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Article

# TNF-alpha and IL-17 cytokine levels in Brazilian patients with ankylosing spondylitis after anti-TNF therapy

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The aim of this study was to evaluate tumor necrosis factor alpha (TNF-α), interleukin (IL)-17A/F levels in the serum of ankylosing spondylitis (AS) patients after anti-TNF therapy, in order to understand how these cytokines are involved in this therapeutic response. Forty-four AS patients were included in the study: thirty using anti-TNF therapy were classified according to their therapy response as responders (15) and non-responders (15) and 14 without anti-TNF therapy were classified as AS control. Fifteen healthy individuals formed the control group. Serum levels of TNF-α were determined using Luminex technology and for IL-17A and IL-17F using ELISA. The non-responder patients presented higher serum levels of TNF-α than the responders and AS control; the same results were found when *HLA-B*27* positive or negative patients were separately analyzed. IL-17A and IL17F serum levels were similar for all groups. According to the clinical disease activity, AS patients with BASDAI ≥4 had higher serum levels of TNF-α than AS patients with BASDAI <4. Positive correlation was found between TNF-α levels and BASDAI. In AS patients, TNF-α serum levels were associated with anti-TNF therapy and disease activity independently of *HLA-B*27*, and IL-17A and IL-17F were not related to anti-TNF treatment.

***Key-words:*** Genetic association studies. Polymorphism, genetic. Spondylitis, ankylosing. Cytokines.

## INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory autoimmune disease that usually starts with an inflammation in the sacroiliac joint and in its chronic phase can lead to calcification and fusion of the spine. The main clinical manifestations include back pain and progressive spinal rigidity. However, inflammation of the hips, shoulders, peripheral joints and fingers/toes can also occur (Zhu *et al*., 2019).

AS is often associated with other conditions, such as uveitis, psoriasis, and inflammatory bowel disease (IBD), with a significant genetic overlap. Susceptibility to AS is polygenic, with more than 100 genes identified. The most important part of disease heritability comes from the *HLA-B*27* allele, but several other HLA alleles and even some outside of the major histocompatibility complex loci have been shown to be involved in the disease (Costantino *et al*., 2018; Vecellio *et al.,* 2019). Thus, the pathogenesis of AS is still unclear and several mechanisms have been proposed to elucidate it, involving not only HLA-B27 but also endoplasmic reticulum aminopeptidases (ERAP) 1 and 2, disorder of the IL-23/IL-17 axis and the abnormality of peptides that induce lymphocyte activation and differentiation. All of them

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lead to the production of cytokines that influence the pathogenesis of AS (Zhu *et al*., 2019).

The gradual clinical worsening in the manifestations of disease is linked to inflammation and bone mineral density of the joints, due to a high production of inflammatory cytokines including tumor necrosis factor alpha (TNF-α) and Interleukin (IL)-17A and IL-17F (Ranganathan *et al*., 2017; Lata, Hettinghouse, Liu, 2019). Different levels of these cytokines have been found in AS patients (Park *et al*., 2007; Milanez *et al*., 2016; Gonzalez-Lopez *et al*., 2017; Limón-Camacho *et al*., 2012).

TNF-α has been reported to be the main cytokine contributing to the early onset of AS disease (Lata, Hettinghouse, Liu, 2019) and has been associated with AS in several studies (Gonzalez-Lopez *et al*., 2017; Limón-Camacho *et al*., 2012; Pedersen, Maksymowych, 2018). Elevated levels of this pro-inflammatory cytokine are associated with tissue destruction and cachexia, which is considered to be the major mediator of acute and chronic inflammation (Lata, Hettinghouse, Liu, 2019). The single nucleotide polymorphism on the promoter region *TNF-308 G>A* (rs18000629) has been associated with AS patients, and it appears to influence their TNF serum level (Kroeger *et al*., 2000; Wilson *et al*., 1997). Our group found that the *TNF-308 GA/AA* genotype was associated with AS regardless of *HLA-B*27* (Rocha Loures *et al*., 2018).

The IL-17 family of cytokines consists of six proteins (IL-17A to IL-17F), and IL-17A and IL-17F have been crucial in chronic inflammatory diseases, including AS (Ruiz de Morales *et al*., 2020). IL-17A and IL-17F can recruit immune cells like neutrophils and monocytes in the inflammatory site and have synergistic actions with other pro-inflammatory cytokines, such as TNF, IL-1β, IFN-γ (Interferon-gamma), GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor), and IL-22, providing a potent inflammatory action (Ruiz de Morales *et al*., 2020). In a previous study, we found that the *IL17A* GA/AA and *IL17F* TC/CC genotypes were associated with AS regardless of sex and *HLA-B*27* (Rocha Loures *et al*., 2018), and these polymorphisms affect the production of IL-17A and IL-17F, respectively (Espinoza *et al*., 2011; Kawaguchi *et al*., 2006).

The IL-23/IL-17 axis has been widely studied and considered a key axis in this disease. Th17 cells are induced by IL-23 and are the most common IL-17-producing lymphocytes subtype (Ranganathan *et al*., 2017). The IL-6 and IL-1β pro-inflammatory cytokines are required for Th17 differentiation and studies have demonstrated an increase in the production of these cytokines in AS patients compared with normal subjects (Ruiz de Morales *et al*., 2020). Of note, TNF-α acts synergically with IL-17 resulting in a potent inflammatory effect (Ruiz de Morales *et al*., 2020, Wen *et al*., 2017). IL-17 is also produced by CD8+ lymphocytes and by tissue-resident innate cells such as NK (Natural Killer), NKT (Natural Killer T-Cells), Tγδ (gamma-delta T cells), and ILC3 (group 3 innate lymphoid cells) after activation. On the other hand, Th17 cells also produce IL-10, an anti-inflammatory cytokine (Ruiz de Morales *et al*., 2020).

Improvements in understanding the AS pathogenesis have allowed for the development of drugs directed to specific biological targets that could interfere in the different stages of the immune response cascade. The treatment of AS involves nonsteroidal anti-inflammatory medications (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), like methotrexate and sulfasalazine, and biologic drugs, like anti-TNF-α (anti-TNF) and anti-IL17 drugs. Anti-TNF drugs, such as infliximab, adalimumab, etanercept, golimumab and certolizumab are widely used for the treatment of this disease because they bind to both membrane-bound and soluble TNF-α (Pedersen, Maksymowych, 2018; Rubbert-Roth *et al*., 2018). Although successful, 30-40% of patients with spondyloarthropathies (SpA) do not respond or respond inadequately to this therapy (Menegatti, Bianchi, Rogge, 2019).

Even though the pathogenesis of AS involves mainly immune T cells, research continues to explore the role of different cytokine levels to predict disease occurrence, development and severity (Zhu *et al*., 2019). Therefore, the aim of this study was to evaluate tumor necrosis factor alpha (TNF-α) and interleukin (IL)-17A and IL-17F levels in the serum of ankylosing spondylitis patients after anti-TNF therapy in order to understand how these cytokines are involved in this therapeutic response.

## MATERIAL AND METHODS

### Sample selection

A total of 44 patients with AS were included in this case-control study. The AS patients were diagnosed through clinical (sex, age, duration and age at onset of back pain), laboratory (HLA-B27, the erythrocyte sedimentation, and C-reactive protein - CRP) and radiological (sacroiliitis) examination according to the ASAS 2009/2011 Criteria (Rudwaleit *et al*., 2009; Rudwaleit *et al*., 2011) by the rheumatologists at the University Hospital of Maringá and all the patients had a follow-up with the same rheumatologists. All patients met ASAS Criteria with lumbar/sacral pain in the morning, or arthritis of peripheral joints and/or tendinitis of these joints. The ASAS Criteria for axial SpA require, as a mandatory criterion, the presence of back pain for ≥ 3 months and symptom onset before the age of 45 and one of the following possible criteria: *i.* the presence of sacroiliitis on imaging studies and at least one SpA feature or *ii.* HLA-B27 positive and two or more features of SpA. Features of SpA include inflammatory back pain, peripheral arthritis, heel enthesitis, dactylitis, psoriasis, acute anterior uveitis, inflammatory bowel disease, good response to non-steroidal anti-inflammatory drugs (NSAIDs), family history of SpA, HLA-B27 positive and elevated C-reactive protein (CRP) (Rudwaleit et al., 2009; Rudwaleit et al., 2011). Patients were recruited in elective consultations with rheumatologists (community-bases).

