

# The 30-kDa band from *Salmonella typhimurium*: IgM, IgA and IgG antibody response in patients with ankylosing spondylitis

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**Objective.** To determine the association of *Salmonella typhimurium* antigens with AS by analysing the IgA, IgG and IgM antibody response to the crude lysate and the 30-kDa band from this micro-organism.

**Methods.** Sera from 28 AS patients, 28 HLA-B27+ healthy relatives, 28 unrelated healthy subjects and 14 RA patients were included. *Salmonella typhimurium* proteins were electrophoretically separated and blotted onto nitrocellulose sheets for immunodetection with sera from AS patients and unrelated healthy subjects. The electroeluted 30-kDa band (p30) and a crude lysate (StCL) from *S. typhimurium* were used as antigen to evaluate the IgM, IgA and IgG (total and subclasses) antibody levels by ELISA. An inhibition assay was carried out to confirm the specificity of IgG response to the p30.

**Results.** Twenty out of 28 AS patients (71.4%) and 4 out of 28 unrelated healthy subjects (14.3%) recognized a 30-kDa band from *S. typhimurium* with IgG antibodies. Six out of 28 AS patients (21.4%) and 4 out of 28 unrelated healthy subjects (14.3%) detected it with IgA antibodies. Recognition of p30 and StCL by both IgA and IgG antibodies was higher in AS patients than in control groups ( $P=0.003$ ,  $<0.001$  and  $0.003$  for IgA and  $<0.001$ ,  $0.003$  and  $0.006$  for IgG). Sera from AS patients have higher percentage of IgG antibodies p30 and IgG3 subclass was higher in AS patients than in control groups. No differences in the IgM response were found.

**Conclusions.** Data presented suggest the association between the p30 and AS.

**KEY WORDS:** Ankylosing spondylitis, *Salmonella typhimurium*, Antibody levels, Autoimmunity.

## Introduction

AS is the major subtype and a main outcome of an inter-related group of rheumatic diseases now named SpAs. AS is a chronic inflammatory disease of unknown aetiology, in which immunogenetic and environmental factors are involved [1]. The former is represented by the association with genes of the MHC, mainly HLA-B27 [2–4]. Further support for a common genetic background comes from the existence of borderline patterns of SpA and from the presence in some families of members with different SpA subtypes. Family clustering is a striking feature that emphasizes the existence of genetic susceptibility factors [5].

Environmental factors are also involved in pathogenesis of SpA. The fact that ReA is triggered by genitourinary infections with *Chlamydia trachomatis* or by enteritis by Gram-negative bacteria such as *Shigella* and *Salmonella*, *Yersinia* and *Campylobacter* provides a solid background for the possible interaction between HLA-B27 and bacteria, but the evidence for triggering infection in other SpAs, including AS, is marginal [6, 7]. Thus, SpAs are multifactor-related diseases whose pathogenesis involves gene–environment interactions [5, 6, 8, 9]. Studies on the association between AS and bacterial infections has been reported. The immune response to enterobacteria such as *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Shigella flexneri* and *Yersinia enterocolitica* has been evaluated and these bacteria have been associated with AS [9–15]. Most studies have been

carried out with *K. pneumoniae* [16–18]; however, its dominant role in AS has not been confirmed [12].

In this context, it has been reported that the behaviour of *S. typhimurium* was modified in HLA-B27 U891-transfected cells [19]; particularly, the infection by *Salmonella* induces more IL-10 and lower TNF- $\alpha$  synthesis in the response of infected cells. Also, *Salmonella* sp. DNA was detected in SF from patients with SpA [20]. On the other hand, lymphoproliferative response of mononuclear cells from SF in the presence of *S. typhimurium* was also reported in patients with ReA and uSpA [21].

The antibody response to *S. typhimurium* in AS patients has been analysed by some authors [10, 12, 22–24]. However, all these studies have been made with the lyophilized bacteria or crude lysate from the bacterium. These studies emphasize the association of this bacterium with AS, but a particular antigen towards which the immunological response is directed in AS patients and could then be involved in the pathogenesis of the disease, has not yet been reported. The aim of this work was to identify the particular antigens from sonicated crude lysate from *S. typhimurium* (StCL) recognized by the humoral immune response in AS patients that could be involved in the immunopathogenesis of the disease.

## Materials and methods

### Patients and controls

We included 28 consecutive patients with AS [25] attending the outpatient clinic of our department. As control group, 28 HLA-B27+ healthy relatives of AS patients, 28 unrelated healthy subjects and 14 patients with RA were included. All participants were informed about the nature of the study and written consents were obtained according to the Declaration of Helsinki. They were bled by venipuncture and the serum was obtained. The study was approved by the local ethics committee.

