RESEARCH ARTICLE

Serum and urinary levels of IL-18 and its inhibitor IL-18BP in systemic lupus erythematosus

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ABSTRACT. Overproduction of inflammation-related cytokines plays an important role in systemic lupus erythematosus (SLE). A crucial cytokine is IL-18, a member of the IL-1 family involved in the regulation of both innate and acquired immune responses. The aim of this study was to evaluate free IL-18 levels in the serum and urine of SLE patients, in order to establish their relationship with other biomarkers of disease activity. Serum and urine levels of IL-18 and IL-18BP were measured by ELISA in 50 SLE patients and in 32 healthy subjects; free IL-18 was calculated using the law of mass action. Serum levels of total IL-18, IL-18BP and free IL-18 were higher in SLE patients than in healthy controls. Total and free serum IL-18 levels were higher in patients with active disease (with nephritis or active non-renal disease), and correlated with the ECLAM score. Urinary levels of total and free IL-18 were higher in patients than in controls, but did not correlate with disease activity. The data collected in this study show that increased levels of both IL-18 and its natural inhibitor IL-18BP, characterise SLE. Despite the overproduction of IL-18BP, free IL-18 is still significantly higher in SLE patients than in controls, and its serum levels are a marker of disease activity.

Keywords: systemic lupus erythematosus, IL-18, inflammation

Systemic lupus erythematosus (SLE) is an autoimmune disorder of unknown etiology, characterised by a wide spectrum of immunological abnormalities that lead to the production of a variety of autoantibodies. Cytokine imbalance has a major role in this immune activation. The overproduction of pro-inflammatory cytokines, driving the expansion of Th1 cells, has been recognised as a common feature of both murine lupus models and human SLE disease [1-3].

A crucial inflammation-related cytokine is IL-18, a member of the IL-1 family involved in the regulation of both innate and acquired immune responses. IL-18 is a critical regulator of Th1 responses, although it is also capable of inducing Th2 cytokines. IL-18 was initially identified as a major inducer of IFN-γ in Th1 and natural killer (NK) cells, with a key role in Th1 activation in synergy with IL-12 [4]. In addition, IL-18 stimulates production of pro-inflammatory cytokines such as TNF-α and IL-1β in mature Th1 cells, monocytes/macrophages, and NK cells, upregulates chemokine production and expression of adhesion and costimulatory molecules, enhances perforin- and FasL-mediated cytotoxicity by NK and T cells, and induces the release of matrix metalloproteinases, activities that are central to the inflammatory reaction and subsequent tissue damage. IL-18 is synthesised as a biologically inactive precursor and subsequently cleaved by caspase-1 to give rise to the mature, active cytokine. Monocytes/macrophages and dendritic cells are the major source of IL-18. IL-18 activity is regulated by IL-18BP, a soluble molecule that binds mature IL-18 with high affinity and prevents its interaction with cell surface receptors [5]. IL-18 indirectly increases the production of its own inhibitor in a feedback loop, through upregulation of the major IL-18BP-inducer IFN-γ [6]. In pathological conditions characterised by high levels of IL-18, IL-18BP levels are also increased, in an attempt to counteract the inflammatory effects of IL-18.

In the MRL *lpr/lpr* murine lupus model, intraperitoneal administration of recombinant IL-18 has been demonstrated to exacerbate renal disease [7]. Increased reactivity to IL-18 is observed in lymphocytes, due to constitutive hyperexpression of the IL-18 receptor accessory chain IL-18Rβ [8]. Up-regulation of IL-18 expression is detected in all affected organs [7, 9], including nephritic

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kidneys [10]. Moreover, *in vivo* inhibition of IL-18 by anti-IL-18 cDNA vaccination, attenuates lymphoproliferation and nephritis and increases lifespan [11].

In the human disease, several studies have detected high levels of the cytokine in patients' serum [12], and especially in those with active nephritis [14-16]. Increased glomerular expression of IL-18 is found in kidney biopsies from SLE patients [16, 17], and the local production of the cytokine has been reported to play an important role in driving the migration of dendritic cells to the kidney [17].

