308A and -238A alleles was extremely low, so their association with SpA was not ascertainable. The two haplotypes resulting from the combination between HLA-B27 and TNFA-857 C and T alleles were both associated with SpA considering patients overall. Interestingly the HLA-B27 positive/-857T haplotype was less frequent in AS than the HLA-B27 positive/-857C haplotype. Once again we hypothesize that a TNFA allele inhibiting TNF release, as the -857T allele, can antagonize inflammatory spondyloarthritis, this effect being evident when in the context of a powerful genetic predisposing factor like HLA-B27.

In the complex scenario of TNF effects in inflammation and autoimmunity one might bear in mind the role of TNF receptors, which genetic variability might underlie

complex autoinflammatory diseases such as TRAPS [110, 111]. For this reason we evaluated whether sequence variations of the gene TNFRSF1A encoding for the TNFR1 could be associated also with SpA. Only a few number of variants were detected in our population of patients and controls and none of them was associated with the diagnosis of AS or PsA. Therefore a role for TNFRSF1A genetics in SpA could be reasonably excluded.

When considering the genetic predisposition to SpA, it was emerged in recent years from GWAS that HLA as well as non-HLA predisposing genes are involved and these include cytokines genes (e.g. IL23R, IL17, IL2R), but also genes encoding proteins of the inflammasome pathway (CARD9, CARD14, CARD16) [88-98]. This pathway is known to be critically involved in autoinflammatory diseases and genetic variants of MEFV gene, encoding for the protein pyrin, have recently been suggested as potentially involved also in SpA. This fits well with the relative frequent finding of SpA among patients with FMF [124-130]. In our study we identified several MEFV variants, half without functional significance and half with potential functional consequences for the predicted aminoacid substitution in the protein. These variants were however sporadic and none was associated with SpA diagnosis. Intriguingly the main MEFV variant that has been demonstrated to be associated with AS, namely the M694V [124-126], was never recorded among our patients. To explain this discrepant result we might take into consideration the followings:

1. The association was described among Turkish population, which ethnicity is different from that of our patients;
2. The selection criteria of the studied population are different: FMF diagnosis with a subsequent search for AS was the inclusion criterion for studies finding an association, while in our study the selection started from the presence of SpA;
3. When an association between M694V and AS was found, all patients were invariably HLA-B27 negative, this further stressing the differences existing between our studied patients and other studied populations.

One of the main problems in the clinical setting of SpA is dealing with interindividual variability in response to therapy, mainly in response to anti-TNFα agents [135-138, 149]. Different drugs belonging to this category are now available and they are frequently used to treat both AS and PsA especially when NSAIDs or DMARDS are not beneficial [36-38]. In our series about 80% AS and 50% PsA were on treatment with anti-TNFα drugs, and among them 13% AS and 25% PsA required in

their disease history the switch from one to at least another drug type for an incomplete response to treatment. This empirical approach is the only one actually proposed since no predictive response biomarkers are validated. Their identification is really necessary also considering the high costs for the health care system of this type of therapy [150]. Among factors that might affect variability in response to therapy, genetics should be considered besides to non-genetic physiological factors (age, sex, weight, and body fat), pathophysiological factors (liver, kidney, and cardiovascular function, and associated diseases), and environmental factors (tobacco and alcohol consumption and concomitant treatments) [139-143]. To enhance the challenge facing SpA treatment and variables that can predict response prior to treatment, we verified whether TNFA or TNFRSF1A genetics is involved. Patients were subdivided according to a reasonably prompt or delayed response to therapy by considering early responder those reaching a BASDAI ≤4 within 10 months and late responder those reaching the same goal after 10 months from the starting therapy. The c.625+10A>G polymorphism of TNFRSF1A gene was associated with a different response to therapy, in particular carriers of the frequent A allele were mainly early responders, while those carrying the rare G allele were mainly late responders. The A allele was previously demonstrated to be correlated with increased serum TNF-α levels in patients with celiac disease [151] and an altered TNF-α/TNFR1 balance consequent to the synthesis of a truncated TNFR1 in patients carrying the rare G allele has been suggested by Ottoboni et al [152]. This polymorphism therefore might affect the response to anti-inflammatory therapy because of its potential effects on the TNF-TNFR pathway. When single drugs were evaluated with the aim to verify whether a difference exists between anti-TNF antibodies and anti-TNF soluble receptors, we could only ascertain anti-TNF antibodies because in our series of patients only few cases were treated with etanercept. TNFRSF1A c.625+10A>G polymorphism was confirmed to influence the response to therapy in case of infliximab, but not in case of adalimumab treatment. This finding, if confirmed in larger series of patients, might support the use of adalimumab instead of infliximab, as first line therapy due to its efficacy independent from genetics.

In conclusion the results of this study indicates the relevant role of TNF-TNFR pathway genetics in the complex network causing SpA and conditioning response to therapy. TNFA was shown to be a predisposing factor for SpA, but mainly for AS, among Italian patients, while genetics of the autoinflammatory gene MEFV appears of no impact in this setting. The haplotype resulting from TNFA-1031C/-308G, potentially

associated with lower TNF production, exerts a protective role in AS, while the TNFRSF1A c.625+10A>G polymorphism emerged as a potential predictor of response to anti- TNFα therapy.

