RESEARCH ARTICLE

Characterization of serum interleukin-15 in healthy volunteers and patients with early arthritis to assess its potential use as a biomarker

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ABSTRACT. As interleukin-15 (IL-15) has been implicated in the pathophysiology of rheumatoid arthritis, we analysed the serum IL-15 (sIL-15) levels in healthy subjects and patients with early arthritis to establish a cut-off point that might serve to define elevated sIL-15. This is an initial step to determine whether sIL-15 has the potential for use as a biomarker for patients with early arthritis. The IL-15 concentration was measured in serum obtained from 161 healthy controls and from 174 patients with early arthritis, and the relationship between the expression of the two IL-15 mRNA variants and the sIL-15 levels was also assessed. In healthy controls, the median sIL-15 value was 0.83 [interquartile range (IQR) 0-8.68] pg/mL; there was no significant difference in the sIL-15 values according to gender [median level in males was 1.99 (IQR: 0-8.68) pg/mL and in females 0.50 (0-8.25) pg/mL: $p = 0.821$]. Moreover, sIL-15 levels did not correlate with age ($r = 0.033$, $p = 0.685$), and they did not display a clear circadian rhythm in healthy donors, with the median values for IL-15 close to zero at each time tested. In the light of these findings, we considered that sIL-15 was elevated if its concentration was above 20 pg/mL, since this cut-off point corresponded to the 90th percentile for this healthy population. We found that 30% of the patients with early arthritis had sIL-15 values > 20 pg/mL. The levels of sIL-15 did not correlate with disease duration in early arthritis patients, nor did they fluctuate with changes in disease activity over the follow-up period. In addition, the high level of sIL15 in patients was not associated with alterations in the alternative splicing of the IL-15 mRNA, favouring the variant that produces the protein with a long signal peptide for secretion. Serum IL-15 levels were increased in a subpopulation of patients with early arthritis, indicating that this measure may serve as a biomarker for this condition. Further studies will be necessary to determine whether the clinical evolution or response to treatment of patients with high sIL-15 levels differs.

Keywords: interleukin-15, rheumatoid arthritis, early arthritis, circadian rhythm

Interleukin-15 (IL-15) is a cytokine that shares certain biological functions with IL-2, and which plays a very relevant role in the innate immune response. IL-15 mRNA is expressed in a variety of tissues and cells, including the placenta, skeletal muscle, kidney, lung, heart, fibroblasts, epithelial cells and activated monocytes. However, it is difficult to measure meaningful quantities of IL-15 in the supernatants of many of these cells, suggesting that post-transcriptional regulatory events critically affect IL-15 expression [1, 2].

Interestingly, IL-15 has been implicated in the pathophysiology of rheumatoid arthritis (RA), and indeed, this cytokine can be detected in the synovial fluid of patients with RA, but not in patients with osteoarthritis or other inflammatory joint diseases [3-5]. In addition, increased levels of IL-15 have been found in the serum of patients with RA, but not in healthy controls [6-9]. On the other hand, IL-15-activated lymphocytes induce macrophages to produce tumour necrosis factor (TNF) *in vitro* [10-13], and the neutralization of IL-15 activity improves arthritis in animal models and in patients with RA [14, 15].

Identifying reliable biomarkers that can predict disease severity and the response to treatment is of particular interest for patients with early arthritis (EA). The aforementioned role of IL-15 in RA and the fact that we

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previously found that only a subpopulation of RA patients display increased serum (s)IL-15 levels [9], supports the hypothesis that sIL-15 might be useful as a biomarker for patients with EA. However, little is known about the behaviour of sIL-15 in the general population, a crucial step prior to defining what may be considered to be an elevation in the levels of sIL-15. Hence, the main purpose of this study was to analyze the levels of sIL-15 levels in healthy individuals, and to determine whether any variations are associated with age, gender or circadian rhythm. In addition, the sIL-15 levels were studied in EA patients, along with their variation with disease activity and other parameters.

DONORS AND METHODS

Patients

Serum was obtained from three groups of individuals that had previously offered their verbal or written consent to be included in the register, and their written, informed consent to participate in the study: a) a group of 18 healthy volunteers, nine men and nine women, aged between 20 and 24 years, who participated in a pharmacokinetic study of an iron compound, and in whom the circadian effect on IL-15 production was assessed; b) a group of 153 patients, 67 men and 86 women, aged between 25 and 90 years (median 58.5 years) scheduled for surgery at the Orthopaedic Surgery and Traumatology Service of our institution, where blood samples were routinely obtained during the preoperative period (none of these patients presented inflammatory disorders or other co-morbid conditions and thus they served as healthy controls); and c) the group of early arthritis patients.

The group of early arthritis patients (group c) included 174 patients from the Early Arthritis Registry of the Hospital Universitario de La Princesa. There were 40 men and 134 women in this group, and while the median age was 50.8 years (IQR: 38.9-64.1), the men were significantly older than the women *(table 1)*. At the end of the follow-up period, 121 (69.5%) patients fulfilled the ACR classification criteria for RA [16], and the remaining 53 patients were diagnosed with undifferentiated arthritis (UA). The distribution of men and women in both diagnostic groups was similar *(table 1)*, although the patients who met the ACR criteria were significantly older than those patients with undifferentiated arthritis *(table 1)*. A more detailed description of this population is shown in *table 1*.