However, in order to obtain a more comprehensive view of the functional role of IL-18 in SLE, evaluation of the cytokine levels in patients' serum and urine is not sufficient. In fact, since IL-18 can be bound and neutralised by its soluble inhibitor IL-18BP, only the fraction of IL-18 that is not bound to IL-18BP is actually free to interact with the membrane receptors on target cells/organs and is thus biologically active. The interaction of IL-18 with IL-18BP has been described in detail, and is a stoichiometric 1:1 interaction with a K_d of 400 pM [18]. Thus, the level of free IL-18 depends on the absolute concentrations of both IL-18 and IL-18BP, and on their affinity of interaction, according to the law of mass action. The assays available to detect IL-18 measure the mature form of the cytokine, both free and complexed with IL-18BP. Indeed, most of the reports about IL-18 levels in SLE refer to total IL-18, while only limited information is available on paired IL-18 and IL-18BP levels [19], or free IL-18 levels [20]. As IL-18 is produced in nephritic kidneys [15-17], the measurement of IL-18 and IL-18BP levels in the urine of SLE patients may allow a more direct estimate of the activity of nephritis.

The aim of this work is to evaluate the free IL-18 levels in serum and urine, by analysis of IL-18 and IL-18BP concentrations, in a cohort of SLE patients, and to establish their relationship with other biomarkers of disease activity.

PATIENTS AND METHODS

Patients

Fifty SLE patients attending the Clinical Immunology and Rheumatology Units of the University of Pisa were included in this study; there were 47 females and 3 males, age range 15-71 years (mean 37.7, median 36).

For all patients, a full clinical and serological evaluation was performed that included measurement of complement levels, anti-dsDNA and anti-C1q antibodies. Anti-dsDNA and anti-C1q antibodies were detected using ELISA as previously described [21]. On the basis of clinical and serological findings, a disease activity score (ECLAM) was calculated.

Thirty two normal subjects served as controls: 30 females and 2 males; age range 22-61 years (mean 38.0, median 34).

Informed consent was obtained from all the subjects and the study was approved by the local Ethics Committee.

Blood and urine sampling

Venous blood was collected by venipuncture, and serum immediately prepared, aliquoted, and stored frozen at - 80°C until assay. Urine was collected within one hour of blood collection, centrifuged to eliminate debris, aliquoted and stored frozen at - 80°C until assayed. To ensure the homogeneity of results, assays of IL-18 and IL-18BP were always performed using new aliquots, not having been subjected to thawing and re-freezing. Repeated testing on both serum and urine aliquots subjected to two, three, or four thawings however, showed that loss of reactivity to antibodies for both IL-18 and IL-18BP was minimal.

Measurement of IL-18, IL-18BP, and calculation of free IL-18

Serum and urine levels of IL-18 and IL-18BP were measured by ELISA using commercially available kits. The IL-18 ELISA (human IL-18 ELISA kit; MBL, Woburn, MA, USA) measures the mature form of IL-18, with no cross-reaction with the uncleaved, biologically inactive IL-18 precursor. Serum samples had to be diluted at least 1:2 before assay, since in undiluted serum the IL-18 content was significantly underestimated. No such problems were experienced with urine samples. Previous results showed that the MBL ELISA could detect IL-18 with the same efficiency in the presence of increasing concentrations of IL-18BP [22]. Detection of IL-18BP was performed using the commercial IL-18BPa ELISA (R&D Systems, Minneapolis, MN, USA). The commercial assay appeared to significantly overestimate IL-18BP concentrations, as judged by a series of samples tested in parallel with the originally developed sandwich ELISA that used *bona fide* human IL-18BP as standard [18]. The IL-18BP standard used in the commercial assay is an IL-18BP-containing chimeric protein of higher molecular weight, thus the declared standard curve values had to be adjusted as a function of the real IL-18BP MW. After this adjustment, the concentrations measured in samples with the commercial kit coincided with those measured against the *bona fide* IL-18BP standard. The cross-reactivity of the commercial assay with different isoforms of IL-18BP is not known. However, it should be noted that IL-18BPa is the major IL-18BP isoform expressed in man [23]. In the case of the IL-18BP ELISA assay, detection was not affected by serum concentrations, and was also reliable in undiluted serum. The presence of IL-18 was found to interfere with IL-18BP detection only at concentrations > 1.6 ng/mL [22]. In the present study, IL-18BP detection was performed at sample dilutions that never contained > 0.1 ng/mL of IL-18.

