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# Humoral Immune Response to *Salmonella* Antigens and Polymorphisms in Receptors for the Fc of IgG in Patients with Ankylosing Spondylitis

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## 1. Introduction

Ankylosing Spondylitis (AS) is the prototype of an interrelated group of rheumatic diseases now named spondyloarthritides (SpA), otherwise known as spondyloarthropathies. Clinical features of this disease include inflammatory back pain, asymmetrical peripheral oligoarthritis, enthesitis, and specific organ involvement, such as anterior uveitis, psoriasis and chronic inflammatory bowel disease (Braun & Sieper, 2007).

AS is a chronic inflammatory disease primarily affecting the spine. Its major clinical features include sacroiliitis, loss of spinal mobility and spinal inflammation. The chronic inflammation leads to fibrosis and ossification, where bridging spurs of bone known as syndesmophytes form, especially at the edges of the inter-vertebral discs, thus producing the ankylosing (Ebringer & Wilson, 2000).

AS is a disease that affects more men than women, with a ratio of 2:1 (Feldtkeller *et al.*, 2003). The prevalence of the disease is between 0.1 and 1.4 % in general population. Studies conducted in different countries have shown that the incidence of AS is between 0.5 and 14 per 100,000 people per year (Braun & Sieper, 2007).

The diagnosis of AS is based more on clinical features than laboratory tests. Table 1 shows the criteria for diagnosing AS, according to the modified New York criteria (van der Linden *et al.*, 1984). Further, in 1990, Amor and colleagues proposed the first set of classification criteria for the entire group of spondyloarthritis, allowing a patient to be classified as having spondyloarthritis whatever the presenting symptoms (Amor *et al.*, 1990). A different set of criteria for the entire group of spondyloarthritis was developed by the European Spondyloarthropathy Study Group (Dougados *et al.*, 1991), with inflammatory back pain and

peripheral arthritis as major entry criteria. Recognition of the drawbacks of criteria focused on a specific subtype, the Assessment of Spondyloarthritis International Society did a large cross-sectional study to propose new criteria on the basis of the two main clinical features identified in daily practice – eg, axial symptoms and peripheral involvement (Dougados & Baeten, 2011).

Clinical criteria	
-	Lower back pain and stiffness for longer than 3 months, which improve with exercise but are not relieved by rest
-	Restriction of the movement of the lumbar spine in both the sagittal and frontal planes
-	Restriction of chest expansion relative to normal values correlated with age and sex
Radiological criterion	
-	Sacroiliitis grade $\geq 2$ bilaterally, or grade 3-4 unilaterally
Definite ankylosing spondylitis is present if the radiological criterion is associated with at least one clinical criterion	

Table 1. Modified New York criteria, 1984, for ankylosing spondylitis (Braun & Sieper, 2007).

<p>Sacroiliitis on imaging* plus one or more features of spondyloarthritis†</p> <p>Or</p> <p>HLA-B27 plus two or more other features of spondyloarthritis†</p>
<p>*Active (acute) inflammation on MRI highly suggestive of sacroiliitis associated with spondyloarthritis or definite radiographic sacroiliitis according to modified New York criteria.</p> <p>†Inflammatory back pain, arthritis, enthesitis (heel), uveitis, dactylitis, psoriasis, Crohn's disease or ulcerative colitis, good response to non-steroidal anti-inflammatory drugs, family history for spondyloarthritis, HLA-B27, or elevated C-reactive protein (a spondyloarthritis feature in the context of chronic back pain).</p>

Table 2. Assessment of Spondyloarthritis International Society (ASAS) classification criteria for axial spondyloarthritis in patients with back pain for 3 months or more and age at onset younger than 45 years (Dougados & Baeten, 2011).

Although AS is of unknown aetiology, it is considered an autoimmune disease in which environmental and genetic factors are involved. There is a strong association with HLA-B27, as approximately 95% of AS patients are positive for this antigen. However, this association does not explain the cause of the disease. It has been reported that the risk of developing AS is about 5% for HLA-B27-positive subjects, but substantially higher for HLA-B27-positive relatives. However, most HLA-B27-positive individuals remain healthy. The HLA-B27 subtypes most clearly associated with AS are HLA-B\*2705 B\*2702, B\*2704 and B\*2707. The HLA-B\*2706 and B\*2709 subtypes do not appear to be associated with AS (Reveille & Arnett, 2005).

In addition, evidence of the importance of HLA-B27-bacteria interaction comes from work in animals, where HLA-B27-transgenic rats developed SpA-like features, but many transgene copies are needed to transfer the disease. Environmental factors also play a role, since HLA-B27-transgenic rats bred in a germ-free environment do not develop the disease, though gut flora contribute to the development of colitis (Braun & Sieper, 2007).

On the other hand, about 10-20% of HLA-B27-positive patients with reactive arthritis develop AS after 10-20 years. A possible central role of bacteria in the pathogenesis of SpA is further supported by the relation between Crohn's disease, HLA-B27 positivity, and ankylosing spondylitis, as 54% of HLA-B27-positive patients with Crohn's disease develop AS, but only 2.6% of HLA-B27-negative patients develop this disease. Leakage of the gut mucosa, as a result of the inflammation caused by colitis such as that found in Crohn's disease, leads to an interaction of the immune system with gut bacteria. In about 50% of patients with AS, chronic macroscopic or microscopic mucosal lesions resembling Crohn's disease have been detected in the gut mucosa (Braun & Sieper, 2007).

In this study, we provide support for the hypothesis of the interaction between an environmental factor (a bacterial antigen) and a genetic factor (a receptor for the Fc fragment of IgG).

## 2. Environmental factors involved in the pathogenesis of ankylosing spondylitis

It has been postulated that infectious agents play a crucial role as triggering factors for some autoimmune diseases, such as rheumatoid arthritis and ankylosing spondylitis. However, the mechanisms by which these microbial antigens become involved in the aetiopathogenesis of the disease remain unknown, though abundant data suggest this possibility.

Humoral immune responses against bacteria such as *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Shigella flexneri*, *Yersinia enterocolitica* and *Campylobacter jejuni* have been analyzed in patients with SpA, and it has been suggested that some microbial agents have a role in the disease.

*Klebsiella pneumoniae* has been considered the main microbial agent implicated as a triggering factor for the aetiopathogenesis of AS (Rashid & Ebringer, 2007). It has been reported that IgA antibodies to *Klebsiella pneumoniae* are significantly elevated in AS patients compared to healthy subjects (Blankenberg-Sprenkels *et al.*, 1998; Tani *et al.*, 1997). Moreover, an association between the heat shock protein (HSP) of 60 kDa from *Klebsiella pneumoniae* and AS has been evidenced because of the significantly higher levels of IgG antibodies in proportion to this protein observed in AS patients compared to control groups (Cancino-Diaz *et al.*, 1998; Parra-Campos *et al.*, 1996), while the cellular immune response, measured as lymphoproliferation (LP) against this protein, has also been reported (Dominguez-Lopez *et al.*, 2000).