The evaluation of the clinical disease activity index BASDAI (Bath Ankylosing Spondylitis Disease Activity Index) was performed according to criterions previously defined (Torres, Ciconelli, 2006). The scores were defined from the visual analogical scale from 0 to 10 (0 = good; 10 = bad): scores <4.0 indicated low clinical activity and ≥4.0 high clinical activity. The use of anti-TNF therapy was previously considered for 30 patients with a severe and active disease, clinically defined as BASDAI ≥4, and with therapeutic failure with the use of nonsteroidal anti-inflammatory drug (NSAIDs) or disease-modifying anti-rheumatic drugs (DMARDs). These patients were classified according to the clinical response to the treatment into two groups: *i.* those who had BASDAI <4 after anti-TNF therapy were classified as responders (n=15), *ii.* those who had BASDAI ≥4 after at least six months using anti-TNF therapy were classified as non-responders (n=15). Fourteen AS patients with BASDAI <4 and without DMARDs or biologic treatments were classified as AS control group. Subsequently, responders, non-responders and AS control groups were further stratified in *HLA-B*27* positive and *HLA-B*27* negative groups. In addition, fifteen individuals without AS were included as a healthy individuals group, selected by the same rheumatologists after clinical, laboratory and radiological analyses. The non-inclusion criteria for patients and controls were individuals with chronic diseases such as diabetes and other inflammatory and autoimmune diseases, infections at the time of collection, recent trauma, previous hospitalization or any other type of non-rheumatic systemic inflammation. The study subjects in the study were recruited from May 2014 to May 2016 and from February to March 2021. All participants were from the northwestern region of Paraná, southern Brazil (22°29'30"-26°42'59"S and 48°02'24"-54°37'38"W) and classified as mixed ethnicity with predominantly European origin, based on the ethnic constitution of the state of Paraná as previously described (Probst *et al*., 2000; Reis *et al*., 2018) and confirmed for our region (Reis *et al*., 2018). This study was approved by the Research Ethics Committee of the State University of Maringá (UEM), number CAAE 27723114. All participants signed the consent form.

### Sample collection and technical procedures

The peripheral blood was collected from individuals in 5 ml tubes with EDTA for DNA extraction and without anticoagulants to obtain the serum. The blood and extracted DNA were stored at -20°C and the serum samples were kept at -80°C until analysis.

Serum levels of TNF-α were determined using Luminex technology with the ProcartaPlex™ Immunoassay (Invitrogen, ThermoFisher Scientific, Inc., Burlington, Ontario, Canada). This immunoassay is based on the principles of the sandwich ELISA, using two highly specific antibodies that bind to different epitopes of one protein for quantitation. This was done using a Luminex

instrument, where the sensitivity of the assay was 0.4 pg/mL. The bound proteins to bead are identified with biotinylated antibodies and streptavidin–R-phycoerythrin (RPE). The Luminex instrument contains two lasers: one to distinguish the spectral signature of each bead and the other to quantify the amount of fluorescence, which is proportional to the amount of protein present in the sample. For the quantification of TNF-α, the standard curve was made by 1:3 serial dilution of the standard antigen (7 dilutions) and samples were thawed and mixed in vortex. The magnetic beads were prepared according to the manufacturer's instructions and 50 µL were added to each well of the plate; after removing the liquid with the aid of the Hand-Held Magnetic Plate Washer, the beads remained at the bottom of the wells. In each well we added 25 µL of standards, controls and samples in duplicate and an additional 25 µL of universal assay buffer was added. For the blanks, 50 µl of universal assay buffer was used. After incubation and shaking of the plate at room temperature, the beads were washed (twice) and 25 µL of detection antibody mix was added. The plate was incubated with shaking at room temperature and after washing the beads the RPE solution was added in each well. The plate was sealed, incubated under shaking at room temperature, and after washing the beads we added 120 µL of reading buffer. The reactions were read after incubation using a Luminex™ instrument. The results were expressed as median fluorescent intensities (MFI) and data were analyzed using the xPONENT® software.

Serum levels of IL-17A and IL-17F were performed using immunoenzymatic assay method (ELISA), with the Human IL-17 platinum ELISA kit (affymetrix eBioscience, North America, USA) in accordance with the manufacturer's instructions. Briefly, an anti-human IL-17A/ IL-17F coating antibody is adsorbed onto microwells and the human IL-17A/IL-17F present in the samples or standards bind to the antibodies adsorbed in the microwells. In this way, a later added biotin-conjugated anti-human IL-17A/IL-17F antibody binds to human IL-17A/IL-17F captured by the first antibody. The unbound biotin-conjugated anti-human IL-17A antibody is removed during a wash step and the streptavidin-HRP added to each well binds to the biotin-conjugated anti-human IL-17A/ IL-17F antibody. After incubation, the unbound Streptavidin-HRP is removed during a wash step and HRP-reactive substrate solution is added to the wells. A colored product is formed in proportion to the amount of human IL-17A/ IL-17F present in the sample or standard. The reaction is stopped by adding acid and the absorbance is measured at 450 nm. A standard curve is previously prepared from serial dilutions of a standard solution containing human IL-17A/IL-17F and the cytokine concentrations in serum samples from patients and controls are then determined. Samples were performed in duplicates. The minimum detectable concentrations for cytokines were 0.18 pg/ml for IL-17A and 0.59 pg/ml for IL-17F.

*HLA-B*27* genotyping was performed using Polymerase Chain Reaction-Sequence Specific Primers (PCR-SSP) (Lara-Armi *et al*., 2020). Two primer mixes were used to assess the presence of *HLA-B*27* allelic group and nine mixes were used to identify the *HLA-B*27* alleles. Primers amplifying a 782 bp fragment in the third intron of HLA-DRB1 were used as the internal control. Same PCR conditions were used for all mixes. The PCR products were analyzed using 2% agarose gel electrophoresis stained with SYBR™ Safe DNA Gel Stain dye (Invitrogen; Carlsbad, CA, USA), after running at 100 V, 300 mA, 150 W for 20 minutes. Visualization and photo documentation were performed on the Quantum ST4 transilluminator (Vilber Lourmat; Collegien, France). Molecular weight markers with 100 base pairs (DNA Ladder, Thermo Fisher; Vilnius, Lithuania) were included to ensure band size.

*TNF-308* (rs1800629), *IL17A* (rs2275913), and *IL17F* (rs763780) genotyping was previously described and validated (Rocha Loures *et al*., 2018; Zacarias *et al*., 2015). Genotyping of *TNF-308* (rs1800629), *IL17A* (rs2275913), and *IL17F* (rs763780) SNPs was performed with DNA samples by PCR-restriction fragment length polymorphism (RFLP). PCR amplification was performed in a total volume of 30 µL mixture containing 100 ng of genomic DNA, 1.0 µM of each primer, 200 µM of each dNTP, 2.0 mM of $MgCl_2$, 3 µL 10x PCR buffer, and 1.5 U Taq DNA polymerase (Invitrogen Life Technologies, Grand Island, NY, USA). PCR products were digested for one hour at 37ºC with NcoI (Thermo Scientific™) for *TNF-308* (rs1800629), EcoNI (Thermo Scientific™)

for *IL17A* (rs2275913) and NlaIII (New England Biolabs) for *IL17F* (rs763780) and then they were separated by electrophoresis in 3% agarose with SYBR™ Safe DNA Gel Stain dye (Invitrogen Life Technologies, Grand Island, NY, USA).