After measurement of the concentrations of both IL-18 and IL-18BP in each sample, the law of mass action was used to calculate free IL-18 (*i.e.*, the fraction of cytokine not bound to its inhibitor IL-18BP), as described by Novick *et al.* [18]. Briefly, by knowing the MW of both IL-18 (18.4 kDa) and IL-18BP (17.6 kDa), that IL-18 and IL-18BP interact at a ratio

of 1:1, and that this interaction has a K_d of 0.4 nM [23], the law of mass action:

$$K_d = \frac{[Ligand]\cdot[Receptor]}{[Ligand \cdot Receptor]}$$

was applied as follows:

$$x = \frac{-b + \sqrt{b^2 - 4c}}{2}$$

where x is $[\text{IL-18}]_{free}$, b is [IL-18BP] – [IL-18] + K_d, and c is – K_d • [IL-18].

Statistical analysis

The Spearman correlation coefficient was used to examine the relationship between the disease activity score and levels of cytokines (total IL-18, IL-18BP and free IL-18), complement component or autoantibodies.
Cytokine levels in SLE patients and healthy controls, and in subgroups of SLE patients (active/inactive), were compared using the Mann-Whitney test.
The non-parametric Wilcoxon matched pairs test was used in a sub-group of eight patients to compare the ECLAM scores and the values of serum IL-18 before and after the treatment of a disease flare.

RESULTS

Fifty SLE patients were consecutively recruited for this study. In 16/50 patients active nephritis was present, and was confirmed in 15 out of 16 by a renal biopsy that showed in 13/15 proliferative nephritis class IV, in 1/15 proliferative nephritis class III and in 1/15 membranous nephritis class V. In 6/50 patients, the disease was active but not at the renal level (hematologic manifestations, brain involvement, myositis or cutaneous manifestations). Twenty eight/50 patients were in an inactive stage of the disease. In 12/50 patients, a secondary, anti-phospholipid syndrome was diagnosed.
Eight patients of our cohort were seen twice, at least three months apart, in two different phases of the disease (the first sample taken during a disease flare, the second sample, in an inactive phase of the disease).
Thirty three patients were treated with steroids at low dosage (prednisone < 10 mg/day); nine received prednisone 15 mg/day. Seventeen patients were treated with hydroxychloroquine, three with azathioprine, two with methotrexate, six with cyclosporine and seven with mycophenolate. In all the active patients, blood and urine samples were collected before the administration of high dose steroids or cyclophosphamide.

IL-18 and IL-18BP in serum and urine

Serum and urine levels of IL-18 and IL-18BP were measured in SLE patients and healthy controls.
Serum levels of both total IL-18 and IL-18BP were higher in SLE patients than in healthy controls (both $p < 0.0001$) *(figure 1A left and central panels)*. Despite the increase in IL-18BP, serum levels of free IL-18 were higher in SLE patients than in controls ($p < 0.0001$) *(figure 1A right panel)*.
Urinary levels of total IL-18 were higher in SLE patients than in healthy controls ($p = 0.0415$; *figure 1B left panel*), while IL-18BP levels did not differ (*figure 1B central panel*). Free urinary IL-18 was significantly higher than in controls ($p = 0.0194$) (*figure 1B right panel*). Urinary cytokines levels did not correlate with urinary protein concentration (data not shown).

IL-18 and disease activity

Total and free serum IL-18 levels were higher in active *versus* inactive patients ($p = 0.0078$ and $p = 0.0074$ respectively), while IL-18BP levels did not differ *(figure 2A)*. Likewise, total and free serum IL-18 levels correlated with the ECLAM score ($p = 0.0019$ and $p = 0.0078$, respectively), while there was no significant correlation between serum IL-18BP and disease severity *(figure 3 upper panels)*. Moreover, the levels of C3 were inversely related to total and free serum IL-18 levels ($p = 0.0077$ and $p = 0.0290$ respectively), while again no significant correlation was found with serum IL-18BP *(figure 3 lower panels)*. In the case of total and free urine IL-18, as for IL-18BP, no differences were detected between active and inactive patients *(figure 2B)* and no correlation was found with disease severity markers (ECLAM, C3) (data not shown). The ECLAM score correlated strongly with the levels of anti-C1q antibodies ($p = 0.0035$) and, to a lesser extent, with anti-dsDNA antibodies ($p = 0.0313$) (data not shown).
Serum IL-18 or IL-18BP levels were no higher in patients with active nephritis than in patients with active non-renal disease *(table 1)*. However, the serum levels of both total and free IL-18 appeared to be significantly higher than those of renal patients. There were no differences in the levels of urinary IL-18 (total or free) or of serum and urinary IL-18BP in the different subgroups of active patients *(table 1)*.