Other HSPs have been associated with HLA-B27-positive subjects, because of the higher levels of IgG antibodies observed, compared to HLA-B27-negative subjects, in particular HSP60 from *Klebsiella pneumoniae* and *Salmonella typhi* (Dominguez-Lopez *et al.*, 2002).

The antibody response against the lipopolisaccharide (LPS) of *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhimurium* and *Salmonella enteritidis* has been evaluated by

ELISA, indicating that only the LPS from *Klebsiella pneumoniae* and *Escherichia coli* are associated with AS, on account of the higher levels of IgG and IgA antibodies observed (Ahmadi *et al.*, 1998).

The association of *Salmonella spp.* with AS is supported by the presence of DNA from *Salmonella sp.* in the synovial fluid of patients with SpA (Pacheco-Tena *et al.*, 2001). On the other hand, the behaviour of *Salmonella typhimurium* is modified by the presence of HLA-B27 in transfected cells, because of the increased production of IL-6, IL-8 and IL-10 and the lower production of TNF $\alpha$  (Ekman *et al.*, 2002; Saarinen *et al.*, 2002).

In our laboratory, we have previously found that 71.4% of patients with AS and 14.3% of healthy subjects recognized a 30 kDa band (p30) from of *S. typhimurium* (p<0.001) by using anti-human IgG in a western-blot analysis. Moreover, the levels of IgA and IgG against a crude extract of *S. typhimurium* were significantly higher in AS patients than in healthy subjects, though no differences in IgM levels were found. When the antibody levels against electroeluted p30 were analyzed, we found that IgG and IgA against p30 were statistically higher in AS patients than in healthy subjects; however, as in the case of the response to the crude extract of *S. typhimurium*, the absorbance obtained in IgM to this antigen showed no significant differences between groups (Zambrano-Zaragoza *et al.*, 2009).

In the sera from AS patients and controls, all four IgG subclasses were found to be involved in the recognition of the p30 from *S. typhimurium*, but the frequency of IgG3 antibodies to p30 was statistically different in AS patients compared to healthy subjects (Zambrano-Zaragoza *et al.*, 2009).

These results showed that a 30 kDa band from *S. typhimurium* is recognized by the IgG antibodies of most AS patients, compared to healthy subjects, and suggest an association between a particular antigen of *S. typhimurium* (p30) and the disease.

An association of *S. typhimurium* with AS has been reported previously (Brown & Wordsworth, 1997; Leirisalo-Repo *et al.*, 2003), but no specific antigen of this bacterium has been reported until now. Nevertheless, in association with other bacterial antigens, certain proteins have been reported to be implicated as triggers of AS (Lahesmaa *et al.*, 1991). Thus, we have reported that the 30 kDa band from *S. typhimurium* could be differentially recognized by the immune response in AS patients, and hence be involved in the immunopathogenesis of AS (Zambrano-Zaragoza *et al.*, 2009). These findings led us to ask whether antigens from *S. enteritidis* are recognized in the same way by patients with AS, or if this response is specific for *S. typhimurium*.

The interaction between HLA-B27 and *S. enteritidis* has been reported by using mouse fibroblasts transfected with HLA-B27, HLA-B7, or beta2-microglobulin only. Although *S. enteritidis* invaded all three of these transfected cells with the same efficiency, more living intracellular *Salmonella* organisms were found in the HLA-B27 transfectants than in the other transfected cell lines, suggesting that the bactericidal effect is impaired in these cells. Moreover, impaired NO production in HLA-B27-transfected cells was indicated as a possible mechanism (Virtala *et al.*, 1997).

Another study, one using transfected human monocytic U937 cell lines, demonstrated that the expression of the HLA-B27 antigen does not influence the uptake of *S. enteritidis* into U937 cells in vitro. It is interesting to note that HLA-B27 markedly impaired the elimination of *S. enteritidis* in the HLA-B27-transfected U937 cells (Laitio *et al.*, 1997).



Considering that *Salmonella typhimurium* is in fact *Salmonella enterica* serovar *typhimurium*, we asked if the antigen recognized by patients with AS (p30) is an antigen specific to *Salmonella typhimurium*, or if it can be found in another serovar of *Salmonella enterica*.

To answer this question, a group of 28 patients with AS treated with non-steroidal anti-inflammatory drugs and sulfasalazine, but without receiving tumour necrosis alpha blockers, and 28 non-AS-related healthy subjects were included to analyze the IgG and IgA humoral immune response against *Salmonella enterica* serovar *enteritidis* (*S. enteritidis*) by western-blot, using similar conditions, and protocols previously reported by us (Zambrano-Zaragoza *et al.*, 2009).

Our results show that 14/28 AS patients recognized a band with a relative molecular mass of 10 kDa (p10) from *S. enteritidis* with IgG antibodies, but that none of the subjects in the healthy group did ( $p < 0.001$ , Figure 1). However, no differences in the recognition of *S. enteritidis* antigens were found when IgA antibodies were detected (Figure 2); suggesting that the antigenic behaviour of *S. enteritidis* and *S. typhimurium* are different, and only some antigens from each serovar could be important for the etiopathogenesis of AS. Additionally, it appears that only IgG antibodies anti- *S. enteritidis* could be important in the association with AS. These results are in agreement with Ahmadi *et al.*, who did not find differences in the antibody levels against *S. enteritidis* in patients with AS (Ahmadi *et al.*, 1998).

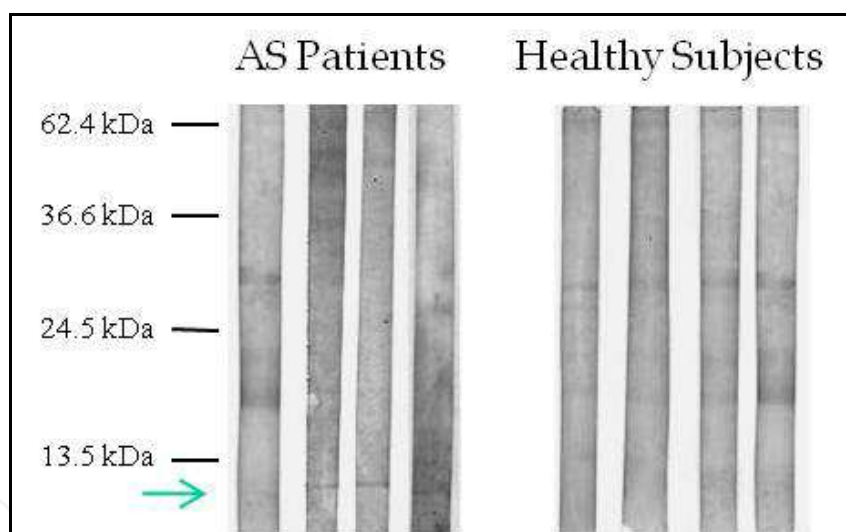


Fig. 1. Representative Western-blot strips showing antigens of *S. enteritidis* recognized by IgG antibodies in AS patients and healthy subjects. The bars on the left indicate the molecular masses of standard markers (kDa); the arrow indicates the recognized 10 kDa band.