6. REFERENCES

1. Moll JM, Haslock I, Macrae IF, Wright V. Associations between ankylosing spondylitis, psoriatic arthritis, Reiter's disease, the intestinal arthropathies, and Behcet's syndrome. Medicine 1974;53:343-64.

2. Giovannini L, Orlandi M, Lodato C, Cioffi E, Tenti S, Bardelli M, et al. One year in review 2015: spondyloarthritis. Clin Exp Rheumatol 2015;33:769-78.

3. Garg N, van den Bosch F, Deodhar A. The concept of spondyloarthritis: where are we now? Best Pract Res Clin Rheumatol 2014;28:663-72.

4. Dougados M, Baeten D. Spondyloarthritis. Lancet 2011;377:2127-37.

5. Dougados M, van der Linden S, Juhlin R, Huitfeldt B, Amor B, Calin A, et al. The European Spondylarthropathy Study Group preliminary criteria for the classification of spondylarthropathy. Arthritis Rheum 1991;34:1218-27.

6. Amor B, Dougados M, Mijiyawa M. Criteria of the classification of spondylarthropathies. Rev Rhum Mal Osteoartic 1990;57:85-9.

7. Rudwaleit M, van der Heijde D, Landewé R, Akkoc N, Brandt J, Chou CT, et al. The Assessment of SpondyloArthritis International Society classification criteria for peripheral spondyloarthritis and for spondyloarthritis in general. Ann Rheum Dis 2011;70:25-31.

8. Stolwijk C, van Onna M, Boonen A, van Tubergen A. Global Prevalence of Spondyloarthritis: A Systematic Review and Meta-Regression Analysis. Arthritis Care Res 2016;68:1320-31.

9. De Angelis R, Salaffi F, Grassi W. Prevalence of spondyloarthropathies in an Italian population sample: a regional community-based study. Scand J Rheumatol 2007;36:14-21.

10. Feldtkeller E, Khan MA, van der Heijde D, van der Linden S, Braun J. Age at disease onset and diagnosis delay in HLA-B27 negative vs. positive patients with

ankylosing spondylitis. Rheumatol Int 2003;23:61-6.

11. Mohan C, Assassi S. Biomarkers in rheumatic diseases: how can they facilitate diagnosis and assessment of disease activity? BMJ 2015;351:h5079.

12. Zochling J. Measures of symptoms and disease status in ankylosing spondylitis: Ankylosing Spondylitis Disease Activity Score (ASDAS), Ankylosing Spondylitis Quality of Life Scale (ASQoL), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), Bath Ankylosing Spondylitis Global Score (BAS-G), Bath Ankylosing Spondylitis Metrology Index (BASMI), Dougados Functional Index (DFI), and Health Assessment Questionnaire for the Spondylarthropathies (HAQ-S). Arthritis Care Res 2011;63 Suppl 11:S47-58.

13. Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. J Rheumatol 1994;21:2286-91.

14. Godfrin-Valnet M, Prati C, Puyraveau M, Toussirot E, Letho-Gyselink H, Wendling D. Evaluation of spondylarthritis activity by patients and physicians: ASDAS, BASDAI, PASS, and flares in 200 patients. Joint Bone Spine 2013;80:393-8.

15. Lukas C, Landewé R, Sieper J, Dougados M, Davis J, Braun J, et al. Assessment of SpondyloArthritis international Society.Development of an ASAS-endorsed disease activity score (ASDAS) in patients with ankylosing spondylitis. Ann Rheum Dis 2009;68:18-24.

16. Van der Heijde D, Lie E, Kvien TK, Sieper J, Van den Bosch F, Listing J, et al. Assessment of SpondyloArthritis international Society (ASAS). ASDAS, a highly discriminatory ASAS-endorsed disease activity score in patients with ankylosing spondylitis. Ann Rheum Dis 2009;68:1811-8.

17. Heuft-Dorenbosch L, Spoorenberg A, van Tubergen A, Landewé R, van ver Tempel H, Mielants H, et al. Assessment of enthesitis in ankylosing spondylitis. Ann Rheum Dis 2003;62:127-32.

18. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44-8.

19. Downie WW, Leatham PA, Rhind VM, Pickup ME, Wright V. The visual analogue scale in the assessment of grip strength. Ann Rheum Dis 1978;37:382-4.

20. Downie WW, Leatham PA, Rhind VM, Wright V, Branco JA, Anderson JA. Studies with pain rating scales. Ann Rheum Dis 1978;37:378-81.

21. Calin A, Garrett S, Whitelock H, Kennedy LG, O'Hea J, Mallorie P, Jenkinson T.A new approach to defining functional ability in ankylosing spondylitis: the development of the Bath Ankylosing Spondylitis Functional Index. J Rheumatol 1994;21:2281-5.