### Antigens

**Bacterial strain.** A typical *S. typhimurium* strain was kindly donated by Laboratorio Estatal de Salud Publica from

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Secretaria de Salud, Tepic, Nayarit, Mexico. This strain was assigned to a serotype by the slide agglutination method with commercial anti-serum according to the manufacturer's (Sanofi Diagnostic Pasteur, Paris, France) instructions and identified as *Salmonella enterica* subsp. *enterica* serovar Typhimurium.

#### Sonicated crude lysate from *S. typhimurium*

Bacteria were cultured in Soy-tripticase broth (Bioxon) at 37°C overnight and the bacterial mass was obtained and washed twice with sterile PBS by centrifugation. The resultant pellet was resuspended in PBS and sonicated (30 cycles at 5 Watts for 30 s in 1 min intervals) in an ice bath. Protein concentration was determined by the Lowry method [26]. The antigenic preparation was named StCL and was kept at -20°C until used.

#### 30-kDa band from *S. typhimurium* (p30)

Pellets obtained from 4 ml of bacterial culture were dissolved and denatured by boiling at 100°C for 15 min, in sample buffer (0.5 M Tris-HCl, 1% 2-mercaptoethanol, 10% glycerol, 1% SDS and crystals of bromophenol blue). *Salmonella typhimurium* proteins were resolved by 12% (1.5 mm) SDS-PAGE under reducing conditions (SDS-PAGE-ME), according to the discontinuous buffer system described by Laemmli [27]. After staining with 0.125% Coomassie blue R-250 (Sigma Chem. Co., St Louis, MO, USA) in methanol:acetic acid:water (5:1:2.75) and unstained with methanol water:acetic acid (5:4:1). A horizontal gel strip corresponding to the 30-kDa band was carefully cut and electroeluted, (electroelution unit, Bio-Rad, CA, USA). Analysis of the p30 electroeluted was done by SDS-PAGE-ME. Protein concentration was determined by the Lowry method [26].

#### Western blot analysis

Western blot was carried out using the procedure previously described by Towbin *et al.* [28]. Briefly, the volume equivalent to 30 µg of StCL was separated by using SDS-PAGE [22].

Proteins were then blotted onto nitrocellulose sheets (0.45 µm) (Amersham Bioscience, CT, USA) using a miniprotean III transfer unit (Bio-Rad). After blocking non-specific binding, Nitrocellulose (NC) strips were incubated with each test serum (from either AS patients or unrelated healthy subjects) diluted at 1/100 in blocking buffer. After appropriate wash, NC strips were incubated with 1/2000 peroxidase-conjugated goat anti-human IgG or goat anti-human IgA for 1 h. The antibody binding was developed with 0.5 mg/ml 3,3'-diaminobenzidine (Sigma Chem. Co.) -0.03% H<sub>2</sub>O<sub>2</sub> in PBS. The relative molecular masses of the recognized antigens were calculated in comparison of their Rf with the standard molecular weight markers (Benchmark Pre-Stained Protein Ladder; Invitrogen, CA, USA).

#### IgA, IgG (total and subclasses) and IgM to the StCL and the 30 kDa from *S. typhimurium* by ELISA

IgA, IgM and IgG (total and subclasses) antibody levels, measured as absorbance, to the p30 or StCL in the sera of AS patients, healthy subjects and RA patients were determined by ELISA. Polystyrene microtitre plates (Costar, NY, USA) were coated with 100 µl of either StCL or electroeluted p30 (both at final concentration of 3 µg protein/ml) in 0.1 M carbonate buffer, pH 9.6. After blocking, 100 µl of 1/400 diluted serum samples were added to the corresponding well and they were incubated 1 h at 37°C. Peroxidase-conjugated goat IgG anti-human IgG, goat anti-human IgA or goat anti-human IgM (Calbiochem, CA, USA), was diluted 1/10 000 and monoclonal mouse anti-human IgG1, IgG2, IgG3 or IgG4 (Zymed Laboratories, CA, USA) was diluted 1/5000 in blocking buffer and 100 µl was added to each well and incubated 1 h at 37°C. Reaction was developed with 0.4 mg/ml *o*-phenyldiamine and 0.01% hydrogen peroxide in citrate-phosphate buffer, pH 5.0. Absorbance was measured at 490 nm in an ELISA reader (microtitre reader model 680, Bio-Rad). To compensate for non-specific background (binding of immunoglobulin to the plate), the absorbance in