During the two-year follow-up period, patients at the EA clinic (EAC) attended four, structured visits. At each visit, the following data were collected and entered into an electronic database: clinical and demographic information; disease duration at the beginning of the follow-up; 28 tender and swollen joint counts (TJC and SJC, respectively); global disease activity on a 100 mm visual analogue scale assessed both by the patient (GDAP) and by the physician; the Spanish version of the Health Assessment Questionnaire [17]; and laboratory tests including erythrocyte sedimentation rate (ESR), the levels of C-reactive protein (CRP), and the rheumatoid factor (RF) assessed by nephelometry. In addition, we obtained serum samples that were frozen at - 80°C until sIL-15 was measured. The study protocol was reviewed and approved by the Local Research Ethics Committee.

Measurement of serum IL-15

The concentration of sIL-15 was measured using a sandwich enzyme immune assay (EIA). Briefly, 96-well, high binding EIA plates (Costar, Cambridge, MA, USA) were coated overnight at 4°C with the MAB647 MAb (anti-IL-15, 50 µL/well: R&D Systems Europe Ltd., Abingdon, UK), diluted to 4 µg/mL in PBS (pH 7.4). Each well was then washed twice with 200 µL of wash buffer [0.05% Tween 20 in PBS (pH 7.4)] and blocked for 1 hour at 37°C by adding 200 µL of PBS containing 2% BSA. Between each step, the wells were washed three times with 200 µL of wash buffer. Subsequently, 50 µL/well of diluent buffer [0.1% BSA, 0.05% Tween20, 1 mg/mL normal mouse immunoglobulin (Calbiochem, San Diego, CA, USA], 20 mM Trizma base, 150 mM NaCl (pH 7.3)], plus 50 µL of each sample and standard dilutions of recombinant human IL-15 (500 to 6.2 pg/mL; R&D Systems) were added to the respective wells (in duplicate) and incubated at room temperature for 2 h. The bound IL-15 was detected with BAM247 (biotinylated anti-IL-15 MAb, 50 µL/well; R&D Systems), and diluted at 200 ng/mL in diluent buffer for 1 h at room temperature. After washing, streptavidin HRP (100 µL/well, 1/5,000 in diluent buffer; Calbiochem) was added to the wells for 20 min at room temperature, and the antibody binding was visualised with 3,3′,5,5′-tetramethylbenzidine (100 µL/well, Chemicon

Table 1
Characteristics of patients with early arthritis at the baseline visit

Data	All patients (n = 174)	Rheumatoid arthritis (n = 121)	Undifferentiated arthritis (n = 53)	P value
Female gender	134 (77)	94 (77.7)	40 (75.5)	NS
Age, years, median (IQR)	50.8 (38.9-64.1)	54.4 (43.2-67.3)	49.4 (35.3-59.9)	0.03
Positive rheumatoid factor	75 (43)	65 (53.8)	9 (17)	< 0.001
Anti-CCP	67 (38.5)	62 (51.2)	5 (9.4)	< 0.001
IL-15 > 20 pg/mL	50 (29)	42 (35%)	8 (15%)	0.008
IL-15 pg/mL, median (IQR)	8.05 (2.6-23.8)	10.6 (4-30)	5.4 (1-15.1)	0.006
DAS28, median (IQR)	4.5 (3.3-5.7)	4.9 (3.8-5.9)	4.7 (2.8-4.7)	< 0.001
HAQ, median (IQR)	1 (0.5-1.625)	1.125 (0.5-1.75)	0.75 (0.375-1.25)	0.005

IQR: interquartile range; anti-CCP: anti-cyclic citrullinated peptide antibodies; DAS28: disease activity score estimated with 28 joint counts; HAQ: health assessment questionnaire.

International Inc., Temecula, CA, USA). The optical density of each well was determined using a microtitre plate reader LP400 (Sanofi Diagnostics Pasteur, Marnes la Coquette, France) set to 450 nm, with a wavelength correction set to 550 nm. Cytokine values were calculated from the standard curve and samples that generated values higher than the highest standard were diluted (1:1) in diluent buffer and assayed again.

As described previously [9], this protocol excluded the possible interference of rheumatoid factor in the immune assay, and although we previously failed to observe significant differences in sIL-15 levels between samples processed in the presence or absence of murine immunoglobulins, we maintained this supplement in the diluent buffer. In fact, increased IL-15 levels were observed in patients positive and negative for rheumatoid factor (data not shown). For this procedure, the intra-assay variability (mean ± standard deviation, SD) was 15.4 ± 21.7% and the inter-assay variability was 21.9 ± 33.4%.

Quantitative analysis of IL-15 mRNA using RT-PCR

To analyse the possible correlation between the expression of the two IL-15 mRNA isoforms and the sIL-15 levels, we studied eleven samples from patients with IL-15 serum levels below the limit of the detection of our EIA (see above). They were compared with samples from four patients with serum IL-15 levels higher than 60 pg/mL. This limit was chosen since it corresponds to the 90th percentile of the distribution of sIL-15 levels in the group of patients with early arthritis *(table 2)*.