22. Daltroy LH, Larson MG, Roberts NW, Liang MH. A modification of the Health Assessment Questionnaire for the spondyloarthropathies. J Rheumatol 1990;17:946-50. Erratum in: J Rheumatol 1991;18:305.

23. Jenkinson TR, Mallorie PA, Whitelock HC, Kennedy LG, Garrett SL, Calin A. Defining spinal mobility in ankylosing spondylitis (AS). The Bath AS Metrology Index. J Rheumatol 1994;21:1694-8.

24. Song IH, Poddubnyy DA, Rudwaleit M, Sieper J. Benefits and risks of ankylosing spondylitis treatment with nonsteroidal antiinflammatory drugs. Arthritis Rheum 2008;58:929-38.

25. Braun J, Sieper J. Therapy of ankylosing spondylitis and other spondyloarthritides: established medical treatment, anti-TNF-alpha therapy and other novel approaches. Arthritis Res 2002;4:307-21.

26. Braun J, Brandt J, Listing J, Rudwaleit M, Sieper J. Biologic therapies in the spondyloarthritis: new opportunities, new challenges. Curr Opin Rheumatol 2003;15:394-407.

27. Prince DS, McGuigan LE, McGirr EE. Working life and physical activity in

ankylosing spondylitis pre and post anti-tumor necrosis factor-alpha therapy. Int J Rheum Dis 2014;17:165-72.

28. Baeten D, Kruithof E, Van den Bosch F, Van den Bossche N, Herssens A, Mielants H, et al. Systematic safety follow up in a cohort of 107 patients with spondyloarthropathy treated with infliximab: a new perspective on the role of host defence in the pathogenesis of the disease? Ann Rheum Dis 2003;62:829-34.

29. Van der Heijde D, Landewé R, Einstein S, Ory P, Vosse D, Ni L, et al. Radiographic progression of ankylosing spondylitis after up to two years of treatment with etanercept. Arthritis Rheum 2008;58:1324-31.

30. Braun J, Sieper J. Ankylosing spondylitis. Lancet 2007;369:1379-90.

31. Daikh DI, Chen PP. Advances in managing ankylosing spondylitis. F1000Prime Rep 2014;6:78.

32. Smith JA. Update on ankylosing spondylitis: current concepts in pathogenesis. Curr Allergy Asthma Rep 2015;15:489.

33. Van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. Arthritis Rheum 1984;27:361-8.

34. Khan MA. Polymorphism of HLA-B27: 105 subtypes currently known. Curr Rheumatol Rep 2013;15:362.

35. Robinson PC, Brown MA. Genetics of ankylosing spondylitis. Mol Immunol 2014;57:2-11.

36. Braun J, van den Berg R, Baraliakos X, Boehm H, Burgos-Vargas R, Collantes-Estevez E, et al. 2010 update of the ASAS/EULAR recommendations for the management of ankylosing spondylitis. Ann Rheum Dis 2011;70:896-904.

37. Van den Berg R, Baraliakos X, Braun J, van der Heijde D. First update of the current evidence for the management of ankylosing spondylitis with non-

pharmacological treatment and non-biologic drugs: a systematic literature review for the ASAS/EULAR management recommendations in ankylosing spondylitis. Rheumatology 2012;51:1388-96.

38. Baraliakos X, van den Berg R, Braun J, van der Heijde D. Update of the literature review on treatment with biologics as a basis for the first update of the ASAS/EULAR management recommendations of ankylosing spondylitis. Rheumatology 2012;51:1378-87.

39. Gladman DD. Psoriatic arthritis. Dermatol Ther 2009;22:40-55.

40. Wright V, Moll JMH. Psoriatric Arthritis. In seronegative polyarthritis. Amsterdam: North Holland Publishing Co., 1976: 169–235.

41. Taylor W, Gladman D, Helliwell P, Marchesoni A, Mease P, Mielants H. CASPAR Study Group. Classification criteria for psoriatic arthritis: development of new criteria from a large international study. Arthritis Rheum 2006;54:2665-73.

42. Cervini C, Leardini G, Mathieu A, Punzi L, Scarpa R. Psoriatic arthritis: epidemiological and clinical aspects in a cohort of 1.306 Italian patients. Reumatismo 2005;57:283-90.

43. Punzi L, Pianon M, Rossini P, Schiavon F, Gambari PF. Clinical and laboratory manifestations of elderly onset psoriatic arthritis: a comparison with younger onset disease. Ann Rheum Dis 1999;58:226-9

44. Gladman DD, Farewell VT. Progression in psoriatic arthritis: role of time varying clinical indicators. J Rheumatol 1999;26:2409-13.

45. Punzi L, Podswiadek M, Oliviero F, Lonigro A, Modesti V, Ramonda R, Todesco S. Laboratory findings in psoriatic arthritis. Reumatismo 2007;59 Suppl 1:52-5.

46. Gudjónsson JE, Kárason A, Antonsdóttir AA, Rúnarsdóttir EH, Gulcher JR, Stefánsson K, Valdimarsson H. HLA-Cw6-positive and HLA-Cw6-negative patients with Psoriasis vulgaris have distinct clinical features. J Invest Dermatol 2002;118:362-5.