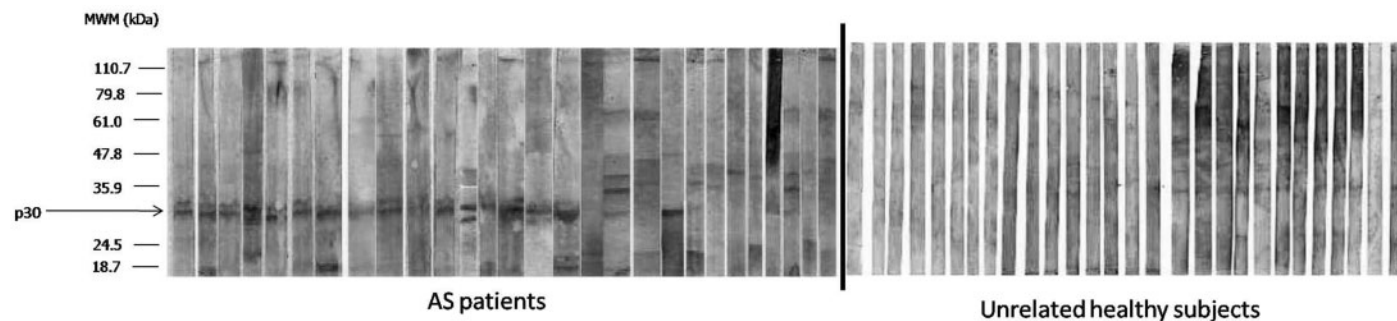


FIG. 1. *Salmonella typhimurium* antigens recognized by IgG antibodies in AS patients and unrelated healthy subjects. The bars on the left indicate the molecular masses of standard markers (kDa); the arrow indicates the recognized 30-kDa band.

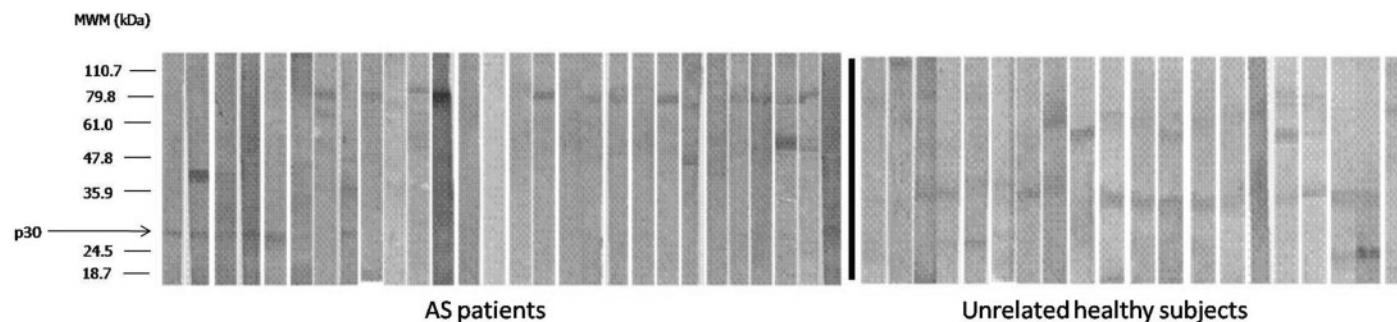


FIG. 2. *Salmonella typhimurium* antigens recognized by IgA antibodies in AS patients and unrelated healthy subjects. The bars on the left indicate the molecular masses of standard markers (kDa); the arrow indicates the recognized 30-kDa band.