Total cellular RNA from PBLs was isolated with the Ultraspec RNA reagent (Biotecx, Houston, Texas, USA), according to the manufacturer's protocol. Blood samples were taken at the patients' first visit, prior to the onset of treatment. The RNA was re-suspended in 0.02 mL of water and stored at - 70°C. Total RNA was converted into cDNA. The reverse transcriptase (RT) reaction was carried out using 2 µg of DNaseI-treated RNA and the ImProm-II Reverse Transcriptase (Promega GmbH, Mannheim, Germany), and gene expression was quantified by SYBR Green real-time PCR (Roche Diagnostics GmbH, Penzberg, Germany) in a DNA Lightcycler rapid thermal cycler system (Roche, Mannheim, Germany). The sequences of the cytokine specific 5′ and 3′ primer pairs were as follows: GAPDH forward: 5′ GAA GGT GAA GGT CGG AGT C 3′; GAPDH reverse: 5′ GAA GAT GGT GAT GGG ATT TC 3′; IL15 forward b: 5′ GGA TTT ACC GTG GCT TTG AGT AAT GAG 3′; IL15 forward a: 5′ GCC TTC ATG GTA TTG GGA AC3′; IL15 reverse: 5′ GAA TCA ATT GCA ATC AAG AAG TG 3′. The results for each IL-15 isoform were normalized to the GAPDH expression and measured in parallel in each sample. The PCR products were resolved in a 2% agarose gel and visualized with GelStat Nucleic Acid Gel Stain (Lonza, Basel, Switzerland).

Statistical analysis

Categorical variables were compared with the chi-squared test (χ^2) or the Mann-Whitney U test. The correlation between age and sIL-15 levels in the group of healthy controls was assessed using the Pearson's product-moment correlation coefficient. The Wilcoxon signed-rank test was used to compare the sIL-15 levels at follow-up visits in the group of patients with RA. The data are expressed as the median and interquartile range (25^{th}-75^{th} percentile, IQR). All the statistical analyses were performed with Stata, version 9.2 for Windows (StataCorp LP, College Station, TX, USA), and unless otherwise specified, statistical significance was set at $p < 0.05$.

RESULTS

Serum IL-15 levels in healthy controls

The sIL-15 levels in the healthy subjects did not follow a normal distribution, but rather, there was a higher

Table 2
Changes in serum IL-15 levels in patients with RA during a 2-year follow-up period

Study subjects	Serum levels of IL-15 (pg/mL)			
	Median	25^{th}-75^{th} percentile	90^{th} percentile	P value when compared with visit 1
All EA patients				
Visit 1 (baseline)	8.05*	2.6-23.8	57.7	-
Visit 2	8.4	3.0-35.1	54.9	0.936
Visit 3	8.4	3.0-23.2	41.6	0.026
Visit 4	6.4	0-17.9	50.3	0.031
RA patients				
Visit 1 (baseline)	10.8	4.4-32.6	61.0	-
Visit 2	11.4	3.1-37.0	68.0	0.808
Visit 3	8.8	3.5-31.7	45.0	**0.007**
Visit 4	7.0	1.0-18.5	65.4	0.101
UA patients				
Visit 1 (baseline)	6.2	1.1-16.3	35.0	-
Visit 2	4.8	2.4-17.5	39.2	0.501
Visit 3	4.5	1.1-19.0	31.0	0.82
Visit 4	6.7	0-15.0	29.5	0.151

The Wilcoxon test for paired data was applied to compare sIL15 levels at baseline with the levels at the following visits. Owing to multiple comparisons, the level of significance was adjusted with Bonferroni's correction to $p < 0.015$.

frequency of values close to zero and a long tail to the right, with a median sIL-15 value of 0.83 (IQR 0-8.68) pg/mL *(figure 1A)*. There were no significant differences in sIL-15 values according to gender *(figure 1B)* as the median value in men was 1.99 (0-8.68) pg/mL compared with 0.50 (0-8.25) pg/mL in women ($p = 0.821$). In addition, the sIL-15 levels did not correlate with age ($r = 0.033$, $p = 0.685$, *figure 1C*). Taking into account these findings, we established a cut-off point of 20 pg/mL to consider sIL-15 levels as elevated, since this value corresponded to the 90th percentile for this healthy population *(figure 1A)*.

Lack of circadian effect on serum IL-15 levels

Some cytokines, such as IL-6 and TNF, show circadian fluctuations in their production, [18]. Hence, we assessed whether the differences in the aforementioned data could

