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The relationship between pharmacological treatment and genetic polymorphisms with clinical and structural severity in ankylosing spondylitis

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DOCTORAL THESIS

The relationship between pharmacological treatment and genetic polymorphisms with clinical and structural severity in ankylosing spondylitis

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TITULO: *Relación entre el tratamiento farmacológico y los polimorfismos genéticos con la gravedad clínica y estructural en la espondilitis anquilosante*

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Dedication

To Ma,
Without your love I would not have become who I am.

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LIST OF PUBLICATIONS

Published articles *in full text* as a result of doctoral research

1. Ruxandra Schiotis, Pilar Font, Alejandro Escudero, Pedro Zarco, Raquel Almodovar, Jordi Gratacos, Juan Mulero, Xavier Juanola, Carlos Montilla, Estefanı´a Moreno, Rafael Ariza Ariza, Eduardo Collantes-Estevez on behalf of REGISPONSER working group*. Usefulness of a centralized system of data collection for the development of an intearnational multicentre registry of spondyloarthritis. Rheumatology (Oxford) 2011;50:132–136 (ISI-factor de impact 4.058)*

2. Ruxandra Schiotis, Nerea Bartolome, Alejandra Sanchez, Magdalena Szczypiorska, Jesus Sanz, Eduardo Cuende, Eduardo Collantes Estevez, Antonio Martınez, Diego Tejedor, Marta Artieda, Anca Buzoianu, Juan Mulero**. Both *Baseline Clinical Factors and Genetic Polymorphisms Influence the Development of Severe Functional Status in Ankylosing Spondylitis. PlosOne 2012; Vol.7(9), e43428 (peer-reviewed, open acces journal, indexed in PubMed, MEDLINE, PubMed Central, Scopus, Web of Science, Google Scholar, factor de impact 4.092)*

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LIST OF ABREVIATIONS

AS	ankylosing spondylitis
ASAS	Assessment of SpondyloArthritis International Society
ASDAS	Ankylosing spondylitis Disease Activity Score
BASDAI	Bath Ankylosis Spondylitis Disease Activity Index
BASFI	Bath Ankylosing Spondylitis Functional Index
BASRI	Bath Ankylosing Spondylitis Radiological Index
CRP	C reactive proteine
DMARDs	Disease-modifying antirheumatic drugs
ESR	Erythrocyte sedimentation rate
HLA	Human leukocyte antigen
IBD	Inflammatory bowel diseases
IFNγ	Interferon-gamma
LD	Linkage disequilibrium
MCH	Major complex of histocompatibility
MRI	Magnetic resonance imaging
mSASSS	Modified Stoke Ankylosing Spondylitis Spine Score
NSAIDS	Non-steroidal antiinflammatory drugs
RA	Rheumatoid arthritis
ReA	Reactive arthritis
SNPs	single nucleotide polymorphisms
SpA	Spondyloarthropaty
SSZ	Sulphasalazine
TNF	Tumor necrosis factor
VAS	Visual analogical scale

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INTRODUCTION

Ankylosing spondylitis (AS) is a common inflammatory rheumatic disease, with world wide distribution, affecting approximately 0.5% of Caucasians. The main clinical feature of AS is sacroiliac involvement which is causing pain and stiffness. In a great number of cases, these symptoms progress to lumbar spinal fusion with characteristic spinal deformities and to hip joint stiffness.

There is strong evidence that in individuals with genetic susceptibility to AS, the disease is caused by a common ubiquitous pathogen. The major histocompatibility complex (MHC) on chromosme 6 is strongly associated with genetic susceptibility to disease, being the major genetic locus of the disease. Although the strongest association is determined by the presence of the HLA B27 antigen in this locus, there is evidence of the contribution of other non-HLA-B genes and non-MHC genes (e.g. IL-23R and ERAP1/ARTIS-1 genes) to susceptibility to disease.

The main concern in the progress of AS is represented by the prevention of disease progression to total ankylosis of the spine and of the adoption of a vicious position. Patients with advanced spinal ankylosis (bamboo spine) present a significant impairment of physical function and thus, have a poor quality of life and low socio-professional integration.

Therefore, the objective of the pharmacological treatment must simultaneously accomplish with two fundamental requirements: to control disease symptoms (disease activity) and to prevent structural damage (radiographic progression of the disease).

The only classes of drugs that have proven effective in controlling disease symptoms are non-steroidal anti-inflammatory drugs (NSAIDs) and more recently, anti-TNF-alpha biological agents. However, according to published studies, 30-40% of patients do not achieve clinical control of the disease after the use of this new pharmacological class, the genetic background probably playing an important role. Radiographic progression, represented by new bone formation in the spine, measured on conventional radiographies, could not be stopped by biological treatment administered for 2 years compared with the historical reference cohort in AS. Some literature data showed that continuous administration of NSAIDs in certain patients may reduce the rate of structural damage after 2 years of treatment.

Thus, until now, there is data comparing biological and NSAIDs treatment in AS only on historical cohorts and the major difference brought by the first study of this thesis is that we evaluated the effectiveness of the type of treatment

administered by direct comparison of patients examined in clinical practice on a daily basis.

Genetic studies provide solid predictive information on the probability that an individual will develop a particular disease and even on its severity. However, the information provided is a probability that can be influenced by both the expression of other genetic factors and the presence of certain environmental factors.

Understanding the genetic background of functional severity in AS, would be of great interest in order to identify in early stages of the disease the patients at risk of developing severe impaired of physical function. Thus, physicians could optimize the preventive and therapeutic approach for each patient from the time of diagnosis, through the objective distribution of expensive biological treatments. Impaired physical function may be partially controlled by appropriate treatment and thus, the purpose of the second study of this thesis was to identify if there is a combination of clinical factors and genetic markers that can predict the individual evolution of functional severity. The results of the study identified a number of clinical factors and genetic polymorphisms predicting severe functional impairment in patients with AS. However, the combination of clinical and genetic factors identified was not sufficient to create an accurate predictive model for severe functional status in patients with AS. Therefore, it is necessary to look for other additional factors.

Moreover, it is necessary to identify patients with AS without clinical response following administration of an expensive treatment which may induce possible severe side effects. Identifying the genetic background associated with poor response to anti-TNF-alpha treatment would prevent its irrational use in patients destined to be non-responders. Few studies have examined the role of genetic markers in the response to anti-TNF-alpha treatment in AS and they have reported conflicting results. Most studies have analyzed the role of HLA-B27 marker or of TNF-alpha gene polymorphisms as potential predictors of response to biological agents. Among the polymorphisms studied in the third study of my thesis, we identified prognostic factors for the lack of clinical response to the first biological agent administered in patients with AS. These genetic variants could interfere with the mechanism of action of TNF-alpha blockers. Our results suggest the existence of a specific genetic profile for an ineffective anti TNF-alpha treatment, and the validation of a genetic model responsible for the inefficiency of the first anti-TNF-alpha agent would be a milestone in facilitating the personalized treatment.

This research was made possible through permantente and rigorous guidance of Mrs. Prof. Anca Buzoianu, Md which thus facilitated my research project to be included in the Sectoral Operational Programme, Human Resources Development 2007 - 2013, by contract HRD 6/1.5/S / 3 - "DOCTORAL STUDIES: through science towards society" supervised by the Babes-Bolyai University,Cluj-Napoca, within the research team of Prof. Simon Simon. Under the careful tracking of two distinguished professors have shaped and subsequently finalized, the bibliographic research in

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detail outlining the framework of the studied topic. This lead to the mobility for 4 months in a prestigious university in Europe., University of Cordoba, Spain. Following pre-doctoral and doctoral partnership with "Reina Sofia" University Hospital, Cordoba, Spain, under the supervision of Prof. Dr. Eduardo Collantes Estevez, out of over 500 patients with AS recorded in the Spanish Register of Spondyloarthritis, REGISPONSER, I have listed those who will be part of the patient group analyzed within the doctoral research, in accordance with inclusion criteria. I also participated to the collection of biological samples to determine HLA-B27 and to identify SNPs that might predict the therapeutic response and the severe functional status. During the traineeship, I worked in the immunology laboratory at "Reina Sofia" Hospital where I learned the PCR technique to determine HLA-B27 and I participated in performing the statistical analysis.

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CURRENT STAGE OF KNOWLEDGE

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1. **Ankylosing spondylitis**

1. 1. Clinical and diagnostic considerations

Ankylosing spondylitis (AS) is the prototype in the group of spondyloarthropathies (SpA), together with reactive arthritis (Reiter's syndrome), psoriatic spondyloarthritis, enteral spondyloarthropathies and undifferentiated spondyloarthritis. Among the many milestones in AS research, perhaps the most important are represented by the revelation of infectious etiology and genetic predisposition. With regard to genetic susceptibility, the discovery from the 1940-50s, human leukocyte antigens (HLAs) and subsequent characterization of major histocompatibility complex (MHC) are seen as the most important issues in understanding SpA. Infectious etiology was initially proposed because of the correlation between AS and reactive arthritis, probably the best understood SpA.

AS affects twice as many men than women and has an estimated prevalence of 0.2 to 0.8%[1]. The first symptoms usually occur in the second and third decade of life and it is thus an important cause of disability for the active population[2,3,4]. AS is characterized by the close relationship between inflammation and new bone formation, phenomena whose progress is only partially known. A particular characteristic of the disease is the location of the inflammatory process which starts from the entheses (the site where tendons, ligaments and joint capsule meet bone), unlike the others inflammatory rheumatic diseases which cause direct inflammation of joint capsule and, unlike AS, prevails erosion processes and joint destruction[5]. Thus, AS tends to progress to fibrosis, ossification and bone proliferation.

1.1.1. Pathogenesis

The pathogenesis of AS is not fully understood. Immune-mediated mechanisms are suggested by the histological and inflammatory aspect of the affected tissues, the high serum levels of acute phase reactants and the close relationship between HLA-B27 and AS. No etiologic agent of the disease has been identified so far, but the relationship between AS, rheumatoid arthritis (RA) and inflammatory bowel disease

(IBD) suggests that enteric bacterial infections may play a trigger role. Some authors consider that the interaction between MHC class I molecules and HLA-B27 on the one hand, and the response mediated by T cells on the other hand, is the key to the understanding of the pathogenesis in AS. There is evidence that the cytokine secretion pattern influences the pathogenesis of SpA[6]. Percentage of T cells secreting tumor necrosis factor-alpha (TNF‑α) and interferon-gamma (IFN-γ) was lower in the peripheral blood of patients with AS and in healthy HLA-B27 positive controls than in HLA-B27 negative patients. Patients with AS showed a higher production of interleukin 10 (IL10) mediated by CD8+ T lymphocytes, compared with patients in control groups. Several studies conducted by Rudwaleit and Hohler**Error! Bookmark not defined.** suggest that impaired production of TNF-alpha and IL10 may be partially determined by genetic polymorphisms. A relative deficit in the synthesis of cytokine-mediated T helper 1 cells (Th1), such as TNF-alpha, may lead to longer persistence of bacterial antigens and thus, the prolonged antibacterial immune response may later trigger an autoimmune response[7].

1.1. 2. Clinical features

Most patients have mild chronic disease with alternate periods of remission and flares of the disease.

In 90% of the cases, the disease begins with an inflammation of the sacroiliac joints. Consequently, the main symptom is the insidious onset of either lower back pain or gluteal pain. Pain improves while walking and it is accompanied by morning stiffness and nocturnal accentuation. During the evolution of the disease, the following impairments can occur: sacroiliac joint stiffness and vertebral involvement at various levels through spondylitis, spondylodiscitis and/or facet joint arthritis. This process can progress to total damage of the spine, resulting in a bamboo spine[8].

Neck pain and characteristic stiffness with throat projection appear in advanced stages of the disease.

Peripheral enthesitis occurs in approximately one third of patients. Common sites are behind the heel (Achilles tendonitis), the heel pad (plantar fasciitis) and the tibial tuberosity. Lesions are often painful, especially in the morning.

Damage of large joints may also occur in approximately 30% of patients, manifested often through asymmetric aseptic arthritis (coxitis), shoulder arthritis (glenohumeral, sternoclavicular and acromioclavicular) or arthritis of the joints of the chest wall (costovertebral joints, costosternal junctions). They occur most often in early stages of the disease. Asymmetric arthritis of other joints, predominantly in the lower limbs, occurs rarely and may be present at any stage of the disease.

Extra-articular manifestations associated with AS occur in approximately 40% of patients, the most common affection being the inflammation of the eye by previous acute uveitis. Patients present unilateral eye pain and congestion, photophobia and

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increased tearing. Up to 60% of patients with AS show subclinical enteral damage. But symptomatic IBD may occur in about 10% of cases. Although it is possible, aortic insufficiency associated with congestive heart failure is rare in patients with AS.

1.1. 3. Clinical examination

The main change observed during the clinical examination of patients is the loss of spinal mobility with a restriction in the lumbar flexion (Schober's test), lumbar spine extension restriction and decreased thoracic expansion amplitude. Limitation of movement is not proportional to the degree of spine damage due to the occurrence of secondary muscle spasm. The presence of sacroiliac joint pain is tested by direct pressure or by movement of the joints, but the presence of pain is not a good indicator of sacroiliitis. Clinical signs of the disease can range from mild spinal stiffness to a completely immobile spine, with possible concomitant presence of severe bilateral hip damage, peripheral joint swelling, or presence of extra-articular manifestations.

Patient status undergoes characteristic changes when there is a natural progression of a severe illness, and they adopt the so-called "question mark posture".

1.1. 4. Paraclinical examination

Unlike RA, measurement of acute phase reactant levels appears to have limited predictive value of disease activity in AS. Otherwise, it was shown that only 50% of patients with active disease have increased levels of C-reactive protein (CRP) and a high erythrocyte sedimentation rate (ESR)[9]. Moreover, studies have shown a lack of correlation between clinical signs of disease activity (nocturnal back pain, morning stiffness) and CRP and ESR values[10]. Mild normocytic normochromic anemia can be detected in some patients. Elevated alkaline phosphatase levels may be present in severe disease.

Radiography is crucial in determining structural damage extension in AS but does not give information on AS activity. In addition, radiological changes are not obvious at the onset of the disease, but occur after the disease causes alterations in bone structure. Thus, it may take several years of disease to progress until there are specific radiological lesions. The earliest visible sacroiliac joint alterations are the blurred edges of subchondral cortical bone, erosions and sclerosis. Bone erosions progress in time, joint space appears larger and then it disappears due to fibrosis and bone formation. Sacroiliac joint changes are usually symmetrical during disease evolution.

Magnetic resonance imaging (MRI) is performed for early detection of sacroiliitis in patients with clinical signs of AS but without changes in sacroiliac radiography. MRI is also useful in monitoring acute and chronic lesion extension of sacroiliac joints and spine[11] during treatment.

1.1. 5. Positive diagnosis

According to Calin et al., low back pain is inflammatory in nature if three of the following characteristics are present:

1. <40 years of age at onset;
2. >3 months lasting pain;
3. insidious onset;
4. morning stiffness;
5. improvement of the pain with exercise.

The diagnosis of AS is based on the modified New York criteria (1984)[12] defining that a patient can be diagnosed with AS if there is:

1. low back pain lasting at least 3 months, which improves with exercise and gets worse during rest;
2. limited mobility of the spine in the frontal and sagital plane;
3. reduction of chest expansion according to the patient's age and gender normal values;
4. radiographic sacroiliitis grade <2 bilateral, or grade <3 unilateral.

Positive diagnosis requires one radiological criteria and at least one clinical criteria.

Patients diagnosed with AS may present a pattern of symptoms grouped into four major types of syndromes with varying degrees of expression in the same patient. According to their frequency:

1) Axial Syndrome (the most important hallmark is the involvement of the axial skeleton with spondylitis and/or sacroiliitis). This is characterized clinically by inflammatory back pain and stiffness, the latter being due to both inflammation and progressive bony ankylosis of the spine,

2) Peripheral syndrome (generally asymmetrical oligoarthritis predominantly of the lower limbs),

3) Enthesitic syndrome and

4) Extra-skeletal syndrome (SpA is associated with several extra-articular manifestations, including inflammatory intestinal lesions, acute uveitis, skin lesions, etc)[13]

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1.1.6. Monitoring disease progression

The goals of treatment are focused, on the one hand on controlling the signs and symptoms of disease (disease activity) and maintaining the physical function of the patient, and on the other hand, on preventing structural damage (radiographic progression of disease) with the purpose to improve the patient's quality of life and maintain their social and economic integration. Thus, internationally validated parameters for patient monitoring are used in clinical practice.

Disease activity score (Bath Ankylosis Spondylitis Disease Activity Index[14]-BASDAI) with values between 0-10, includes 6 questions on five main symptoms of AS, assessed using a visual analog scale (VAS) from 0 to 10.

1. Fatigue (VAS 0-10)
2. Spinal pain (VAS 0-10)
3. Peripheral joint pain or swelling (VAS 0-10)
4. Pain at pressure on enthesitis (VAS 0-10)
5. Morning stiffness severity (VAS 0-10)
6. Morning stiffness duration (VAS 0-10)

BASDAI disease activity score is obtained by calculating the arithmetic mean of the last two questions, sum it up with the first four questions and divide the result by five.

This score defines an active disease if the calculated BASDAI score value is >4.

In recent years, the Assessment of SpondyloArthritis International Society (ASAS) validated a new disease activity score called Ankylosing Spondylitis Disease Activity Score (ASDAS). ASDAS was designed by ASAS, in analogy with the Disease Activity Score (DAS) for RA and it is a composite index with continuous values. ASDAS formula combines, in a weighted logarithm, three elements of BASDAI score (spinal pain, morning stiffness duration, arthralgia/peripheral arthritis) with global patient assessment, all of these features having values between 0-10, and inflammatory parameters (CRP in mg/l or ESR in mm/h)[15]. The three threshold values for disease activity levels were selected as: inactive disease (ASDAS score <1.3), moderate disease activity (ASDAS score 1.3-2.1), high disease activity (ASDAS score 2.1–3.5), and very high disease activity (ASDAS score >3.5).

Impairment of functional capacity in patients is assessed with Bath Ankylosing Spondylitis Functional Index[16] (BASFI), consisting of 10 questions, the answers being assessed using VAS, with possible values between 0 (easy to evaluate) and 10 (impossible to evaluate). Score value is the arithmetic mean of the 10 questions (table 1). The first 8 questions assess functional limitations due to disease progression and the last 2 questions assess the patient's ability to cope with everyday life.