In spite of the controversial role of bacterial antigens in the etiopathogenesis of AS, specifically the Gram negative bacterial antigens, we found a second candidate to be associated with AS, the p10 that could be a relevant antigen for patients with AS. Moreover, we also found that the humoral immune response to *S. typhimurium* is different to those for *S. enteritidis*, the antigens recognized and the antibody isotypes produced against those bacteria are different, because neither the 30 kDa band from *S. enteritidis* nor the 10 kDa band from *S. typhimurium* were recognized.

Considering that western blot is not a quantitative test, as it shows only the frequencies of recognition of some antigens, so a quantitative test must be used to determine the possible association of this antigen (p10) with AS.

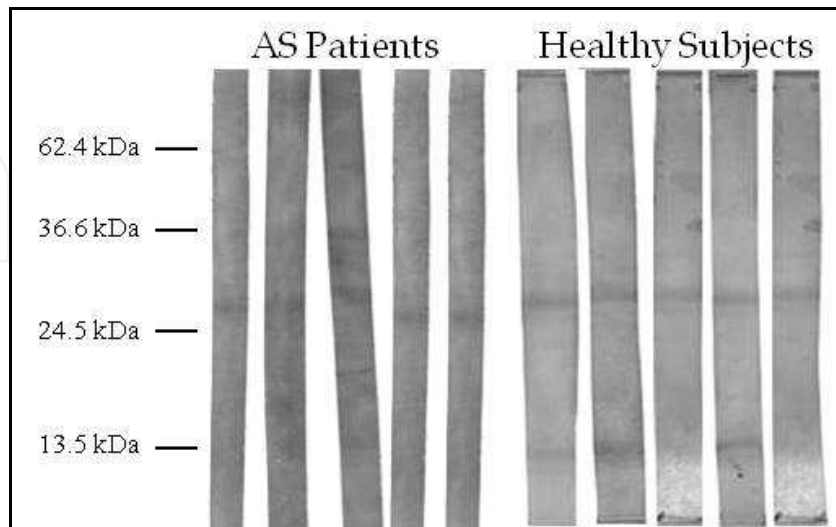


Fig. 2. Representative Western-blot strips showing antigens from *S. enteritidis* recognized by IgA antibodies in AS patients and healthy subjects. The bars on the left indicate the molecular masses of standard markers (kDa).

To do this, we analyzed the IgG and IgA antibody levels against either a crude extract or the electroeluted 10 kDa band (p10) from *S. enteritidis* using ELISA, as we have done for *S. typhimurium* (Zambrano-Zaragoza *et al.*, 2009).

We did not find any statistically significant differences in the antibody levels against either the crude extract or p10 (Figures 3 and 4), indicating that neither the total antigens nor the p10 of *S. enteritidis* are associated with AS.

Although different bacteria have been associated as possible triggers of AS, in this study we argue that the antigenic differences present in related bacteria, such as *S. typhimurium* and *S. enteritidis*, could be differentiated by the immune response of patients with AS, and thus be involved in the aetiopathogenesis of the disease.

These results indicate that not all species of *Salmonella* are associated with the disease. As has been reported, the HSP60 of *S. typhi* (Dominguez-Lopez *et al.*, 2009), and *S. typhimurium*, in particular, the p30 (Zambrano-Zaragoza *et al.*, 2009), are indeed associated with the illness. However, the relationship between the antibody levels observed and the mechanisms involved in the pathogenesis of AS has not yet been elucidated.

Considering that the differences in the IgG immune response observed could be due to a IgG subclass, we explored the IgG subclass that recognizes the p30 of *S. typhimurium* and found that patients with AS produce more IgG3 than healthy subjects (Zambrano-Zaragoza *et al.*, 2009), which suggests that the humoral immune response and, in particular, the IgG3 antibody levels, could play a role in the pathogenesis of AS.

IgG subclasses have been shown to be involved in autoimmunity, because of differences in the expression of Fc gamma receptors that explain the clearance of immune complexes from

the body, and its role in the inflammatory response observed. We found that in both patients and controls recognition of the p30 of *S. typhimurium* by serum antibodies was due to all of the IgG subclasses, but that the frequency and levels of IgG3 antibodies against p30 were higher in the AS group.

These results suggest that in the humoral immune response against a microbial antigen (p30) in a susceptible individual, the IgG3 antibodies produced against this protein could be involved in the pathogenesis of AS, and that the relationship between the humoral immune response observed and the inflammatory process could be explained by differences in the expression of specific polymorphisms of receptors for the Fc of IgG. However, this suggestion must be explored much more thoroughly. In this case, the p30 antigen could cross-react with a putative auto-antigen, and the resulting antibody response could be responsible for maintaining the inflammatory process, probably through the receptors for the Fc of IgG.

Host genetics are better understood in AS compared to other types of SpAs. The strong link between AS and HLA-B27 has been known for years; the HLA-B27 is the major risk factor associated with AS, this molecule is present in many genetically diverse population, however other genes than HLA-B27 have been analyzed and associate with AS, such as endoplasmic reticulum endopeptidase I (ERAP1), interleukin 23 receptor (IL23R), and tumour necrosis factor receptor 1 (TNFR1) (Dougados & Baeten, 2011). Perhaps the definitive missing link lies in the recently discovered genetic contributions of AS and how these genes might co-localise with HLA-B27 in the presence of certain stool microflora (Carter, 2010).