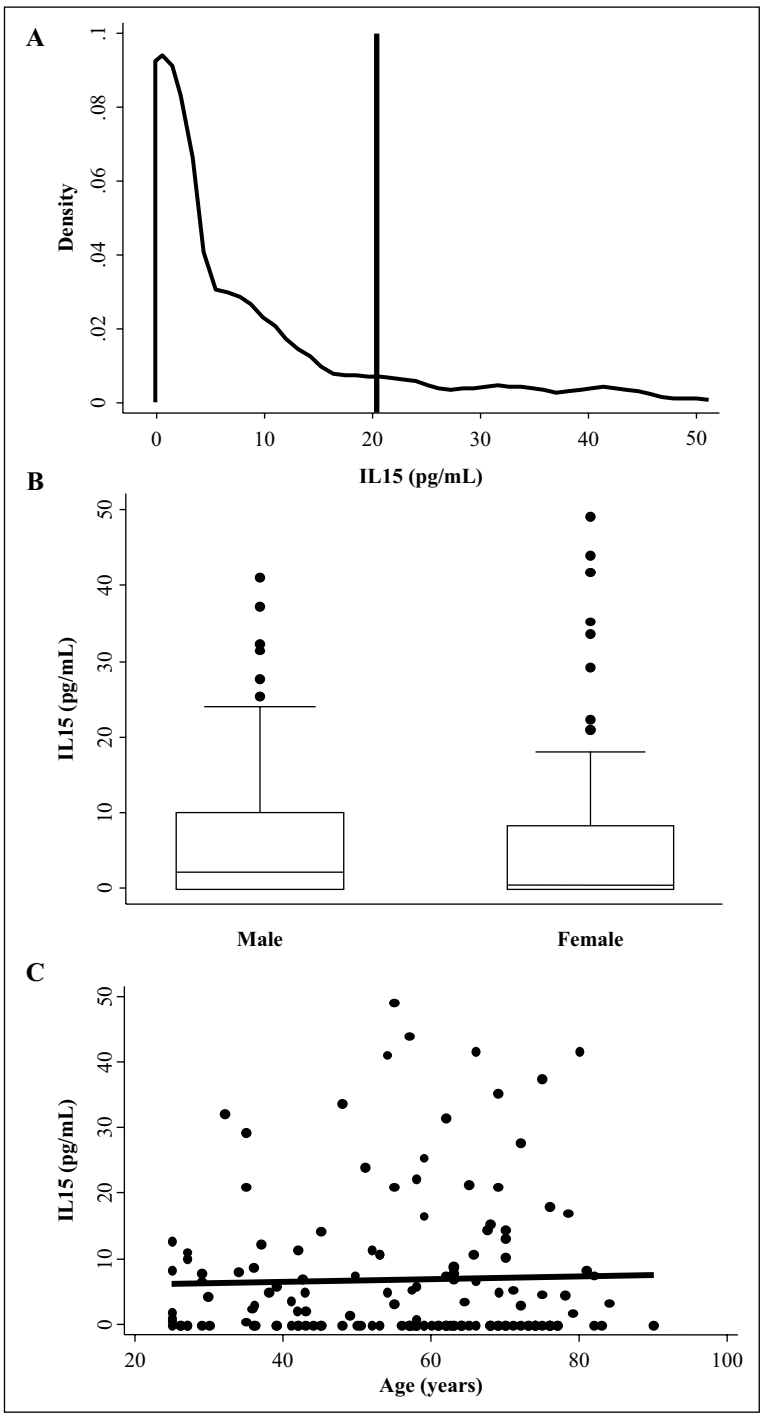

Figure 1

A) Distribution of serum IL-15 levels (sIL-15) in the population of healthy subjects. **B**) There are no differences in sIL-15 between men and women. Data are presented as the interquartile range (p75 upper edge of the box, p25 lower edge, p50 midline in the box), as well as the p95 (upper line from the box) and p5. Dots represent the outliers. **C**) There is no correlation between sIL-15 levels and age. Dots represent the values of sIL-15 for each patient *versus* their age. The solid black line represents the local (bandwidth 0.8) least-squares weighted regression, using the lowess command of Stata 10 for Windows.

be due to the collection of samples during a particular period of the circadian cycle that naturally corresponds to low cytokine production. Accordingly, we measured sIL-15 in samples obtained over a 24-hour period in 18 healthy volunteers. In all cases there was no clear-cut circadian rhythm associated with the sIL-15 values, and the median values of sIL-15 were close to zero at all times *(figure 2)*. However, increasing sIL-15 values were observed in two subjects during the study period *(figure 2)*, although the pattern of sIL-15 production in these cases still failed to follow a circadian cycle.

Serum IL-15 levels in early arthritis patients

In the EA group, 30% of patients had sIL-15 values above 20 pg/mL, the cut-off point that corresponded to the 90th percentile of the healthy population *(figure 3A)*. The proportion of patients with increased sIL-15 was greater in patients fulfilling the ACR criteria for RA than in those with UA (34.7% *versus* 15.1%; *table 1* and *figure 4*). In addition, sIL-15 levels in the early arthritis group were higher than those in healthy controls whose sIL-15 concentrations were above the cut-off point of 20 pg/mL *(figure 3A)*. This was also the case in both the RA and UA subgroups, although the difference was greater in those fulfilling the ACR criteria for RA *(figure 4)*.
Serum IL-15 levels did not correlate with disease duration from the beginning of symptoms to the first visit to the EAC *(figure 3B)*. In addition, sIL15 levels did not fluctuate as the disease activity changed as a result of treatment during the follow-up period *(figure 3C)*.The variations in sIL-15 levels during the two-year follow-up of EA patients are shown in *table 2*. A slight trend towards a decrease over time was observed in the whole early arthritis group, although it was not statistically significant *(table 2)*. On average, this decrease was less than 3 pg/mL over the follow-up period, and it was only significant in the RA subgroup of patients at the 3rd visit *(table 2)*.