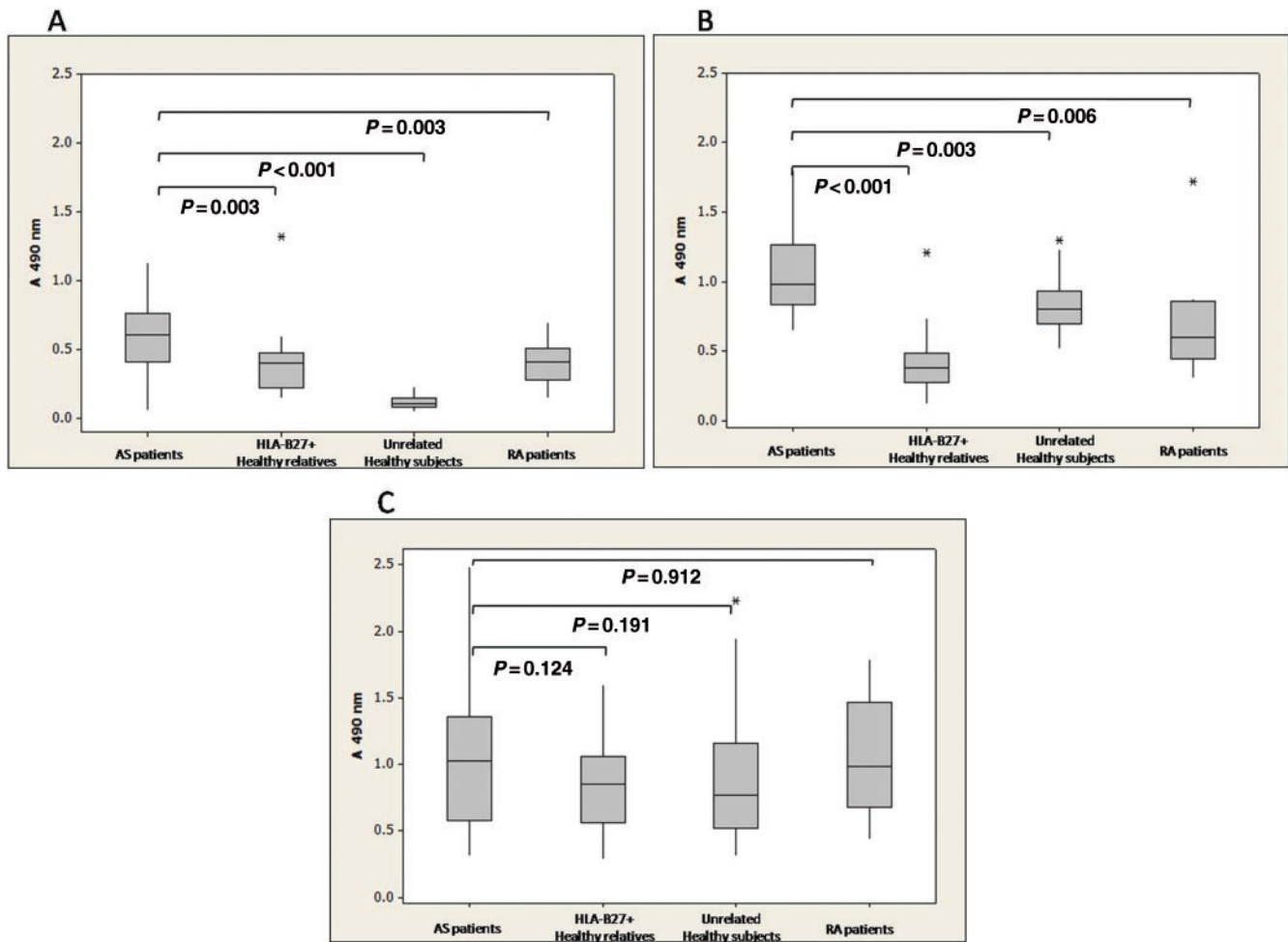


FIG. 3. Antibody levels to StCL in AS patients and control groups. Levels of IgA (A), IgG (B) and IgM (C) antibodies determined by ELISA, assayed with 0.3  $\mu$ g of antigen/well, sera were diluted 1/400 in triplicates. The graphic shows the median  $A_{490\text{nm}}$  value of each group and the percentile at 25 and 75%. Statistical analysis was done with Student's *t*-test.

wells not coated with antigen (negative control) was subtracted from the absorbance produced in antigen-coated wells. For IgG subclasses, ratios were calculated by reference to the highest value obtained for each IgG subclass.

#### Inhibition assays

In order to further illustrate the relationship between AS and p30, the percentage of antibodies anti-p30 relative to the antibodies anti-StCL was determined in an inhibition assay. In this one, serum from 10 AS patients, 10 *HLA-B27+* healthy relatives, 10 unrelated healthy subjects or 6 RA patients were diluted 1/1000 and incubated with an excess or without of the electroeluted p30 for 90 min at 37°C. Later, the mixture was used in ELISA as described above but plates were sensitized with 0.01  $\mu$ g of StCL. Percentages of inhibition were calculated by reference to the control without electroeluted p30 and plotted on graphs.

#### Statistical analysis

Differences in the frequencies of recognition of *Salmonella* antigens were analysed by the chi-square test. Differences in antibody levels measured as absorbance or ratios for IgG subclasses were analysed by the Student's *t*-test. In all cases, Minitab 14 software (Pennsylvania, USA) was used.

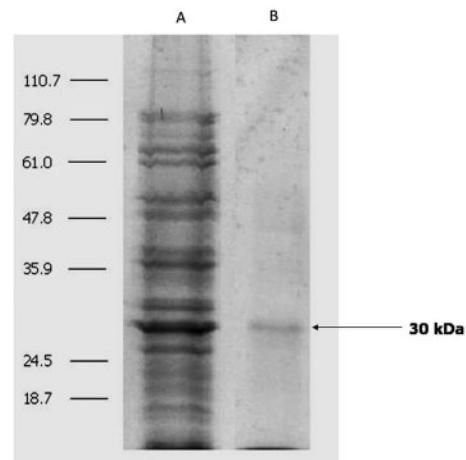


FIG. 4. Analysis of the electroeluted p30. (A) StCL. (B) Electroeluted p30. The bars on the left indicate the molecular masses of standard markers (kDa).