### Statistical analyses

The data were analyzed by the software BioEstat 5.3. Categorical variables were described as numbers and percentages. The Shapiro Wilk test was used for normality testing. Variables with a normal distribution were presented as mean ± standard deviation (SD), and comparisons between groups were performed using Student's t test. Non-normal quantitative variables were presented as median and range, and comparisons between groups were performed using the Mann-Whitney test. Correlation was assessed using the Spearman test. The analyses were done between the following groups: responders *vs*. non-responders, responders *vs*. AS controls, non-responders *vs*. AS controls. The association between *TNF308* (rs18000629), *IL17A (*rs2275913) and *IL17F* (rs763780) genotype and cytokine concentration were defined for responders, non-responders, and AS controls. Samples with cytokine concentrations below the sensitivity level of the test were replaced by the lower detection threshold value for each analyte. *P* values less than 0.05 were considered statistically significant. The analysis and results were adjusted for sex and age.

## RESULTS

The characteristics and clinical data of AS patients and healthy individuals were shown in Table I. The patients' mean age was 44.34 ± 14.42 years and 56.81% of the patients were male.

**TABLE I -** Characteristics and clinical data of AS patients and AS controls and Healthy individuals

| Variable | Patients N=44 | Responders N=15 | Non-responders N=15 | AS control N=14 | Healthy individuals N=15 |
|---|---|---|---|---|---|
| Age mean + SD (year) | 44.34 ± 14.42 | 44.93 ± 15.33 | 43.73 ± 14.13 | 44.35 ± 14.8 | 39.26 ± 11.59 |
| Sex n (%) | | | | | |
| Male | 25 (56.81) | 8 (53.30) | 7 (46.67) | 10 (71.43) | 9 (60.00) |
| Female | 19 (43.19) | 7 (46.67) | 8 (53.30) | 4 (28.57) | 6 (40.00) |
| BASDAI mean ± SD | 3.64 ± 1.80 | 2.26 ± 1.31* | 5.6 ± 1.19* | 3.02 ± 0.49 | |
| HLA-B27 n (%) | | | | | |
| Presence | 27 (61.36) | 11 (73.33) | 8 (53.33) | | |
| Absence | 17 (38.63) | 4 (26.66) | 7 (46.67) | | |
| Anti-TNF n (%) | N=30 | N=15 | N=15 | | |
| Adalimumab | 16 (53.33) | 10 (66.66) | 6 (40.00) | | |
| Certolizumab | 1 (3.33) | 1 (6.67) | 0 | | |
| Etanercept | 8 (26.66 | 1 (6.67) | 7 (46.67) | | |
| Golimumab | 2 (6.66) | 2 (13.33) | 0 | | |
| Infliximab | 3 (10.00) | 1 (6.67) | 2 (13.33) | | |
| NSAID n (%) | 26 (59.09) | 8 (53.3) | 6 (40.00) | 13 (92.85) | |

**TABLE I -** Characteristics and clinical data of AS patients and AS controls and Healthy individuals

| Variable | Patients N=44 | Responders N=15 | Non-responders N=15 | AS control N=14 | Healthy individuals N=15 |
|---|---|---|---|---|---|
| DMARD n (%) | 14 (31.81) | 8 (53.3) | 6 (40.00) | | |
| Sulfasalazine | 14 (31.81) | 8 (53.3) | 6 (40.00) | | |
| Methotrexate | 1 (2.27) | 0 | 1 (6.66) | | |
| Age of disease onset (year) | 34.88 ± 15.19 | 38.13 ± 16.59 | 30.73 ± 12.46 | 35.85 ± 16.30 | |
| Disease duration (months) | 48 (2-348) | 48 (9-40) | 84 (12-348) | 20 (2-324) | |

AS: Ankylosing spondylitis; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index. DMARD: disease-modifying anti-rheumatic drugs. N: number of individuals. NSAID: non-steroidal anti-inflammatory agent. SD: standard deviation. Age of disease onset is show as mean + SD (year). Disease duration is show as median (range: minimum-maximum).

*Responders *vs.* Non-responders $P<0.0001$.

The cytokine levels in the serum of AS patients and healthy individuals are shown in the Figure 1 and in supplementary Table I. As *HLA-B*27* was an important biological factor for predisposition to AS, the cytokine levels were also analyzed separately for the *HLA-B*27* positive and for *HLA-B*27* negative patients (Figures 2 and 3, respectively, and supplementary Table I).

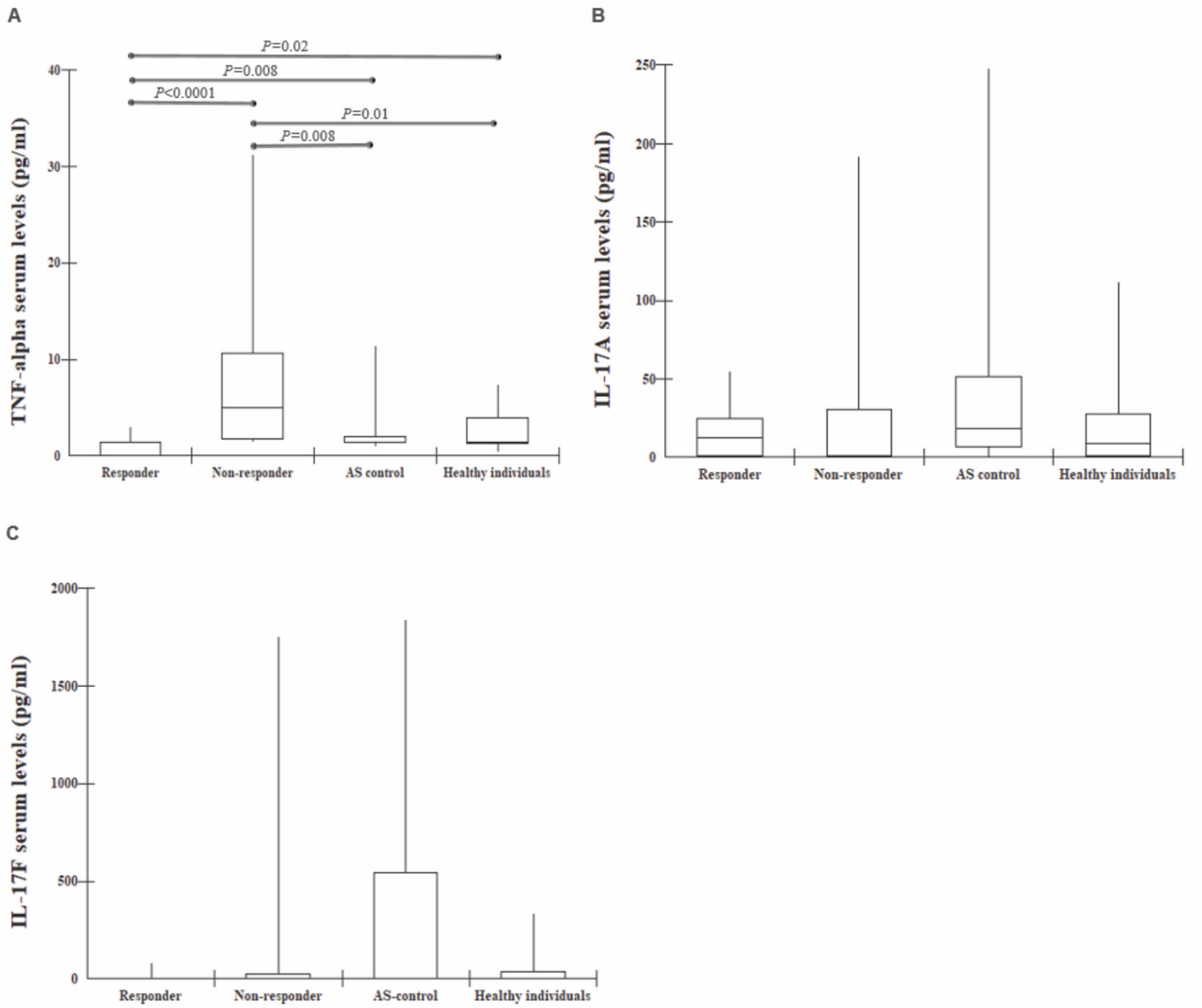



**FIGURE 1 –** Serum levels of TNF-α, IL-17A and IL-17F cytokines in responders, non- responders, AS control and healthy individuals groups.