Expression of IL15 mRNA variants in peripheral blood lymphocytes of patients with early arthritis

We assessed whether the alternative splicing of IL-15 mRNA could explain the differences in sIL-15 levels in patients with EA. Two different variants of human IL-15 mRNA exist, and the precursor protein encoded by each variant differs in the signal peptide sequence. The authentic IL-15 mRNA encodes a precursor protein with a 48-amino acid signal peptide (long signal peptide, LSP), while the alternatively spliced form of IL-15 mRNA, generated by retention of exon "A" *(figure 5A)*, has a premature termination codon that provides an alternative initiation codon. The new signal peptide generated is only 21 amino acids long (short signal peptide, SSP), and the associated IL-15 produced from this SSP isoform is not secreted, but rather it is stored intracellularly, accumulating in the nucleus and cytoplasm [19-21].

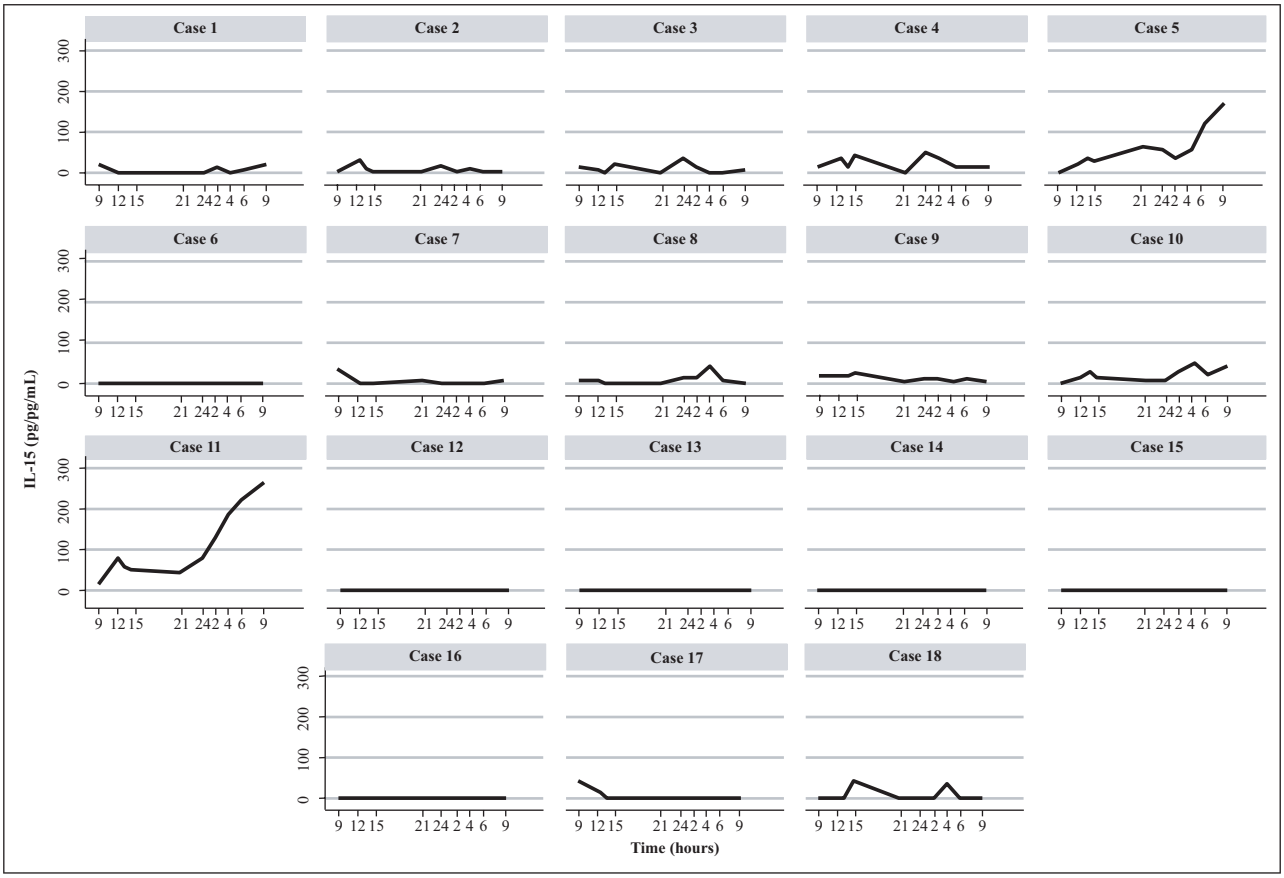

Figure 2
Serum IL-15 levels do not follow a circadian pattern. Blood samples from 18 healthy volunteers were obtained at 9:00, 12:00, 13:00, 14:00, 21:00, 24:00 and 2:00, 4:00, 6:00 and 9:00 on the next day, these latter samples corresponding to 26:00, 28:00, 30:00 and 33:00 hours, respectively.