Table 1. BASFI score questions

1. Putting on your socks or tights without help or aids?
2. Bending forward from the waist to pick up a pen from the floor without an aid?
3. Reaching up to a high shelf without help or aids?
4. Getting up out of an armless dining room chair without using your hands or any   other help?
5. Getting up off the floor without any help from lying on your back?
6. Standing unsupported for 10 minutes without discomfort?
7. Climbing 12-15 steps without using a handrail or walking aid (one foot on each step)?
8. Looking over your shoulder without turning your body?
9.   Doing physically demanding activities (e.g. physiotherapy exercises, gardening or sports)?
10. Doing a full day activities at home or at work?

Structural damage is monitored with radiographic progression scores, such as Bath Ankylosing Spondylitis Radiological Index[17] (BASRI), with values between 2 to 16 or modified Stoke Ankylosing Spondylitis Spine Score[18] (mSASSS), with values between 0 to 72 (table 2).

Table 2. Radiographic scoring methods in AS

New York criteria for sacroiliitis (mean score of both SI joints is used in the BASRI)
0 = normal
1 = suspicious (without certain changes)
2 = mild changes (minimal sacroiliitis, defined as loss of sacroiliac joint contour, light juxta articular sclerosis, minimal erosions, possible joint space narrowing)
3 = moderate changes (moderate sacroiliitis, defined as sclerosis of both sides of scaroiliac joint, joint side deletion, erosive changes, joint space narrowing,)
4 = severe changes (complete fusion of sacroiliac joint – ankylosis)

BASRI- hip (mean score of both hips– included in **BASRI-total**), value 0-4
0 = normal
1 = suspicious (focal joint space narrowing)
2 = mild changes (circumferential loss of joint space >2 mm)
3 = moderate changes (circumferential loss of joint space ≤2 mm or bone apposition <2 cm)
4 = severe changes (bone deformation or bone apposition ≥2 cm)
Grade shall be increased by 1 if 2 of the following changes are present: bone erosions, osteophytes, protrusion.

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BASRI spinal score, value 2-12

0 = normal

1 = suspicious (without certain changes)

2 = mild changes (any number of erosions, sclerosis of square vertebrae, with or without syndesmophytes in ≤ 2 vertebrae)

3 = moderate changes (syndesmophytes in ≥3 vertebrae, with or without fusion that affects the 2 vertebrae)

4 = severe changes (fusion involving ≥3 vertebrae)

mSASSS (value 0–72) (posterior column of the lumbar spine is evaluated, as well as the posterior column of the cervical spine from the lower side of the C2 vertebrae to the upper side of the T1 vertebrae, in lateral position)

0 = normal

1 = erosions, sclerosis, square vertebrae

2 = syndesmophytes

3 = fusioning syndesmophytes

1. 2. Ankylosing spondylitis treatment

The optimal therapeutic approach in patients with AS is to combine non-pharmacological with pharmacological treatment and, in case they are insufficient, to use orthopedic corrections. The main goal of the treatment is to reduce or even to prevent structural damage, while maintaining normal functionality, and to eliminate patient symptoms.

1.2.1 Non-pharmacological treatment

1.2.1.1. Physical therapy

Physical therapy is the most important non-pharmacological treatment method in AS and for a long time it has been the only available treatment. Its main objectives are to prevent and/or delay spinal mobility impairments and secondary, to prevent physical disability and patient symptoms. Appropriate daily exercises are essential in AS management. Patients may have different approaches, but it seems that guided physical therapy supervised by a specialist is more effective than individual exercises performed at home. It turned out that when performed regularly, physiotherapy improves physical function, reduces pain and improves overall evaluation of disease scoring[19].

Thus, it is recommended that patients with predominantly axial disease to be managed by a physiotherapist, at least in the first years of disease, in order to learn specific exercises. Rehabilitation program may be effective enough to return to work, and hence leads to economic benefits. Physical therapy may be supplemented by other procedures (balneotherapy and electrotherapy). They should be used between the flares, during the whole course of the disease, in combination with pharmacological treatment.

1.2. 2. Pharmacological treatment

1.2.2.1. Nonsteroidal anti-inflammatory drugs (NSAIDs)

For many years, the only drugs available for the treatment of AS were nonsteroidal anti-inflammatory drugs (NSAIDs). Efficacy of NSAIDs in AS was well established[8], therefore symptom relief (such as morning pain and stiffness) within 48 hours of NSAIDs therapy or rapid recurrence of pain after interrupting the therapy, has been defined by Amor et al. as an element in the classification criteria for SpA since 1990[20].

The fundamental step in the pharmacological treatment of AS is the long-term administration of NSAIDs, differing from other inflammatory rheumatic diseases where NSAIDs are only adjuvants (Figure 1).
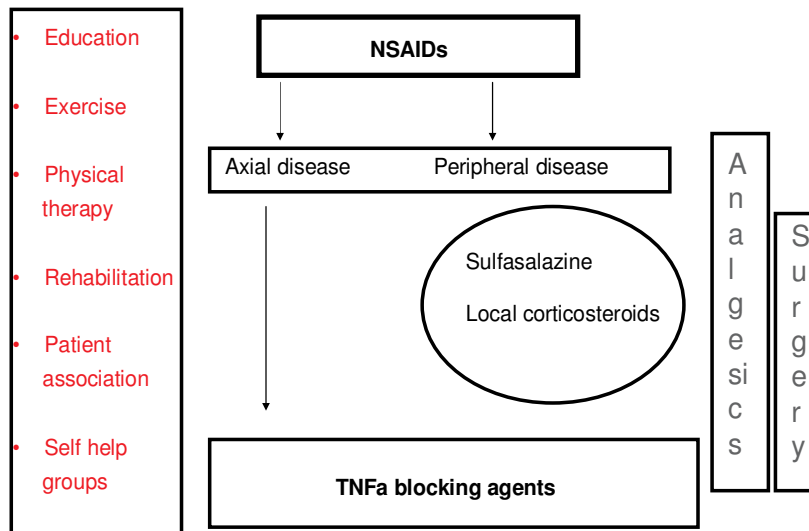
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Figure 1. Summary of disease management recommendations in AS, according to ASAS. NSAIDs are first-line of tretament for patients with AS. In patients with peripheral disease, when NSAIDs are ineffective, sulphasalazine may be administrated. Another option is represented by corticosteroid infiltrations. For patients who do not respond to NSAIDs (patients with axial diasease), corticosteroid infiltrations or sulphasalazine (patients with peripheral arthritis), anti-TNF-alpha treatment could be initiated.

There are studies that only recommend NSAIDs therapy in active (symptomatic) stages of disease, but current recommendations are of continuous NSAIDs therapy. This recommendation is based on their possible disease-modifying effect. Hence, a recent study demonstrated a reduced radiographic progression, thus inhibiting bone formation, in the subgroup of patients with AS who received continuous treatment with high doses of NSAIDs and maintained elevated CRP[21] values throughout the study, compared to those with discontinuous treatment[22].

Observing the inhibition of new bone formation in patients treated with NSAIDs can be explained by the inhibition of prostaglandin synthesis (especially prostaglandin E2) which is mediated by cyclooxygenase 2 (COX-2). Prostaglandin E2 is able to stimulate new bone formation by stimulating osteoblastic replication and differentiation. Prostaglandins also stimulate the increase in blood supply in the new bone formation site through vasodilatation and angiogenesis. Similarly, NSAIDs were able to delay ectopic bone formation by bone morphogenetic protein 7, in an

experimental murine model, indicating an important role of COX-mediated prostaglandin synthesis in new bone formation[23].

In addition, there is evidence of NSAIDs effect on bone mineral density (BMD) in multicenter studies performed on patients with osteoporosis, showing different evolution in men and women. Thus, treatment with NSAIDs has reduced BMD in men and increased in women, more probably due to inhibition of proinflammatory status in postmenopausal women, while in men, the demineralized bone effect of NSAIDs was explained by their direct inhibition of prostaglandins[24].

Therefore, continuous administration of high doses of NSAIDs (Table 3) may be preferred in patients with AS, although this may increase the risk of side effects such as gastrointestinal, cardiovascular and renal toxicity. An in-depth discussion of these adverse reactions is particularly important because it is the only chronic rheumatic disease where continuous NSAID treatment is medically justified, given the lack of effectiveness of other classes of disease-modifying antirheumatic drugs (DMARDs) in inflammatory rheumatism.

Digestive and cardiovascular risks of continuous administration of NSAIDs have been investigated in detail, recently suggesting that the benefits of such a treatment outweigh the risks in patients with AS[25]. Song et al. showed that severe gastrointestinal adverse effects could be annually expected in about 1-3% of patients with AS treated continuously with classic, non-selective NSAIDs, while severe cardiovascular adverse effects would be seen in 1-2% of treated patients, regardless of the type of NSAIDs used (non-selective or COX-2 selective). These severe side effects were dose dependent and more common in patients with cardiovascular and gastrointestinal risk factors.

Most studies showed no significant difference in efficacy or safety between different NSAIDs, even though aspirin and other salicylates showed a lower efficiency in the control of disease symptoms. There are also no significant differences in efficacy between agents with long or short duration of action, or between COX-2 selective agents and non-selective agents[26], but only COX-2 selective NSAIDs are indicated in patients with IBD[27].

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Table 3. Recommended doses of NSAIDs in ankylosing spondylitis

Drug	Half life time (hours)	Maximum tolerated dose(mg)
Aceclofenac	~ 4	200
Celecoxib	8-12	400
Diclofenac	~2	125-150
Etoricoxib	~22	90
Ibuprofen	1,8-3,5	2400-3200
Indomethacin	~2	150-200
Ketoprofen	1,5-2,5	200-300
Meloxicam	~20	15
Naproxen	10-18	1000
Piroxicam	30-60	20

1.2.2.2. Disease-modifying antirheumatic drugs (DMARDS) and corticosteroids in AS

Unlike RA, there is no evidence that disease-modifying antirheumatic drugs (DMARDs) such as methotrexate, sulfasalazine, leflunomide are effective in treating axial disease, while there is some evidence showing that sulfasalazine (SSZ) is effective in controlling disease-associated peripheral arthritis[25]. Sulfasalazine is the best studied DMARD in the treatment of AS. A recent meta-analysis published in Cochrane review analyzed 12 randomized, placebo-controlled studies, which showed some benefits of SSZ in reducing peripheral symptoms of ESR and improving morning stiffness, but found no benefit in improving physical function, pain, spinal mobility and disease activity[28].

In case of enthesitis or refractory sacroiliac pain, trigger point injection of glucocorticoids can be considered.

1.2.2.3. Anti-TNF-alpha biological therapy

Patients with remaining active disease despite treatment with at least two consecutive NSAIDs in maximum tolerated dose for at least 4 weeks, or with restrictions to this treatment, are suitable for the initiation of anti-TNF-alpha biological therapy[29].

Thus, the only therapeutic alternative to axial disease are TNF-alpha blocking agents, refractory to NSAIDs.

TNF-alpha is a key proinflammatory cytokine in the innate immune response. It is produced by monocytes and macrophages and, to a lesser extent, by T cells. Inflammatory response of TNF-alpha is regulated by IL-1, IL12, IL18 interleukins, cytokines which strongly induce interferon-gamma (IFNγ), also amplifying Th1-type proinflammatory response, resulting from the activation of CD4+ T lymphocytes[30]. Studies have revealed elevated levels of TNF-alpha and other cytokines in serum of patients with AS, compared to patients with non-inflammatory low back pain. Inflammatory infiltrate in biopsies taken from the sacroiliac joints also revealed the presence of a high level of TNF-alpha mRNA, suggesting a physio-pathological role of this cytokine in AS, which is shown later, and in synovial fluid of peripheral joints[31].

TNF-alpha-mediated intracellular signaling is achieved by interaction with cell receptors. These receptors are present on almost all cells. There are two distinct but structurally identical types of TNF-alpha receptors, which are referred to as p55 and p75. These receptors form dimers at the cell surface. Each subunit of the dimer binds one molecule of trimeric TNF-alpha, thus originating intracellular signal transduction. Receptors are also activated after binding to lymphotoxin alpha (TNF-beta), a cytokine with structure and biological actions similar to TNF-alpha. Although TNF-alpha p55 and p75 receptor subtypes have functional similarities, studies on knockout mice for TNF-alpha receptor showed that p75 receptor plays a significantly more important role than p55 in the fight against TNF- alpha-mediated proinflammatory response.

Soluble forms of p55 and p75 receptors were found in synovial fluid and serum of patients with rheumatic diseases. Their role of endogenous inhibitors of TNF-alpha results in a decrease in inflammatory immune response. (Figure 2)

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Figure 2. Image of binding and neutralization of TNF-alpha (sTNFR-soluble receptor for TNF-alpha, anti-TNF-alpha monoclonal antibody) adapted from Mease PJ. Ann Rheum Dis 2002, 61:298-304
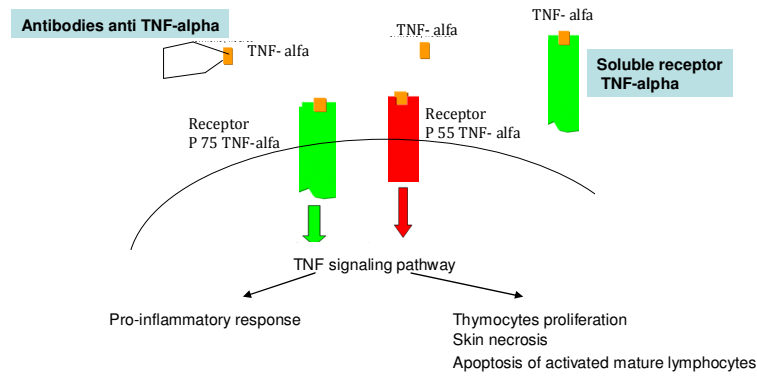
Until now, three anti-TNF-alpha biological agents have been recorded on the Romanian market, with a fourth antibody pending for approval (golimumab).

Infliximab (Remicade®) is a chimeric anti-TNF-alpha monoclonal antibody (with a 25% murine variable region and a 75% human constant region). Binding epitope of TNF-alpha is of murine origin, while the human fragment is represented by IgG. Food and Drug Administration (FDA) approved it for use in rheumatic diseases in October 1998.

Each infliximab molecule is able to bind two trimeric TNF-alpha molecules (occupying all 6 possible binding sites) forming a relatively stable complex. Infliximab binds to both soluble TNF-alpha, free in the plasma, and to cell membrane. Infliximab does not bind to lymphotoxin alpha (TNF-beta). It produces in vitro complement-mediated cell lysis. The half-life is 8 to 9.5 days. Infliximab does not bind to plasma proteins. The maximum effect is reached after 6-12 weeks of treatment.

Murine variable region can lead to the formation of human anti-infliximab antibodies, which significantly limits its therapeutic effectiveness. Formation of anti-mouse antibody increases infliximab clearance. The association with methotrexate in

the therapy of RA reduces the frequency of anti-infliximab antibodies. It is administered intravenously at 0-2-6 weeks, and then every 8 weeks. The dose is individualized according to patient and disease. Thus, the recommended dose in the therapy for AS is of 5mg/kg. If the results are unsatisfactory, the dose may be increased up to 10 mg/kg or the interval between doses may be decreased to 6 weeks.

Infliximab is provided as lyophilized powder in vials. Each vial contains 100 mg infliximab. The solution obtained by dissolving is slowly administered intravenously within 2 hours.

Etanercept (Enbrel®) is a dimeric TNF-alpha soluble receptor fusion protein. Etanercept is composed of human p75 receptor (TNF-RII) attached to Fc region of human immunoglobuline G1. It was approved by the FDA for the treatment of rheumatic diseases in November 1998. Etanercept binds reversibly to both TNF-alpha and TNF-beta. By competitive binding, soluble dimer (TNF-RII) binds to two of the three existing binding loci of trimeric TNF-alpha molecule, thus preventing p75 cell receptor binding.

The half-life is 3 to 5.5 days. It does not bind to plasma proteins. Effect onset accurs after 2-4 weeks. Maximum effect is reached after 3-6 months of treatment. Etanercept is administered subcutaneously in adults with AS, 25 mg twice a week or 50 mg once a week. Etanercept has been approved for pediatric use in juvenile idiopathic arthritis. It is provided as 25 mg and 50 mg solution in prefilled syringes.

Adalimumab (Humira™) is an anti-TNF-alpha IgG1 specific human monoclonal antibody generated by phage display technology. Adalimumab was approved by the FDA for the treatment of rheumatic diseases in December 2002. Adalimumab binds to both soluble TNF-alpha and transmembrane receptor, resulting in a stable complex. Adalimumab produces in vitro complement-mediated lysis of TNF-alpha bound cells. The half-life is 10-20 days. It does not bind to plasma proteins. Its effect installs after 2-4 weeks of treatment, reaching its peak after 3 months of treatment. Associatiion with methotrexate reduces adalimumab clearance by about 30-40%. Adalimumab is provided as 40mg solution in prefilled syringes. Use of adalimumab in the therapy of AS is subcutaneous, 40mg every 2 weeks.

Given the high cost of this therapy and its potential severe side effects, patients should be carefully selected for these therapies. It is also important to choose right from the start those patients who can reach the maximum therapeutic response (disease remission).

Initiation of anti-TNF-alpha treatment is given by the following ASAS recommendations:

1. Definite diagnosis of AS (based on New York criteria);

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2. Active disease for at least 4 weeks, defined by BASDAI score ≥ 4 (scale of 0-10) combined with the expert rheumatologist's opinion based on the patient's clinical examination;

3. refractory disease defined by failure after administration of 2 NSAIDs for a total of 4 weeks, failure to intra-articular administration of glucocorticoids (if there is any indication) or failure of the administration of SSZ in patients with peripheral joint impairment;

4. Identify patients with contraindications for biological tratementul.

Choosing a specific biological agent must consider the possible risks of infection (especially tuberculosis), the presence of exta-articular manifestations (uveitis, IBD), comorbidities and patient preference for subcutaneous or intravenous administration.