**A:** Levels of tumor necrosis factor (TNF)-α. **B:** Levels of interleukin (IL)-17A. **C:** Levels of IL-17F. The Mann–Whitney test was used for comparisons.

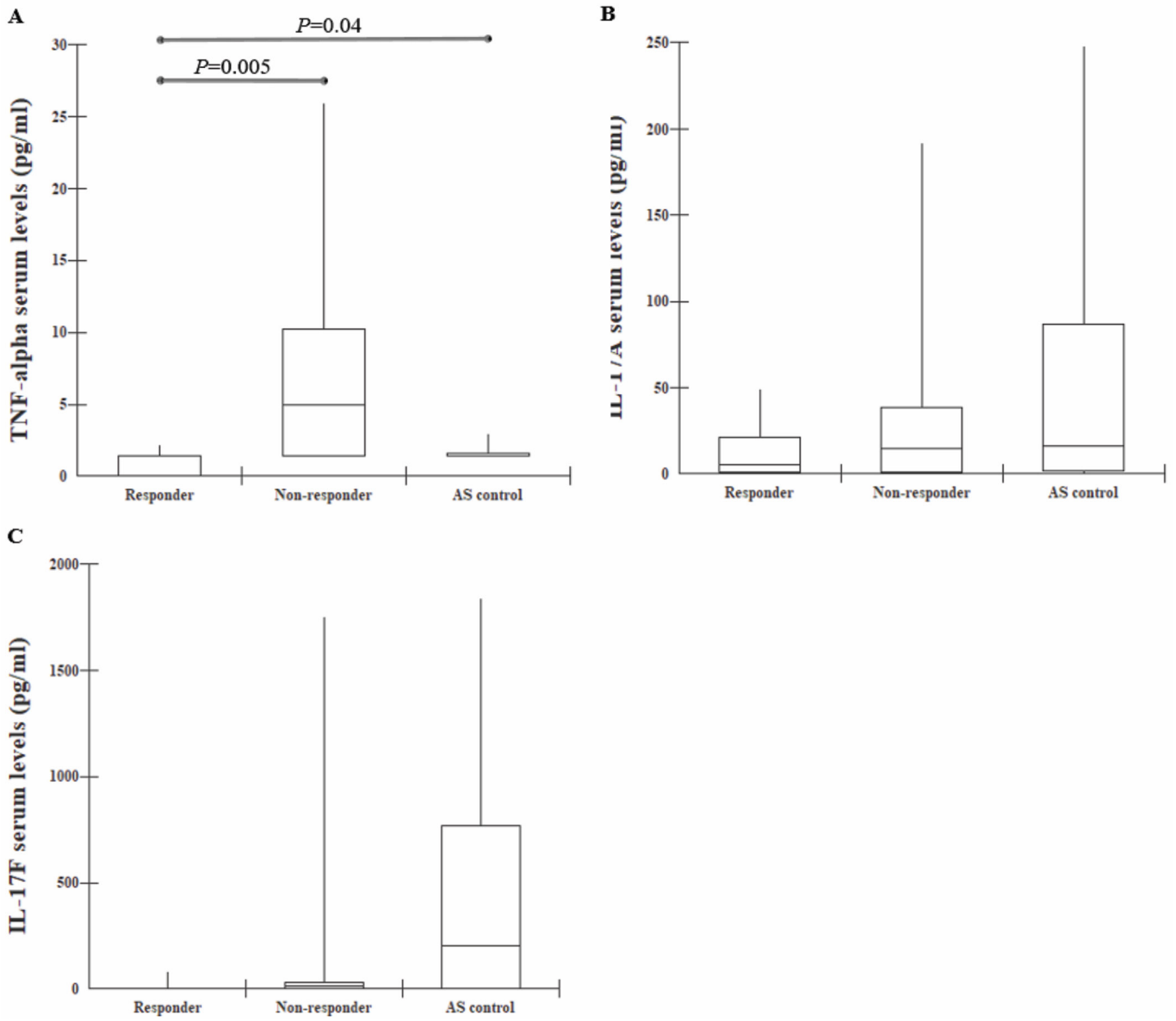



**FIGURE 2 –** Serum levels of TNF-α, IL-17A and IL-17F in *HLA-B*27* positive patients (responders, non- responders and AS controls).

**A:** Levels of TNF-α; **B:** Levels of IL-17A; **C:** Levels of IL-17F. The Mann–Whitney test was used for comparisons.

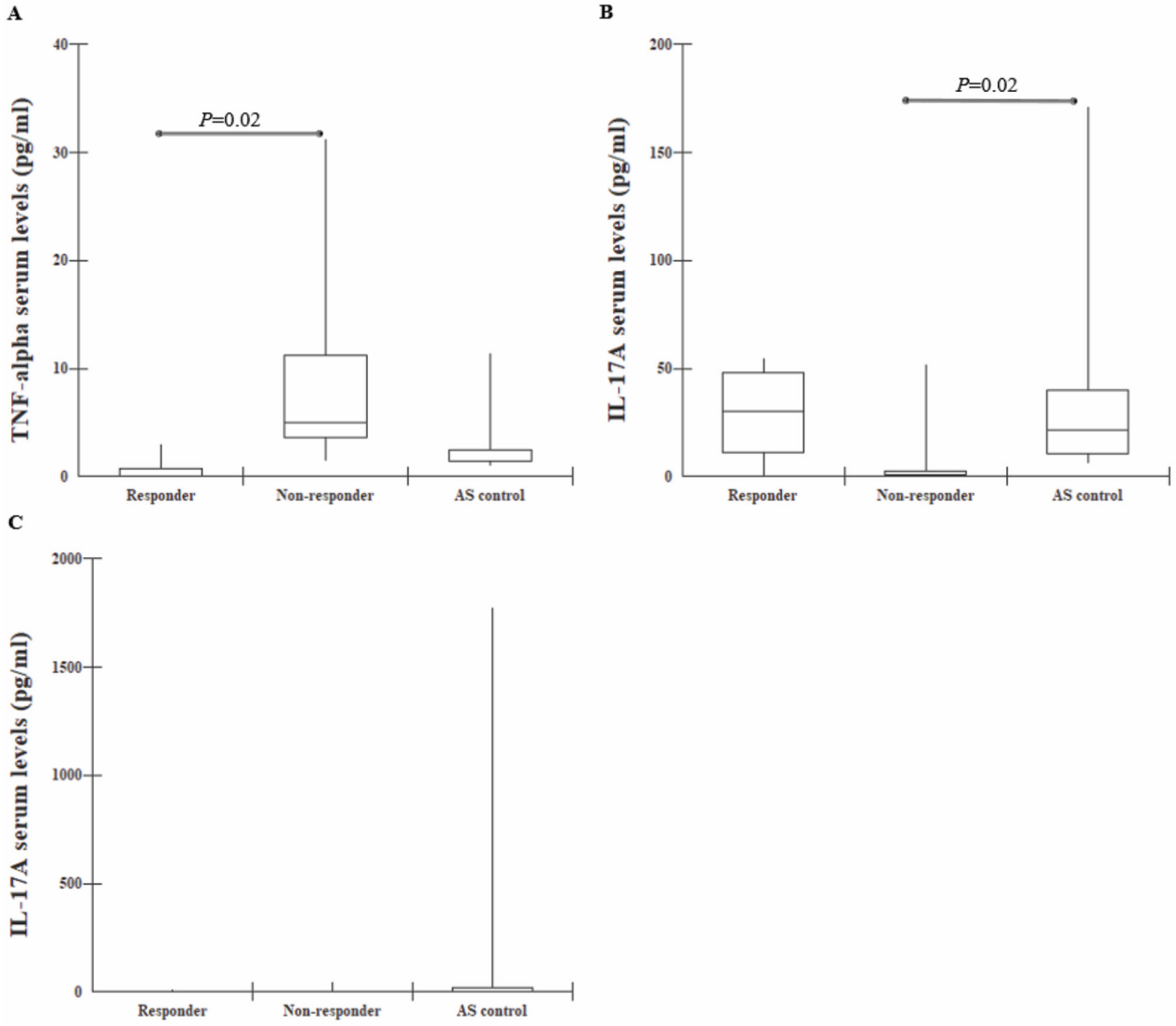



**FIGURE 3 –** Serum levels of TNF-α, IL-17A and IL-17F cytokines in *HLA-B*27* negative patients (responders, non- responders, AS controls).