47. Rahman P, Elder JT. Genetic epidemiology of psoriasis and psoriatic arthritis. Ann Rheum Dis 2005;64 Suppl 2:ii37-9; discussion ii40-1.

48. O'Rielly DD, Rahman P. Genetics of psoriatic arthritis. Best Pract Res Clin Rheumatol 2014;28:673-85.

49. Gladman DD, Helliwell PS, Mease PJ; Group for Reasearch and Assessment of Psoriasis and Psoriatic Arthritis. GRAPPA at the European League Against Rheumatism (EULAR) 2008. J Rheumatol 2009;36:656-8.

50. Gossec L, Smolen JS, Ramiro S, de Wit M, Cutolo M, Dougados M, et al. European League Against Rheumatism (EULAR) recommendations for the management of psoriatic arthritis with pharmacological therapies: 2015 update. Ann Rheum Dis 2016;75:499-510.

51. Gossec L, Smolen JS. Treatment of psoriatic arthritis: management recommendations. Clin Exp Rheumatol 2015;33:S73-7.

52. Ramos M, López de Castro JA. HLA-B27 and the pathogenesis of spondyloarthritis. Tissue Antigens 2002;60:191-205.

53. Chatzikyriakidou A, Voulgari PV, Drosos AA. What is the role of HLA-B27 in spondyloarthropathies? Autoimmun Rev 2011;10:464-8.

54. Benjamin R, Parham P. Guilt by association: HLA-B27 and ankylosing spondylitis. Immunol Today 1990;11:137-42.

55. Atagunduz P, Appel H, Kuon W, Wu P, Thiel A, Kloetzel PM, Sieper J. HLA-B27-restricted CD8+ T cell response to cartilage-derived self peptides in ankylosing spondylitis. Arthritis Rheum 2005;52:892-901.

56. López de Castro JA. HLA-B27 and the pathogenesis of spondyloarthropathies. Immunol Lett 2007;108:27-33.

57. Breban M, Fernández-Sueiro JL, Richardson JA, Hadavand RR, Maika SD, Hammer RE, Taurog JD. T cells, but not thymic exposure to HLA-B27, are required for

the inflammatory disease of HLA-B27 transgenic rats. J Immunol 1996;156:794-803.

58. May E, Dorris ML, Satumtira N, Iqbal I, Rehman MI, Lightfoot E, Taurog JD. CD8 alpha beta T cells are not essential to the pathogenesis of arthritis or colitis in HLA-B27 transgenic rats. J Immunol 2003;170:1099-105.

59. Schröder M, Kaufman RJ. ER stress and the unfolded protein response. Mutat Res 2005;569:29-63.

60. Colbert RA. HLA-B27 misfolding: a solution to the spondyloarthropathy conundrum? Mol Med Today 2000;6:224-30.

61. Turner MJ, Sowders DP, DeLay ML, Mohapatra R, Bai S, Smith JA, et al. HLA-B27 misfolding in transgenic rats is associated with activation of the unfolded protein response. J Immunol 2005;175:2438-48.

62. Tran TM, Dorris ML, Satumtira N, Richardson JA, Hammer RE, Shang J, Taurog JD. Additional human beta2-microglobulin curbs HLA-B27 misfolding and promotes arthritis and spondylitis without colitis in male HLA-B27-transgenic rats. Arthritis Rheum 2006;54:1317-27.

63. Bird LA, Peh CA, Kollnberger S, Elliott T, McMichael AJ, Bowness P. Lymphoblastoid cells express HLA-B27 homodimers both intracellularly and at the cell surface following endosomal recycling. Eur J Immunol 2003;33:748-59.

64. Kollnberger S, Bird L, Sun MY, Retiere C, Braud VM, McMichael A, Bowness P. Cell-surface expression and immune receptor recognition of HLA-B27 homodimers. Arthritis Rheum 2002;46:2972-82.

65. Chan AT, Kollnberger SD, Wedderburn LR, Bowness P. Expansion and enhanced survival of natural killer cells expressing the killer immunoglobulin-like receptor KIR3DL2 in spondylarthritis. Arthritis Rheum 2005;52:3586-95.

66. Van Kuijk AW, Reinders-Blankert P, Smeets TJ, Dijkmans BA, Tak PP. Detailed analysis of the cell infiltrate and the expression of mediators of synovial inflammation and joint destruction in the synovium of patients with psoriatic arthritis: implications for

treatment. Ann Rheum Dis 2006;65:1551-7.

67. Bal A, Unlu E, Bahar G, Aydog E, Eksioglu E, Yorgancioglu R. Comparison of serum IL-1 beta, sIL-2R, IL-6, and TNF-alpha levels with disease activity parameters in ankylosing spondylitis. Clin Rheumatol 2007;26:211-5.

68. Rudwaleit M, Siegert S, Yin Z, Eick J, Thiel A, Radbruch A, et al. Low T cell production of TNFalpha and IFNgamma in ankylosing spondylitis: its relation to HLA-B27 and influence of the TNF-308 gene polymorphism. Ann Rheum Dis 2001;60:36-42.