#### Results

We included 28 consecutive patients with AS [25]; 22 were males and 6 females; 22 had adult and 6 juvenile-onset disease; their mean age  $\pm$  s.d. was  $31.9 \pm 10.1$  years; *HLA-B27* was present in

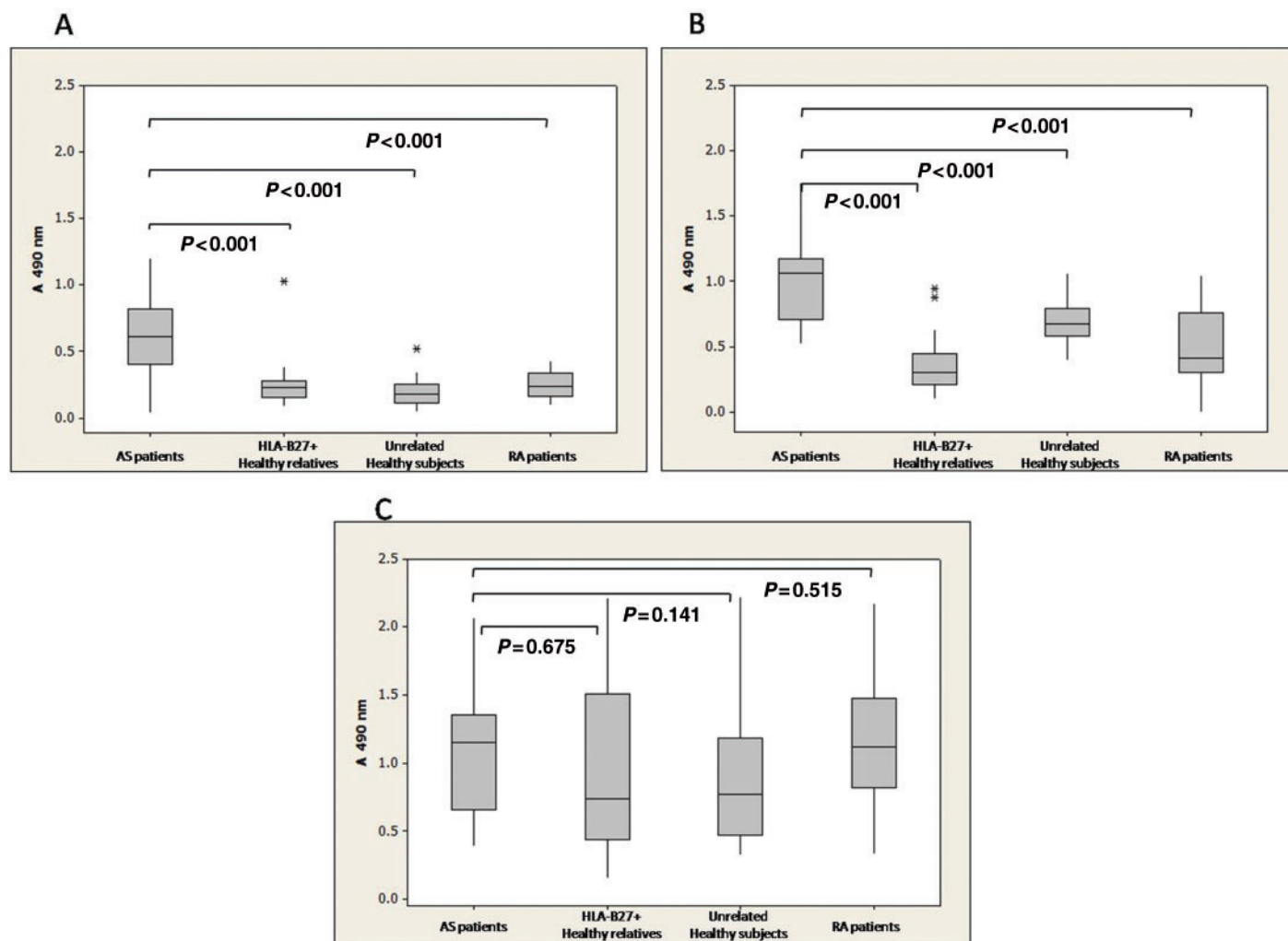


FIG. 5. Antibody levels to p30 in AS patients and control groups. Levels of IgA (A), IgG (B) and IgM (C). Antibodies determined by ELISA, assayed with  $0.3 \mu\text{g}$  of antigen/well, sera were diluted 1/400 in triplicate. The graphic shows the median  $A_{490\text{nm}}$  value of each group and the percentile at 25 and 75%. Statistical analysis was done with Student's *t*-test.

18 (85.7%) of 21 tested. All patients were treated with NSAIDs, 22 with SSZ, 12 with prednisone and 2 with MTX; none was receiving TNF- $\alpha$  blockers. Patients with RA were included as a control disease, five males and nine females; their mean age  $\pm$  s.d. was  $42 \pm 7.8$  years. Treatment included MTX, SSZ, diclofenac and paracetamol. For healthy subjects, their mean age was  $\pm$  s.d. was  $26.6 \pm 6.4$  years.

There was no history of previous infection by *Salmonella* in any group. However, *Salmonella* infection represents a major health problem and *S. typhimurium* is the most frequent serotype of *Salmonella* isolate in Mexico [29, 30], and then all subjects included in this study had the same risk of exposure to *Salmonella*.