**A:** Levels of TNF-α; **B:** Levels of IL-17A; **C:** Levels of IL-17F. The Mann–Whitney test was used.

Serum levels of TNF-α in AS non-responder group were higher (median=5.05 pg/ml) than responders (median=0.33 pg/ml; $P$ <0.0001) and AS controls (median=1.39 pg/ml; $P$= 0.008). Still, the responder group had lower serum levels than AS control ($P$=0.008). For healthy individuals the TNF-α median concentration was 1.39 pg/ml. Similar results were observed for *HLA-B*27* positive and for *HLA-B*27* negative patients: non-responders had higher levels of TNF-α (median=5.00 pg/ml and 5.05 pg/ml, respectively) than responders (median=0.33 pg/m; $P$=0.005 and $P$=0.02). Patients who were responders and *HLA-B*27* positive had lower levels of TNF when compared with AS control ($P$=0.04).

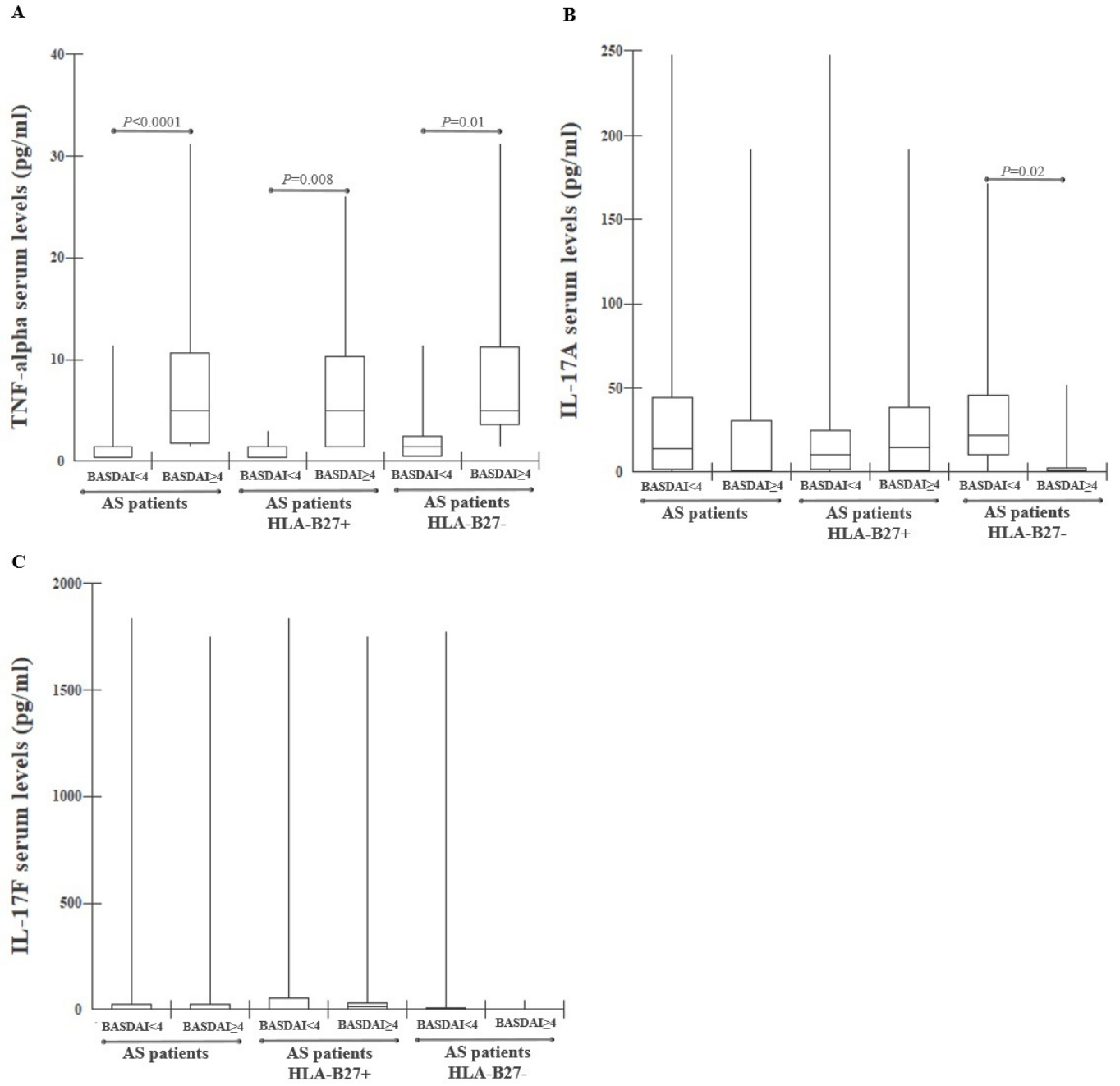
IL-17A cytokine serum levels were the same in non-responders (median=1.15 pg/ml), responders (median=12.31 pg/ml), and AS control (median=18.09 pg/ml) and for healthy individuals group (median=9.13 pg/ml). Similar results were found when *HLA-B*27* positive patients were analyzed. Non-responders who were *HLA-B*27* negative had lower IL-17A serum levels (median=0.95 pg/ml) than AS controls (median=21.43 pg/ml; $P$=0.02).

No significant differences were observed for IL-17F serum levels between responders (median=0.59 pg/ml), non-responders (median=0.59 pg/ml), AS controls (median=1.27 pg/ml), and healthy individuals (median=0.59 pg/ml). Similar serum levels of IL-17F were also observed for *HLA-B*27* positive and for *HLA-B*27* negative patients for all analyzed AS groups.

Because clinical symptoms of the disease were directly associate with BASDAI, the TNF-α, IL-17A and IL-17F levels were analyzed separately in AS patients who had Corrigir para BASDAI≥4 (responder + AS control) and BASDAI≥4 (non-responders). The AS patients with BASDAI≥4 presented higher TNF-α levels compared to AS patients with BASDAI <4 (median=5.05 pg/ml *vs* 1.39 pg/ml; *P*=0.0001), and the results were the same for *HLA-B*27* positive or negative AS patients. IL-17A and IL-17F serum levels were similar among AS patients who had BASDAI <4 or ≥4. Nevertheless, IL-17A cytokine levels were lower in *HLA-B*27* negative patients with BASDAI≥4. Data are shown in the Figure 4 and supplementary Table II.