69. Verweij CL. Tumour necrosis factor gene polymorphisms as severity markers in rheumatoid arthritis. Ann Rheum Dis 1999;58 Suppl 1:I20-6.

70. El-Tahan RR, Ghoneim AM, El-Mashad N. TNF-α gene polymorphisms and expression. Springerplus 2016;5:1508.

71. Chung WT, Choe JY, Jang WC, Park SM, Ahn YC, Yoon IK, et al. Polymorphisms of tumor necrosis factor-α promoter region for susceptibility to HLA-B27-positive ankylosing spondylitis in Korean population. Rheumatol Int 2011;31:1167-75.

72. Li B, Wang P, Li H. The association between TNF-alpha promoter polymorphisms and ankylosing spondylitis: a meta-analysis. Clin Rheumatol 2010;29:983-90.

73. Lee YH, Song GG. Lack of association of TNF-alpha promoter polymorphisms with ankylosing spondylitis: a meta-analysis. Rheumatology 2009;48:1359-62.

74. Poddubnyy DA, Märker-Hermann E, Kaluza-Schilling W, Zeidler H, Braun J, Listing J, et al. Relation of HLA-B27, tumor necrosis factor-α promoter gene polymorphisms, and T cell cytokine production in ankylosing spondylitis -- a comprehensive genotype-phenotype analysis from an observational cohort. J Rheumatol 2011;38:2436-41.

75. Vargas-Alarcón G, Casasola-Vargas J, Rodríguez-Pérez JM, Huerta-Sil G, Pérez-Hernández N, Londoño J, et al. Tumor necrosis factor-alpha promoter polymorphisms in Mexican patients with spondyloarthritis. Hum Immunol 2006;67:826-32.

76. Shiau MY, Lo MK, Chang CP, Yang TP, Ho KT, Chang YH. Association of tumour necrosis factor alpha promoter polymorphisms with ankylosing spondylitis in Taiwan. Ann Rheum Dis 2007;66:562-3.

77. Nossent JC, Sagen-Johnsen S, Bakland G. Tumor necrosis factor-α promoter -308/238 polymorphism association with less severe disease in ankylosing spondylitis is unrelated to serum TNF-α and does not predict TNF inhibitor response. J Rheumatol 2014;41:1675-82.

78. Manolova I, Ivanova M, Stoilov R, Rashkov R, Stanilova S. Association of single nucleotide polymorphism at position -308 of the tumor necrosis factor-alpha gene with ankylosing spondylitis and rheumatoid arthritis. Biotechnol Biotechnol Equip 2014;28:1108-1114.

79. Höhler T, Grossmann S, Stradmann-Bellinghausen B, Kaluza W, Reuss E, de Vlam K, et al. Differential association of polymorphisms in the TNFalpha region with psoriatic arthritis but not psoriasis. Ann Rheum Dis 2002;61:213-8.

80. Höhler T, Schäper T, Schneider PM, Meyer zum Büschenfelde KH, Märker-Hermann E. Association of different tumor necrosis factor alpha promoter allele frequencies with ankylosing spondylitis in HLA-B27 positive individuals. Arthritis Rheum 1998;41:1489-92.

81. Milicic A, Lindheimer F, Laval S, Rudwaleit M, Ackerman H, Wordsworth P, et al. Interethnic studies of TNF polymorphisms confirm the likely presence of a second MHC susceptibility locus in ankylosing spondylitis. Genes Immun 2000;1:418-22.

82. McGarry F, Walker R, Sturrock R, Field M. The -308.1 polymorphism in the promoter region of the tumor necrosis factor gene is associated with ankylosing spondylitis independent of HLA-B27. J Rheumatol 1999;26:1110-6.

83. Fraile A, Nieto A, Beraún Y, Vinasco J, Matarán L, Martín J. Tumor necrosis factor gene polymorphisms in ankylosing spondylitis. Tissue Antigens 1998;51:386-90.

84. Kaijzel EL, Brinkman BM, van Krugten MV, Smith L, Huizinga TW, et al.

Polymorphism within the tumor necrosis factor alpha (TNF) promoter region in patients with ankylosing spondylitis. Hum Immunol 1999;60:140-4.

85. Gallo E, Cabaleiro T, Román M, Abad-Santos F, Daudén E. Study of genetic polymorphisms in the tumor necrosis factor α promoter region in Spanish patients with psoriasis. Actas Dermosifiliogr 2012;103:301-7.

86. Giardina E, Hüffmeier U, Ravindran J, Behrens F, Lepre T, McHugh NJ, et al. Tumor necrosis factor promoter polymorphism TNF*-857 is a risk allele for psoriatic arthritis independent of the PSORS1 locus. Arthritis Rheum 2011;63:3801-6.

87. Lv K, Chen R, Cai Q, Fang M, Sun S. Effects of a single nucleotide polymorphism on the expression of human tumor necrosis factor-alpha. Scand J Immunol 2006;64:164-9.

88. Australo-Anglo-American Spondyloarthritis Consortium (TASC), Reveille JD, Sims AM, Danoy P, Evans DM, Leo P, Pointon et al. Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. Nat Genet 2010;42:123-7.