#### Western blot analysis

In order to establish possible differences in antigen recognition, a first approach was carried out by western blot analysis in two groups: AS patients and unrelated healthy subjects. By using anti-IgG antibodies we found that 20 out of 28 AS patients (71.4%) and 4 out of 28 unrelated healthy subjects (14.3%) recognized a 30-kDa band from *S. typhimurium* (Fig. 1,  $P < 0.001$ ). When anti-IgA antibodies were used, 6 out of 28 AS patients (21.4%) and 4 out of 28 unrelated healthy subjects (14.3%) recognized this antigen (Fig. 2,  $P = 0.485$ ).

#### IgA, IgG and IgM antibodies to StCL

The antibody levels against StCL were higher in AS patients than in control groups (*HLA-B27+* healthy relatives, unrelated healthy subjects and RA patients) for IgA ( $P = 0.003$ ,  $< 0.001$  and  $0.003$ , respectively; Fig. 3A) and IgG ( $P < 0.001$ ,  $0.003$  and  $0.006$ , respectively; Fig. 3B). No differences in the IgG levels between unrelated healthy subjects and RA patients ( $P = 0.234$ ) but *HLA-B27+* healthy relatives have higher levels than unrelated healthy subjects and RA patients ( $P = 0.001$  and  $0.017$ , respectively). For IgA antibodies, RA patients showed higher IgA levels than both the groups of healthy subjects ( $P < 0.001$ ). No differences in the IgM levels were found in the groups analysed (Fig. 3C).

#### IgA, IgG and IgM antibodies to 30-kDa antigen

After the p30 was electroeluted, purity was confirmed by SDS-PAGE (Fig. 4). The absorbance observed for IgA and IgG against the p30 were statistically higher in AS patients than they were in control groups ( $P < 0.001$ ; Fig. 5A and B, respectively). No differences in the IgA levels were found between control groups ( $P = 0.163$ ,  $0.974$  and  $0.095$ , respectively) but unrelated healthy subjects showed higher IgG levels than *HLA-B27+* healthy relatives and RA patients ( $P < 0.001$  and  $0.030$ ,

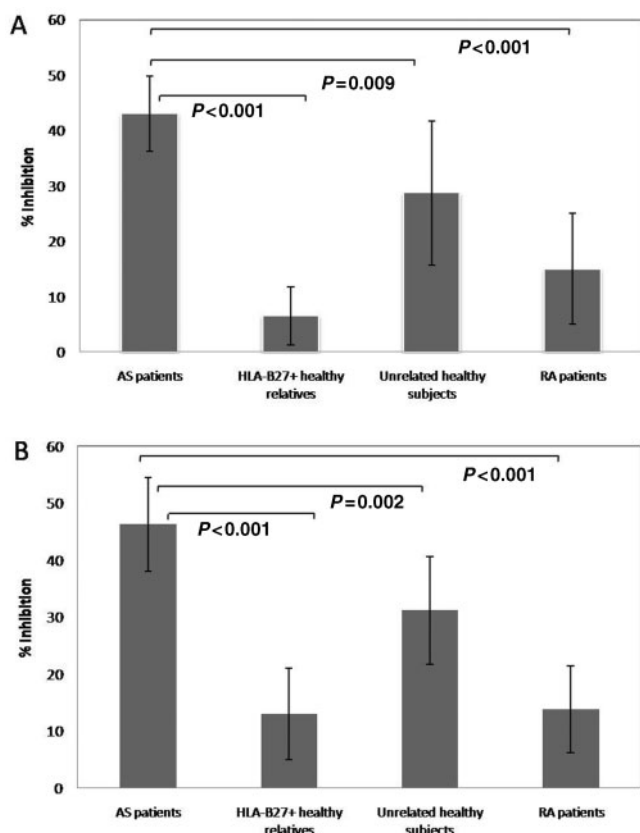


Fig. 6. Inhibition assays for IgG antibodies to StCL. Sera from AS patients or control groups were diluted 1/1000 and incubated with 0.05 (A) or 3.0 µg (B) or without electroeluted p30. Inhibition was determined by ELISA with 0.01 µg of StCL/well. Statistical analysis was done with the Student's *t*-test.

respectively). As was observed in the case of the response to the StCL from *S. typhimurium*, the IgM levels did not show significant differences between the groups (Fig. 5C).

#### Inhibition assay

In order to support the antibody to p30 that is significantly in the response to *S. typhimurium* antigens in AS patients, an inhibition assay was carried out in serum from patients and controls. An excess of electroeluted p30 was used (0.05 and 3 µg/ml; Fig. 6A and B, respectively). Inhibition of the recognition of the *S. typhimurium* antigens by electroeluted p30 was observed for all groups; however, a significantly higher inhibition was observed in sera from AS patient.