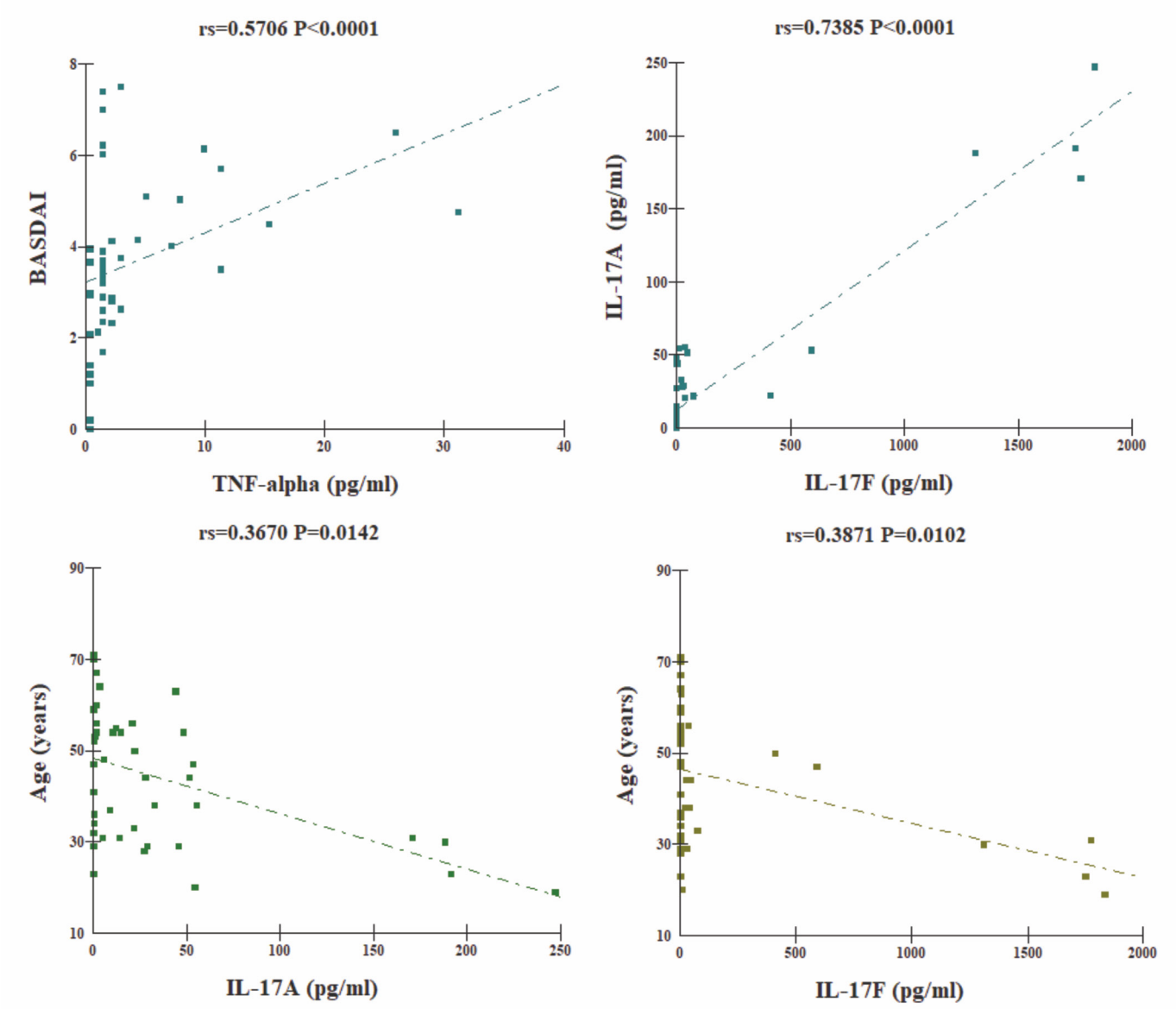
Correlation analysis among BASDAI, age and cytokine serum levels were realized (supplementary Table III) and significant results are shown in Figure 5. A statistically significant positive correlation was found between serum levels of TNF-α and BASDAI (rs=0.5706, *P* <0.0001) and between IL-17A and IL-17F (rs=0.7385, *P* <0.0001). The levels of IL-17A (rs=0.3670, *P*=0.0142) and IL-17F (rs=0.3801, *P*=0.0102) were inversely correlated with age in AS patients.

The *TNF-308* (rs18000629), *IL17A* (rs2275913) and *IL17F* (rs763780) genotyping for all individuals analyzed in this study was previously performed (Rocha Loures *et al*., 2018)and the correlation between genotypes and cytokine serum levels was done (supplementary Table IV and Figure 6). No correlation was found between all analyzed genotypes and cytokine levels in the serum of AS patients.




**FIGURES 4 –** Serum levels of cytokines for AS patients and for *HLA-B*27* positive and negative patients after stratifying according to BASDAI (BASDAI <4 and BASDAI ≥4).

**A:** Levels of TNF-α; **B:** Levels of IL-17A; **C:** Levels of IL-17F. The Mann–Whitney test was used for comparisons.




**FIGURES 5 –** Correlation analysis among BASDAI, age and serum levels of TNF-α, IL-17A and IL-17F in the AS patients. Only results with statistical significance were shown. Correlation was determined using Spearman test.