89. Brown MA. Genetics of ankylosing spondylitis. Curr Opin Rheumatol 2010;22:126-32.

90. Costantino F, Talpin A, Evnouchidou I, Kadi A, Leboime A, Said-Nahal R, et al. ERAP1 Gene Expression Is Influenced by Nonsynonymous Polymorphisms Associated With Predisposition to Spondyloarthritis. Arthritis Rheumatol 2015;67:1525-34.

91. Daryabor G, Mahmoudi M, Jamshidi A, Nourijelyani K, Amirzargar A, Ahmadzadeh N, et al. Determination of IL-23 receptor gene polymorphism in Iranian patients with ankylosing spondylitis. Eur Cytokine Netw 2014;25:24-9.

92. Karaderi T, Keidel SM, Pointon JJ, Appleton LH, Brown MA, Evans DM, Wordsworth BP. Ankylosing spondylitis is associated with the anthrax toxin receptor 2 gene (ANTXR2). Ann Rheum Dis 2014;73:2054-8.

93. Ma X, Liu Y, Zhang H, Qiu R, Zhao H, Xin Q, et al. Evidence for genetic

association of CARD9 and SNAPC4 with ankylosing spondylitis in a Chinese Han population. J Rheumatol 2014;41:318-24.

94. Jordan CT, Cao L, Roberson ED, Pierson KC, Yang CF, Joyce CE, et al. PSORS2 is due to mutations in CARD14. Am J Hum Genet 2012;90:784-95.

95. Zinovieva E, Bourgain C, Kadi A, Letourneur F, Izac B, Said-Nahal R, et al. Comprehensive linkage and association analyses identify haplotype, near to the TNFSF15 gene, significantly associated with spondyloarthritis. PLoS Genet 2009;5:e1000528.

96. Yamazaki K, McGovern D, Ragoussis J, Paolucci M, Butler H, Jewell D, et al. Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. Hum Mol Genet 2005;14:3499-506.

97. Takedatsu H, Michelsen KS, Wei B, Landers CJ, Thomas LS, Dhall D, et al. TL1A (TNFSF15) regulates the development of chronic colitis by modulating both T-helper 1 and T-helper 17 activation. Gastroenterology 2008;135:552-67.

98. Pointon JJ, Harvey D, Karaderi T, Appleton LH, Farrar C, Stone MA, et al. The chromosome 16q region associated with ankylosing spondylitis includes the candidate gene tumour necrosis factor receptor type 1-associated death domain (TRADD). Ann Rheum Dis 2010;69:1243-6.

99. Chadwick W, Magnus T, Martin B, Keselman A, Mattson MP, Maudsley S. Targeting TNF-alpha receptors for neurotherapeutics. Trends Neurosci 2008 ;31:504-11.

100. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003;10:45-65.

101. Van Hauwermeiren F, Vandenbroucke RE, Libert C. Treatment of TNF mediated diseases by selective inhibition of soluble TNF or TNFR1. Cytokine Growth Factor Rev 2011;22:311-9.

102. Karaderi T, Pointon JJ, Wordsworth TW, Harvey D, Appleton LH, Cohen CJ, et

al; Australo-Anglo-American Spondyloarthritis Consortium. Evidence of genetic association between TNFRSF1A encoding the p55 tumour necrosis factor receptor, and ankylosing spondylitis in UK Caucasians. Clin Exp Rheumatol 2012;30:110-3.

103. Davidson SI, Liu Y, Danoy PA, Wu X, Thomas GP, Jiang L, et al; Australo-Anglo-American Spondyloarthritis Consortium., Visscher PM, Brown MA, Xu H. Association of STAT3 and TNFRSF1A with ankylosing spondylitis in Han Chinese. Ann Rheum Dis 2011;70:289-92.

104. Yousaf N, Gould DJ, Aganna E, Hammond L, Mirakian RM, Turner MD, et al. Tumor necrosis factor receptor I from patients with tumor necrosis factor receptor-associated periodic syndrome interacts with wild-type tumor necrosis factor receptor I and induces ligand-independent NF-kappaB activation. Arthritis Rheum 2005;52:2906-16.

105. Lehmann P, Salzberger B, Haerle P, Aksentijevich I, Kastner D, Schoelmerich J, et al. Variable intrafamilial expressivity of the rare tumor necrosis factor-receptor associated periodic syndrome-associated mutation I170N that affects the TNFR1A cleavage site. Mod Rheumatol 2010;20:311-5.

106. Lobito AA, Gabriel TL, Medema JP, Kimberley FC. Disease causing mutations in the TNF and TNFR superfamilies: Focus on molecular mechanisms driving disease. Trends Mol Med 2011;17:494-505.

107. Aganna E, Hammond L, Hawkins PN, Aldea A, McKee SA, van Amstel HK, et al. Heterogeneity among patients with tumor necrosis factor receptor-associated periodic syndrome phenotypes. Arthritis Rheum 2003;48:2632-44.