In recent years, several placebo-controlled open studies have shown the similar effectiveness of anti-TNF-alpha agents in controlling active disease. In these studies, 50-70% of patients with AS have obtained an improvement of more than 50% of the disease activity score (BASDAI), response rate which is comparable to results obtained with anti-TNF-alpha therapy in other chronic inflammatory diseases, showing that patients respond differently to the same treatment[32,33,34,35]. This variability suggests that among patients with AS, there might be individual genetic mechanisms regulating the response to treatment, responsible, in part, for the lack of clinical response to treatment. There are conflicting results on the radiographic progression under treatment with biological agents. This can be explained by the fact that study results regarding the effect of TNF-alpha blockers on bone formation in AS include patients who are non-responsive to the treatment with NSAIDs. Thus, some researchers have reported either a decrease in radiographic progression in case of long term administration of these agents (eight years) [36], or on the contrary, rapid radiographic progression and formation of new syndesmophytes after 2 years in vertebral regions where the inflammatory process was initially completely inhibited by anti-TNF-alpha therapy[37]. Most studies investigating the role of anti-TNF-alpha agents on radiographic progression in AS have shown that these drugs were not able to inhibit structural destruction[38,39].

1.2. 3 Clinical monitoring of the response to biological therapy

Clinical monitoring of the response to biological therapy uses both ASAS criteria and BASDAI score.

Patients with at least 50% or 2 unit (scale of 0-10) improved BASDAI score measured when the biological therapy is initiated, also considering the opinion of the rheumatologist regarding further treatment, are considered to be responsive to biological therapy.

The new Assessment in Spondyloarthritis International Society (*ASAS*) classification criteria for AS consider the following aspects of the disease: 1. physical function, 2. pain, 3. inflammation, and 4. patient global assessment. Given this, ASAS 20 response is defined as improvement of two units in at least 3 of the four domains mentioned, and absence of worsening in the fourth domain.

Discontinuation of treatment in non-resposive patients can be considered after 12 weeks of treatment. Choosing another anti-TNF-alpha agent is possible in non-responsive patients and has proved to be effective[40]. Neutralizing antibody formation may be involved in the loss of initial response (these are considered to be secondary non-reponsive patients) and patients may reach an important response to treatment with the second TNF-alpha blocker. This does not happen so frequently in primary non-responsive patients, where a different mechanism occurrs, patients presenting hypersensitivity to biological therapy[41, 42].

Clinical and biological predictive factors of response to anti-TNF-alpha therapy were studied in different cohorts of patients with AS. Thus, young age, high levels of CRP, good functional status, presence of entheses at onset, were all associated with good response to anti-TNF-alpha treatment[43].

2. Genetics of ankylosing spondyltis.

2. 1. Single nucleotide polymorphisms and pharmacogenomics

2.1.1. Single nucleotide polymorphisms (SNPs)

Research has shown that the genomes of two randomly selected patients contain only 0.1% difference or variation. This variation is called polymorphism and is due to mutations. Over 80% of these mutations consist of single nucleotide polymorphisms (SNPs). An SNP is a substitution of a nucleotide base with another. Both versions can be seen in the general population with a frequency greater than 1%[44]. These polymorphisms occur once in every 100-300 pairs of nucleotide bases. An example of SNP is the following: individual A has a GAATTC sequence, while individual B has an AAGCTT sequence, the polymorphism is represented by the A/G base change.

This implies that the human genome consits of about 10 to 30 million SNPs. More than 4 million SNPs have been identified, and the information was made available through the efforts of SNP Consortium (TSC-HapMap). Many of these SNPs have unknown associations. The compilation of all SNPs by the National Center for Biotechnology Information (NCBI) produced a subset of SNPs defined as a non-

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redundant set of markers which are used for marking reference sequences of the human genome and are called reference SNPs (rsSNPs). Over 2.6 million SNPs have been designated so far as rsSNPs (http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi).

Several comparative studies on identical twins suggest that SNPs are one of the many factors associated with susceptibility to common diseases, determinants of human traits peculiarities and differences in response to treatment.

Unlike mutations, SNPs are not necessarily located within genes, and they may also not always affect protein function.

SNPs are divided into two main categories:

1. Linked SNPs (also called indicative SNPs) do not reside within genes and do not affect protein function. Nevertheless, they do correspond to a particular drug response or to the risk for getting a certain disease.

2. Causative SNPs affect the way a protein functions, correlating with a disease or influencing a person's response to medication. Causative SNPs come in two forms:

a. Coding SNPs, located within the coding region of a gene, change the amino acid sequence of the gene's protein product.

b. Non-coding SNPs, located within the gene's regulatory sequences, change the level of gene expression and, therefore, how much RNA and protein is produced.

As described in the initial reports of the human genome project[45], 50% of SNPs are located in the encoded region of the genes, being referred to as cSNPs. Half of these cSNPs result in "missense" mutations, and the remaining 50% are silent mutations that do not change the encoded amino acid sequence.

"Missense" mutations can be neutral, as they produce no detectable phenotypic changes, and may lead to differences in individual human characteristics, different response to treatment, or different susceptibility to disease. cSNPs are thus directly responsible for susceptibility to a particular disease or a particular response to drug treatment. Some of these cSNPs have been associated with significant changes in metabolism and in the effects of the drugs which are being used[46]. These SNPs can be used as markers in association studies, in order to identify disease-causing genes. In such studies, it is assumed that two similar alleles (gene and polymorphism) are inherited together. Therefore, comparing genetic variations between patients and control individuals without disease may provide a method to identify loci responsible for susceptibility to the disease[47].

2.1.2. Pharmacogenetics and pharmacogenomics

Although polymorphisms have been described for several genes that encode proteins involved in the metabolism, transport, and action mechanisms of drugs,

using this knowledge in clinical routine practice is limited. Except for a few examples of enzymes involved in drug metabolism, the contribution of genetic polymorphisms to individual differences in therapeutic rasponse is not well understood[48].

The aim of pharmacogenomics is to provide new strategies in order to optimize therapy, based on individual genetic factors which are responsible for the effectiveness and toxicity of the treatment.

SNPs can be used to distinguish between patients who may or may not benefit from a specific treatment. This advantage to divide the population into groups of response to treatment allows targeting of specific populations for whom the treatment would be the most beneficial.

The term pharmacogenomics was introduced to reflect recent move from genetics to genomics using the genome in identifying genes that cause a particular disease or a particular response to tratment. Pharmacogenomic approach may allow administration of specific therapies targeted to genetically defined subsets of patients and may lead to the classification of the treatment and disease at molecular level. Moreover, the identification of new genes may provide new drug targets. With the complete availability of human genome sequence, individualized treatment may soon become a reality. Genomic information may allow a more accurate prediction of an individual's response to treatment and an appropriate dose selection to achieve optimal therapeutic response and to minimize side effects and toxicity.

Thus, the effort to identify SNPs serves as a milestone for developing pharmacogenomics and the emerging field, personalized medicine, which means choosing the right drug, in the right dose, for the right person, at the right time.

2. 2. The role of HLA-B27 in the etiology and pathogenesis of ankylosing spondylitis

Marked variability in the severity of clinical presentation of patients with AS may be related to the variable contribution of genetic factors involved in disease occurrence, as it is proved today that a large number of diseases have a multifactorial determinism, meaning that both genetic and environmental factors contribute to their etiology and/or clinical severity.

The genomic region most commonly associated with susceptibility to AS is represented by MHC class I. Thus, for over 30 years, the main genetic component in relation to AS was HLA-B27 antigen. Histocompatibility antigen, HLA-B27 is found in over 80% of European patients, while its incidence in the general population is of about 8%[49], and from these carriers, only 1-5% develops AS. Concordance rate for AS in HLA-B27-positive monozygotic twins is significantly higher (63%) than in dizygotic twins (24%). Thus, this low association indicates that HLA-B27 and possibly other genes encoded by MHC are not sufficient to explain genetic

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susceptibility to AS[50]. Patients with AS who are HLA-B27 negative comprise about 5-10% of all patients, and they tend to have different clinical characteristics than HLA-B27 positive patients.

HLA-B27 antigen is directly related to the pathogenesis of AS, being present in all human races. The geographic distribution of the disease follows HLA-B27 prevalence in different populations. The disease is more common in Northern countries, where the prevalence of HLA-B27 reaches 15% of the population and it is rare in Africans and Asians, where its prevalence ranges between 1-4%. Thus, the prevalence of the disease in Norway is 1.4%, while in Japan it is under 0.04%[51],[52]. HLA-B27 positivity does not constitute a "per se"diagnosis of AS, although when facing compatible clinical symptoms, its positivity supports the diagnosis, but its negativity does not exclude it, and therefore, in the early 90s Amor included it as a classification criterion for sSpA[20]. Disease association with HLA-B27 is one of the strongest associations between a HLA molecule and a specific pathology. The importance of HLA-B27 determination is represented by those cases with high clinical suspicion of AS, but where radiographic changes have not appeared yet.

Family studies have suggested that there is only a 40% contribution of HLA-B27 to the overall risk of disease[53].

There are several theories accepted to date on HLA-B27 contribution to susceptibility to disease.

1. Arthritogenic peptide theory, suggests that the occurrence of AS results from HLA-B27 capacity to bind to a number of antigenic peptides starting from the intestinal and/or urinary tract.

2. Formation of HLA B27 homodimers theory, according to which, heavy chains of HLA-B27 molecule fold poorly in the endoplasmic reticulum, thus being withheld here and generating pro-inflammatory cytokine response. This assumption also includes, as a possible trigger mechanism, cell surface expression of HLA-B27 homodimers. According to this theory, they will behave as ligands for natural killer cells.

3. HLA-B27 as an autoantigen theory, according to which HLA-B27 will be presented by HLA class II heterodimers (DR, DQ, DP), CD4+ T lymphocytes.

2. 3. Contribution of other genetic factors to the etiology and pathogenesis of AS

In recent years, improvements in study genotyping and design have revolutionized the field of genetics of common diseases, having a major impact on AS, where 13 genes, others than HLA-B, were identified in association with the risk of disease.

In addition, genetic association studies have revealed loci responsible for non-MHC susceptibility to AS and other SpAs. In recent years, Genome-Wide Association Studies (GWAS) have identified ERAP1 gene variants (endoplasmic reticulum aminopeptidase 1) with rs27434, IL23R polymorphism (IL-23 receptor), with rs11209026 polymorphism, ANTXR2 (anthrax toxin receptor 2) with rs4333130 and IL1R2 polymorphism (IL-1 receptor type II), with rs2310173 polymorphism, as well as 2p15 intergenic regions (rs10865331) and 21q22 intergenic regions (rs2242944) as being associated with AS[54],[55].

More replication and haplotype analysis studies conducted on cohorts with different ethnic backgrounds confirmed that ERAP1 and IL23R polymorphisms have the strongest association, among candidate genes outside MHC, with susceptibility to AS[56],[57],[58],[59].

The study of candidate genes for susceptibility to AS described CYP2D6 (cytochrome P450, subfamily IID, polypeptide 6) and TNFR1 (TNF receptor 1, rs4149577)[60],[61] as genes involved in disease incidence.

2. 4. Genetic factors and prognosis of ankylosing spondylitis

Genetic factors not only influence susceptibility to disease but also its prognosis. Family studies have shown that functional and radiographic severity in AS have a significant genetic component[62],[63]. There is published data in the literature showing the association of HLA-B27 with a worse functional prognosis, with a higher disease activity score, with extra-articular manifestations of the disease, which is reflected in higher quantities of biological drugs in these patients[64].

The presence of HLA-B27 in patients with ankylosing spondylitis showed their tendency towards a better response to treatment, having a 50 times better Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) response rate than HLA-B27 negative patients[65].

There are also reports looking for SNPs associated with severe radiological outcome in patients with AS. This led to the association between a variant of VEGF gene haplotype and radiographic severity in AS, described by Seo et al[66], but the association was lost as a result of the correction after disease duration. On the other hand, a recent study by Ward et al.[67] showed the association between MHC alleles and the severity of radiographic damage of the spine, using the 75th percentile as threshold in order to define radiographic severity. Another study conducted on Spanish population with AS identified a combination of clinical and genetic factors, consisting of four SNPs located in the MHC and in variations of beta 1-adrenergic receptor genes (ADRB1) and NEL-like protein 1 (NELL1) that may predispose to radiographic severity of the disease[68] .

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However, compared with RA, there are few studies on AS analyzing specific genes inside and outside the MHC, genes that might be involved in the functional and radiographic severity and the lack of response to treatment, and study results have been contradictory[69,70]. Possible causes of divergence between the results of genetic association studies have been either genetic heterogeneity of populations used for the study, the small number of individuals studied, or the linkage imbalance (LD) of the genes involved[71].

Until now, no genetic markers that influence response to treatment in AS have been identified. With the wide use of expensive anti-TNF-alpha biological therapies, the need for objective prognostic markers for response or lack of response to treatment in patients with AS is growing. These genetic markers may be selected either from genes encoding drug receptor proteins, from proteins involved in drug metabolism, or from genetic markers linked to the pathogenesis of the disease.

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PERSONAL CONTRIBUTION

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1. Work (background) hypothesis

Short and long term objectives of the treatment is to control the inflammatory process in the spine, pelvis, peripheral joints and entesis with the final target of preventing or slowing the structural damage, measured radiographically. Therefore the main purpose of the treatment is to maintain physical function of patients within acceptable parameters and their socio-professional insertion. The main concern of the physicians in the set of the pharmacological treatment is to administrate therapies that act primarily on radiological progression because it causes through the structural changes generated (sindesmofite, bony bridges, or total ankylosis of the spine), the impairment in patients` physical function.

Although the evidence confirmed that the only classes of drugs effective in controlling clinical signs of disease are the NSAIDs – as first stage of treatment, and anti-TNF-alpha agents – as the second stage of treatment - there is, however, a significant proportion of patients who maintained high activity parameters in spite of long term therapy with even biological agents. On the other hand, long influence on radiological progression following the administration of NSAIDs and anti-TNF-alpha agents is not well known and characterized. Moreover, the majority of the information was obtained from the patients included in clinical trials and less evidence about their effectiveness on real patients provided by the disease registries, is available.

It was found that genetic factors influence both susceptibility and the prognosis of AS. Family studies have shown that functional and radiographic severity in AS has an important genetic component. Therefore, understanding the genetic basis of functional severity in AS would be of major value to differentiate at early stages patients at high risk of severe functional impairment and patients with a lower risk. Thus, clinicians could better select and optimize the preventive and therapeutic approach for each patient as of the time of diagnosis of the disease by objectively distributing high cost treatments.

On the other hand, pharmacogenomic studies, focusing on genes involved in AS etiology and pathogenesis, in order to analyze the role of allelic polymorphisms in the individual difference in treatment response to TNF-alpha inhibitors, are lacking in AS. Taking into 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 a treatment.

Thus, patients with AS could benefit from this project because this work has the following objectives:

1. Comparative analysis of the effect of long-term treatment with NSAIDs, on the one hand, and anti-TNF-alpha biologic agents, on the other hand, on radiological progression in daily AS patients.

2. Identifing the clinical and genetic factors predictive of severe functional status among patients with AS by analyzing candidate genes polymorphisms.

3. Finding of genetic determinants of clinical non-response to anti-TNF-alpha biologic therapy by analyzing candidate genes polymorphisms.

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2. General methodology

Health care managers need reliable instruments to help them distribute and allocate health and social resources objectively and fairly. These instruments should be flexible and provide real time data, and they should easily incorporate any change in practice and scientific knowledge. Disease registries are most suitable for this task, as they provide real time data on the frequency, geographic and temporary distribution, as well as on the pattern of the disease[72]. They inform about the case mix in different locations and provide an enlightening tool for assessing the impact of the disease and clinical practice variability. Furthermore, disease registries are an ideal source of random samples for cohort studies or for case-control studies, the correct setting to test medical hypothesis[73]. The experience that we have so far from the registries developed over the last few years showed their usefulness in describing the epidemiological aspects, clinical pattern, disease activity, structural damage, response to therapy, impairment degree in quality of life and socioeconomic impact associated with inflammatory rheumatic diseases, in our case focused on patients with Spondyloarthropathies

REGISPONSER, which is a dynamic data base registry, was initiated in April of 2004, by Spondyloarthropathies study group of the Spanish Society of Rheumatology (GRESSER). Thereby, REGISPONSER (*Registro Español de Espondiloartritis de la Sociedad Española de Reumatologia)* that is, The Spanish National Registry of Spondyloarthropathies, is composed of a large enough cohort of patients enabling us to determine a well-defined picture of patient characteristics and the progress of their diseases even from onset.

The originality of this registry has been the creation of a virtual network of researchers set upon a computerized internet database accessible to all participating members no matter of the city or country they belong (http://regisponser.ser.es/). Each centre has individual access to the registry either for investigation or for managing purposes. The on-line application is easy accessible via a standard internet browser. No additional software is needed to be installed on the user's computer. Once the self administered patients` validated questionnaires are achieved, the data is introduced in the electronic data sheet under strict quality control by filters that detect data inconsistencies, for assuring the uniformity of data collection. The information contained in these electronic CRF was agreed by all the investigators before starting the data base. The electronic CRF provides the basic minimum data set required for the complete definition of the patient and his illness, according to the recommendations of ASAS[74] (table 4). A specific code (login and password) is

assigned to each investigator to access the electronic CRF and random codes are given to each introduced case. In this way, the system meets the rules of the protection of personal data. All submitted data are collected in a central computer, where they are safely stored in the database. The data can be exported for authorized users as a local database for further processing.

Table 4. Data collected by the electronic CRF.

Socio-demographic data	Clinical data			Variables of disease assessment	Treatment in the last 2 weeks	Working conditions
	First signs and symptoms	**Comorbidities**	**Diagnostic data**			
Date of birth				BASDAI	NSAID	fully employed
Gender	Date of first symptoms	Hypertension	Year of diagnostic	BASFI	DMARDs	wok disability
				Mander enthesis index		
Race	Articular symptoms	Diabetes	Clinic form		anti-TNFalpha	unemployed
		Ischemic heart disease		Metrologic index: Mod.Schober test,		
		Dilslipidemia		Occiput/tragust to wall distance		
	Extra-articular symptoms related to the disease (uveitis, IBD, psoriasis)	Gastro-duodenal ulcer		Fingers to floor distance		
		Cerebral-vascular disease		Cervical rotation Lateral spinal flexion Chest expantion		
Marital status			HLA-B27			
	Associated infection	Peripheral vascular disease		BASRI-total		
	(uretritis, balanitis, cervicitis)			m-SASSS		
Profession		Drinking, Smoking	ESSG criteria			
Study degree	Trauma associated	COBP	Amor criteria	DNA bank for selected groups		
Average income	Family history of SpA	Infections	Hip involvement	NSJ/NPJ		
		Neoplasm Demyelinating diseases		VAS-patient		
	Treatment before the inclusion in the registry	Liver disease		VAS-physician		
Living conditions		Depression		VAS-night back pain		
		Cytopenias		ASQoL		
		Renal failure		SF-12		
		Amiloidosis		ESR, CRP		
		Atlanto-axoidal subluxation		MRI sacro-iliac and spine		

NSJ- number swollen joints, NPJ- number painful joints.