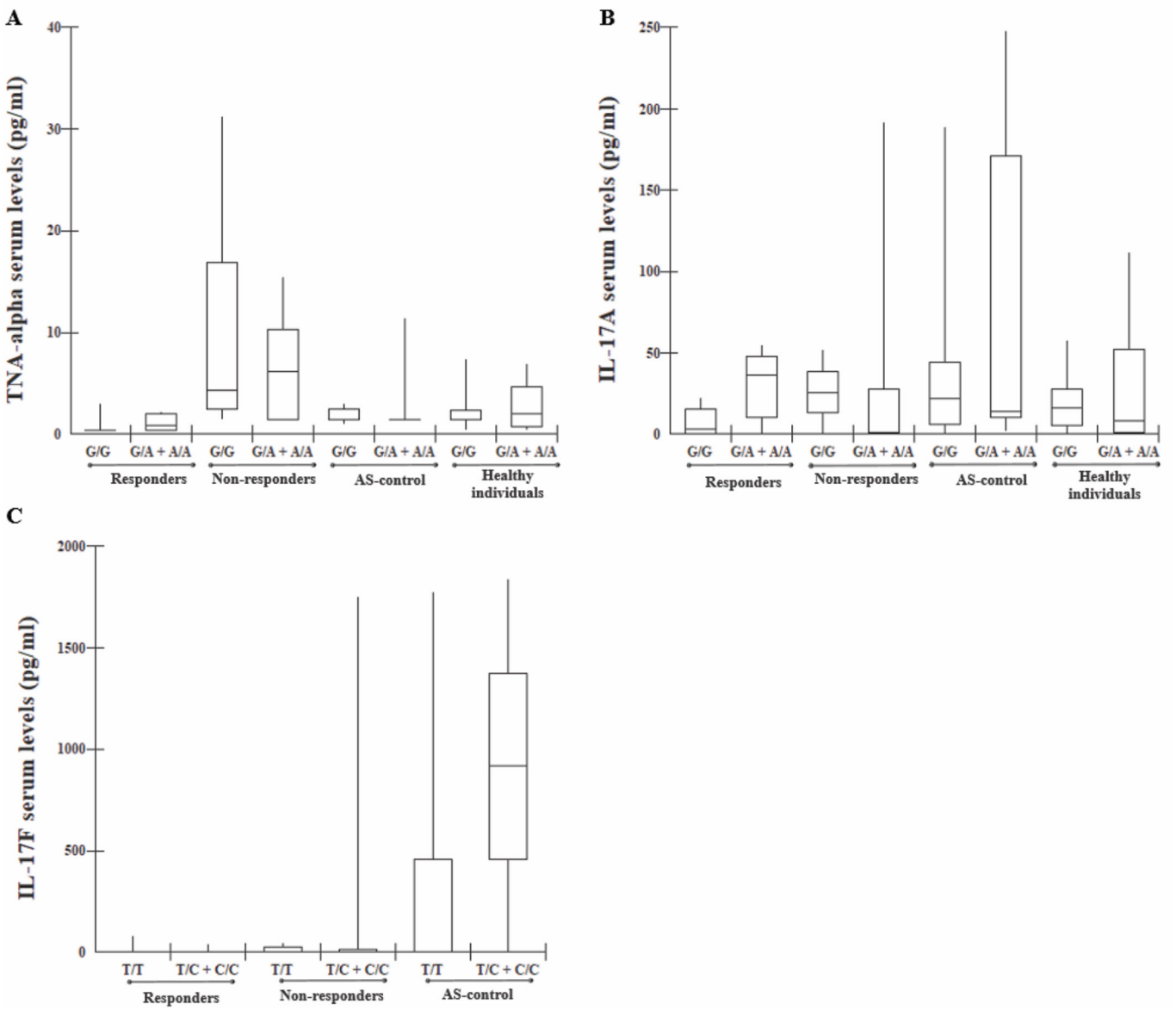



**FIGURE 6 –** Correlation between genotype and cytokine serum levels in AS patients after anti-TNF therapy.

**A:** Correlation between TNF-308 (rs18000629) genotype and TNF-α serum levels; **B:** Correlation between IL-17A (rs2275913)) genotype and IL-17A serum levels; **C:** Correlation between IL-17F (rs763780) genotype and IL-17F serum levels. The Mann–Whitney test was used for comparisons. There was no statistical difference between the data analyzed and p-values were not shown.

## DISCUSSION

There is a great interest in elucidating the possible pathways related to the pathogenesis of AS, which could lead to the discovery of new targets and therapeutic agents for its treatment (Tutuncu *et al*., 1994). The introduction of anti-TNF therapy has been effective in reducing inflammation and clinical symptoms in AS, in addition to being an alternative in case of failure or intolerable adverse effects of DMARD and non-steroidal anti-inflammatory drugs (NSAIDs). However, some patients do not receive the benefit of the clinical efficacy of anti-TNF and are non-responsive and represent 30–40% of patients with AS. Understanding how TNF inhibitors affect the immune system in patients is limited (Menegatti, Bianchi, Rogge, 2019). In this

way, our results confirm the important role of TNF-α in the immunopathogenesis and in the clinical course of AS. The main finding of this study was that higher serum levels of the TNF-α were associated with a worse response to anti-TNF treatment and higher disease activity, independently of *HLA-B*27.*

The early stages of AS are characterized by a strong inflammatory process and TNF-α has been the main cytokine related to the course of the disease (Lata, Hettinghouse, Liu, 2019). Gonzalez-Lopez *et al.* (2017) found that AS patients with BASDAI 5,2 ± 2,4 (mean ± standard deviation) presented elevated levels of TNF-α compared to controls, and 29% of them were undergoing anti-TNF therapy. Limón-Camacho *et al.* (2012) found that TNF-α was elevated in AS patients with BASDAI ≥6 when compared with both responder patients for anti-TNF therapy and controls. Xueyi *et al.* (2013) also found that serum levels of TNF-α were positively correlated with BASDAI score, however before anti-TNF-α therapy. It is known that the use of anti-TNF therapy in AS patients reduces the severity of the disease symptoms (Lata, Hettinghouse, Liu, 2019), however, a considerable number of patients do not perceive the clinical benefits of this therapy (Pedersen, Maksymowych, 2018). In our study, some patients did not go into disease remission when treated with anti-TNF, and TNF-α serum levels were high in AS with BASDAI≥4 (non-responder group), thus anti-TNF therapy seems not to be sufficient to completely block the inflammatory process in these patients.

Susceptibility to AS is polygenic and polymorphisms in the *TNF* gene could lead to increased production of TNF-*α*. Individuals with the *TNF-308 G>A* single nucleotide polymorphism are better producers of this cytokine (Wilson *et al*., 1997; Abraham, Kroeger, 1999) and it occurs when guanine (G) is substituted for adenine (A) within this regulatory region (Wilson *et al*., 1997; Abraham, Kroeger, 1999; Banday *et al*., 2016). A previous study of our group demonstrated that the *TNF-308 G/A* and *A/A* genotypes were risk factors for AS, independent of *HLA-B*27* (Rocha Loures *et al*., 2018). The patients in these studies were not the same as in our previous study, therefore, in order to evaluate the involvement of these genotypes in the production of TNF-α, the *TNF-308 G>A* genotypes were compared between the responders, non-responders, and AS control. However, no association was found probably due to the small sample number.

Regarding IL-17A, our results suggest that serum levels of this cytokine were not related to the response to anti-TNF treatment. However, IL-17A levels were associated with disease activity in AS patients who were *HLA-B*27* negative after anti-TNF therapy. In the literature, the results were contradictory. Milanez *et al.* (2016) found that AS responders had higher plasma levels of IL-17A compared with non-responders after 24 months of TNF blockade. On the other hand, Xueyi *et al.* (2013) found a significant decrease in circulating Th17 cells and in the IL-17 serum levels after anti-TNF-α therapy in AS responders, and an increase in frequencies of Th17 cells and serum levels of IL-17 in non-responders. These authors also found that serum levels of IL-17 correlated positively with the BASDAI score before anti-TNF-α therapy (Xueyi *et al*., 2013). IL-17 interaction with the receptor (IL-17R) initiates signaling pathways leading to activation of the transcription factors which induce transcription of several genes, among them, TNF-α (Ruiz de Morales *et al*., 2020).

We also found that serum levels of IL-17A and IL-17F were inversely correlated with the age of patients with AS, where older patients had lower levels of these cytokine. As expected, the levels of IL-17A and IL-17F directly correlated with each other. A hypothesis that could explain is aging and the decline of the adaptive immune response (Weiskopf *et al*., 2009).

A previous study by our group demonstrated that *IL17A GA/AA* and *IL17F TC/CC* genotypes are associated with AS, independently of sex and *HLA-B*27* and that other studies showed that genetic polymorphisms of *IL17A* G197A and *IL17F* T7488C affect the production of IL-17A and IL-17F, respectively (Espinoza *et al*., 2011; Kawaguchi *et al*., 2006; Braga *et al*., 2021). Our study evaluates the involvement of these genotypes in the production of IL-17A and IL-17F, but no association was found. These cytokines are extremely evanescent, and despite care in sample processing, this is definitely an important bias and can explain the absence of difference of IL-17A and IL-17F cytokines levels between the two groups. Another important factor is the smaller number of patients in our sample.

Clinical trials have been carried out with drugs that neutralize IL-17A (secukinumab and ixekizumab) and IL-17A and IL-17F (bimekizumab) which have shown clinical benefits in AS patients (Braun *et al*., 2017). The efficacy and safety of secukinumab has been confirmed in 80% of AS patients (Ruiz de Morales *et al*., 2020). These findings suggest that other mechanisms, in addition to IL-17 and TNF- α, may be involved in the immunopathogenesis of AS and further studies should be carried out to try to understand which immune components are involved in non-responsiveness to treatment.

Lack of data for cytokine levels in patients with AS before starting anti-TNF therapy was a limitation of this study. Moreover, knowledge regarding the levels of cytokines in the target tissues of the disease could provide extra information.

## CONCLUSIONS

This study showed that TNF-α cytokine production is involved in the failure of the anti-TNF response in AS patients. In addition, TNF-α was associated with disease activity in AS patients, independently of *HLA-B*27*. The cytokines IL-17A and IL-17F were not related to anti-TNF treatment responses in AS.