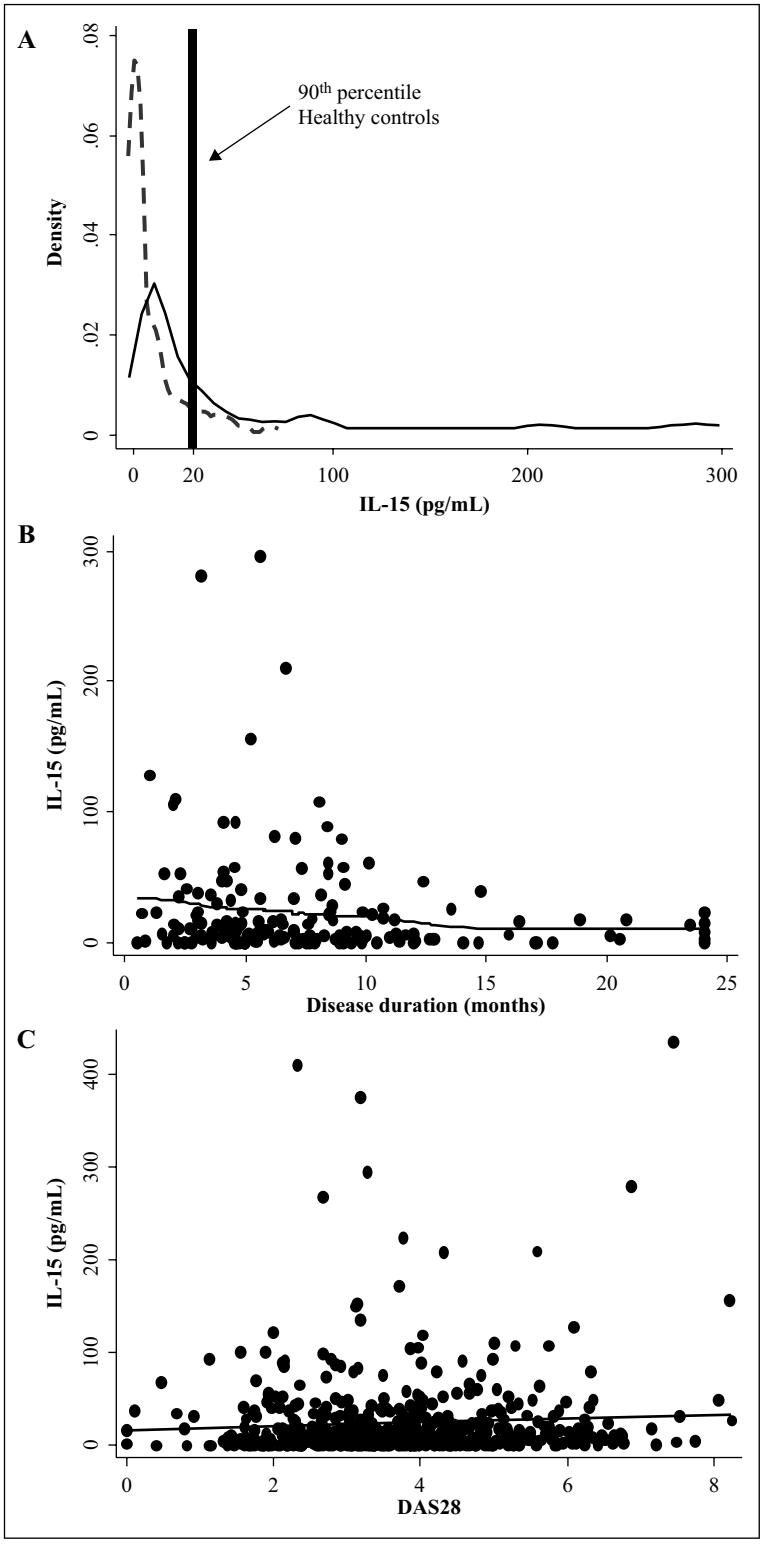

Figure 3

Serum IL-15 levels (sIL-15) in patients with early arthritis (EA). **A**) There are more EA patients with increased sIL-15 levels (solid black line) than controls (dotted line). **B**) The sIL-15 levels do not correlate with disease duration or with **C**) disease activity (DAS28: disease activity score estimated with 28 joint counts), over the follow-up period. Dots represent the values of sIL-15 for each patient *versus* their disease duration (panel B) or disease activity (panel C). The solid black line represents the local (bandwidth 0.8) least-squares weighted regression using the lowess command of Stata 10 for Windows.

To measure the levels of the two IL-15 mRNA variants, we performed RT-PCR using specific primers *(figure 5A)*. Accordingly, we found that expression of the two IL-15 mRNA variants appeared to be higher in patients with increased sIL-15 than in those in which this cytokine was not detected *(figure 5B)*. However, these differences were only significant for the SSP IL-15 mRNA variant. Since both variants were increased in patients with higher sIL-15, we investigated whether the ratio between both IL-15 mRNA variants was weighted towards the LSP