IL-18 in follow-up of SLE patients

In eight of the active patients, a second evaluation was performed, when treatment had induced disease remission. Data in *figure 4* show serum IL-18 (total and free) and IL-18BP, and the ECLAM score in flare and remission. All three cytokine parameters showed a significant and concomitant reduction after treatment, with a p of 0.0039, 0.0391 and 0.0273 respectively.

DISCUSSION

The data collected in this study show that increased levels of both IL-18 and its natural inhibitor IL-18BP characterise SLE. Despite the overproduction of IL-18BP, free IL-18 is still significantly higher in SLE patients than in healthy subjects, and thus able to exert biological effects. The increase in free IL-18 is present in most SLE patients, including those with inactive disease, but is more obvious in patients with active disease. Free IL-18 levels, in fact, correlate positively with the ECLAM score and negatively with a known serological marker of disease activity, serum C3.
Thus, IL-18 may play a role both in inducing the immune system abnormalities typical of SLE, and in triggering the

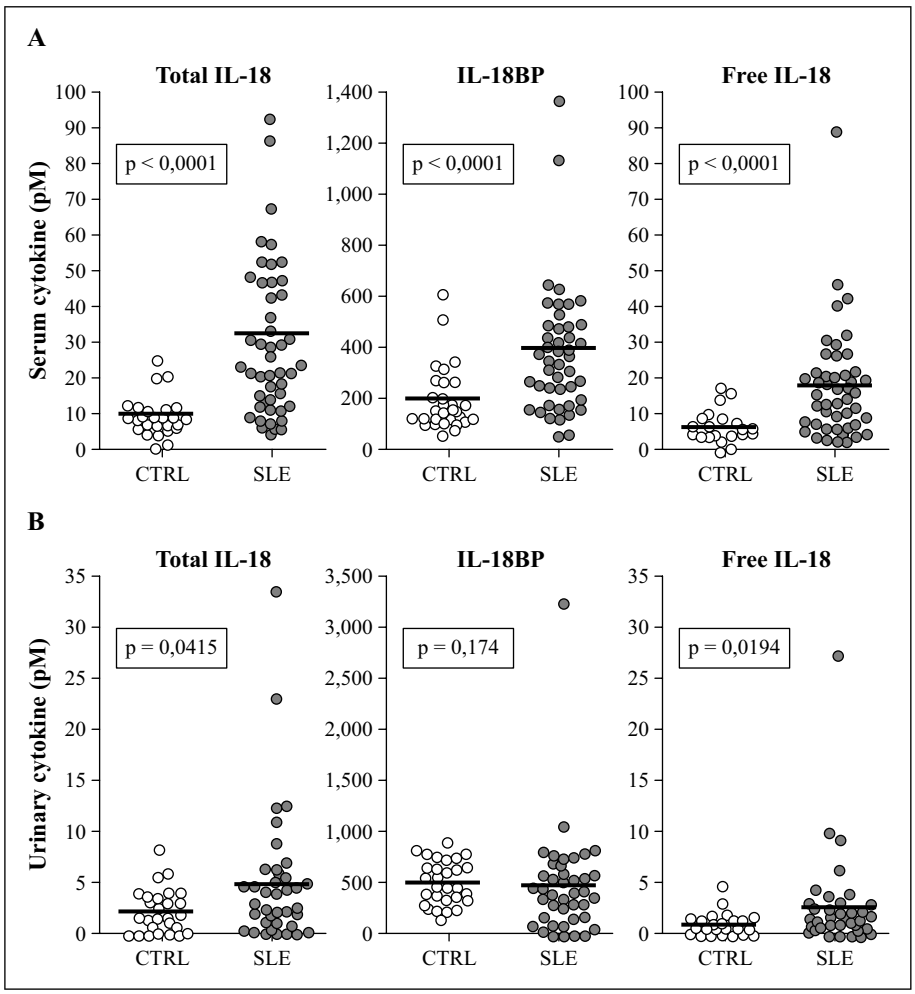

Figure 1
IL-18 and IL-18BP in serum and urine of SLE patients and healthy subjects.
A) Total IL-18, IL-18BP, and free IL-18 serum levels in SLE patients and in healthy subjects.
B) Total IL-18, IL-18BP, and free IL-18 urinary levels in SLE patients and in healthy subjects.
The concentration of total IL-18 and IL-18BP was measured in serum samples: the amount of biologically active, free IL-18 in serum and urine samples from patients and controls was calculated as described. Bars show mean levels.