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At its first beginning (2004- 2005) the registry (REGISPONSER I) comprised of twelve reference Spanish rheumatology departments selected from all the centers who accepted to participate in the project, based on their experience in treating these patients. These centres from 8 different cities represent a broad sociodemographic spectrum of the population attended at the Spanish Health System. The average population covered by the participating hospitals is 800,000 (range 300,000 to 1,100,000), and includes urban and rural zones. The participating rheumatologists were asked to include all consecutive patients fulfilling the inclusion criteria up to a minimum of 100 per center.

REGISPONSER I main purposes were to create, to develop and to exploit the Spanish National Registry of Patients with Spondyloarthritis and its specific objective was to know the characteristics of the spanish patients with spondyloarthritis (sociodemographic, clinical, radiological, laboratory and treatment features) in a cross-sectional study ("photography"). REGISPONSER I included **1379** patients fulfilling ESSG classification criteria and the results were already published[75]. The most important goal achieved by REGISPONSER I has been the growing of the interest of the spanish rheumatologists in spondyloarthritis and so, in this form "to increase the visibility of the SpA in Spain".

After the data acquiring system was validated an invitation was sent to all those Spanish rheumatology centers that met the minimum requirement criteria and so, other 19 centers joined the project. Thus, by the end of March 2007, in the registry were included **2367** patients. This phase of the project is known as "Universalization".

REGISPONSER II helped both to increase the knowledge over spondyloarthropathies in Spanish population and also to incite developing new projects. Regisponser II collected information from 31 Spanish rheumatologic departments, belonging to 19 provinces which cover a wide spectrum of Spanish habitants with different social, economic and occupational conditions.

REGISPONSER II is a dynamic project that collects standardized socio-demographic, clinical, biological, radiological, genetic and treatment data relevant to spondyloarthropathies (SpAs) spectrum. The authenticity of this project consists in the requirement that all the patients included to fulfill both ESSG and Amor criteria for SpAs. Several studies were developed from REGISPONSER II and the results have already been published[76],[77],[78].

A number of new prospective secondary projects are now in progress:

1) **REGISPONSER - early** that comprise of a cohort of patients with Spondyloarthropathies with less than 2 years of disease evolution (150 patients)[79],

2) **REGISPONSER-AS** designed for the cohort of patients with AS that contains 529 patients. In this cohort we are also evaluating some of the most important genetics biomarkers known to be related with the disease susceptibility and

pathogenesis. It is provided that each selected patient to accomplish 5 visits (one every year). For the statistical analysis statistical programs Stata and SPSS are used. The analysis plan varies depending on the sub-studies.

REGISPONSER is the first dynamic SpA data base composed of cohorts with a significant number of patients distributed by specific diagnosis, which provides basic specific information of the sub-cohorts useful for patients' evaluation in rheumatology ambulatory consulting. One of the most important achievements was the incorporation of self administered questionnaire into daily clinical practice leading to a uniformity of data collection that further enable the comparison and analysis of data obtained from different sources. REGISPONSER is providing rheumatologists, on one hand, demographic, socio-economic and clinical data and on the other, information about evolution of the disease in each patient by means of clinical, biological and radiographic assessments. The efficiency of the treatment administrated to the patients can also be evaluated, as can be the implication of genetic factors in disease evolution and in response to different therapies.

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3. First study.

Influence of NSAIDs and anti TNF- alpha agents on radiographic progression in ankylosing spondylitis.

3.1. Introduction

Ankylosing spondylitis (AS) is a chronic progressive inflammatory disease with pathognomonic radiological features such as sindesmophites and bony bridges which determine a negative impact on patients` physical function and quality of life. AS can lead to a significant proportion of patients towards fused sindesmofite progression with the emergence of pathognomonic radiological change (bamboo spine) and secondary reduced spine mobility in patients. The goal of the pharmacological treatment must accomplish at least two fundamental requirements: to control the symptoms (disease activity) and to prevent structural damage (disease progression). But for patients with AS this goal is difficult to achieve as it could not be identified until now a true relationship between disease activity (signs and symptoms) and structural destruction[80].

In contrast with other inflammatory rheumatic diseases, pharmacologic therapeutic options in AS are limited to nonsteroidal anti-inflammatory drugs (NSAIDs) and to tumor necrosis factor α (TNF) blockers[81]. Consequently, Assessment of SpondyloArthritis international Society (ASAS) recommends the use of NSAIDs as the first-line drug treatment in patients with symptomatic disease[82]. Patients who are still active despite treatment with minimum of two NSAIDs at maximum recommended or tolerated anti-inflammatory dose for minimum 4 weeks in total or, in who is contraindicated such a treatment, a TNF- α blocker may be started[83].

Past evidence demonstrated that NSAIDs reduce signs and symptoms in AS however, their influence on disease progression is not well estabished but, there is some recent evidence that continuous therapy may reduce the structural damage[84].

Over the past years placebo controlled and open trials have shown an important response in active AS of all available TNF-α antagonists (adalimumab, golimumab, etanercept, infliximab). In these trials TNF-α antagonist reduced disease activity in most patients, reflected in achieving of BASDAI50 clinical response, in 50 to 70% of the treated patients[85] . Radiographic progression measured by new bone formation on plain radiographies was not stopped at 2 years, when compared with the historical OASIS cohort - Outcome *in Ankylosing Spondylitis International Study* (OASIS)[86,87,88]. Interestingly, in other two studies which assessed radiographic

damage at 2 years it was found a tendency for less radiographic progression in the patients treated with TNF- α blockers compared with OASIS cohort[38,39.]

3.2. Work hypothesis

The objective of our study is to assess the long term influence on the rate of radiographic progression of NSAIDs and TNF-α blockers in a cohort of AS patients seen in daily clinical practice. The secondary objective was to study the evolution of disease activity and physical function of the patients in both treatment groups.

3.3. Patients and methods

3.3.1. Patients selection and study design

All patients belong to Spanish Register of Spondyloarthropaties - REGISPONSER. Of all patients with SpA we have selected for this study, only those who met the modified New York criteria for AS[12]. Patients with AS had to fulfill in addition the following three inclusion criteria: a) a follow-up period since inclusion visit in REGISPONSER of at least three years b) continuous treatment only with NSAIDs at recommended dosage during 3 years and/or c) continuous treatment with a anti-TNF-α agent during 3 years.

3.3.2. Data collection

Registry data that were used as variables for this study were demographic, clinical, radiological as well as pharmacologic treatment. Disease parameters were assessed annually in the same session for all patients and included, metrological measures, registers of the C-reactive protein (CRP) level (mg/l), erythrocyte sedimentation rate (ESR), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), Bath Ankylosing Spondylitis Radiological Index (BASRI). Ankylosing Spondylitis Disease Activity Index (ASDAS) was calculated using the recent ASAS approved CRP formula[89].

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3.3.3. Radiographs scoring

Radiographs were scored by the local rheumatologists who were blinded for the clinical data of the patients and who were previously trained with the regard of radiological scoring system to be applied which included both spine and hips.

The Bath Ankylosing Spondylitis Radiology Index for the spine (BASRI-spine) was applied to anteroposterior and lateral lumbar radiographs and to lateral cervical radiographs. Lumbar spine was defined as the lower border of T12 to the upper border of S1 and cervical spine was defined as the lower border of C1 to the upper border of C7. For the lumbar spine, anteroposterior and lateral radiographs were both examined and the image showing more significant change was scored (range 0-4). For the cervical spine, only the lateral view was scored (range 0-4). Grading of sacroiliac joints was performed according to the established scoring system (range 0-4)[12]. The BASRI-spine is the sum of the mean score of the right and left SI joints plus the scores of the lumbar spine and the cervical spine so, the range of the BASRI-spine in patients fulfilling AS the criteria is 2–12. The score for the hip is based on the same grading system that is applied for BASRI spine (range 0-4).The BASRI-hip score is the mean of the right and left hips. BASRI spinal scores with the addition of BASRI-hips are combined into BASRI-total, range 2-16.

3.3.4. Statistical analysis

Statistical analysis was performed with the SPSS v19.0 software (SPSS, Chicago, IL, USA). Data of the patient population in the study were shown as mean and standard deviation (±SD) for the quantitative variables and as absolute number and relative frequencies (%) for qualitative variables. Baseline characteristics of the patients in the two treatment groups (NSAIDs and anti-TNF-α blockers) were compared with the χ^2 test for categorical variables and with the t-test for continuous variables. Radiographic progression was compared between the patients from the two treatment cohorts using ANCOVA, adjusted for baseline BASRI score. Logistic regression statistical analysis was used to search for predictors of radiological progression. analiza statistică. The dependent variable was considered increased radiological progression rate (1-yes, 0-no). Variables were then entered into the model using Wald's statistic test. We used retrograde binary logistic model with step by step elimination of variables up to a value of $p \geq 0.15$. Hosmer-Lemeshow test statistic was used to test the statistical model discrimination capacity obtained. Comparisons were bilateral and all p values <0.05 were considered statistically significant.

3.3.5. Primary outcome

The primary outcome was to find out the proportion of patients in each treatment group who showed increased structural damage at 3 years follow-up during continuous treatment with either NSAIDs or anti-TNF-α agents. The cut-off value of radiographic progression was calculated by the difference between follow-up and baseline mean values of BASRI (BASRI spine and BASRI total) in each treatment group. Significant radiographic progression was defined as a worsening of the BASRI score by ≥ 3 units over 3 years.

3.3.6. Ethical approval

This study was approved by the Committee of Ethical and Sanitary Investigation of Reina Sofia University Hospital. Each patient signed an informed consent at inclusion in REGISPONSER-AS, according to the fundamental principles established in the Declaration of Human Rights in Helsinki.

3.4. Results

3.4.1 Patient and disease characteristics at baseline

One hundred eighty eight (188) AS patients fulfilled the inclusion criteria. The two treatment groups included 130 patients with continuous treatment with NSAIDs at recommended dosages and 58 patients with continuously treated with biologic therapies (BT). Table 1 comparatively shows the baseline clinical characteristics of the disease at the inclusion visit in REGISPONSER of the studied cohorts. Mean age at the diagnosis of the disease in both cohorts was 26.1 ± 8.1 years with an average disease evolution since diagnosis of 17± 9.2 years. The time of the disease evolution since first symptoms (symptom duration) was of 25.8 ±10.45 years in NSAIDs cohort and 23 ±8.8 years in BT cohort (p= SN). There was no statistical significant difference in demographic and clinical parameters between the two treatment groups at baseline with the exception of peripheral arthritis which was more frequent in BT treatment group and in BASDAI which was higher in NSAIDs group (table 5). HLA-B27 was found to be approximately in the same proportion of patients in both treatment groups.

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At inclusion visit, 37 patients (65%) received infliximab, 15 patients (25%) etancercept and 6 patients (10%) adalimumab. None of the patient was treated with golimumab. Of the 58 patients within BT cohort, 46 patients already were receiving anti- TNF-α treatment at inclusion visit in REGISPONSER, and in 12 patients BT was started at inclusion visit. Mean anti-TNF- α treatment in the former patients at baseline was 1.72 ±1.25 years

Table 5. Baseline* clinical characteristics of the two treatment groups

Characteristics	NSAID group (n=130)	BT group (n=58)	*P* value
Male n(%)	103 (79.2)	40 (70)	SN
HLA B27 n(%)	115 (88.5)	46 (80)	SN
Age, mean ± SD years	52 (9.9)	49.3 (9.2)	SN
IBP n(%)	130(100)	57 (98.3)	SN
Peripheral arthritis n(%)	32 (24.6)	35 (60)	<0.001
Uveitis n(%)	28 (21.5)	15 (25.9)	SN
Dactilitis n(%)	8 (6.2)	4 (7)	SN
Enthesitis n(%)	43 (34.1)	21 (36.2)	SN
Psoriasis n(%)	7 (5.4)	7 (12.1)	SN
IBD n(%)	4 (3.1)	4 (7)	SN
Family history n (%)	71 (55.6)	22 (38)	SN
Schober test (cm), mean ± SD	2.57 (1.6)	2.54 (1.8)	SN
Occiput to wall (cm), mean ± SD	6.27 (6.8)	6.8 (7.1)	SN
CRP (mg/l), mean ± SD	9.8 (1.31)	6.8 (1.92)	SN
ESR (mm/h), mean ± SD	17.6 (14)	22.5(22)	SN
BASDAI, mean ± SD	4.5 (2.3)	3.6 (2.4)	0.021
BASFI, mean ± SD	4.05 (2.7)	4.5 (2.6)	SN
BASRI-total, mean ± SD	8.1 (4.2)	9.4 (3.8)	SN
BASRI-spine, mean ± SD	7.2(3.3)	8.2 (3.1)	SN

*Baseline refers to the inclusion visit in the REGISPONSER, n value is the number of patients in each treatment group, IBP= inflammatory low back pain; IBD= inflammatory bowel disease

3.4.2. The changes in radiographic scores (structural damage)

At the evaluation visit after 3 years of monitoring, significant increase of structural damage was detected in the NSAIDs cohort, both in terms of total radiological score and in the spine score (BASRI total 8.72 (± 4) vs. 8.18 (± 4.2), p = 0.001); BASRI spine 7.58 (± 3) vs. 7.20 (± 3.3), p = 0.009). BT cohort of patients did not show statistically significant (SN) radiological progression at the assessment visit (table 6).

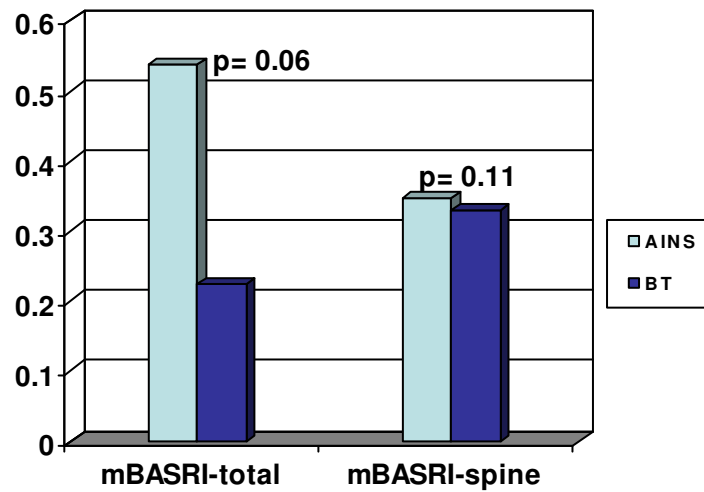*Table 6. Radiologic progression in the two treatemtn groups.*

Paramenter	Group NSAIDs		P value	Group BT		P value
	Baseline visit* (N=130)	Follow-up visit (N=130)		Baseline visit* (N=58)	Follow-up visit (N=58)	
BASRI spine (2-12)	7.2 (±3.31)	7.58 (±3.11)	0.009	8.21 (±3.17)	8.54 (±2.95)	SN
BASRI total (2-16)	8.18 (±4.21)	8.72 (±4)	0.001	9.4 (±3.83)	9.6 (±3.47)	SN

*Baseline refers to the inclusion visit in the REGISPONSER, n value is the number of patients in each treatment group. Values are expressed in mean and standard deviation (SD), SN- statistically non-significant.

Next we examined whether the difference in radiological progression of the two cohorts could be dependent on the treatment administered. For this we calculated the rate of change after 3 years BASRI radiologic score for each treatment group separately. Mean change in BASRI-total score (mBASRI-total) after 3 years was 0.54 (± 1.82) units for patients taking NSAIDs versus 0.23 (± 2.39) units in the group treated with BT, p = 0.067 (figure 3). Regarding the rate of change in BASRI-spine score (mBASRI-spine), the differences were less important. The group of patients treated with NSAIDs showed a change in score of 0.347 (± 1.49) units versus 0.33 (± 1.95) units in patients treated with BT, p = 0.113 (figure 3).

These rates were then considered as threshold values for radiological progression of the disease in the 2 treatment groups.

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Figure 3. Rate of radiological progression (mean change in BASRI score) in the treatment groups.

Although we found a tendency for less radiological progression rate close to statistical significance at 3 years for mBASRI-total in the group treated with biological agents, compared with the group treated with NSAIDs, in the analysis of covariance, after adjusting with baseline BASRI score we could not confirm a statistically significant difference in radiological progression rate, according to the treatment administered to the two groups of patients (p = 0.77 for BASRI-total, p = 0.46 for BASRI-spine).

Significant radiographic progression at follow-up.

Twenty-eight patients with AS (48.3%) of BT cohort and 61 (47%) patients in the NSAIDs treatment group showed increased radiographic progression rate (over the average of the progression rate of BASRI total for the treatment group), p =SN. Twenty-three patients (40%) of BT treatment group and 51 (40%) patients in the NSAIDs group had a BASRI- spine progression greater than the average radiological progression for each group (p = SN).

At the evaluation visit, significant radiological progression of at least 3 units was identified in 6 patients in the BT group (10.3%) and in 9 patients in the NSAIDs group (7%) (p = 0.43).

Predictors of radiological progression

We then sought to identify predictors of increased radiographic progression rate. Thus, we introduced as independent variables (predictors) in the logistic regression analysis beside the type of treatment administered (NSAIDs and BT), the following: sex of patients, *HLA B27* status, time of evolution of the disease, baseline inflammatory parameters (ESR, CRP), baseline radiologic score (BASRI-spine and BASRI-total). In the final model, the predictors of increased radiographic progression rate after 3 years of follow up in order of decreasing odds ratio (OR) were: baseline value of radiologic score in the spine, baseline value of total radiological score and male gender of patients (table 7).

Table 7. Predictive factors of high rate of radiological progression in AS.

Parameter	P value	O.R 95% C.I[#]
BASRI total baseline	0.001	0.607(0.45-0.82)
BARSRI spien baseline	0.03	1.489 (1.04- 2.14)
Male gender	0.04	0.450(0.21-0.96)

[#] to test the goodness to fit of the model we used Hosmer - Lemeshow Test (Chi^2= 2.26, p =0.972) and Omnibus test (p= 0 .000).