108. Turner MD, Chaudhry A, Nedjai B. Tumour necrosis factor receptor trafficking dysfunction opens the TRAPS door to pro-inflammatory cytokine secretion. Biosci Rep 2012;32:105-12.

109. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003;10:45-65.

110. McDermott MF, Aksentijevich I, Galon J, McDermott EM, Ogunkolade BW, Centola M, et al. Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. Cell 1999;97:133-44.

111. Stojanov S, McDermott MF. The tumour necrosis factor receptor-associated periodic syndrome: current concepts. Expert Rev Mol Med 2005;7:1-18.

112. Milhavet F, Cuisset L, Hoffman HM, Slim R, El-Shanti H, Aksentijevich I, et al. The infevers autoinflammatory mutation online registry: update with new genes and functions. Hum Mutat 2008;29:803-8.

113. Goris A, Fockaert N, Cosemans L, Clysters K, Nagels G, Boonen S, et al. TNFRSF1A coding variants in multiple sclerosis. J Neuroimmunol 2011;235:110-2.

114. Rigante D, Cantarini L, Imazio M, Lucherini OM, Sacco E, Galeazzi M, et al. Autoinflammatory diseases and cardiovascular manifestations. Ann Med 2011;43:341-6.

115. Van Hauwermeiren F, Vandenbroucke RE, Libert C. Treatment of TNF mediated diseases by selective inhibition of soluble TNF or TNFR1. Cytokine Growth Factor Rev 2011;22:311-9.

116. Amigues I, Stojanovic KS, Bansal A, Gagnard A, Tebib JG. Ankylosing spondylitis associated with auto-inflammatory syndrome mutation. Joint Bone Spine 2011;78:530-1.

117. French FMF Consortium. A candidate gene for familial Mediterranean fever. Nat Genet 1997;17:25-31.

118. Masters SL, Simon A, Aksentijevich I, Kastner DL. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease. Annu Rev Immunol 2009;27:621-68.

119. Touitou I. The spectrum of Familial Mediterranean Fever (FMF) mutations. Eur J Hum Genet 2001;9:473-83.

120. Marek-Yagel D, Bar-Joseph I, Pras E, Berkun Y. Is E148Q a benign polymorphism or a disease-causing mutation? J Rheumatol 2009;36:2372.

121. Ozkurede VU, Franchi L. Immunology in clinic review series; focus on autoinflammatory diseases: role of inflammasomes in autoinflammatory syndromes. Clin Exp Immunol 2012;167:382-90.

122. Heller H, Gafni J, Michaeli D, Shahin N, Sohar E, Ehrlich G, et al. The arthritis of familial Mediterranean fever (FMF). Arthritis Rheum 1966;9:1-17.

123. Garcia-Gonzalez A, Weisman MH. The arthritis of familial Mediterranean fever. Semin Arthritis Rheum 1992;22:139-50.

124. Akar S, Soysal O, Balci A, Solmaz D, Gerdan V, Onen F, et al. High prevalence of spondyloarthritis and ankylosing spondylitis among familial Mediterranean fever patients and their first-degree relatives: further evidence for the connection. Arthritis Res Ther 2013 28;15:R21.

125. Maraş Y, Akdoğan A, Kisacik B, Kiliç L, Yilmaz E, Tufan A, et al. MEFV mutation frequency and effect on disease severity in ankylosing spondylitis. Turk J Med Sci 2014;44:203-7.

126. Akkoc N, Gul A. Familial Mediterranean fever and seronegative arthritis. Curr Rheumatol Rep 2011;13:388-94.

127. Yigit S, Inanir A, Karakus N, Kesici E, Bozkurt N. Common Mediterranean fever (MEFV) gene mutations associated with ankylosing spondylitis in Turkish population. Dis Markers 2012;33:113-8.

128. Cosan F, Ustek D, Oku B, Duymaz-Tozkir J, Cakiris A, Abaci N, et al. Association of familial Mediterranean fever-related MEFV variations with ankylosing spondylitis. Arthritis Rheum 2010;62:3232-6.

129. Durmus D, Alayli G, Cengiz K, Yigit S, Canturk F, Bagci H. Clinical significance of MEFV mutations in ankylosing spondylitis. Joint Bone Spine 2009;76:260-4.

130. Cinar M, Dinc A, Simsek I, Erdem H, Koc B, Pay S, et al. The rate and significance of Mediterranean fever gene mutations in patients with ankylosing spondylitis: a three-month, longitudinal clinical study. Rheumatol Int 2008;29:37-42.

131. Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. Pharmacol Ther 2008;117:244-79.

132. Horiuchi T, Mitoma H, Harashima S, Tsukamoto H, Shimoda T. Transmembrane TNF-alpha: structure, function and interaction with anti-TNF agents. Rheumatology 2010;49:1215-28.

133. Scallon B, Cai A, Solowski N, Rosenberg A, Song XY, Shealy D, Wagner C. Binding and functional comparisons of two types of tumor necrosis factor antagonists. J Pharmacol Exp Ther 2002;301:418-26.

134. Braun J, Brandt J, Listing J, Rudwaleit M, Sieper J. Biologic therapies in the spondyloarthritis: new opportunities, new challenges. Curr Opin Rheumatol 2003;15:394-407.