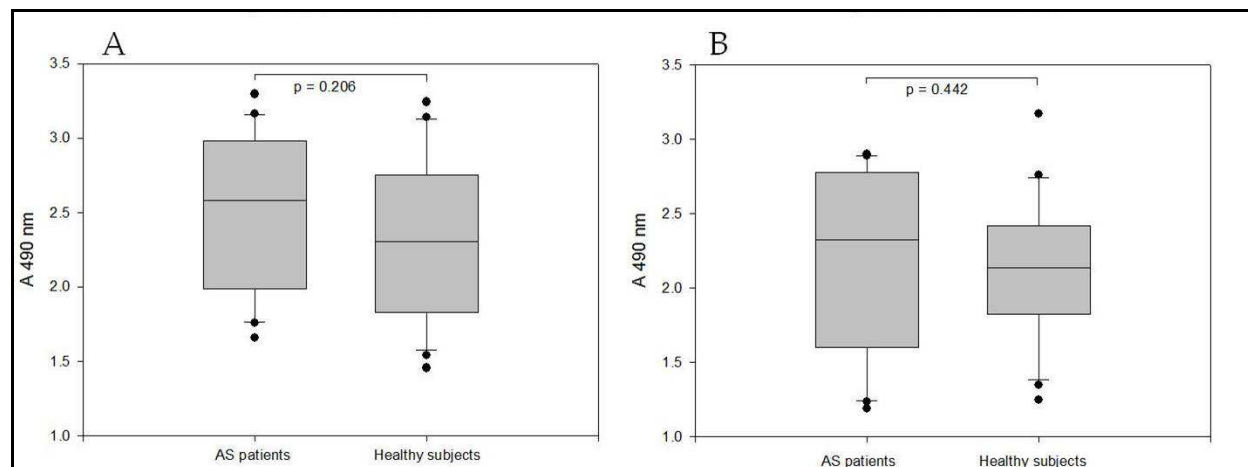


Fig. 3. Antibody levels against a crude extract of *S. enteritidis* in AS patients and healthy subjects. Levels of IgG (panel A) and IgA (panel B). The graph shows the median A490nm value for each group and the percentile at 25 and 75%. Non-statistically significant differences were found in both studies.

### 3. Receptors for the Fc of IgG

Specific receptors for most immunoglobulin isotypes have been described. IgG represents the dominant antibody in plasma, while the receptors for the Fc of IgG (FcγR) play important roles in the initiation and regulation of many immunological and inflammatory



processes, thus providing a crucial link between humoral and cellular immune responses. Ligation of these receptors triggers a variety of signals to develop effectors of the immune response, such as macrophage phagocytosis, antibody dependent cellular cytotoxicity (ADCC), neutrophil activation, cytokine release, degranulation and the inhibition of B cell activation (Dijstelbloem *et al.*, 2001; Ravetch & Bolland, 2001; van der Pol & van de Winkel, 1998).

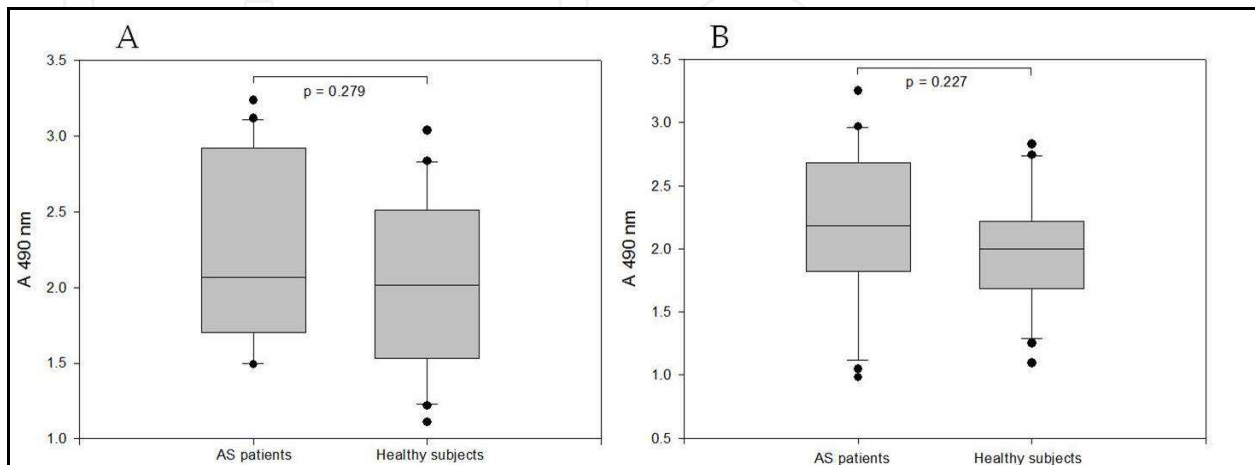


Fig. 4. Antibody levels against the p10 of *S. enteritidis* in AS patients and healthy subjects. Levels of IgG (panel A) and IgA (panel B). Antibodies determined by ELISA, assayed with 0.3  $\mu$ g of antigen/well, and sera diluted to 1/400 in triplicate. The graph shows the median A490nm value for each group and the percentile at 25 and 75%. Non-statistically significant differences were found in both studies.

Three classes of Fc $\gamma$ R have been reported on the surface of immune system cells Fc $\gamma$ RI (CD64) that can bind to monomeric IgG, Fc $\gamma$ RII (CD32) and Fc $\gamma$ RIII (CD16), which then bind to immune-complexes.

These receptors can be divided into two types: activating (Fc $\gamma$ RI, Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa), characterized by the presence of an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain; and inhibiting (Fc $\gamma$ RIIb), which contain an immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic domain (Dijstelbloem *et al.*, 2001).

Most cell types express both activating and inhibitory receptors. Therefore the cellular response depends on the relative expression of activating and inhibitory receptors. This ratio is also influenced by the cytokine environment (Cohen-Solal *et al.*, 2004). Moreover, the presence of allelic variants, with different affinities for selective the antibody isotypes support their role in the regulation of the inflammatory process due to the humoral immune response.

Three polymorphisms in the Fc $\gamma$ R genes that affect the IgG binding affinities have been described: first, a G>A point mutation in Fc $\gamma$ RIIa (rs1801274) that causes an H-131-R substitution; second, a T>G SNP at nucleotide 559 (rs396991) in Fc $\gamma$ RIIIa that results in a V-158-F substitution; and, third, two combinations of five nucleotides (141, 147, 227, 277 and 349) in the exon 3 of IIIb that encode two isophorms, called Fc $\gamma$ RIIIb-NA1 and Fc $\gamma$ RIIIb-NA2 (Willcocks *et al.*, 2009).

These haplotypes have distinct affinities for the different IgG subclasses. In this context, FcγRIIa-131H has a higher affinity for human IgG3 than FcγRIIa-131R. FcγRIIIa-158V has a higher affinity for IgG1 and IgG3 than FcγRIIIa-158-F, and FcγRIIIb-NA1 internalizes human IgG1- or IgG3-opsonized particles more efficiently than FcγRIIIb-NA2 (Dijstelbloem *et al.*, 2001).

Interest in the FcγR in the context of autoimmunity is rooted in mechanisms of immune complex handling (Salmon & Pricop, 2001), although these molecules participate in a much broader range of cell functions. In the case of rheumatic diseases such as AS, the antibody immune response has been described in several studies. However, the link between these antibodies and the pathogenesis of the diseases is still unclear.

The FcγR offer a link between the humoral and inflammatory responses because of their differences in affinity for the different IgG subclasses and cellular distribution. Hence, the hypothesis is that if AS patients develop an immune response against bacterial antigens that depends predominantly on IgG3 antibodies, as we have reported previously (Zambrano-Zaragoza *et al.*, 2009), then these antibodies will bind to a haplotype of FcγR to promote inflammation.