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**SUPPLEMENTARY TABLE I -** TNF and IL-17 levels in serum of AS patients after anti-TNF therapy and in healthy individuals

| Cytokines | Groups | Responders | | Non-responders | | AS controls | | Healthy individuals | | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|
| TNF-α | AS patients | N= 15 | 0.33 (0.33-2.89) | N= 15 | 5.05 (0.33-31.14) | N= 14 | 1.39 (1.39-11.30) | N= 15 | 1.39 (0.33-7.24) | <0.0001[a]<br>0.008[bd]<br>0.02[c]<br>0.01[e] |
| | *HLA-B*27*+ | N= 11 | 0.33 (0.33-2.14) | N= 7 | 5.00 (1.39-25.94) | N= 8 | 1.39 (1.39-2.89) | | | 0.005[a]<br>0.04[b] |
| | *HLA-B*27*- | N= 4 | 0.33 (0.33-2.89) | N= 8 | 5.05 (1.39-31.14) | N= 6 | 1.39 (1.00-11.30) | | | 0.02[a] |
| IL-17A | AS patients | N= 15 | 12.31 (0.18-54.29) | N= 15 | 1.15 (0.18-191.55) | N= 14 | 18.09 (0.18-247.38) | N= 15 | 9.13 (0.18-111.37) | ns |
| | *HLA-B*27*+ | N= 11 | 4.91 (0.18-48.23) | N= 7 | 14.75 (0.29-191.55) | N= 8 | 16.38 (0.18-247.38) | | | ns |
| | *HLA-B*27*- | N= 4 | 30.43 (0.18-54.29) | N= 8 | 0.75 (0.18-51.55) | N= 6 | 21.43 (5.57-170.98) | | | 0.02[d] |
| IL-17F | AS patients | N= 15 | 0.59 (0.59-74.1) | N= 15 | 0.59 (0.59-1749.9) | N= 14 | 1.27 (0.59-1833.8) | N= 15 | 0.59 (0.59-328) | ns |
| | *HLA-B*27*+ | N= 11 | 0.59 (0.59-74.1) | N= 7 | 11.73 (0.59-1749.86) | N= 8 | 206.05 (0.59-1833.77) | | | ns |
| | *HLA-B*27*- | N= 4 | 0.59 (0.59-9.23) | N= 8 | 0.59 (0.59-100.14) | N= 6 | 1.27 (0.59-1771.95) | | | ns |

Data are expressed as median (range: minimum-maximum) and pg/mL. N: number of individuals. AS: Ankylosing spondylitis. ns: not significant. *HLA-B*27+:* presence of *HLA-B*27; HLA-B*27-:* absence of *HLA-B*27;* [a] Responders *vs.* Non-responders; [b] Responders *vs.* AS control; [c] Responders *vs.* Healthy individuals; [d] Non-responders *vs.* AS control; [e] Non-responders *vs.* Healthy individuals.

**SUPPLEMENTARY TABLE II -** Cytokine levels in serum of AS patients classified according to BASDAI

| Cytokines | Groups | BASDAI <4 | BASDAI ≥4 | *P* value |
|---|---|---|---|---|
| TNF-α | AS patients | 1.39 (0.33-2.89) | 5.05 (1.39-31.14) | <0.0001 |
| | AS *HLA-B*27* positive | 1.39 (0.33-2.89) | 5.00 (1.39-25.94) | 0.008 |
| | AS *HLA-B*27* negative | 1.39 (0.33-11.30) | 5.05 (1.39-31.14) | 0.01 |
| IL-17A | AS patients | 14.06 (0.18-54.29) | 1.15 (0.18-51.55) | ns |
| | AS *HLA-B*27* positive | 10.63 (0.18-247.38) | 14.75 (0.29-191.55) | ns |
| | AS *HLA-B*27* negative | 21.90 (0.18-170.98) | 0.75 (0.18-51.55) | 0.02 |
| IL-17F | AS patients | 0.59 (0.59-1833.77) | 0.59 (0.59-1749.86) | ns |
| | AS *HLA-B*27* positive | 0.59 (0.59-1833.77) | 11.73(0.59-1749.86) | ns |
| | AS *HLA-B*27* negative | 0.59 (0.59-1771.95) | 0.59 (0.59-45.14) | ns |

Data are expressed as median (range: minimum-maximum) and pg/ml. N: number of individuals. AS: Ankylosing spondylitis. ns: Not significant. The number of individuals for each group: AS patients BASDAI <4: N=29; AS patients BASDAI ≥4: N=15; AS patients *HLA-B*27* positive BASDAI <4: N=19; AS patients *HLA-B*27* positive BASDAI ≥4: N=8; AS patients *HLA-B*27* negative BASDAI <4: N=10; AS patients *HLA-B*27* negative BASDAI ≥4: N=7.

**SUPPLEMENTARY TABLE III -** Correlation analysis among cytokine serum levels, BASDAI, and age in AS patients

| | AS patients N=44 | | AS patients *HLA-B*27*+ N=27 | | AS patients *HLA-B*27*- N=17 | |
|---|---|---|---|---|---|---|
| **Variable** | **rs** | ***P*** | **rs** | ***P*** | **rs** | ***P*** |
| Age x BASDAI | -0.0644 | ns | -0.1711 | ns | 0.0959 | ns |
| Age x TNF-α | -0.0153 | ns | -0.1734 | ns | 0.2424 | ns |
| Age x IL-17A | -0.3670 | 0.0142 | -0.5009 | 0.0077 | -0.2127 | ns |
| Age x IL-17F | -0.3871 | 0.0102 | -0.5503 | 0.0029 | -0.2448 | ns |
| BASDAI x TNF-α | 0.5706 | <0.0001 | 0.4163 | 0.0307 | 0.6382 | 0.0058 |
| BASDAI x IL-17A | -0.0734 | ns | 0.0327 | ns | -0.3428 | ns |
| BASDAI x IL-17F | 0.0130 | ns | -0.0079 | ns | -0.0304 | ns |
| TNF-α x IL-17A | 0.1112 | ns | 0.1464 | ns | 0.0405 | ns |
| TNF-α x IL-17F | 0.2533 | ns | 0.2161 | ns | 0.3760 | ns |
| IL-17A x IL-17F | 0.7385 | <0.0001 | 0.7357 | <0.0001 | 0.7497 | 0.0005 |

AS: Ankylosing spondylitis. Correlation was determined using Spearman test. ns: Not significant.

**SUPPLEMENTARY TABLE IV -** Correlation between *TNF-308* (rs18000629), *IL17A* (rs2275913) and *IL17F* (rs763780) genotypes and serum levels of TNF-α, IL-17A and IL-17F, respectively, in AS patients after anti-TNF therapy

| **Genotype** | | **Groups** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **N** | **Responders** | **N** | **Non-responders** | **N** | **AS control** | **N** | **Healthy individuals** |
| ***TNF-308* (rs18000629)** | **G/G** | 9 | 0.33 (0.33-2.88) | 7 | 4.32 (0.33-31.14) | 7 | 1.39 (1.00-2.89) | 8 | 1.39 (0.33-7.24) |
| | **G/A + A/A** | 6 | 0.86 (0.33-2.14) | 8 | 6.10 (1.39-15.34) | 7 | 1.39 (1.39-11.29) | 7 | 2.00 (0.33-6.90) |
| ***IL17A* (rs2275913)** | **G/G** | 9 | 2.96 (0.18-21.92) | 2 | 25.86 (0.18-51.55) | 9 | 22.12(0.18-188.35) | 8 | 16.14(0.52-57.32) |
| | **G/A + A/A** | 6 | 36.59 (0.18-54.29) | 13 | 1.15 (0.18-191.55) | 5 | 14.06 (1.60-247.38) | 7 | 8.44 (0.54-111.37) |
| ***IL17F* (rs763780)** | **T/T** | 9 | 0.59 (0.59-74.10) | 8 | 1.05(0.59-45.14) | 12 | 1.27 (0.59-1771.95) | 14 | 0.59 (0.59-328.00) |
| | **T/C + C/C** | 6 | 0.59 (0.59-36.85) | 7 | 0.59 (0.59-1749.86) | 2 | 917.18 (0.59-1833.97) | 1 | 71.05 |

Data are expressed as median (range: minimum-maximum) and pg/mL. N: number of individuals. There was no statistical difference between the data analyzed and p-values were not shown.