135. Maneiro JR, Souto A, Salgado E, Mera A, Gomez-Reino JJ. Predictors of response to TNF antagonists in patients with ankylosing spondylitis and psoriatic arthritis: systematic review and meta-analysis. RMD Open 2015 18;1:e000017.

136. Rudwaleit M, Listing J, Brandt J, Braun J, Sieper J. Prediction of a major clinical response (BASDAI 50) to tumour necrosis factor alpha blockers in ankylosing spondylitis. Ann Rheum Dis 2004;63:665-70.

137. Vastesaeger N, van der Heijde D, Inman RD, Wang Y, Deodhar A, Hsu B, et al. Predicting the outcome of ankylosing spondylitis therapy. Ann Rheum Dis 2011;70:973-81.

138. Rudwaleit M, Claudepierre P, Wordsworth P, Cortina EL, Sieper J, Kron M, et al. Effectiveness, safety, and predictors of good clinical response in 1250 patients treated with adalimumab for active ankylosing spondylitis. J Rheumatol 2009;36:801-8.

139. Song GG, Seo YH, Kim JH, Choi SJ, Ji JD, Lee YH. Association between TNF-α (-308 A/G, -238 A/G, -857 C/T) polymorphisms and responsiveness to TNF-α blockers in spondyloarthropathy, psoriasis and Crohn's disease: a meta-analysis. Pharmacogenomics 2015;16:1427-37.

140. Liu J, Dong Z, Zhu Q, He D, Ma Y, Du A, et al. TNF-α Promoter Polymorphisms Predict the Response to Etanercept More Powerfully than that to Infliximab/Adalimumab in Spondyloarthritis. Sci Rep 2016;6:32202.

141. Pierik M, Vermeire S, Steen KV, Joossens S, Claessens G, Vlietinck R, Rutgeerts P. Tumour necrosis factor-alpha receptor 1 and 2 polymorphisms in inflammatory bowel disease and their association with response to infliximab. Aliment Pharmacol Ther 2004;20:303-10.

142. Matsukura H, Ikeda S, Yoshimura N, Takazoe M, Muramatsu M. Genetic polymorphisms of tumour necrosis factor receptor superfamily 1A and 1B affect responses to infliximab in Japanese patients with Crohn's disease. Aliment Pharmacol Ther 2008;27:765-70.

143. Schiotis R, Sánchez A, Escudero A, Bartolomé N, Szczypiorska M, Font P, et al. Candidate's single-nucleotide polymorphism predictors of treatment nonresponse to the first anti-TNF inhibitor in ankylosing spondylitis. Rheumatol Int 2014;34:793-801.

144. Marchenko YV, Carroll RJ, Lin DY, Amos CI, Gutierrez RG. Semiparametric analysis of case–control genetic data in the presence of environmental factors. Stata Journal Volume 8 Number 3; 2008.

145. Coates LC, FitzGerald O, Helliwell PS, Paul C. Psoriasis, psoriatic arthritis, and rheumatoid arthritis: Is all inflammation the same? Semin Arthritis Rheum 2016. pii: S0049-0172:30064-6.

146. Miele L, Vallone S, Cefalo C, La Torre G, Di Stasi C, Vecchio FM, et al. Prevalence, characteristics and severity of non-alcoholic fatty liver disease in patients with chronic plaque psoriasis. J Hepatol 2009;51:778-86.

147. Elahi MM, Asotra K, Matata BM, Mastana SS. Tumor necrosis factor alpha -308gene locus promoter polymorphism: an analysis of association with health and disease. Biochim Biophys Acta 2009;1792:163-72

148. Skoog T, van't Hooft FM, Kallin B, Jovinge S, Boquist S, Nilsson J, et al. A common functional polymorphism (C>A substitution at position -863) in the promoter region of the tumour necrosis factor-alpha (TNF-alpha) gene associated with reduced circulating levels of TNF-alpha. Hum Mol Genet 1999;8:1443-9.

149. Plasencia C, Pascual-Salcedo D, Nuño L, Bonilla G, Villalba A, Peiteado D, et al. Influence of immunogenicity on the efficacy of long term treatment of spondyloarthritis with infliximab. Ann Rheum Dis 2012;71:1955-60.

150. McLeod C, Bagust A, Boland A, Dagenais P, Dickson R, Dundar Y, et al. Adalimumab, etanercept and infliximab for the treatment of ankylosing spondylitis: a systematic review and economic evaluation. Health Technol Assess 2007;11:1-158, iii-iv.

151. Rossi E, Basso D, Zambon CF, Navaglia F, Greco E, Pelloso M, et al. TNFA Haplotype Genetic Testing Improves HLA in Estimating the Risk of Celiac Disease in Children. PLoS One 2015;10:e0123244.

152. Ottoboni L, Frohlich IY, Lee M, Healy BC, Keenan BT, Xia Z, et al. Clinical relevance and functional consequences of the TNFRSF1A multiple sclerosis locus. Neurology 2013;81:1891-9.