### 3.1.1 Polymorphisms in FcγR and autoimmunity

FcγR have been implicated in the pathogenesis of autoimmunity in different ways: 1) by maintaining the level of self-tolerance and increasing the activation threshold of autoreactive B cells; 2) by facilitating the elimination of some autoreactive B cells during development; 3) FcγR ligation can affect dendritic cell maturation; and, 4) FcγR are critical for the elimination of circulating IgG immune complexes (Willcocks *et al.*, 2009).

On the other hand, an allelic variant of FcγR has been reported to be present in normal populations, but without exercising any impact on the normal functions of the immune system. However, some isophorms present in certain environmental and genetic contexts can contribute to the development of autoimmune diseases.

Genetic variants of FcγR have been associated with different autoimmune and infectious diseases. Evidence supporting the role of the heterogeneity of FcγR in Systemic Lupus Erythematosus (SLE) has been controversial. SLE is the prototype of immune-complex-mediated autoimmune diseases and several studies on associations with FcγRIIa and FcγRIIIb have been published (Hong *et al.*, 2005; Michel *et al.*, 2000; Ni *et al.*, 2000).

In cases of myasthenia gravis, patients have been found to have a higher frequency of the FcγRIIIb-NA1 allele than FcγRIIb-NA2, compared to a control group (Raknes *et al.*, 1998).

In other autoimmune diseases, such as Wegener's granulomatosis, it has been reported that either FcγRIIa-131-RH or FcγRIIIa-158-VF represent an inheritable risk factor for the development of the disease (Dijstelbloem *et al.*, 1999), while other authors have reported an association with FcγRIIIb-NA1 (van der Pol & van de Winkel, 1998).

In the case of rheumatic autoimmune diseases, it has been reported that FcγRIIIa-158VV is overrepresented in RA patients, FcγRIIIa-158VF was higher in healthy controls, and FcγRIIIa-158-FF is equally distributed in both populations. However, no association between FcγRIIIa-158-VV and clinical parameters was found (Nieto *et al.*, 2000).

### 3.1.2 Polymorphisms in FcγR in ankylosing spondylitis

Considering our previous findings, in which the humoral immune response against *S. typhimurium* was directed principally against p30, and the IgG subclass involved was mainly IgG3, we hypothesized that the relationship between the humoral response observed and the pathogenesis of the disease could be due (at least in part) to FcγR polymorphisms, because of the differences in IgG subclass affinity reported.

To test this hypothesis, the genotypification of FcγRIIa, FcγRIIIa and FcγRIIIb was carried out in a small cohort of 35 patients with AS and 120 non-AS-related individuals using the primers reported (Table 3) in a PCR protocol.

Receptor	Name	Sequence	Ref
FcγRIIa	EC2-131R	5'-CCA GAA TGG AAA ATC CCA GAA ATT CTC TCG-3'	(Raknes <i>et al.</i> , 1998)
	EC2-131H	5'-CCA GAA TGG AAA ATC CCA GAA ATT CTC TCA-3'	
	TM1	5'-CCA TTG GTG AAG AGC TGC CCA TGC TGG GCA-3'	
FcγRIIIa	KIM-G(V)	5'-TCT CTG AAG ACA CAT TTC TAC TCC CTA C-3'	(Van Den Berg <i>et al.</i> , 2001)
	KIM-1(F)	5'-TCT CTG AAG ACA CAT TTC TAC TCC CTA A-3'	
	A013	5'-ATA TTT ACA GAA TGG CAC AGG-3'	
FcγRIIIb	NA1	5'-CAG TGG TTT CAC AAT GTG AA-3'	(Raknes <i>et al.</i> , 1998)
	NA2	5'-CAA TGG TAC AGC GTG CTT-3'	
	NA-REV	5'-ATG GAC TTC TAG CTG CAC-3'	

Table 3. PCR primers used for genotypification of FcγR.

As internal controls, primers to either an amplified 270 bp from the TCR Vα22 (Ctrl-1 and Ctrl-2) gene, or a 439 bp fragment of the human growth hormone gene (HGH-1 and HGH-2) were used (Table 4).

Name	Sequence	Ref
HGH-1	5'-CAG TGC CTT CCC AAC CAT TCC CTT A-3'	(Raknes <i>et al.</i> , 1998)
HGH-2	5'-ATC CAC TCA CGG ATT TCT GTT GIG TTT C-3'	
Ctrl-1	5'-GAT TCA GTG ACC CAG ATG GAA GGG-3'	
Ctrl-2	5'-AGC ACA GAA GTA CAC CGC TGA GTC-3'	

Table 4. PCR primers used as internal controls.

All participants were informed as to the nature of the study and written consent was obtained in accordance with the Helsinki Declaration. Blood samples were taken by venipuncture. The study design was previously approved by the local Ethics Committee.

Genomic DNA from each individual tested was extracted from whole peripheral blood using the easy DNA kit (Invitrogen). The FcγRIIa genotypes were determined using the amplification refractory mutation system-PCR (Raknes *et al.*, 1998). For each sample, two independent PCR reactions were carried out. PCRs were performed by adding 100 ng of genomic DNA, 200 μM of each dNTPs, 3 mM MgCl<sub>2</sub>, 20 ng of each control primer (Ctrl-1 and Ctrl-2), 200 ng of EC2-131-R or EC2-131-H primer and their respective reaction, and 2.0 U of *Taq* polymerase (Invitrogen) to a 50 μL solution containing PCR buffer 1X (Invitrogen). PCR conditions were as follows: Denaturation for 5 min at 94°C, 45 cycles of 94°C for 45

seconds, 63°C for 30 seconds and 72°C for 90 seconds; and a final extension step at 72°C for 10 min. A 980 bp fragment was observed in 2% agarose gels together with the 270 bp fragment of the internal control (TCR  $\alpha$ 22 gene). Figure 5 shows a typical reaction.