disease exacerbations. Indeed, this picture is apparently typical of SLE, as for instance in rheumatoid arthritis (RA), the levels of IL-18 and IL-18BP do not correlate with clinical measures of disease activity, nor with response to treatment of patients with early RA [24]. This also suggests that perhaps SLE should be considered to be a non-typical Th1 disease. In fact, IL-18 is allegedly involved in both Th1 and Th2 responses, and anti-TNF-α therapy is rarely used in SLE [2].

Active SLE patients present a wide spectrum of manifestations, and several organs may be involved. However, by analysing subsets of patients characterised by a different pattern of organ involvement, it was evident in this study that IL-18 levels do not differ within the different subsets, and, in particular, do not distinguish patients with active nephritis from patients with an extra-renal flare. This observation, although limited by the small number of active patients without nephritis, suggests that the dysregulation of IL-18 is a prominent feature of the disease rather than a marker of kidney involvement. Thus, at variance with data proposing urinary IL-18 as biomarker for acute renal injury [25, 26], no correlation between IL-18 and active nephritis could be seen in SLE patients.

These results extend the data obtained in previous studies, which were however, mostly based on the measurement of total IL-18. In a large cohort of SLE patients, Calvani *et al.* [15] detected high IL-18 levels in patients as compared to controls and especially in those affected by diffuse proliferative and membranous glomerulonephritis. No association between cytokine level and other organ involvement was found, and the inflamed kidney was suggested to be the main source of IL-18. However, the number of patients with non-renal flare was not reported, making difficult a comparison with the population of patients included in our study. A correlation between serum IL-18 levels and disease activity was reported by other authors in Caucasians [27] as well as in other ethnic groups [13, 14].

A parallel evaluation of IL-18 and IL-18BP levels was conducted in three studies [16, 19, 20]. In 16 patients with SLE nephritis, increased levels of both molecules and an increased IL-18/IL-18BP ratio were detected, as compared with controls or patients affected by primary