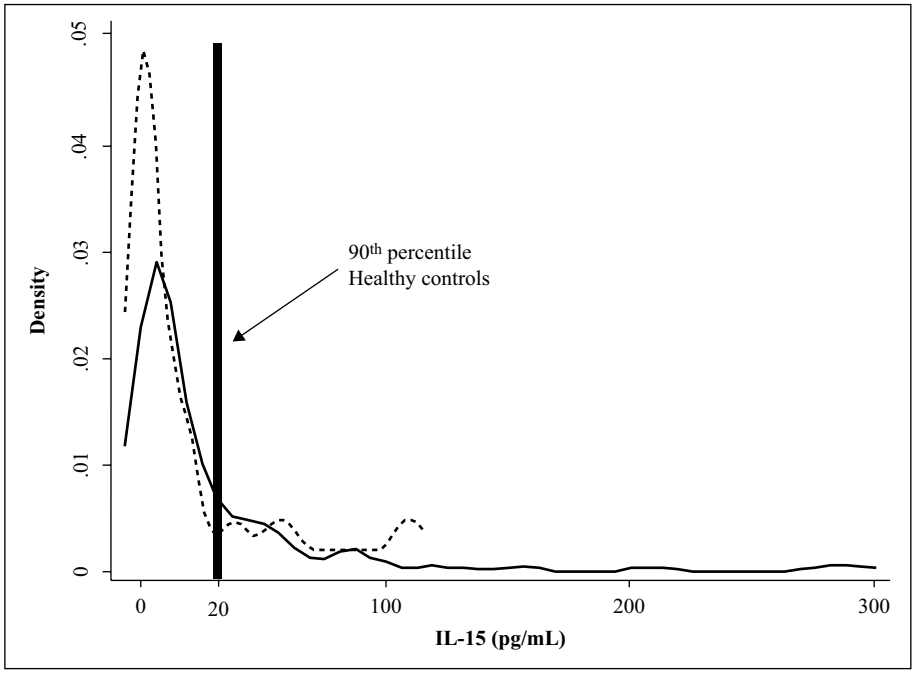

Figure 4
Distribution of IL-15 serum levels in patients with rheumatoid arthritis (solid black line) and patients with undifferentiated arthritis (dotted line).

precursor protein in patients with high sIL-15. In most of the EA samples tested, both cDNA variants were amplified by RT-PCR, although they were differentially expressed in each sample *(figure 5C)*. However, the ratio between the two amplification products obtained by RT-PCR (643 bp/524 bp) was very similar in both groups of patients *(figure 5C)*.

DISCUSSION

The present study provides two relevant findings: about 30% of patients with EA have sIL-15 levels higher than the cut-off point that can be considered normal in the general population; the behaviour of this cytokine suggests that it may be a reliable biomarker for this subpopulation of patients.

Our data indicate that the presence of sIL-15 in the general population is extremely rare, since in 70% of the healthy controls, the sIL-15 levels were below the threshold for detection of our assay (6.5 pg/mL). Indeed, in only 10% of healthy subjects were the sIL-15 values above 20 pg/mL, which led us to select arbitrarily this 90th percentile as the upper limit for normal sIL-15 values. Considering this cut-off value, 30% of the patients with early arthritis had high sIL-15 levels, and although this finding was more frequent in patients that fulfilled the ACR classification criteria for RA, the sIL15 levels do not seem to be useful for the diagnosis of early RA. Nevertheless, the detection of this cytokine had many characteristics that suggest it may be a reliable biomarker. Firstly, in contrast to other cytokines, it did not suffer circadian variations and thus, it can be measured at any time of the day. Secondly, no important differences in sIL-15 levels were found according to age, gender or disease duration. Thirdly, variations in sIL-15 according to disease activity were not evident and treatment did not seem to induce any relevant changes in the serum levels of this cytokine, such that sIL-15 can be measured both in naïve and treated patients. Therefore, sIL-15 seems to be useful as a biomarker of a subpopulation of patients with EA rather than as an acute phase reactant.

In this regard, we previously observed a significant correlation between sIL15 and the number of disease-modifying antirheumatic drugs (DMARDs) prescribed to patients [9]. If we accept that the number of DMARDs used in one patient can be considered as a surrogate variable of disease severity, it is possible that those patients with EA and increased sIL15 may correspond to a more aggressive phenotype of the disease. This hypothesis seems reasonable since IL-15 appears to be involved in some of the mechanisms underlying the perpetuation of TNF production [10-12], the survival of human Th17 cells [22], and the production of IL-17 [6], as well as in the proliferation of B lymphocytes and the promotion of osteoclast differentiation [23]. All these events contribute significantly to the persistence of synovitis and osteoclast formation that leads to bone destruction in RA.

On the other hand, as regards the factors involved in the modulation of sIL-15 levels, it is well known that both IFN-γ and TNF induce the expression of membrane-anchored IL-15 [11, 12]. Indeed, we have shown that treatment with TNF antagonists can moderately decrease sIL-15 levels [24]. However, it is very difficult to trigger the production of soluble IL-15 *in vitro* and it has been proposed that its induction is under strict, post-transcriptional control [1]. Here, we have shown that the presence of high sIL-15 levels is not related to a switch in IL-15 mRNA transcription towards the LSP