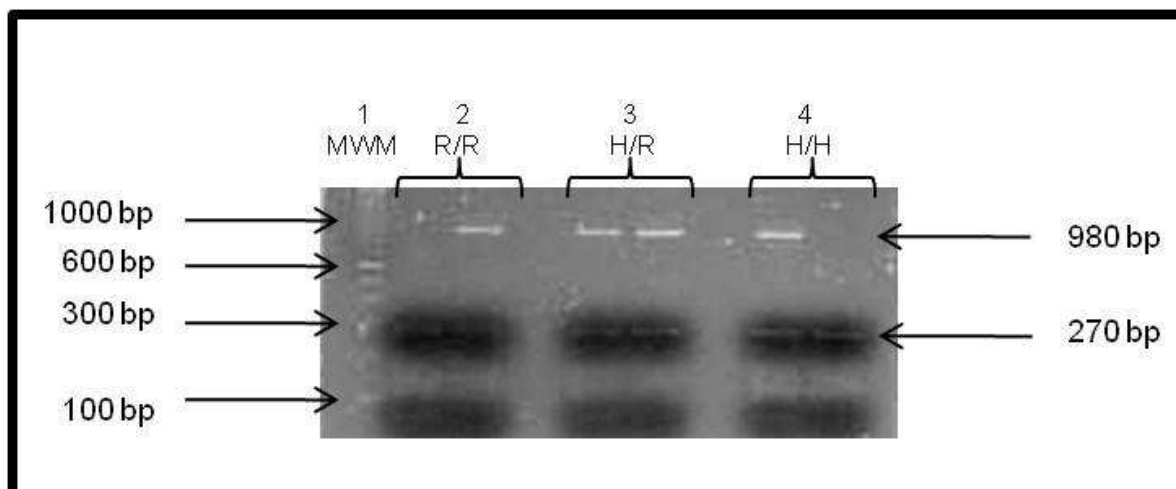


Fig. 5. A representative agarose gel (2%) of PCR products obtained from the amplification of Fc $\gamma$ RIIIa, showing the different genotypes: 1) molecular weight markers; 2) a homozygotic Fc $\gamma$ RIIIa-131RR subject; 3) a heterozygotic Fc $\gamma$ RIIIa-131HR subject; and, 4) a homozygotic Fc $\gamma$ RIIIa-131HH subject. In all cases, the 980 bp band corresponds to the fragment of Fc $\gamma$ RIIIa, and the 270 bp to the internal control.

In order to determine the Fc $\gamma$ RIIIa genotypes, two reactions were carried out for each sample, according to the method described by Van Der Berg *et al.*, (Van Den Berg *et al.*, 2001). PCRs were performed by adding, 100 ng of genomic DNA, 200  $\mu$ M of each dNTPs, 6 mM MgCl<sub>2</sub>, 20 ng of each control primer (Ctrl-1 and Ctrl-2), 200 ng of KIM-G(V) or KIM1(F) primer in their respective reaction, and 2.0 U of *Taq* polymerase (Invitrogen) to a 50  $\mu$ L solution containing PCR buffer 1X (Invitrogen). PCR conditions were as follows: Denaturation for 10 min at 95°C, 37 cycles of 95°C for 30 seconds, 57°C for 20 seconds and 72°C for 25 seconds; and a final extension step at 72°C for 7 min. A 160 bp fragment was detected for the Fc $\gamma$ RIIIa and a 270 bp fragment for the internal control used. In Figure 6 the different patterns obtained are shown.

The Fc $\gamma$ RIIIb genotyping was done in one single reaction to amplify the 141 bp (NA1) and/or 219 bp (NA2) fragments of the receptor, and the 480 bp of the internal control (HGH, Figure 7). PCRs were performed by adding, 100 ng of genomic DNA, and 200 ng of each primer (NA1, NA2, and HGHC) to a 25  $\mu$ L solution containing 1 bead of pure-taq ready to go PCR (GE healthcare). PCR conditions were as follows: Denaturation for 3 min at 94°C, 30 cycles of 94°C for 1 min, 57°C for 2 min and 72°C for 1 min; and a final extension step at 72°C for 10 min.

Genotypes and allele frequencies were obtained by direct count. Differences in genotypes and allele frequencies between patients and controls were compared using the Chi square test. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated to estimate the effect of different alleles. All analyses were carried out using the OpenEpi v 2.0 software, with  $p \leq 0.05$  set as the level of statistical significance, and results are shown in Table 5.

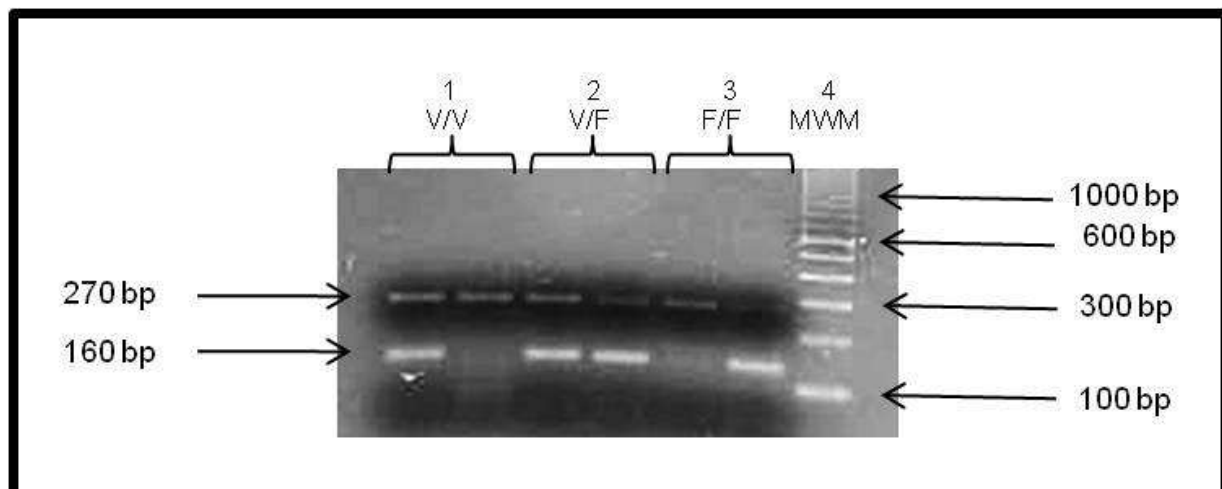


Fig. 6. A representative agarose gel (2%) of PCR products obtained from the amplification of Fc $\gamma$ RIIIa, showing the different genotypes for: 1) a homozygotic Fc $\gamma$ RIIIa-158VV subject; 2) a heterozygotic Fc $\gamma$ RIIIa-158VF subject; 3) a homozygotic Fc $\gamma$ RIIIa-158FF subject; and, 4) molecular weight markers. In all cases, the 160 bp band corresponds to the fragment of Fc $\gamma$ RIIIa, and the 270 bp to the internal control.

For Fc $\gamma$ RIIIa polymorphisms, our results show that 16/35 patients and 29/120 healthy subjects were homozygotic 131-HH (OR = 2.642; IC= 1.205-5.796;  $p= 0.013$ ); eight out of 35 patients and 42/120 healthy subjects were homozygotic 131-RR (OR=0.5503; IC=0.230-1.318;  $p=0.1765$ ); and 11/35 patients and 49/120 healthy subjects were heterozygotic 131-HR (OR=0.664; IC=0.298-1.480;  $p= 0.3170$ ). These results suggest that the homozygotic 131-HH of Fc $\gamma$ RIIIa allele could be associated with AS. Moreover, the frequency of allele H was significantly higher in AS patients compared to healthy controls (OR= 1.980; 95%CI= 1.149-3.412,  $p= 0.0138$ ).