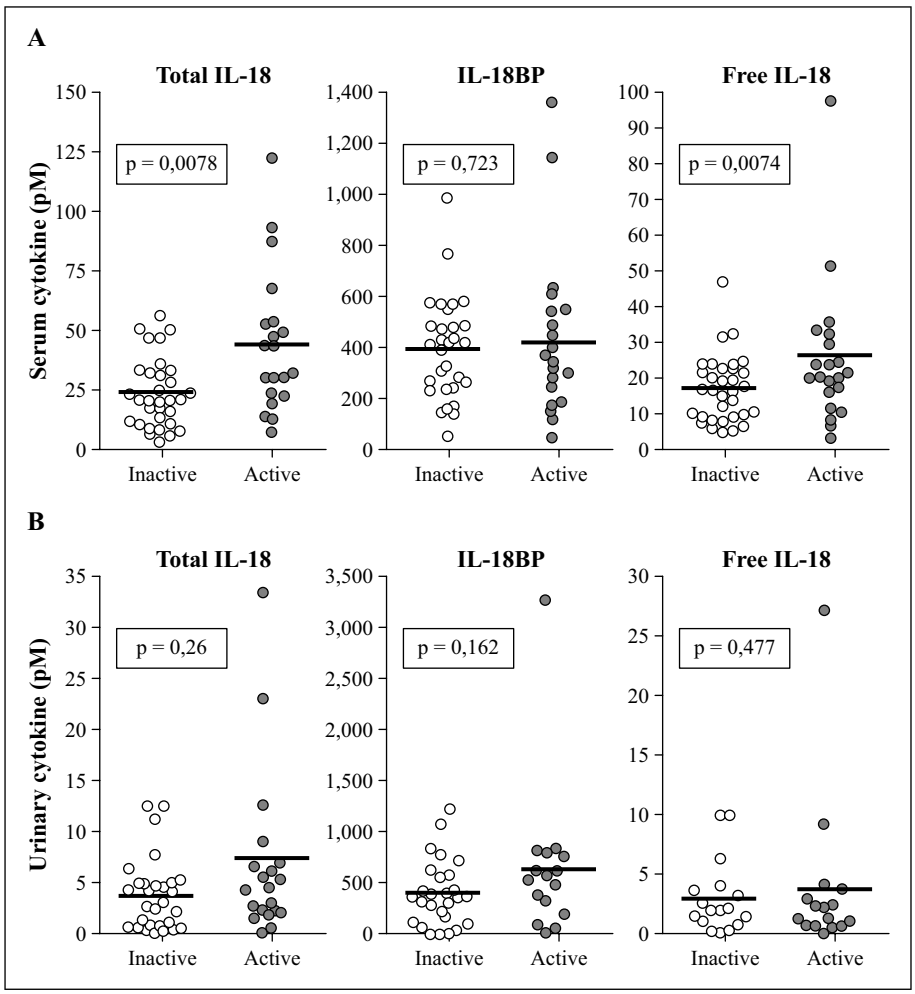

Figure 2
IL-18 and IL-18BP levels in serum and urine of SLE patients with active and inactive disease. Serum levels of total and free IL-18 were measured and calculated as described. Bars show mean levels.

nephrotic syndrome [19]. However, the relevance of such an increase is hard to evaluate in terms of IL-18 signalling and functional activity, since free IL-18 was not calculated. Indeed, variations in the IL-18/IL-18BP ratios do not correlate with the extent of interaction between the two molecules (which depends on the actual concentrations of the two factors), thus the ratio does not provide any meaningful information on the level of free, active IL-18. In another study, IL-18BP expression (at the mRNA level only) was measured by real-time PCR in peripheral leukocytes, probably including all white blood cells, and by immunohistochemistry on renal biopsies of lupus nephritis patients [16]. The slightly lower mRNA expression in leukocytes from lupus patients *versus* controls was, however, not confirmed by parallel measuring of the circulating IL-18BP levels, while the IL-18BP protein was found in lupus kidneys but not in control organs. No SLE patients without active nephritis were included in the study, making difficult to establish an association between nephritis and disease activity. Another very recent study has evaluated free serum IL-18 levels in a retrospective study on 48 SLE patients followed longitudinally [20], with results similar to those we report in the present study. In fact, increased levels of free IL-18, despite the overproduction of IL-18BP, were detected in the whole group of patients, and especially in ones with active disease. Despite a lower number of serum samples tested, our study discloses a much clearer association of free serum IL-18 with disease activity, as shown by the correlation with the ECLAM score.

IL-18BP overproduction is detected not only in SLE, but also in other autoimmune and inflammatory disorders [28, 29], and can be interpreted as a feedback mechanism to limit excessive IL-18 activity. Such a feedback is not observed in autoinflammatory diseases such as Schnitzler syndrome, in which increased IL-18 levels are not counterbalanced by overproduction of IL-18BP [30].

Urinary cytokines are currently evaluated as potential biomarkers for lupus nephritis. Local production by infiltrating inflammatory cells has been demonstrated for several cytokines, and the analysis of urine components offers the possibility to directly evaluate pathological events within the kidney. Among the inflammatory chemokines that mediate leukocyte infiltration and that play an important role in the progression of nephritis, urinary levels of CCL2 [31], soluble TNF receptor [32] and IL-12 [33] appear to be sensitive and specific markers of lupus nephritis. The presence of IL-18 and IL-12,