3.4.3. Evolution of disease acitvity and functional status of the AS patients in the two treatemtn groups.

Determination of disease activity measured with BASDAI score and ASDAS score, showed that at inclusion visit the group of patients taking NSAIDs had higher values of disease activity. At the end of the 3 years of follow up it was observed that this treatment was not able to control signs of the inflammation in patients who further recorded increased disease activity parameters (table 8).

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We noted a clear effect in controlling inflammatory activity of the disease in the group of patients treated with BT over the study period, reflected in maintaining low levels of both BASDAI and ASDAS score (table 8).

Regarding the evolution of physical function of patients in the 2 groups it was found that after 3 years of follow-up of patients treated with NSAIDs experienced a significant deterioration in functional status measured with the BASFI score (p <0.001). Patients in the biological agents treatment group maintained their physical function during the 3 years of follow-up (p=SN).

Table 8. Evolution of disease acitvity and functional status of the AS patients.

Parameter	Group NSAIDs		P Value	Group BT		P Values
	Baseline visit* (N=130)	Follow-up visit (N=130)		Baseline visit* (N=130)	Follow-up visit (N=130)	
BASDAI (0-10)	4.48 (2.3)	4.39 (2.2)	SN	3.6 (2.4)	2.95 (2.02)	0.07
ASDAS	2.71 (1.1)	2.52 (1.04)	SN	2.27 (1.2)	2.1 (0.9)	SN
BASFI (0-10)	4.02 (2.7)	4.79 (2.8)	0.000	4.48 (2.6)	4.51 (2.8)	SN

*Baseline refers to the inclusion visit in the REGISPONSER, n value is the number of patients in each treatment group. Values are expressed in mean and standard deviation (SD), SN- satistically non-significant.

3.5. Discussion

Although prospective rheumatologic registries have been initiated in several countries, there are only few published treatment reports on AS patients and neither of them directly compared the influence of NSAIDs and TNF-α blockers on structural damage of the disease [90,91,92].This paper is the first that assessed, in a large cohort of well characterized patients and up to 3 years of follow-up, the effectiveness of NSAIDs and the TNF α- blockers on structural damage in AS.

In this analysis we have shown that continuous administration of the NSAIDs on the one hand and anti-TNF-alpha biological agents, on the other hand, did not stop the structural destruction of the disease, which progressed radiologically at the end of the 3-year follow-up. However, in the group of patients treated with anti-TNF-alpha therapy, we found a slower rate of progression without statistically significance at the end of the 3 years of the study, both in the spine radiological damage and in the total damage (BASRI-spine p = SN, BASRI-total, p = SN). However, patients treated continuously during the 3 years of follow up only with NSAIDs showed a rapid radiological progression of the disease, statistically significant both in spine and in total damage (BASRI - spine 7.2 (± 3.31) vs. 7.58 (± 3.11), p = 0.009; BASRI-total 8.18 (± 4.21) vs. 8.72 (± 4), p = 0.001).

In addition, the average in total radiological progression in patients with AS treated with NSAIDs is superior 0.54 (± 1.82) to the average rate of progression in the patients on anti-TNF treatment 0.23 (± 2.39), although without statistical significance.

Furthermore, approximately 50% of patients in both treatment groups showed a high rate of radiological progression, higher than the average rate of structural destruction found at the end of the study, with higher values of BASRI score in the cohort of patients with NSAIDs (p = SN).

Interestingly, in the study performed by Wanders et al., it was found that continuous treatment for 2 years with NSAIDs (celecoxib) could slow down the radiological progression of AS in spine, compared with on demand treatment in periods of disease flare[6], supporting the hypothesis of a much older retrospective study conducted with phenylbutazone in patients with AS[7]. Nevertheless, the results of the study of Wanders et. al. are controverste as the difference between total dose of NSAIDs in both groups was very low. The results of other two studies in patients with AS were recently published. To the patients were given continuously for 2 years

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treatment with NSAIDs. The results of this german and duch researchers indicated that slow radiological progression rate could be recorded in the subgroup of patients who experienced high levels of CRP over time **Error! Bookmark not defined.**,[93]

With regard to biological agents and their relationship with radiological progression in AS, the literature data showed that TNF-alpha inhibitors may slow the progression of structural disease. Thus, in the studies of Baraliakos et al. it was identified that 21% of patients with AS have progressed after the first two years of continuous biological treatment while, in the next 2 years the proportion of patients who had radiological progression was only 15%[94],**Error! Bookmark not defined.**. This effect of reducing the rate of disease progression with anti TNF-alpha treatment was statistically significant after 8 years of treatment, compared with patients in the Herne cohort.**Error! Bookmark not defined.**.

However, the results should be interpreted with caution because in the scoring of the radiographs multiple readers who knew the sourcing of patients were used (biological treatment) but, they had no information on the chronological order of radiographs, which can introduce major errors of intrerpretation[95] .

However, by directly comparing the two types of treatment (NSAIDs vs BT), our study showed that there is no statistically significant difference in the rate of radiological progression of the disease according to type of treatment administrated. Moreover, the type of treatment recommended to patients did not represent a predictive factor of increased structured damage in multiple regression analysis, as the only parameters that predicted severe radiographic porogression were baseline structural damage and of male gender of the patients.

It is shown that radiographic progression of the disease leads to reduced mobility of the spine and consecutive impaired physical function in patients with AS. According to data published in the study by Machado P et al. [96] this hypotesis was true for advanced disease while in the early stages, the functionality was determined by the severity of disease activity. Thus, our results show that NSAIDs poorly controlled inflammatory activity of the disease, in contrast to anti-TNF agents which effectively mantained low disease activity for a long time.

An important finding of our study was that physical function significantly deteriorated in patients continuous treated with NSAIDs only, while physical function of patients in the BT group was mantained at the same level as the inclusion visit although they were not in the early stages of the disease, as patients had an average disease evolution since diagnosis of 17± 9.2 year.

Our patients belong to a series of daily life follow-up cohort from different rheumatologic centers and consequently, the decision to treat with a certain drug class depended on rheumatologist scientific opinion without specific instructions in this direction which could be interpreted as a limitation of our study. Although we compare patients who were treated differently, the comparison was assessed while the treatment was done at the same time for all patients. Until now, we only have data that

have compared biologic treatment to historical cohorts and the important difference to all other existing data up to now is that, we studied treatment effectiveness on daily patients; this could be a strength of our study.

Another limitation of our study would be the use of BASRI as the scoring method of radiographic damage and not m-SASSS (modified Stoke Ankylosing Spondylitis Spine Score) but, at the time of the conception of the registry, BASRI was the only validated radiographic tool in AS[97]. REGISPONSER was not specially designed and powered for the investigation of the influence of NSAIDs and TNF-alpha blockers on radiographic progression thus, the doses and the exact duration of treatment with NSAIDs were not strictly recorded in the registry.

Up to this point, we have data that compared the biological treatment with NSAIDs in AS only with historical cohorts and tha major difference brought by our our study is the effectivness of the treatement administrated was assessed by direct comparison of patients consulted daily clinical practice. This could be one of the strengths of our study.

3.6. Conclusions

1. Distribution of patients in this study was homogenous without statistically significant differences in most demographic and clinical parameters of disease.

2. Patients treated with NSAIDs had disease activity score at baseline significantly higher than patients on anti-TNF-alpha agents. Furthermore, our analysis shows that in patients with AS, continuous administration of NSAIDs has not effectively controlled inflammatory process reflected in maintaining high values of BASDAI and ASDAS scores at assessment visit.

3. Patients in both treatment groups analyzed in our study showed radiographic progression of disease after 3 years despite continuous treatment, irrespective of the treatment administered NSAIDs or anti-TNF-alpha biologic agents. However, our results show that treatment with anti-TNF-alpha agents does not lead to a higher progression rate than treatment with NSAIDs.

4. Patients treated with NSAIDs have registred at the end of the study a significant deterioration in functional status while, patients treated with biological agents physical function remained unaltered.

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5. We confirmed that baseline radiographic damage and male gender are predictors of high radiographic progression among patients with AS. The presence of HLA B27 was not associated with radiological progression rate.

6. Randomized controlled trials spanning on a longer survelliance time would be useful to certify that through effective control of inflammatory activity there might be prevented radiological progression in AS.

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4. Second study.

The influence of clinical factors and genetic polymorphisms in the development of severe functional status in ankylosing spondylitis.

4.1. Introduction

Ankylosing spondylitis (AS) is a chronic progressive inflammatory disease affecting the spine and peripheral joints. It is largely confirmed that susceptibility to AS is genetically determined with *HLA-B27* as a major genetic contributor to the disease[98,99,100] and that environmental factors also play a role in susceptibility to the disease. In the last few years, several other genes have been reported to be involved in AS susceptibility manly the polymorphisms in *endoplasmic reticulum aminopeptidase 1 (ERAP1)* gene and in *interleukin 23 receptor (IL23R) gene*[101,102,103].

Although the assessment of physical function is only one of several aspects of assessing disease severity, it is one of the most important measures of structural damage outcome in AS, as it directly influences the quality of life of patients and the economic costs of the disease[104,**Error! Bookmark not defined.**]. Impairment of physical function can be subdivided into a reversible and an irreversible component. In this concept the reversible component is due to disease activity (signs and symptoms of the disease) and the irreversible component is due to structural damage that has occurred as a consequence of the disease, such as syndesmophytes and vertebral bridging. Functional severity was found to be independently determined by both the reversible factors such as- disease activity and the irreversible factors such as- structural damage [80] but, the loss of functional capacity in each patient was not predictable from early disease stages[105].

There is evidence that several clinical parameters such as hip involvement, disease duration, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels, smoking, and lower socioeconomic status are associated with worse

function[106]. Nevertheless, much of the variability in disease functional severity in AS remains unexplained, suggesting that genetic factors could have a greater influence than environmental factors on AS progression[105]. A genetic component has been demonstrated for AS functional severity[107]. However, very little is known about the specific genes or genetic markers inside and outside the major histocompatibility gene complex (MHC) involved in the functional component of the disease [108,109,110].

Understanding the genetic basis of functional severity in AS would be of major value to differentiate at early stages patients at high risk of severe functional impairment and patients with a lower risk. Thus, clinicians could better select and optimize the preventive and therapeutic approach for each patient as of the time of diagnosis of the disease by objectively distributing high cost treatments.

4.2. Work hypothesis

Taking into consideration the fact that impairment of physical function may be partially controlled by the appropriate treatment, the aim of our study was to identify the baseline clinical and genetic factors that determine individual development of functional severity in AS. We have also investigated if the combination of clinical and genetic markers can predict individual progression towards a severe functional status in AS patients.

4.3. Material and Methods

4.3.1 Patients with AS and the design of the study

We performed a cross-sectional association study on Spanish AS patients which were recruited from 25 hospitals which participated in the Spanish National Spondyloarthropathies Registry (REGISPONSER)**Error! Bookmark not defined.**. Patients fulfilled the modified New York Criteria for AS[12] and had at least 10 years of follow-up from the first symptoms of the disease. Baseline characteristics of the patients at the beginning of the disease were recorded as potential prognostic predictors. Specifically, clinical and demographic data, sex, age at disease onset, family history of spondyloarthropathies (SpA), initial symptoms of SpA (inflammatory low back pain, neck pain, enthesitis, dactylitis, tarsitis, sacroiliac syndrome, coxitis, lower limb arthritis, and upper limb arthritis), and the number of initial SpA symptoms.

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Functional phenotype

To measure functional impairment we used the BASFI score, standardized by adjusting for disease duration since first symptoms, denominated here BASFI/t. BASFI is a validated index that focuses on 10 questions pertaining to function, measured on a visual analog scale (VAS). The mean of the 10 questions generates the score, with 10 denoting worst possible functional status[16]. There are no validated threshold values for classifying AS patients into mild or severe categories according to their BASFI score standardized by disease duration (BASFI/t). Therefore, to define functional severity we performed four association analyses using the χ2-test with different criteria. Based on the opinion of the clinicians who participated in this study, who estimated that approximately 25% to 40% of AS patients had severe functional damage, we defined as severe functional status the values of BASFI/t in the top 60th (p60), 65th (p65), 70th (p70) or 75th (p75) percentiles. The cut-off values for BASFI/t severe phenotype for each percentile were: 0.19 for p60, 0.21 for p65, 0.22 for p70, and 0.25 for p75 respectively.

4.3.2 HLA-B27 typing and SNP genotyping

Genomic DNA was isolated from saliva samples using the Oragene™ DNA Self-Collection kit (DNA Genotek Inc., Ottawa, Canada), according to the manufacturer's extraction protocol. All samples were tested for the presence of the HLA-B27 allele by conventional PCR using the primers reported by Olerup et al.[111]

After an extensive bibliographic search we selected 384 SNPs distributed in 190 genes to be analyzed in this study. We selected all the SNPs previously reported in Caucasians as associated with AS or with other SpA (psoriatic arthritis, juvenile idiopathic arthritis, reactive arthritis, undifferentiated arthritis, and inflammatory bowel disease–associated spondyloarthropathy). Besides those SNPs, we included some SNPs in genes described in the literature as associated to other autoimmune diseases and to bone-related disorders, since we considered them as potential candidates to be implicated in AS severity. Finally, we included tag SNPs in genes from the metabolic pathways of the two most important genes considered to be involved in AS: ERAP1 and IL-23R.The tag SNPs were selected from the HapMap CEU panel (the minor allele frequency at each locus was required to be >0.05 in Caucasian population, with an r2-value of <0.8 between adjacent markers). A tag SNPs represents polimorphisms localized regions of genome characterized by high

frequency of linkage desequilibrum (LD). SNP genotyping was performed using the Illumina Golden gate genotyping platform (Illumina, Inc., San Diego, CA, USA)[112].

4.3.3 Statistical analysis

Statistical analysis was performed with SPSS v 15.0 (SPSS, Chicago, IL, USA) and SVS v 7.3.1 (Golden Helix Inc., Bozeman, Montana, USA) softwares.

All quantitative data are presented as mean and standard deviation (± SD) and all qualitative data as absolute frequencies and percentages. To assess the association between clinical variables and BASFI/t severe phenotype an unvaried analysis was performed using the chi-square (χ2) test for categorical variables and the unpaired *t* test for continuous variables.

A test for deviation from Hardy-Weinberg equilibrium (HWE) was performed for each SNP. Pruning of the initial genotype dataset with default parameters (exclusion of SNPs with poor genotype cloud clustering, of SNPs with call-rate <85%, of SNPs with severe deviation from HWE (P<.0001) and of samples with call rate <85%) led to 456 samples and 344 SNPs**Error! Bookmark not defined.**. Association test between allele and genotype frequencies and BASFI/t severe phenotype was performed by the chi-square (χ2) test. P-values were calculated using a single-value permutation test (1000 permutations). The minor allele frequency at all loci was above 10%...Logistic regression analysis was used to discard weather the baseline clinical factors associated with severe function could be confounding for the association between BASFI/t severe phenotype and the SNPs genotypes. P values of 0<.05 were considered statistically significant and p values of (0.05≥p< 0.1) borderline

Clinical factors and SNPs were then studied by means of multivariate logistic regression. Individual *P* values of the SNPs and of the clinical variables were ranked and only those most significantly associated with the severe functional phenotype were included in the multivariate analysis as potential predictors (P< 0.1 in the allele frequencies association test).The multivariate analysis was performed for all four defined classifications of functional severity (p60, p65, p70, and p75). BASFI/t was considered as dependent variable and baseline clinical variables and SNPs were included as predictors. The predictive discrimination of the models was tested both by Hosmer-Lemeshow statistic and the receiver operating characteristic curve (ROC) with 95% confidence interval (CI). An area under the ROC curve (AUC) above 0.75 was considered as an indicator of a good predictive precision of the model.

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4.3.4 Ethical approval

This study was approved by the Ethics Committee of "Reina Sofia" University Hospital, Córdoba and "Puerta de Hierro Majadahonda" University Hospital, Madrid, Spain. Each patient signed an informed consent form upon inclusion in REGISPONSER-AS, in accordance with the fundamental principles set out in the Declaration of Human Rights in Helsinki.

4.4. Results

Demographic and clinical characteristics of the AS population.

The studied cohort included 456 AS patients (348 males and 108 females) with a mean age of 50.8 ± 10.5 years, 26.1 ± 9.1 years at disease onset and 34.6 ± 11.4 years at diagnosis. The average time of evolution, from disease onset, was 24.7 ± 10.1 years. *HLA-B27* was positive in 84.9% of the patients and 19.3% had a family history of spondyloarthropathies (SpA). Patients had mean BASFI at baseline 4.0 ± 2.8, with mean BASFI/t (years) of 0.17 ± 0.13. (table 9).

Table 9. Basal clinical and demographic characteristics of the study AS cohort.

Parameter	AS patients n=456
Gender males/females	348/108
Age, mean ± SD (years)	50.80 ± 10.5
Age at disease onset, mean ± SD (years)	26.06 ± 9.08
Duration of the disease since diagnosis, mean ± SD (years)	16.20 ± 10.04
Duration of the disease since first symptoms, mean ± SD (years)	24.70 ± 10.11
Family history (%)	88 (19.3%)
HLA-B27 positive (%)	386 (84.6%)
BASFI, mean ± SD (years)	4.04 ± 2.80
BASFI/duration AS, mean ± SD (years)	0.17 ± 0.13
BASRI-t, mean ± SD (years)	8,06 ± 4,18

Of the baseline clinical variables analyzed, the association with BASFI/t severe phenotype for neck pain and older age at disease onset was found to be statistically significant. A slight association was also found for low back pain (p60) and HLA-B27 (p65 and p70) (Table 10).

Table 10. Baseline clinical variables associated with functional severity in AS.

	p value			
Clinical Variable	**BASFI/t p75**	**BASFI/t p70**	**BASFI/t p65**	**BASFI/t p60**
Age at disease onset	<0.001	<0.001	<0.001	<0.001
Neck pain	0.040	0.002	0.004	0.011
Low back pain	SN	SN	SN	.030
HLA-B27	SN	0.050	0.049	SN

SN- statistically non-significant.