For Fc $\gamma$ RIIIa, 7/35 AS patients and 5/120 healthy subjects had the 158-VV genotype (OR= 5.75; IC= 1.698-19.47;  $p= 0.002$ ); 14/35 AS patients and 51/120 healthy subjects showed the 158-FF genotype (OR= 0.902; IC= 0.419-1.942;  $p=0.792$ ); and 14/35 and 64/120 showed the 158-VF genotype (OR= 0.737; IC = 0.364-1.569;  $p= 0.0427$ ). Our results suggest that the homozygotic 158-V of Fc $\gamma$ RIIIa could be associated with AS.

In the case of Fc $\gamma$ RIIIb, we found that 9 out of 35 AS patients and 16/120 healthy subjects had NA1/NA1 genotype (OR=2.25; IC=0.894-5.662;  $p= 0.080$ ); 15/35 AS patients and 64/120 healthy subjects had the NA2/NA2 genotype (OR=0.656; IC=0.307-1.402;  $p= 0.275$ ); and 11/35 AS patients and 40/120 healthy subjects were NA1/NA2 (OR= 0.917; IC=0.408-2.057;  $p=0.833$ ). No significant association was found between the NA1 and NA2 haplotypes of Fc $\gamma$ RIIIb and AS.

In spite of the statistical differences among groups, it is important to emphasize that the study population is small and, therefore, the results must be taken as only indicative of the possible role of these polymorphisms in the pathogenesis of AS; they are by no means conclusive.

Fc $\gamma$ RIIIa which is expressed in most immune cells has two allelic variants with different affinities for IgG subclasses. The mutation is G519A that results in an amino acid



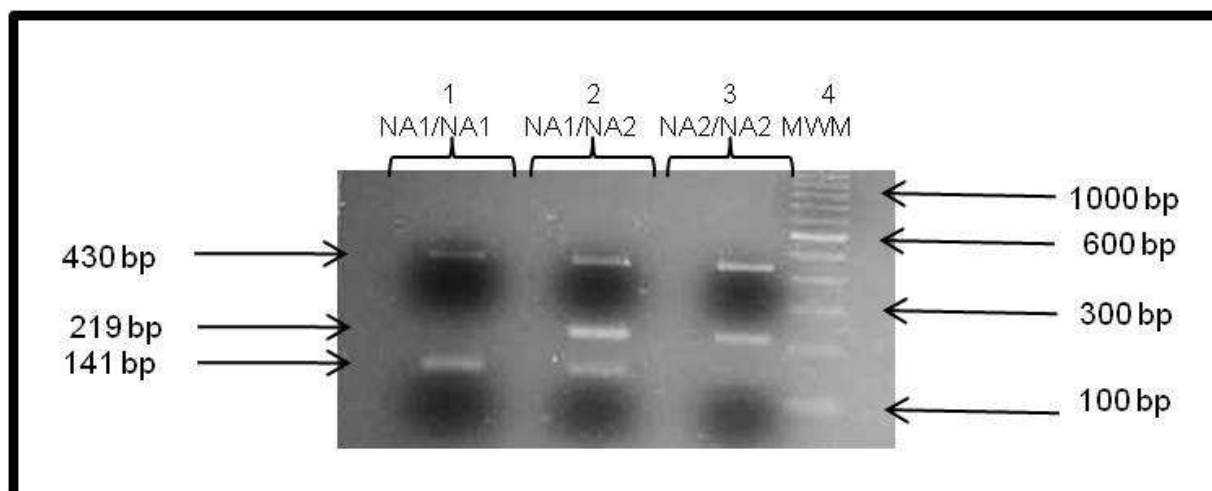


Fig. 7. A representative agarose gel (2%) of PCR products obtained from the amplification of Fc $\gamma$ RIIIa, showing the different genotypes: 1) a homozygotic Fc $\gamma$ RIIIb-NA1 subject; 2) a heterozygotic Fc $\gamma$ RIIIb-NA1/NA2 subject; 3) a homozygotic Fc $\gamma$ RIIIb-NA2 subject; and, 4) molecular weight markers. In all cases, the 141 and 219 bp bands correspond to the NA1 and NA2 amplification products, respectively, while the 439 bp indicates the internal control.

substitution at position 131. The 131-H variant has higher affinity for IgG3 than 131R. Fc $\gamma$ RIIIa, which is expressed on mononuclear phagocytes and natural killer cells, also has two co-dominantly expressed alleles, which differ at amino acid position 158 in the extracellular domain (valine or phenylalanine, respectively). Fc $\gamma$ RIIIa allelic variants differ in IgG1 and IgG3 binding; VV homozygotes bind to IgG1 and IgG3 more avidly than do FF homozygotes, and here we report that AS patients show mostly the VV genotype, compared to the control group (Dijstelbloem *et al.*, 2001).

As we have reported, all IgG subclasses anti-p30 are produced by AS patients, with higher levels compared to healthy controls (Zambrano-Zaragoza *et al.*, 2009). These data altogether with those of Fc $\gamma$ R polymorphisms suggest that IgG3 could be important in maintenance of the inflammatory process observed in AS, because of the presence of the allelic variants Fc $\gamma$ RIIIa-131-HH and Fc $\gamma$ RIIIa-158-VV in the group of AS patients, and then could contribute to the pathogenesis of the disease. Additionally IgG2 anti-p30, that is also produced by AS patients (Zambrano-Zaragoza *et al.*, 2009), could have a synergic action with IgG3 in the Fc $\gamma$ RIIIa, because the higher affinity of the 131-HH allelic variant for IgG2.

It has been reported that mononuclear cells infiltrate the cartilaginous structures of sacroiliac joints and inter-vertebral discs, leading to destruction and ankylosis (Braun & Sieper, 2007). Thus, inflammation and the cellular immune response could be modulated through the Fc $\gamma$ R. Therefore, we propose that one link between the humoral immune response against an environmental antigen such as p30 and AS could be through the Fc $\gamma$ R and, more specifically, the 131-HH genotype of Fc $\gamma$ RIIIa, and the 158-VV genotype of Fc $\gamma$ RIIIa, which is involved in inflammation mediated by immune complexes.