#### IgG subclasses anti-p30 in AS patients and control groups

In the sera from AS patients and controls, the four IgG subclasses were found to be involved in the recognition of the p30 from *S. typhimurium*. However, we found that AS patients have higher levels of IgG3 than control groups ( $P < 0.001$ , 0.006 and 0.025, respectively; Fig. 7C). RA patients showed higher IgG1 levels than AS patients, HLA-B27+ healthy relatives and unrelated healthy subjects ( $P = 0.006$ , 0.002 and 0.001, respectively). Finally, HLA-B27+ healthy relatives have higher levels of IgG2 than AS patients, unrelated healthy subjects and RA patients ( $P = 0.001$ , 0.027 and 0.030, respectively) and IgG4 than unrelated healthy subjects and RA patients ( $P < 0.001$  and 0.006; Fig. 7B and D, respectively).

#### Discussion

AS is a chronic inflammatory disease, in which immunogenic and environmental factors are involved [1, 6, 31]. HLA-B27 is one of the most important molecules associated with AS [2, 3, 6]. However, bacterial infections are very important factors in triggering the disease [22]. *Salmonella typhimurium* has been associated with AS because of its particular behaviour in cells transfected with HLA-B27 [19, 32]. It has been reported that the HLA-B27 molecule transfected into fibroblasts affects the survival of *Salmonella* in these fibroblasts. NO production is inhibited as well [32]. The infection of transfected HeLa cells with HLA-B27 induces the expression of *c-fos* and MCP-1 in an antigen-independent pathway [33]. In addition, the presence of *Salmonella* sp. DNA in SF cells of patients with SpAs has been reported [20]. On the other hand, humoral immune response to *S. typhimurium* has been reported in different studies with contradictory results [10–14], and it has been reported that the OmpP from this bacterium shares homology with five amino acids of HLA-B27 in a non-linear fashion [15]. Considering these data, in this work we looked for the antigens from *S. typhimurium* that could be associated with AS.

Our results showed that a 30-kDa band from *S. typhimurium* is recognized by IgG antibodies from most AS patients, compared with unrelated healthy subjects (Fig. 1). Recognition of this band by IgA antibodies did not show significant differences between groups. These results suggest the association of a particular antigen from *S. typhimurium* with the disease. As has been reported, antibody levels to some bacterial antigens have been associated with the disease [12, 15, 18, 22, 24], and to confirm the association of AS with this antigen, the antibody levels to the StCL and p30 were determined and compared with unrelated healthy subjects and with HLA-B27+ healthy relatives and RA patients, which were included as control groups as well.

We found that the sera from AS patients have higher levels of IgA (Fig. 3A) and IgG (Fig. 3B) antibodies to an StCL from *S. typhimurium* than in all control groups. These results are in partial agreement with those reported [10, 12], in which the IgG level to *S. typhimurium* in AS patients is higher than in healthy subjects. According to these studies, in this work no differences in the IgM response to this bacterium between AS patients and control groups were found (Fig. 3C), and Pacheco-Tena *et al.* [20], have reported the presence of *Salmonella* DNA in the SF of patients with SpA, although the bacteria has not been isolated from these patients. Higher levels of IgG than IgA were observed in all cases, which correlates with data found on western blot analysis, because a lower number of bands are recognized by IgA antibodies than by IgG antibodies.

AS patients showed a higher humoral immune response to *S. typhimurium* antigens; however, the role of the antibodies to p30 was addressed to confirm the association between this antigen and AS. Levels of IgA and IgG antibodies to the p30 from *S. typhimurium* correlate with those observed to the StCL in which the AS patients have a higher IgA and IgG response to this band compared with all control groups. (Fig. 5A and B, respectively). No differences between groups in the IgM levels to this antigen (Fig. 5C) were observed.

IgG levels to some enterobacterial antigens, particularly the HSP60 from *K. pneumoniae*, has been reported. An association between AS and this protein has been found [18]. However, the antibody response to this protein is shared with the other members of the HSP60 family, particularly the HSP60 from *K. pneumoniae*, *S. typhi* and *Y. enterocolitica* [34], suggesting that the response to the HSP60 from *K. pneumoniae* could be due to the conserved portions of the HSP used. On the other hand, the association of *S. typhimurium* and AS has not been conclusive [10–12]. The association of the OmpH from this bacterium with AS has not been supported by the presence of antibodies or cellular immune response to the peptide involved in the share sequence [15].