In the allele frequencies test, from the SNPs analyzed, we identified 24 polymorphisms associated with functional severe phenotype in at least one of the patient classifications. Two SNPs showed consistent association with BASFI/t and were significantly associated in all four patient classifications: rs2542151 in the protein tyrosine phosphatase non-receptor type 2 (*PTPN2*) gene [p60 (*P*=0.046), p65 (*P*=0.006), p70 (*P*=0.002) and p75 (*P*=0.001)] and rs2254441 in the proline-serine-threonine phosphatase-interacting protein 1 (*PSTPIP1*) gene [p60 (*P*=0.036), p65 (*P*=0.017), p70 (*P*=0.010) and p75 (*P*=0.001)]. Five SNPs (*rs10065172. rs2268624, rs4986790, rs4986791, rs3736228*) were associated with BASFI/t in three of the four patient classifications and eight polymorphisms (*rs6887695, rs17481856, rs2280153, rs1217414, rs4958847, rs2227982, rs17551710, rs1248634*) in two classifications. The other nine polymorphisms (*rs6822844, rs11959820, rs3117222, rs660895, rs1061622 rs13151961, rs27044, rs6254, rs743572*) were found significantly associated with BASFI/t for only one of the patient classifications (Table 11).

As we found that age at disease onset and neck pain at onset were the clinical factors associated with BASFI/t severe phenotype, they were entered in the logistic regression modeling as covariates. The results of the genotype frequencies test showed that the SNP *rs2542151* in the *PTPN2* gene was significantly associated to BASFI/t in three of the four patients' classifications after adjustment for age at disease onset and neck pain (table 11).

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There were other four SNPs with significant or borderline genotype associations in two of the patients' classifications after adjustment for age at disease onset and neck pain, *rs2254441* in the *PSTPIP1* gene, *rs2268624* in the *TGFB3* gene, and *rs4986790* and *rs4986791* in the TLR4 gene. The rest of SNPs were not associated to BASFI/t in the genotype test after correction for age at disease onset and neck pain at onset.

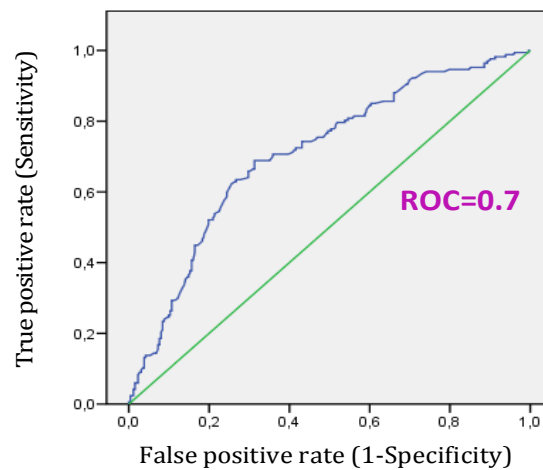**Table 11. SNPs associated with functional severity in AS.**

SNP code	Gene symbol	Risk allele	Allele frequencies test, p value§			
			BASFI/t p75	BASFI/t p70	BASFI/t p65	BASFI/t p60
rs2542151	*PTPN2*	C	0.001*	0.002*	0.006*	0.046#
rs2254441	*PSTPIP1*	A	0.001*	0.010†	0.017#	0.036#
rs2268624	*TGFB3*	G	0.002*	0.017*	0,032#	NS
rs4986790	*TLR4*	G	0.008†	0.009†	0.037#	NS
rs4986791	*TLR4*	A	0.011*	0.006*	0.031#	NS
rs10065172	*IRGM*	G	NS	0,014#	0,012#	0.006*
rs3736228	*LRP5*	A	0.049#	0,051#	0,056#	NS
rs6887695	*IL12B*	C	NS	0.038#	0.037#	NS
rs17481856	*ERAP1*	G	NS	0.035#	NS	0.038#
rs2280153	*WISP3*	G	NS	NS	0.032#	0.014*
rs1217414	*PTPN22*	G	NS	0.049#	NS	0.034†
rs4958847	*IRGM*	G	NS	NS	0.042#	0.027#
rs2227982	*PDCD1*	A	NS	0.019†	0.044#	NS
rs17551710	*COL6A1*	A	0.043#	NS	NS	0.048#
rs1248634	*DLG5*	A	NS	0.047#	0.043#	NS
rs6822844	*IL21*	A	NS	NS	NS	0.031#
rs11959820	*PPARBC1B*	C	NS	NS	NS	0.017*
rs3117222	*LOC646702*	G	NS	NS	NS	0.018*
rs660895	*HLA-DRB1*	G	NS	NS	0.044†	NS
rs1061622	*TNFRSF1B*	A	NS	NS	NS	0.050#
rs13151961	*KIAA1109*	G	NS	NS	NS	0.042#
rs27044	*ERAP1*	C	NS	NS	NS	0.020*
rs6254	*PTH*	A	0.025#	NS	NS	NS
rs743572	*CYP17A1*	A	0.032#	NS	NS	NS

§- The p-values shown correspond to the allele frequencies test corrected by a single-value permutation test; NS- nonsignificant statistically

The symbols represent the results of the genotype frequencies association test before and after a logistic regression analysis using the age at disease onset and neck pain as covariates: * Significant genotype association (p<0.05) after logistic regression analysis with adjustment for age at disease onset and neck pain; † Borderline genotype association (0.05≥p<0.1) after logistic regression analysis with adjustment for age at disease onset and neck pain; # Significant genotype association (p<0.05) was lost after logistic regression analysis with adjustment for age at disease onset and neck pain.

For further investigate the association between clinical-genetic variables and functional severe phenotype in AS, we performed a multivariate analysis using BASFI/t (severe/mild) as dependent variable and as covariates the clinical and genetic variables most significantly associated with BASFI/t ((p<0,1 in the allele frequencies association test). We did not find any model with a good predictive accuracy for BASFI/t for any of the patient classifications, p60, p65, p70, and p75 as none of the ROC curves of the predictive models attained an area under the curve (AUC) above 0.75. (Fig.4)

Figure 4. ROC curve of the clinico-genetic predictive model of functional severity in AS.

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4.5. Discussion

This is a pioneering study performed on daily clinical patients which sought the clinical and genetic factors of influence in the development of functional severity in AS. Maintaining a good functional status is the main aim of pharmacologic treatment in current clinical practice. Reliable markers that could be applied early in the disease course to identify patients with potential severe functional outcome would be of major value for clinicians to consequently select the most suitable treatment strategy. The results of our study found that older age and neck pain at disease onset and several new SNPs, particularly *rs2542151* (*PTPN2*) and *rs2254441* (*PSTPIP1)*, are predictors of severe functional impairment in AS. However, the combination of the genetic and clinical factors identified in our study was not sufficient to develop a predictive model with a good accuracy for AS functional outcome; therefore, additional predictors are required.

We found neck pain and older age at disease onset as the main clinical variables associated with severe AS functional status. Neck pain at onset, which in AS may arise from either mechanical or inflammatory lesions, presented a consistent association in all four patient classifications studied [p75 (*P*=0.040), p70 (*P*=0.002), p65 (*P*=0.004), p60 (*P*=0.011)]. To the best of our knowledge, this is the first study which has found neck pain as a predictor for AS functionality. There is conflicting evidence about the role of age at disease onset as a predictor of disease severity, with some studies finding an association and others failing to support this[113,114,115].]. Patients included in our study were young adults at disease onset, with mean age of 26.1 ± 9.1 years. Interestingly, we found a statistically significant association between older age at disease onset and BASFI/t severe phenotype (p75, p70, p65, p60; *P*< 0.001). This result is in accordance with a recent study which found that the likelihood of developing more severe radiographic damage was greater among patients with an older age at disease onset[Error! Bookmark not defined.]. The authors suggested that this association could result if patients with an older age at disease onset are asymptomatic early in their illness or if they have occasional or milder symptoms, which might cause them to underestimate the duration of their disease. Supporting previous studies[70,116], we found that *HLA-B27* was poorly associated with functional severity; thus, we only identified statistically significant association in two of the percentiles studied [p65 (*P*=0.050), p70 (*P*=0.049)]. Surprisingly, we did not find coxitis at baseline to be associated with development of severe physical function

as found in several previous studies that sought clinical predictors of functional disability in AS [117,118].

Recent genome-wide association studies have provided valuable evidence supporting the involvement of genetic factors in the pathogenesis and prognosis of autoimmune diseases. The two major SNPs found in this study to be associated with development of severe physical function are located in the autoinflammatory genes *PTPN2* and *PSTPIP1.* Our study found a consistent association between AS functional severity and the C allele of the SNP *rs2542151* in the *PTPN2* gene*. PTPN2* is a remarkable gene, since it appears to influence most cells involved in the development of the immune system[119]. *PTPN2* encodes the T cell protein tyrosine phosphatase TCPTP, a key negative regulator of inflammatory responses. Abnormalities in tyrosine phosphorylation have been found to be involved in the pathogenesis of numerous human diseases, such as developmental defects, neoplastic disorders, immunodeficiency, and autoimmunity[120]. The C allele of the SNP *rs2542151* at *PTPN2* gene*,* which we found to influence AS functional severity, was previously found to be associated with other autoimmune diseases, such as Crohn's disease, type 1 diabetes, and rheumatoid arthritis [121]. The fact that the SNP *rs2542151* in *PTPN2* is associated with different aspects of autoimmune diseases suggests that these diseases, including AS, could share common pathogenic mechanisms.

Another consistent association between poor physical function of AS patients and SNPs was found for the A allele of the polymorphism *rs2254441* in the *PSTPIP1* gene. *PSTPIP1* is a cytoskeleton-associated adaptor protein that regulates innate and adaptive immune responses. Although this pathway has been traditionally related to diseases associated with pyoderma gangraenosum such as aseptic abscesses syndrome (PAPA syndrome) and chronic inflammatory bowel disease (IBD)[122], the SNP *rs2254441* has been recently reported as associated with psoriatic juvenile idiopathic arthritis, a disease included in the spondyloarthropathies group together with AS and IBD [123].

In addition to SNPs in *PTPN2* and *PSTPIP1* genes, a milder association with BASFI/t severe phenotype was observed for SNPs transforming growth factor beta 3 (TGFB3) and Toll-like receptor 4 (TLR4). Regarding TGFß3, high serum levels of this protein have been linked to osteoporosis risk[124] and polymorphisms in *TGFß* have been found to be associated with ossification of the posterior longitudinal ligament of the spine (OPLL) in the Japanese population [125]. To the best of our knowledge this is the first report which linked risk from allele G of *rs2268624* in *TGFß3* with development of functional severity in AS. The association of the SNPs *rs4986790* and *rs4986791* in the *TLR4* gene could represent the involvement of the innate immune

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system in the progression of AS. Dysregulation of Toll-like receptor (TLR)-related pathways, specifically upregulation of TLR4 and TLR5, has been reported in AS[126].

The strength of this study lies in the large number of clinically well characterized AS patients. However, our study has some limitations. First, we performed a cross-sectional study in which we did not analyze the treatment administered to patients as a possible factor that influences physical outcome, since we did not have reliable data about which patients were on a specific therapy prior to their inclusion in the study. The lack of information about the treatment could have introduced some bias. Secondly, in spite of the clinical and genetic factors found to influence AS functional prognosis, we could not achieve a good predictive model for development of severe functional status by combining these factors. Thus, further research in this area in other cohorts or in prospective studies is needed to confirm which of these genetic markers in combination with clinical factors could identify an accurate predictive model for AS functional severity.

Moreover it is required the validation of these results in other cohorts of patients with AS.

4.6. Conclusions

1. Analysis of predictive factors for severe functional status of patients with AS showed that patients with late-onset disease and those with cervical spine pain at initiation of disease have significant association with severe functional status.

2. The presence of inflammatory back pain at onset and *HLA B27* were associated to a lesser extent with the severity of deterioration in physical function.

3. Unlike previously published data, we did not find the presence of coxitis at onset associated with functional severity.

4. We confirmed the existence of genetic predisposition in the evolution toward severe functional status, by identifying a total of 24 polymorphisms associated with severity of impairment in physical function. The strongest association was registered polymorphisms *rs2542151* (PTPN2) and *rs2254441* (PSTPIP1).

5. In logistic analysis there were identified clinical and genetic factors associated with functional severity, but we could not build with tose factors a model with good predictive power for severe functional status in AS.

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5. Third study.

Candidate single nucleotide polymorphisms predictors of treatment non-response to the first anti-tumor necrosis factor in ankylosing spondylitis.

5.1. Introduction

Ankylosing spondylitis (AS) is an inflammatory rheumatic disease in which the inflammatory process mainly involves the spine and, to a lesser extent, the peripheral joints. One of the most important clinical challenges is to control the inflammation and, therefore, to maintain AS patients symptom-free. Several studies have strongly confirmed the implication of the pro-inflammatory cytokine TNF-alpha in the pathogenesis of AS[127,128]. Hence, after a documented failure to previous nonsteroidal anti-inflammatory drugs (NSAIDs), anti TNF-alpha agents are being administrated in patients with active disease, according to Assessment of SpondyloArthritis international Society (ASAS) recommendations.

Anti TNF-alpha agents act by inhibiting the binding of TNF-alpha to its receptors and therefore interfere with TNF-alpha signaling transduction pathways. Placebo-controlled randomized trials revealed (RTCs) similar efficacy in controlling active disease for all four accepted TNF-alpha inhibitors (infliximab[129], etanercept[130], adalimumab[131] and golimumab[132]).

Clinical markers of response to anti TNF alpha agents [Error! Bookmark not defined.,133] have been investigated in several RTCs. Nevertheless, patient selection for RTC's is not always representative of patients to whom drugs are prescribed in clinical practice. Thus, data from observational registries provide valuable knowledge about daily patients. Recent evidence from observational studies has reported that raised erythrocyte sedimentation rate (ESR) level, higher C-reactive protein (CRP) level, lower BASFI, younger age at baseline, male gender, peripheral arthritis, and concurrent use of disease modifying anti-rheumatic drug (DMARDS), primly methotrexate, showed association with BASDAI 50 clinical response[134,135]. Nevertheless, there is a high percentage of AS patients who remained disease-active

despite long-term anti-TNF-alpha treatment, in which case genetic background may play an important role[136].

In contrast with rheumatoid arthritis (RA), few studies have analyzed the role of genetic markers in the response to anti TNF-alpha treatment in AS patients and the results were contradictory. Most studies analyzed either *HLA-B27* status of the patients or *TNF-alpha gene* polymorphisms as potential predictor factors of response to biological treatment in AS [137,138]

Pharmacogenomic studies, focusing on genes involved in AS etiology and pathogenesis, in order to analyze the role of allelic polymorphisms in the individual difference in treatment response to TNF-alpha inhibitors, are lacking in AS. Taking into 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.

5.2. Work hypothesis

The aim of our study was to identify SNPs predictors of treatment non-response to the first TNF-alpha inhibitor in Spanish AS patients from daily clinical practice.

5.3. Patients and Methods

5.3.1 Patients and study design

We performed a longitudinal multicenter study on AS patients enrolled in the REGISPONSER registry**Error! Bookmark not defined.**. Out of all REGISPONSER patients, 529 patients fulfilled the modified New York Criteria for AS [12].(REGISPONSER AS cohort). For this study we only selected the patients with AS who started their first TNF-alpha inhibitor according to ASAS recommendation, during the interval between two REGISPONSER-AS scheduled visits. The decision to commence a particular agent depended on the decision of the attending rheumatologist together with the patient's specific preference. Until 2006 patients were treated with either infliximab or etanercept, since adalimumab has been approved for AS treatment in Spain in 2006.

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None of the patients had been treated with Golimumab. In this study patients could receive concomitant medication with either NSAIDs or DMARDs (sulphasalazine, methotrexate) as prescribed by their rheumatologist.

Baseline characteristics of the disease, prior anti TNF-alpha treatment, together with baseline and follow-up parameters of disease activity were registered and analyzed in our study. They included patient clinical and demographic data such as gender, age, ethnicity, age at disease onset, family history of SpA, initial SpA symptoms (low back pain, enthesitis, dactylitis, coxitis, uveitis, peripheral arthritis, psoriasis, inflammatory bowel disease), and co-morbidities. Disease activity parameters were assessed with i) BASDAI (on a scale 0-10), ii) erythrocyte sedimentation rate (ESR), iii) C-reactive protein (CRP, mg/L). ASAS-endorsed disease activity score (ASDAS) was calculated using the accepted formula with CRP.

Definition of non-response

We classified patients as "responders" if they achieved BASDAI 50 clinical response at the assessment visit and "non-responders" if they failed to achieve BASDAI 50 clinical improvement at the assessment visit, according to ASAS guidelines (a 50% improvement of the initial BASDAI). The primary outcome was to identify genetic polymorphisms associated to patients who commenced TNF-alpha blockers and did not achieve BASDAI 50 improvement criteria at the assessment visit.

5.3.2 Genotyping

Genomic DNA was isolated from saliva samples using the Oragene™ DNA Self-Collection kit (DNA Genotek Inc., Ottawa, Canada), following the manufacturer's extraction protocol. All samples were tested for the presence of *HLA-B27* allele with PCR using the primers reported by Olerup et al.[111] After an extensive bibliographic search, we selected candidate SNPs previously reported to be associated with susceptibility or pathogenesis of AS and with other SpAs (psoriatic arthritis, juvenile idiopathic arthritis, reactive arthritis, undifferentiated arthritis and inflammatory bowel disease–associated spondyloarthropathy), SNPs associated with autoimmune and bone-related diseases, and SNPs from the metabolic pathways of the *IL-23 receptor* (IL-23R) and *endoplasmic reticulum aminopeptidase 1* (ERAP1) genes. In

total, 384 candidate SNPs distributed in 190 genes were analyzed in this study. SNP genotyping was performed using the Illumina Golden gate genotyping platform (Illumina, Inc., San Diego, CA, USA)[112].

5.3.3 Statistical analysis

Statistical analysis was performed with SPSS v19.0 software (SPSS, Chicago, IL, USA) and SVS software v7.3.1 (Golden Helix Inc., Bozeman, Montana, USA). Patient population data in the study were shown as mean and standard deviation (±SD) for quantitative variables and as absolute number and relative frequencies (%) for qualitative variables. We compared clinical characteristics of treatment response groups with the χ^2 test for categorical variables and with the unpaired student *t* test for continuous variables.

A test for deviation from Hardy-Weinberg equilibrium (HWE) was performed for each SNP using the Helix Tree software version 7.3.1. Pruning of the initial genotype dataset with default parameters (exclusion of SNPs with poor genotype cloud clustering, of SNPs with call-rate <85%, of SNPs with severe deviation from HWE (P<0.0001), and of samples with call rate <85%) led to 456 samples and 345 SNPs being analyzed. Measures of pairwise linkage disequilibrium (LD) (r^2>0.8) were determined using Haploview version 4.1 (Whitehead Institute for Biomedical Research, Cambridge, MA)[139]. All SNPs reported in this study had minor allele frequency (MAF) >10%.