There is convincing evidence that imbalanced immune responses are responsible for autoimmune diseases such as arthritis, multiple sclerosis, and systemic lupus erythematosus (SLE). It is also widely accepted that many factors, including genetic and environmental components, are involved in the initiation and severity of autoimmune symptoms. Thus,

Polymorphisms	AS (n=35)	Controls (n=120)	p	OR	95% CI
FcγRIIa					
- Genotype					
HH	16 (45.7%)	29 (24.2%)	0.01347	2.642	1.205-5.796
RR	8 (22.9%)	42 (35%)	0.176	0.550	0.223-1.318
HR	11 (31.4%)	49 (40.8%)	0.317	0.664	0.298-1.480
- Allele					
H	43 (61.4%)	107(44.6%)	0.0138	1.980	1.149-3.412
R	27 (38.6%)	133 (55.4%)			
FcγRIIIa					
- Genotype					
VV	7(20%)	5 (4.2%)	0.002	5.75	1.698-19.47
FF	14(40%)	51 (42.5%)	0.792	0.902	0.419-1.942
VF	14 (40%)	64 (53.3%)	0.427	0.737	0.364-1.569
- Allele					
V	28(40%)	74 (30.8%)	0.151	1.495	0.862-2.595
F	42 (60%)	166 69.2%)			
FcγRIIIb					
- Genotype					
NA1/NA1	9 (25.7%)	16 (13.33%)	0.080	2.25	0.894-5.662
NA2/NA2	15 (42.9%)	64 (53.33%)	0.275	0.656	0.307-1.402
NA1/NA2	11 (31.4%)	40 (33.33%)	0.833	0.917	0.408-2.057
- Allele					
NA1	29 (41.4%)	72(30%)	0.073	1.650	0.952-2.860
NA2	41 (58.6%)	168 (70%)			

Table 5. Genotype and allele frequencies of FcγRIIa, FcγRIIIa and FcγRIIIb polymorphisms in patients with AS and controls.

identifying these components could prove helpful in gaining further insight into these diseases and developing novel immunotherapeutic strategies to interfere with chronic inflammation.

On the basis of these findings, we proposed a hypothetical model of interaction between two factors: one environmental, the other genetic (Figure 8). In this model, the immune response against *S. typhimurium* in a susceptible subject leads to the recognition of p30 and the production of IgG3 antibodies. p30 should cross-react with a putative auto-antigen, such that the humoral immune response is maintained by this putative auto-antigen, and it is responsible of the maintenance of the antibody levels.

Then, in the susceptible subject, the FcγRIIa-131-HH and FcγRIIIa-158-VV polymorphism, which have a higher affinity for IgG3, promote and maintain (at least in part) the inflammatory response observed, as well as the ankylosis that is due mainly to the inflammatory response that trigger new bone formation (Braun & Sieper, 2007). However, it is necessary to enlarge the study population in order to confirm both the genetic data and the association of this polymorphism with AS.

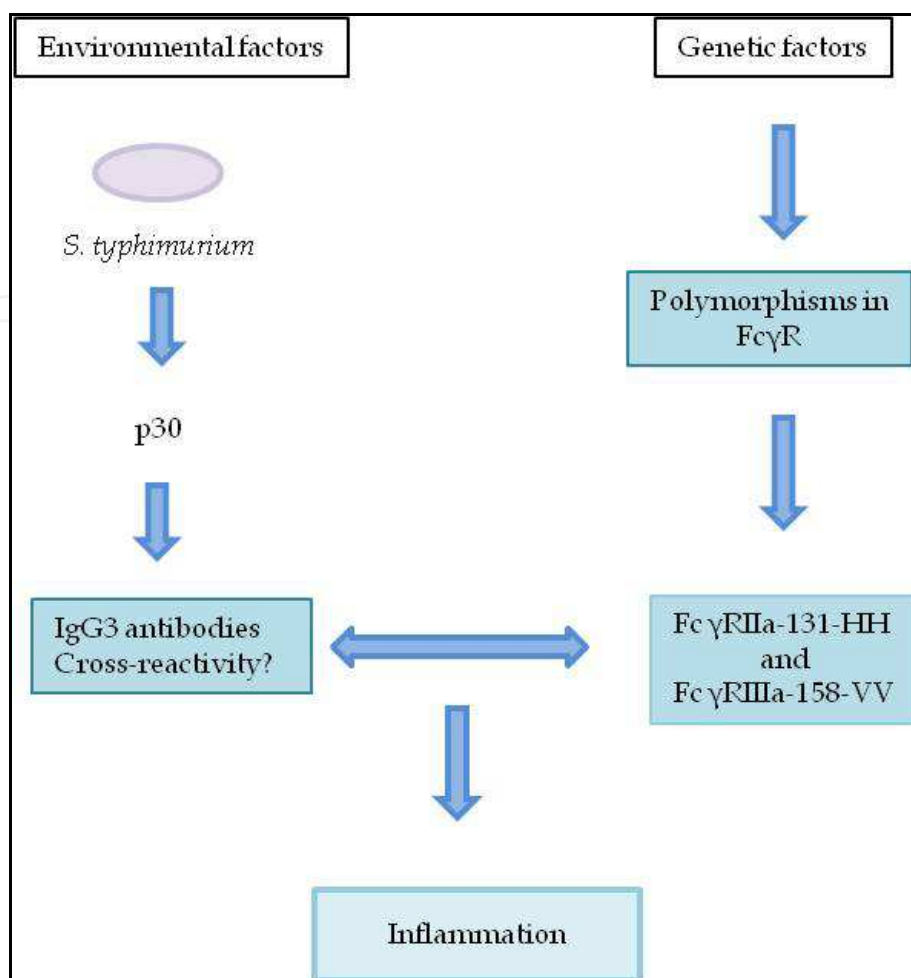


Fig. 8. A hypothetical model of the interaction between the environmental (the p30 from *S. typhimurium*), and the genetic (allelic variants FcγRIIA-131-HH, and FcγRIIIa-158-VV) factor in the inflammatory process observed in AS.

#### 4. Conclusion

In this chapter, we proposed a link between the humoral immune response in a susceptible individual and a genetic factor, FcγRIIA-131-HH, and FcγRIIIa-158-VV.

The immune response against *S. typhimurium* appears to be strain-specific, because of the difference observed with the humoral immune response against *S. enteritidis*. Patients with AS produce IgG3 antibodies against the p30 of *S. typhimurium*, in contrast to healthy controls. Moreover, patients with AS have higher frequencies of FcγRIIA-131-HH and FcγRIIIa-158-VV, which suggests a link between these environmental and genetic factors.

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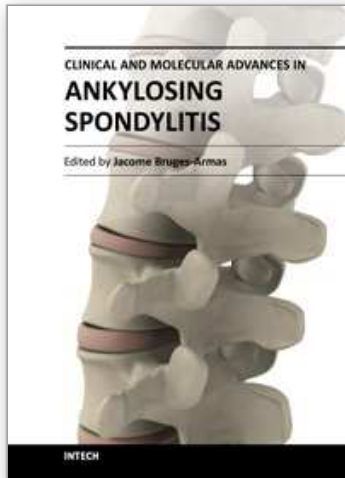
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The first section of the book entitled Clinical and Molecular Advances in Ankylosing Spondylitis is a review of the clinical manifestations of Ankylosing Spondylitis (AS) and Spondyloarthritis (SpA). The book includes chapters on Bone Mineral Density measurements, two chapters on the temporomandibular joints, axial fractures, clinical manifestations, diagnosis, and treatment. Molecular genetics and immune response are analyzed in the second section of the book; information on HLA-B\*27, other MHC genes and the immune response of AS patients to bacteria is reviewed and updated. Two chapters are dedicated to recent information on non-MHC genes in AS susceptibility, and to new data on disease pathways generated from gene expression studies on peripheral blood.

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