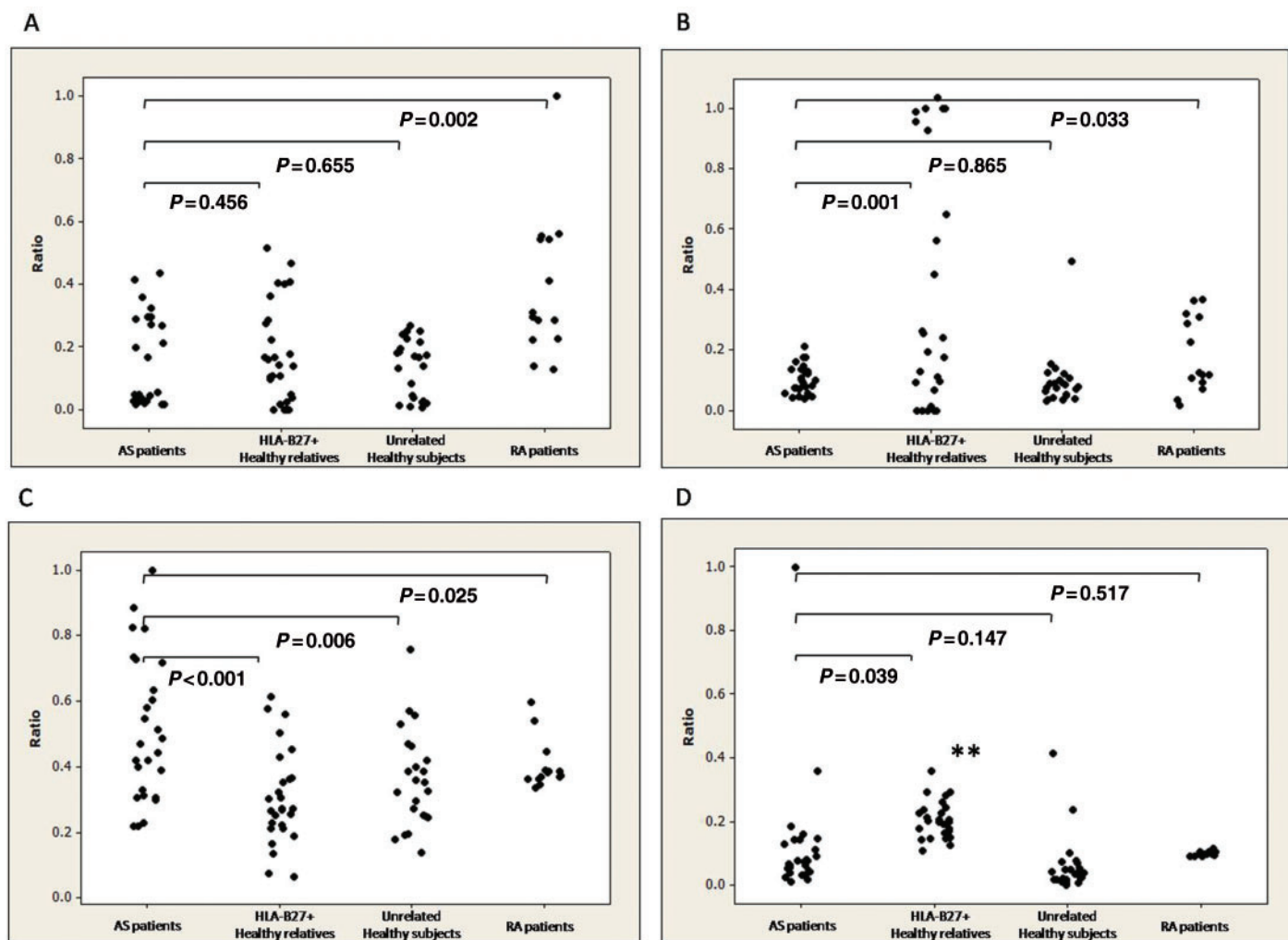


FIG. 7. Individual values for the ratio of IgG subclass levels anti-p30 from *S. typhimurium* in AS patients and control groups. Ratios were calculated with the highest absorbance value for each IgG subclass (0.86 for IgG1; 0.783 for IgG2; 0.2 for IgG3 and 0.82 for IgG4). Antibodies were determined by ELISA, with 0.3  $\mu$ g of antigen/well, sera were diluted 1/400 in triplicate. Statistical analysis was done with the Student's *t*-test.

Here, we report that the 30-kDa band from *S. typhimurium* could be differentially recognized by the immune response in AS patients, and involved in the immunopathogenesis of AS.

In order to confirm the specificity of the response to the p30, an inhibition test was carried out in sera from AS patients and healthy subjects by using an excess of electroeluted p30 to block the interaction with antigens from StCL. Our results showed that AS patients have significantly higher inhibition percentages than control groups (Fig. 6), and therefore the p30 is more immunogenic in AS patients than in the control groups.

IgG subclasses have been involved in autoimmunity diseases such as SLE and RA, in which aberrant expression or the allelic variants of Fc $\gamma$  receptors with altered functionality have been observed that contributes to the pathogenesis of these diseases [35]. We found that in both patients and controls, the recognition of the p30 by serum antibodies was due to all the IgG subclasses (Fig. 7), but the IgG3 antibodies were higher in the AS group than in control groups. These results suggest that differences in the response to this bacterium, particularly the IgG3 antibodies, could be involved in AS, possibly because of differences in Fc $\gamma$  receptors polymorphisms, but this suggestion should be explored.

The findings reported in this work support the possible participation of this antigen in the disease, probably by a cross-reaction with a self-antigen, not identified yet.

### Rheumatology key messages

- The p30 is the antigen from *S. typhimurium* that could be involved in the pathogenesis of AS.
- Differences in IgG subclasses against the p30 could be involved in the inflammatory response observed.

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