An association test between allele frequencies and the treatment response groups was performed with the χ^2 test. The magnitude of allele association was expressed as odds ratio (OR) with a 95% confidence interval (CI) (OR>1 indicates a risk allele, and OR<1 indicates a protective allele). *P*-values were adjusted using a single value permutation test (1000 permutations).The probability of non-response was modeled using logistic regression, considering as dependent variable the variable "non-responder" (1= yes, 0= no) and as independent variables the polymorphisms identified statistically significant associated with non-response to treatment in the allele frequency χ^2 test after adjustment. Independent variables were introduced as genotypes groups (1- homozygote and heterozygote for risk allele, 0- homozygote without risk allele) after dominance or recessive behavior for each SNPs was tested.   The genotypes which showed association with non-response to treatment in the univariate analysis (*P*<0.05) were entered into the multiple backward logistic regression through the Wald statistic test. We used elimination until *P*≥ 0.15; the reduced model was compared with the initial model using the likelihood ratio test. We tested the discrimination of the final model via the Hosmer-

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Lemeshow statistic and the receiver operating characteristic (ROC) curve with 95% CI. To analyze the predictive power of our genetic model of non-response to biological treatment, AUC-ROC was used (Analyse-it software v2.09, Leeds, UK). Contrasts were all bilateral and *P*-values <0.05 were considered statistically significant.

5.3.4. Ethical approval

This study was approved by the Ethics Committee of Reina Sofia University Hospital and of Puerta de Hierro Majadahonda University Hospital, Madrid, Spain. Each patient signed an informed consent upon inclusion in the REGISPONSER-AS, according to the fundamental principles established in the Declaration of Human Rights in Helsinki.

5.4. Results

5.4.1. Response status to anti-TNF alpha treatment

Among the 529 REGISPONSER-AS patients, 121 AS patients fulfilled the inclusion criteria. Sixty-eight (56.2%) were responders and fifty-three (43.8%) were non-responders to anti TNF-alpha treatment at the assessment visit after applying BASDAI 50 improvement criteria compared with baseline (initiation of TNF-alpha treatment). Baseline clinical and demographic characteristics are summarized in Table 12. The mean age of studied patients at treatment initiation visit was 47.7 ± 9.5 years, with mean age at onset of the disease of 26.6 ± 10.6 years and mean disease duration since first symptoms of 21.1 ± 8.9 years. There were no statistically significant differences in baseline clinical and demographic characteristics between the two treatment response groups, with the exception of those patients who did not respond to anti TNF-alpha treatment which had a statistically significantly older age at disease onset (28.4 ± 8.7 *vs.* 24.2 ± 9.4; *P*=0.021). Mean anti TNF-alpha treatment duration between initiation and the assessment visit for all patients was 12 ± 3 months. After applying ASDAS formula we did not find statistically significant differences in baseline disease activity between the two treatment response groups. Interestingly, at baseline the non-responders group had a trend of lower inflammation biomarkers than patients which responded to biological treatment (Table 13) but at the assessment visit they showed significantly higher ESR (mm/h) (22.8 ± 22.4 *vs.* 13.87 ± 13.1; *P*=0.016) and CRP (mg/L) (9.27 ± 8.5 *vs*. 5.09 ± 4.9;

P=0.003) values than responders (Table 2). Infliximab, etanercept, and adalimumab were used in 62 (51.2%), 34 (28.1%) and 25 (20.7%) patients, respectively. There were no statistically significant differences between the response groups for concomitant DMARDs use (sulphasalazine, metothrexate) (data not shown). However, a statistically significant difference was observed between the two groups regarding NSAID use, as nonresponders were more frequent NSAIDs consumers (44 [83%] *vs.* 40 [73.5%]; *P*=0.048).

Tabel 12. Baseline characteristics and disease activity in AS patients at inclusion visit.

Clinical variables and disease activity at baseline	*All patients N= 121*	*Responders N=68*	*Non-responders N=53*	*P-value*
Age, (±SD) (y)	47.7 ± 9.5	46.3 ± 10.2	49.6 ± 8	NS
Age at disease onset, (±SD) (y)	26.6 ± 10.6	24.2 ± 9.4	28.4 ± 8.7	0.021
Males, n (%)	89 (73.6)	48 (70.6)	41 (77.4)	NS
Race Caucasian, n (%)	112 (99.1)	64 (100)	48 (98)	NS
Disease duration, (±SD) (y)	21.1(±8.9)	21.5 (±10.4)	21.16(±6.6)	NS
Inflammatory back pain, n (%)	118 (97.5)	65 (95.6)	53 (100)	NS
Peripheral arthritis, n (%)	55(45.8)	31 (46.3)	24 (45.3)	NS
Coxitis, n (%)	5 (4.1)	4 (5.9)	1 (1.9)	NS
Entesitis, n (%)	46 (38.3)	25 (37.3)	21 (39.6)	NS
Uveitis, n (%)	27 (22.7)	15 (22.4)	12 (23.1)	NS
Dactilitis, n (%)	10 (8.8)	6 (8.8)	4 (7.8)	NS
Psoriasis, n (%)	14 (11.7)	6 (8.8)	8 (15.4)	NS
Colitis, n (%)	17 (14)	13 (19.1)	4 (7.5)	NS
Family history of SpA, n (%)	21 (20.2)	11 (18.6)	10 (22.2)	NS
HLA-B27 positivity, n (%)	94 (77.7)	54 (79.4)	40 (75.5)	NS
ESR (mm/h) (±SD)	32.3 ± 4.8	32.8 ± 25.7	31.7 ± 23.8	NS
CRP (mg/L) (±SD)	17.6 ± 17.2	20 ± 20.1	14.3 ± 12	NS
BASDAI(±SD)	5.68 ± 2.05	5.04 ± 2.1	6.3 ± 1.7	0.003
ASDAS (±SD)	3.6 ± 0.9	3.5 ± 0.9	3.84 ± 0.7	NS

*Data are expressed in mean and standard deviation (±SD), unless otherwise specified. ESR, erythrocyte sedimentation rate; HLA, human leukocyte antigen; NS, not statistically significant; SD, standard deviation; y, years

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Tabel 13. Disease activity parameters in the treatment response groups at evaluation visit.

Parameter	Responders N= 68	Non-responders N= 53	P value
BASDAI, (±SD)	1.76 ± 1.03	5.60 ± 1.74	<0.001
ESR (mm/h), (±SD)	13.87 ± 13.12	22.80 ± 2.43	0.016
CRP (mg/L), (±SD)	5.09 ± 4.98	9.27 ± 8.57	0.003
ASDAS, (±SD)	1.70 ± 0.67	3.20 ± 0.74	<0.001

* Data are expressed in mean and standard deviation (±SD), unless otherwise specified. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; SD, standard deviation

2. SNPs association with treatment response status to the first TNF-alpha inhibitor agent.

From the 345 SNPs tested for association with individual response to anti TNF-alpha agents, 13 polymorphisms showed significant association with treatment response status assessed with BASDAI50. The genotype frequencies of the SNPs associated with response status to anti TNF-alpha treatment are shown in table 14. Three of the SPNs belong to c*almoduline 1 gene (CALM1)* and they were identified to be in linkage disequilibrium (LD) (figure 5).

Risk alleles of SNPs associated with non-response status to anti TNF-alpha treatment and their OR with 95% confidence intervals (CI) are listed in table 15. After adjustment with a single value permutation test, 4 SNPs did not remain significantly associated with treatment response status, two of them were SNPs in the *CALM1* gene; therefore, 9 independent associations were finally identified.

Figure 5. Linkage disequilibrium (LD) in *CALM-1* gene SPNs: rs2300496, rs2300500, rs3213718 obtained with Haploview software. The darkeness cell shows the highest LD among the SNPs.
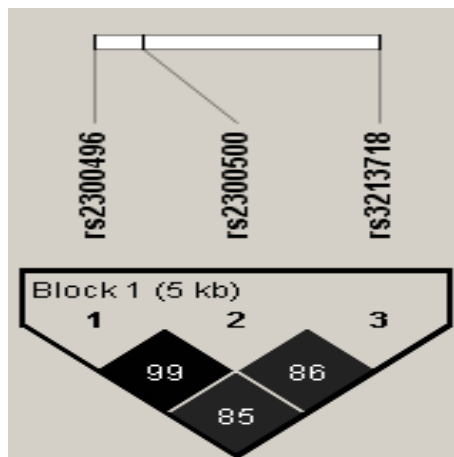

Table 14. Distribution of genotype frequencies associated with treatment response status to the first anti-TNF-alpha agent in AS.

SNP	Gene	Genotype	Nonresponders[a], n(%)	Responders[a], n(%)	Minor allele	MAF*
rs755622	MIF	CC	31 (58.5)	55 (80.9)		
		CG	19 (35.8)	13 (19.1)	G	0.15
		GG	3 (5.7)	0 (0)		
rs917997	IL18RAP	AA	5 (9.4)	3 (4.4)		
		AG	26 (49.1)	18 (26.5)	A	0.24
		GG	22 (41.5)	47 (69.1)		
rs1061622	TNFRSF1B	TT	25 (47.2)	47 (69.1)		
		TG	24 (45.3)	19 (27.9)	G	0.22
		GG	4 (7.5)	2 (2.9)		
rs4343	ACE	TG	3 (5.7)	12 (17.6)		
		GG	5 (9.4)	36 (52.9)	A	0.4
		GG	23 (43.4)	17 (20.5)		

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SNP	Gene	Genotype	Nonresponders[a], n(%)	Responders[a], n(%)	Minor allele	MAF*
rs4355801	TNFRSF11B	AA	28 (52.8)	25 (36.8)		
		AG	19 (35.8)	28 (41.2)	G	0.36
		GG	6 (11.3)	15 (22.1)		
rs6060369	UQCC	AA	26 (49.1)	21 (30.9)		
		AG	22 (41.5)	35 (51.5)	G	0.37
		GG	5 (9.4)	12 (17.6)		
rs3740691	ARFGAP2	AA	8 (15.1)	6 (8.8)		
		AG	29 (54.7)	31 (45.6)	A	0.37
		GG	13 (24.5)	31 (45.6)		
rs764481	CYP2D6	AA	7 (13.2)	13 (19.1)		
		AG	14 (26.4)	27 (39.7)	A	0.33
		GG	32 (60.4)	28 (41.2)		
rs331377	ASPN	AA	12 (22)	27 (39.7)		
		AG	26 (49;1)	28 (41.2)	G	0.45
		GG	15 (28.3)	13 (19.1)		
rs3213718	CALM1	AA	26 (49.1)	21 (30.9)		
		AG	20 (37.7)	33 (48.5)	G	0.39
		GG	7 (13.2)	14 (20.6)		
rs1800896	IL10	AA	18 (34)	10 (14.7)		
		AG	25 (47.2)	41 (60.3)	G	0.48
		GG	9 (17)	16 (23.5)		
rs2300496	CALM1	AA	24 (45.3)	20 (29.4)		
		AC	20 (37.7)	30 (44.1)	C	0.42
		CC	9 (17)	18 (26.5)		
rs2300500	CALM1	CC	9 (17)	18 (26.5)		
		CG	20 (37.7)	30 (44.1)	C	0.42
		GG	24 (45.3)	20 (29.4)		

[a]Non-responders / Responders - reduction in BASDAI 50 according to ASAS criteria , * MAF- minor allele frequency

Table 15. Allelic association test of non-response to anti-TNF-alpha treatment in AS patients according to BASDAI50 clinical response.

Snp	Gena	Alela de risc OR (IC 95%)	X² test *P neajustat*	X² test *P ajustat**
rs755622	***MIF***	G, 2.92 (1.41-6.03)	0.002	0.003
rs917997	***IL18RAP***	A, 2.4 (1.32- 4.35)	0.003	0.005
rs1061622	***TNFRSF1B***	G, 2.12 (1.15- 3.91)	0.014	0.009
rs4343	***ACE***	G, 1.82 (1.05- 3.13)	0.029	0.016
rs4355801	TNFRSF11B	A, 1.79 (1.04- 3.08)	0.031	0.051
rs6060369	***UQCC***	A, 1.77 (1.03- 3.02)	0.035	0.032
rs3740691	***ARFGAP2***	A, 1.76 (1.03- 3.02)	0.035	0.031
rs764481	CYP2D6	G, 1.77 (1.02- 3.09)	0.040	0.077
rs331377	***ASPN***	G, 1.7 (1.01- 2.84)	0.041	0.043
rs3213718	***CALM1***	A, 1.72 (1.01- 2.92)	0.043	0.044
rs1800896	***IL10***	A, 1.69 (1.01- 2.84)	0.044	0.029
rs2300496	CALM1	A, 1.68 (1.002 - 2.83)	0.048	0.055
rs2300500	CALM1	G, 1.68 (1.002-2.83)	0.048	0.055

*P-values adjusted by a single value permutation test (1000 permutation). Significant SNPs after single value permutation test are marked in bold,

All 9 polymorphisms that showed significant association with non-response status to biological treatment, in the allele frequencies association test were entered as genotypes in the multivariate model. We found that: rs917997 in the *IL18RAP* gene (OR: 3.35, 95% CI:1.38- 8.15), rs755622 (OR: 3.14, 95% CI: 1.19- 8.22) in the *MIF* 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* gene, rs1061622 (OR: 2.46, 95% CI: 1.00- 6.04) in the *TNFRSF1B* gene, were independent predictors of non-response of the first anti-TNF-alpha agent (table 16).

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Tabel 16. Logistic regression analysis to predict genetic non-response to biologic treatment in AS.

Snp	Gene	Risk Genotype	Univariate analysis OR (95% CI), P	Multivariate analysis& OR(95%CI), P
rs755622	*MIF*	GG+CG	3 .002 (1.33- 6.78), 0.008	3.14 (1.19- 8.22), 0.019
rs917997	*IL18RAP*	AA+AG	3.15 (1.49-6.78), 0.003	3.35 (1.38- 8.15), 0.007
rs1061622	*TNFRSF1B*	GG+TG	2.5 (1.19-5.28), 0.016	2.46 (1.00- 6.04), 0.048
rs4343	*ACE*	GG	2.40 (1.09-5.27), 0.028	
rs6060369	*UQCC*	AA	2.15 (1.02-4.53), 0.043	
rs3740691	*ARFGAP2*	AA+AG	2.38 (1.08-5.26), 0.031	2.90 (1.12- 7.51), 0.002
rs331377	*ASPN*	GG+AG	2,25 (1.005-5.04), 0.049	
rs3213718	*CALM1*	AA	2.15 (1.02-4.53), 0.043	
rs1800896	*IL10*	AA	3.02 (1.24-7.29), 0.014	3.09 (1.04- 9.15), 0.041

&- to asses the goodness to fit of the model Hosmer - Lemeshow test and the receiver operating characteristic (ROC) curve with 95% CI were used.

The genetic model obtained of non-response to treatment has a predictive power, as indicated by the ROC AUC, of 0.77 (95% CI: 0.68 - 0.86) in figure 6. The distribution of the probabilities obtained with the prediction model for the responders and non-responders patients is shown in figure 7. The median value for the probability in the responder group was 0.32, whereas higher value was obtained for non responders to biological treatment, reaching 0.57.

Figure 6. ROC plot of the predicitve genetic model of non-response to biological treatment.

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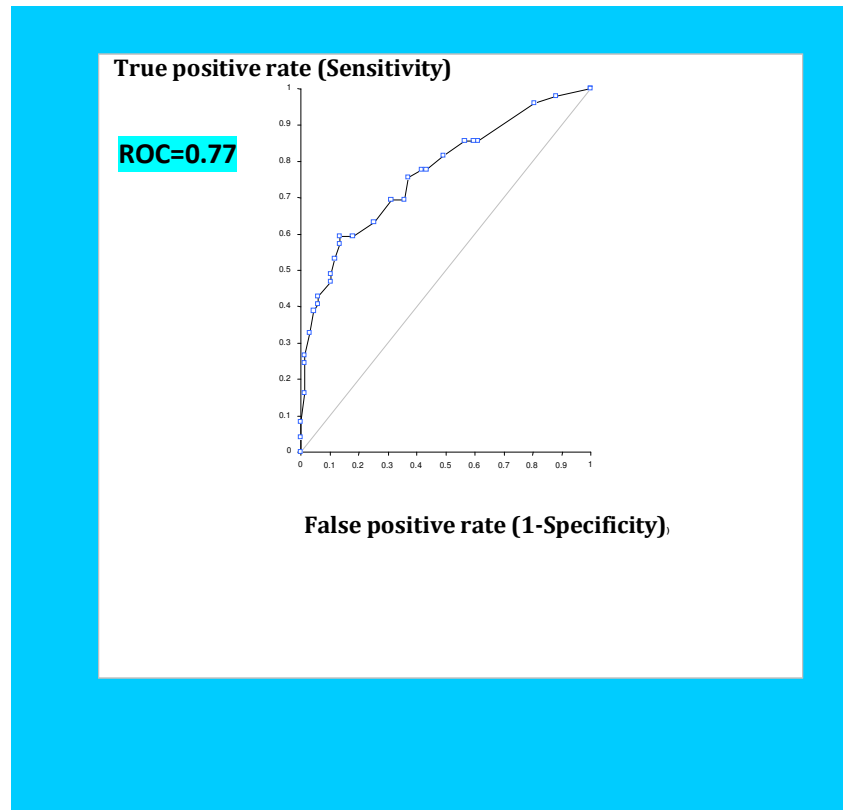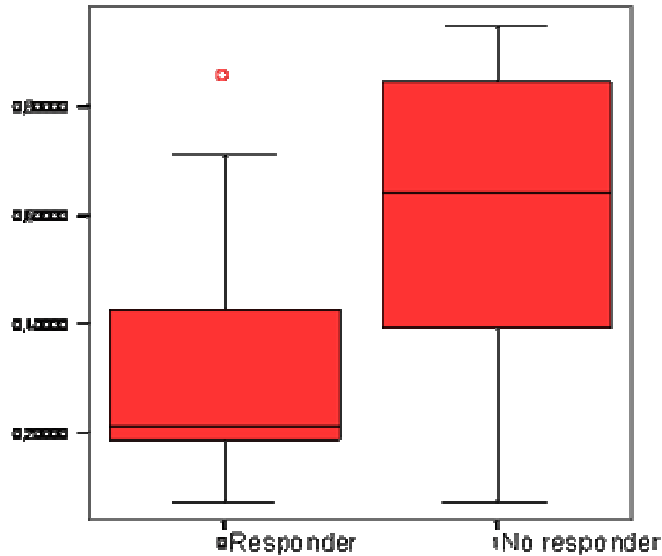
89

Figure 7. Probability distribution of lack of response in the predictive genetic model (responders and non responders to biological treatment).