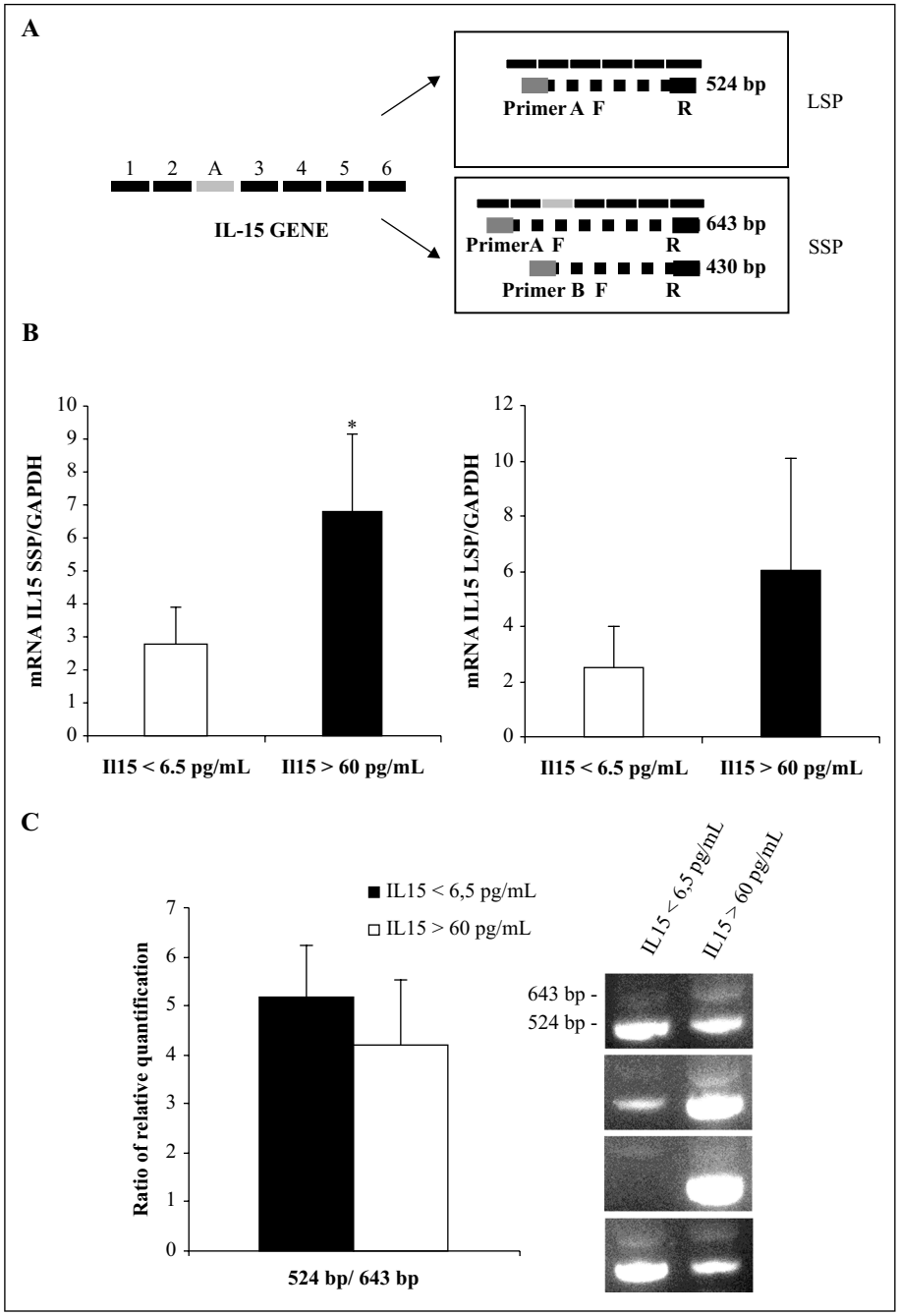

Figure 5

The alternative splicing of IL-15 mRNA does not explain the variation in sIL-15 levels. **A**) The forward primer "a" (exon 1) with the reverse common primer (exon 6) amplified a product of 524 bp as expected for the authentic IL-15 mRNA (LSP IL-15 precursor protein), while the forward primer "b" (alternative exon A) with the common reverse primer generated a 430 bp product. If the alternative form of IL-15 mRNA existed, the PCR product with the forward primer "a" would be 643 bp (SSP IL-15 precursor protein). **B**) The patients with high sIL-15 express more SSP and LSP IL-15 mRNA variants than patients with no detectable sIL-15. The data are shown as the amount of each IL-15 mRNA variant adjusted to GAPDH mRNA considered as the housekeeping control. * $p < 0.05$ Mann-Whitney test. **C**) There are no differences in the ratio between the two IL-15 mRNA variants (LSP and SSP), between patients with high or low sIL-15. The lower band was 524 bp in size, corresponding to the LSP IL-15 precursor transcript, and the upper band was 643 bp corresponding to the precursor transcript of the alternatively spliced SSP IL-15 isoform.

IL-15 mRNA variant. By contrast, we found that our patients expressed both mRNA variants more strongly than patients with no detectable sIL-15. It is possible that genetic variations at the promotor level may be involved, although no association has been observed between genetic variations in the IL-15 gene and the risk of developing RA [25].

As occurs in other conditions such as cancer, the value of biomarkers is not restricted to diagnostic accuracy, but might also be relevant in assessing therapeutic response or detection of severe disease. Our findings suggest that the measurement of sIL15 may be useful for early identification of a subpopulation of patients with recent-onset arthritis with a putative, characteristic RA clinical course. However, further studies are necessary to determine whether this subset of patients behaves differently in terms of their clinical evolution or response to treatment.

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The remaining authors have no conflicts of interest to disclose.

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