For each box it is indicated median value, first and third quartil, minimun and maximum value.

5.5. Discussion

In this study we searched for pharmacogenomic markers responsible for non-response to anti TNF-alpha agents in previously untreated AS patients. Our candidate gene study led to the identification of 5 SNPs in 5 different genes as being predictive factors of non-response to the first biological treatment. These genetic variants could alter the effectiveness of the anti TNF-alpha drugs: rs755622 in the *macrophage migration inhibitory factor (MIF)* gene, rs917997 in the *interleukin 18 receptor accessory protein (IL18RAP) gene*, rs1800896 in the *IL10 gene,* rs1061622 in the *tumor necrosis factor receptor superfamily, member 1B (TNFRSFB1)* and *rs3740691* in *ADP-ribosylation factor GTPase activating protein 2* (*ARFGAP2) gene.*

The percentage of BASDAI 50 responders to TNF-alpha agents in our study was 56.2%, close to the previous findings[135,136],therefore, it supports the need to search for objective predictors of treatment response, beyond clinical and demographic factors. REGISPONSER-AS patients belong to daily clinical practice but, interestingly, the clinical characteristics of the two treatment response status groups did not differ. Moreover, no difference was encountered in AS disease duration or in *HLA B27* status in the two treatment activity groups (responders 54 [79.4%] *vs.* non-responders 40 (75.5%), *P*=NS). Nevertheless, patients with non-response to treatment had a statistically significant older age at disease onset, (24 [±9.4] *vs.* 28 (±8.7), OR=1.048, CI=1.006-1.091, *P*=0.025) and had lower levels of baseline inflammatory biomarkers, consistent with previous studies regarding clinical predictors of treatment response in AS [133,135].

Our study showed that polymorphism rs917997 risk allele A, in the *IL18RAP* gene, was the strongest predictor of non-response to biological treatment. *IL18RAP* gene encodes the ß chain of the heterodimeric receptor for IL-18 (IL18Rß) and it is responsible for signal transduction in response to IL-18. IL-18 is one of the most effective cytokines in regulating NK cell activity and Th1-mediated immune responses and, therefore, defense against infection with intracellular microbes through the induction of IFN-gamma[140]. Defective IL-18 receptor function has been reported in patients with systemic-onset juvenile idiopathic arthritis together with a nonfunctional IL-18/NK cell axis [141]. Allele A in rs917997 was strongly associated with celiac disease susceptibility and it has also shown association with inflammatory bowel disease[142,143] . Risk allele A in rs917997 correlated with lower levels of mRNA IL18RAP expression, suggesting that individuals with risk allele A have reduced IL18Rß, leading to low IFN-gamma secretion [142]. Our study revealed a statistically significant likelihood of non-response to anti TNF-alpha treatment for rs917997 risk allele A in *IL18RAP* gene (OR= 3.35 [1.38- 8.15], *P*=0.007), suggesting a link between non-response to anti TNF-alpha agents and impairment in the efficacy of IL-18 signaling that may generate low production of IFN- gamma and TNF-alpha. Thus anti-TNf-alpha agents would lack their specific ligand.

Moreover, our findings showed that a polymorphism in the *MIF* gene, located on chromosome 22q11.2 contributes to the absence of response to treatment. MIF cytokine is released by many immunologic effectors cells after exposure to microbial products and pro-inflammatory cytokines and promotes the production of pro-inflammatory mediators, including TNF-alpha. The minor allele, allele G, of the SNP rs755622 in the *MIF* gene predicts non-response to treatment in our study (OR= 3.14 [1.19- 8.22], *P*=0.019). This SNP located in the gene's promoter region, was previously reported to be associated with treatment response. Specifically, rs755622 risk allele G has been previously associated with relapse to local steroid treatment in

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psoriasis arthritis patients[144]. Studies in RA patients demonstrated that carriers of the minor allele of rs755622 have higher levels of circulating MIF and higher levels of radiological joint damage[145]. Thus, we may speculate that in AS patients, rs755622 risk allele G reflects a more active disease associated with higher inflammatory activity because of increased MIF levels.

Previous studies demonstrated that polymorphism rs1800896 located on chromosome 1q31-q32, within the *IL10 gene* promoter region, influences IL-10 cytokine plasma levels, which were significantly higher in patients homozygous for the G allele[146]. IL-10 was demonstrated to inhibit the production of inflammatory mediators, and can be considered as a natural immunosuppressant of TNF-α[147]. Low IL-10 producer genotype (AA) in RA patients was found to predispose to development of anticyclic citrullinated peptide antibodies (anti-CCP) positivity RA disease with reduced response to prednisone treatment[148]. Our study identified that AS patients carrying the risk allele A of rs1800896, were non-responders to anti TNF-alpha treatment (OR= 3.09 [1.04- 9.15], *P*=0.041), sustaining the association of low level producer IL-10 genotype with the risk of lack of response to treatment.

The actions of TNF-alpha are mediated by means of binding to two distinct cell surface receptors, namely tumor necrosis factor receptor I (TNF-RI) and II (TNF-RII, also known as TNFRSF1B or p75). The two receptors appear to promote distinct TNF-alpha-induced cellular responses, although both are capable of inducing the nuclear factor-κB (NF-κB) pathway. In addition to membrane bound forms, both TNF receptors can exist as soluble proteins (sTNFRs) and can act as natural inhibitors of TNF-alpha by preventing soluble TNF-alpha from binding to membrane-bound TNF receptors. TNF-RII is necessary for antigen-driven differentiation and survival of T-cells, being an important co-stimulator for T cell activation and for optimal IL-2 and IFN-gamma induction[149]. The levels of both soluble and membrane-bound TNF-RII were high in patients with RA[150,151]. In our analysis we identified the SNP rs1061622 in the *TNFRSF1B (TNFRII)* gene, located on chromosome 1p36, which encodes the p75 receptor, strongly influenced on treatment response status. In RA patients, it was shown that rs1061622 affected the level and functions of soluble TNF receptor p75 and, consequently, influenced the response to anti TNF-alpha treatment[152,153]. Patients with *TNF-RII* GG genotype at rs1061622 expressed the lowest sTNF-RII levels and exhibited a poorer response to anti TNF-alpha therapy[153,154]. In this study we identified the rs1061622 risk allele G to influence the of non-response likelihood to anti TNF-alpha treatment (OR= 2.46 [1.00- 6.04], *P*=0.048), consistent with the previous results in RA patients.

As with other previous reports on the pharmacogenomics of response to anti TNF-alpha agents, our study is limited by a relatively small sample size lack and

by a lack of an independent cohort to validate the observed genetic associations with biologic treatment non-response. We chose to present our results based on BASDAI, as this represents the main tool in daily clinical practice to assess disease activity. Nevertheless, parameters that measure the disease's inflammatory activity more objectively may provide a more accurate means of assessing treatment response. In this context, the ASDAS scoring system has been shown to be a more powerful tool than BASDAI 50 in patients with high CRP and may reflect the inflammatory process and the efficacy of biological treatment better [155,156]. Future studies will need to investigate whether ASDAS cut-offs facilitate the identification of the same genetic markers associated with lack of response to biological treatment.

5.6. Conclusions

1. Consistent with previously published data, our study found that the percentage of patients achieving BASDAI 50 clinical response after 12 months of follow-up was only 56.2%.

2. We found no statistically significant differences between baseline characteristics (clinical and demographic) of the two groups of treatement response (responders vs. non-responders). Distribution of *HLA B27* gene in the two groups showed no statistically significant difference.

3. Although the contribution of genetic factors to the lack of response to anti-TNF-alpha therapy in AS is not yet well defined, our results suggest that there is a specific genetic profile for anti-TNF-alpha in which treatment is ineffective and the validation of the genetic model of inefficiency to first anti-TNF alpha agent would be turning point in facilitating individualized treatment.

4. In this study we analyzed a total of 384 SNPs to identify predictive factors of lack of response to biological treatment, studied in a number of 121 patients diagnosed with AS. We identified among the candiadate polymorphisms, 5 polymorphisms located in five different genes as prognostic factors for the lack of clinical response to first biological agent in patients with AS as follows: rs755622 in the *macrophage migration inhibitory factor (MIF)* gene, rs917997 in the *interleukin 18 receptor accessory protein (IL18RAP) gene*, rs1800896 in the *IL10 gene,* rs1061622 in the *tumor necrosis factor receptor superfamily, member 1B (TNFRSFB1)* and *rs3740691* in *ADP-ribosylation factor GTPase activating protein 2* (*ARFGAP2) gene.*

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5. In our study of assessing efficacy to the first anti-TNF treatment in AS patients using a candidate SNPs approach, we developed a genetic model of non-response. Modelul genetic de non-răspuns la administrarea primului agent anti TNf-alfa obținut este un model robust, având puterea predictivă ridicată, demonstrată de aria de sub curba ROC de 0.77 (95% IC: 0.68 - 0.86).

In conclusion, the contribution of genetic factors accounting for treatment response to anti TNF-alpha agents is not yet well known. Our findings suggest that there are certain genetic profiles for which TNF-alpha blockers are ineffective and validation of a genetic model that predict response status to the first anti TNF-alpha treatment would be a changing point in facilitating individualized therapy. After AS patients fail their first TNF-alpha inhibitor, physicians are faced with trying a second TNF-alpha inhibitor as, so far, they represent the only effective biological treatment for AS[157,158]. It would be interesting to study in the next step whether polymorphisms associated with non-response to the 2nd and/or the 3rd TNF-alpha inhibitor are the same genetic markers responsible for non-response to the 1st inhibitor. The ability to determine whether a patient is genetically a non-responder to a TNF-alpha inhibitor despite good clinical prognostic factors would make these treatment decisions more rational.

Replication of these genetic biomarkers in other independent and much larger cohorts would be of major value to confirm the robustness of the results, especially because TNF-alpha blockers are the only currently available biological therapy for treating AS.

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6. General discussion

The only classes of drugs that have been proven effective in controlling disease activity in AS are the NSAIDs and anti-TNF-alpha biological agents. It is not yet well documented the link between the control of the inflammatory process and disease structural progression in the patients. MRI studies have identified the development of syndesmophytes in the sites of spine where the inflammatory process was controlled by medication.[159]. Moreover, according to data recently published syndesmophytes seem to occur even in the vertebrae in that inflammatory changes. were not previously proven[160]. The administration of biological agents in early stages, pre-radiographic form, showed that in the absence of structural damage, anti-TNF-alpha biologic therapy inhibits radiographic progression of the disease towards full blown AS[161]. Thus, long-term studies are required to investigate whether early administration of anti TNF-alpha agents can influnce the development of structural damage.

The identificationof the patients at increased risk to develop severe functional impairment would be of major importance for clinicians to choose appropriate treatment strategy for each patient. In addition, in a percentage of 30-40% of patients sustained treatment with anti-TNF-alpha agents can not control disease activity despite long term adiministration, in which genetic component could be the responsible factor for this problem. Thus, valid and reproducible prognostic markers that can be used form early stages of the disease still need to be identified. Although many studies have identified clinical and demographic predictors of treatment response to date, few publications have exploited genetic prognostic factors. Hence, the objective prognostic of genetic data of severe impairment in physical function and lack of response to biological treatment can be used for counseling patients about their disease and its evolutionary course.

Remains to be investigated whether genetic polymorphisms may constitute such markers that can reliably predict which patients will respond to biological treatment and who will be a non-responders or which patients will encounter marked disease severity. For these analysis of large cohorts of patients is required, which is possible today because of the existence of disease registers. Future genetic studies will need to carefully select the features that could introduce heterogeneity in the analysis of genetic data.

Also, a future genetic study of response to treatment should use a more powerful tool in monitoring therapeutic response and disease activity, as the new activity score, ASDAS, which is not yet widely used in daily practice. For statistical

analysis of raw genetic data there can be use a more robust statistical tests. We conducted random allelic permutation tests (1000 permutation test). We have not used the Bonferroni adjustment, as it is sweetening conservative, particularly in the way in which is testing the null hypothesis. Acorrdingly, all null assumptions must be true at the same time. In our case, given the small number of patients it would have been difficult to apply it.

The groups of patients analyzed in these three studies were, however, consistent in terms of clinical and demographic characteristics, with a balanced distribution of *HLA B27* antigene, which gave power to the statistical analysis. One aspect, however, is required to be mentioned in connection with the cohort of patients studied, namely the length of the disease evolution since first symptoms, on average over 20 years, in the patients included in REGISPONSER register which could affect the interpretation of treatment response.

Defying a genetic test for identify AS patients who will not respond to anti TNF-alpha treatment would be huge help both in terms of pharmaco-economics and pharmaco-vigilance.

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7. General conclusions (synthesis)

At the end of the 3 years of the study, the group of patients treated with the biological agents had a slower progression rate, without statistical significance, both in the spine and in total radiological damage.

Patients treated with NSAIDs recorded at the end of the 3 years of follow-up, statistically significant radiological progression both in the spine and in the total radiological damage.

The covariance analysis performed showed that radiological progression at the end of the 3 years of monitoring occurs regardless of the type of treatment administered NSAIDs or biological agents.

Our results showed that treatment with anti-TNF-alpha agents does not lead to a progression rate greater than treatment with NSAIDs.

We confirmed that baseline structural destruction and male gender are predictors of high radiographic progression in patients with AS.

The presence of *HLA B27* antigen was not associated with radiological progression rate.

The presence of *HLA-B27* was not associated with the type of response to anti-TNF-alpha biological treatment, thus confirming the data in the literature.

Our results confirm that the evolution toward severe functional status in AS is associated with both clinical and genetic factors.

We report that advanced age at onset, neck pain, polymorphisms *rs2542151 (PTPN2)* and *rs2254441 (PSTPIP1)* are potential predictors of progression to severe functional impairment in patients with AS.

Consistent with previously published data, our study found that the percentage of patients achieving clinical criteria BASDAI 50 responders after 12 months of follow-up was only 56.2%.

We identified five polymorphisms located in 5 different genes as prognostic factors for the lack of clinical response to the first biological agent in AS patients: rs755622 in *macrophage migration inhibitory factor (MIF)* gene*,* rs917997 in *interleukin 18 accessory receptor protein (IL18RAP)*, rs1800896 in *interleukin 10 (IL10)* gene, rs1061622 in *tumor necrosis factor superfamily, 1B (TNFRSFB1)* gene and rs3740691 in *rs3740691* in *ADP-ribosylation factor GTPase activating protein 2 (ARFGAP2)* gene.

We obtained by combining the above 5 polymorphisms a robust genetic model of non-response to he first anti-TNF-alpha agent with high predictive power, as indicated by the area under the ROC curve of 0.77 (95% CI: 0.68 - 0.86).

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8. Originality and innovative contributions of the thesis

The originality of this research resides primarily in the multidisciplinary approach, with international support, of studing one controversial topic of current interest in assessing ankylosing spondylitis, analyzed boyh from the perspective of rheumatologist and clinical pharmacologist.

The studying of these themes emerged from the pre-doctoral internship conducted between the years 2007-2008 at the department of Rheumatology of "Reina Sofia" University Hospital in Cordoba, Spain, under the direction of Professor Collantes Estevez Eduardo, Md researcher of reference in spondyloarthropathies. Consequently, the study was conducted on patients belonging to the Spanish registry spondyloarthropathies, called REGISPONSER. Patients although consulted in daily practice are well characterized clinical, biological, genetic and therapeutic.

For genetic analysis Genomic DNA was isolated from saliva samples of patients with ankylosing spondylitis, in contrast to the majority genome studies that have used DNA from blood samples.

Another innovative aspect is that we performed the analysis of a large number of genetic polymorphisms (384 SNPs) in relation to functional prognosis and response to treatment of patients with ankylosing spondylitis. An original aspect consists in the selection of the genetic polymorphism analyzed which was performed after an extensive bibliographic research which included all polymorphisms described to date to be associated with SpA. Furthermore, SNPs genotyping was performed using Ilumina platform. Illumina GoldenGate genotyping platform (Illumina, Inc., San Diego, CA, USA) is a flexible, pre-optimized system which is using a discriminatory DNA polymerase and ligase to analyze simultaneously 96 or 384-3072, SNPs loci. The protocol can be performed manually or automatically.

In addition, specially designed software to analyze genomic informatione, SVS software v 7.3.1 (Golden Helix Inc., Bozeman, Montana, USA) was used, for proper analysis of polymorphisms. There is a package included in the system for analyzing SNPs which offers an adequate testing tool for genotypic association. These tests can be performed simultaneously with the application of multiple testing corrections (permutation tests). Genetic models include allelic analysis tests, genotypic tests and additive genetic model, dominant and recessive. SVS Package 7.3.1 SNPs analysis incorporates the appropriate technologies for performing regressions, linear and

logistic regression, with covariates both SNPs and other types of covariates. This program also allows the exploration of linkage disequilibrium.

Another innovative aspect is the study of the effectivness of treatment on radiological progression during a long period of time (3 years), unlike previous studies in which the duration of follow-up of patients did not exceed, in the many cases, two years. Until now, we only have data that compared biologic treatment to historical cohorts and the important difference to all other existing data up to now is that, we studied treatment effectiveness on daily patients. We performed a pioneering comparative analysis of the two classes of drugs accepted for the treatment of ankylosing spondylitis, non-steroidal anti-inflammatory drugs and anti-TNF-alpha agents in relation to their effect on radiological progression of the disease. Thus, we demonstrated for the first time that treatment with biological agents does not accelerate the rate of radiographic progression compared with NSAIDs.

Our analysis in which it was demonstrated the involvement of both clinical factors and SNPs in the prognosis of functional status of the patients with ankylosing spondylitis was published in an ISI journal.

It is necessary to mention as innovative aspect the developing of the first large genetic study in which we showed the involvement of genetic polymorphisms in the lack of response to biological treatment. Thus, we identified SNPs predictive of non-response to anti-TNF-alpha therapy among genes that can alter the mechanism of action of biological agents. An important aspect was the achieving a genetic model with high predictive power of non-response to the first biological agent in patients with ankylosing spondylitis.

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