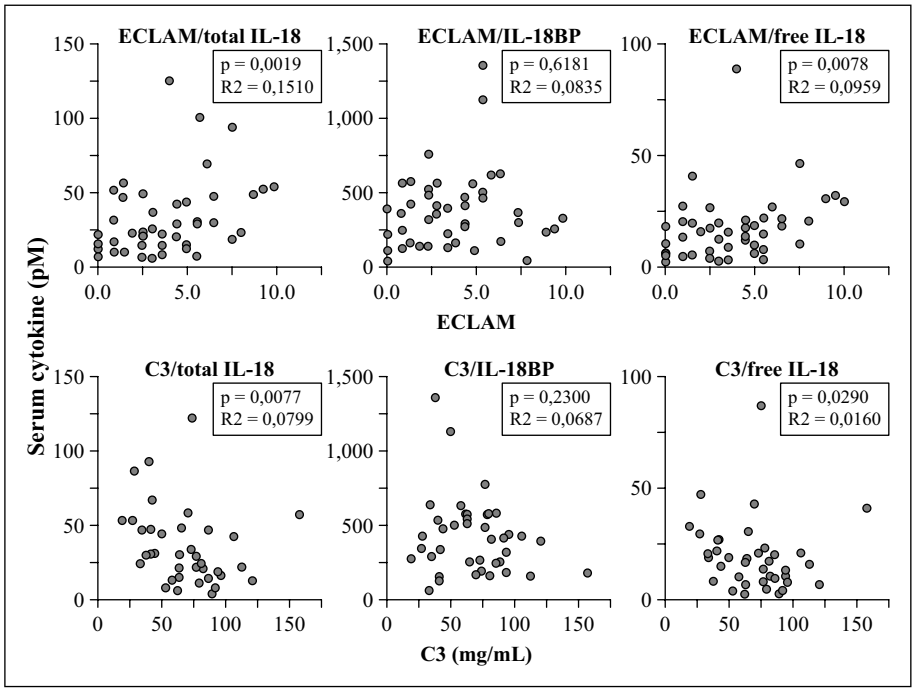

Figure 3
Correlation of serum levels of IL-18 and IL-18BP with disease parameters.
Correlation was assessed between the serum levels of total IL-18 (left panels), IL-18BP (centre panels), and free IL-18 (right panels) with disease activity measured by the ECLAM score (upper panels), and with C3 levels (lower panels).

Table 1
Serum and urinary levels of cytokines in active renal *versus* non-renal SLE patients

Group	No.	Levels (pM) of		
		Total IL-18	IL-18BP	Free IL-18
Serum				
Active renal	16	31.7 (7.9-53.5)	376.3 (127.8-1141)	18.5 (3.5-32.5)
Active non-renal	6	77.4* (22.1-122)	286.5 (108-1003)	24.5# (16.1-88.5)
Urine				
Active renal	10	3.9 (0-33.7)	520.0 (0-831)	1.6 (0-27.3)
Active non-renal	6	5.2 (2.6-23.2)	567.0 (318-3262)	1.8 (0.7-9.3)

Concentrations of cytokines are reported as mean pM value; range is indicated in parentheses.
* p = 0.0027 *vs* renal patients.
# p = 0.001 *vs* renal patients.

as well as IFN-γ in kidneys of lupus nephritis [34], suggests that the kidney damage may have a Th1-dependent basis. Both IL-18 and IL-18BP can be detected in urine, and urinary IL-18 has proved to be a sensitive predictor of acute renal failure [25, 26] and an indicator of disease activity in minimal-changes glomerulonephritis [35].

The present study is the first report on urinary levels of IL-18 and IL-18BP in SLE. Both total and free IL-18 are increased in the urine of SLE patients as compared with controls, while IL-18BP levels are not significantly different. In both the human disease and in murine models of SLE, IL-18 mRNA and protein expression have been detected in kidneys, mostly produced by infiltrating mononuclear cells, but also detectable in tubular epithelial cells [10, 17, 34, 36]. However, in lupus-prone MRL-*lpr/lpr* mice, the levels of free IL-18 in the kidney were found to be high at disease onset, but decreased thereafter, thus not correlating with late-stage nephritis [36]. While it is conceivable that locally-produced IL-18 is, at least, in part filtered and excreted, the finding that similar levels of urinary IL-18 were detected in active or inactive patients *(table 1)* indicates a lack of correlation between urinary IL-18 and disease activity.

In conclusion, this study shows that increased serum levels of total and free IL-18 are a feature of SLE and correlate with disease activity, while urinary levels do not represent an additional tool for the diagnosis and staging of lupus nephritis. A group of IFN-inducible chemokines (CCL2, CXCL10 and CCL19) have proved very useful as biomarkers of disease activity in SLE, and could be used for identifying patients at risk of exacerbation [37]. Serum IL-18, the inducer of IFN-γ and itself chemotactic for some types of leukocytes, also proves useful as biomarker in SLE, able to provide information on the course of the disease. Follow-up studies are currently ongoing to test the value of serum IL-18 in monitoring SLE patients, in particular for predicting disease flares and for the consequent optimisation and tailoring of drug therapy.

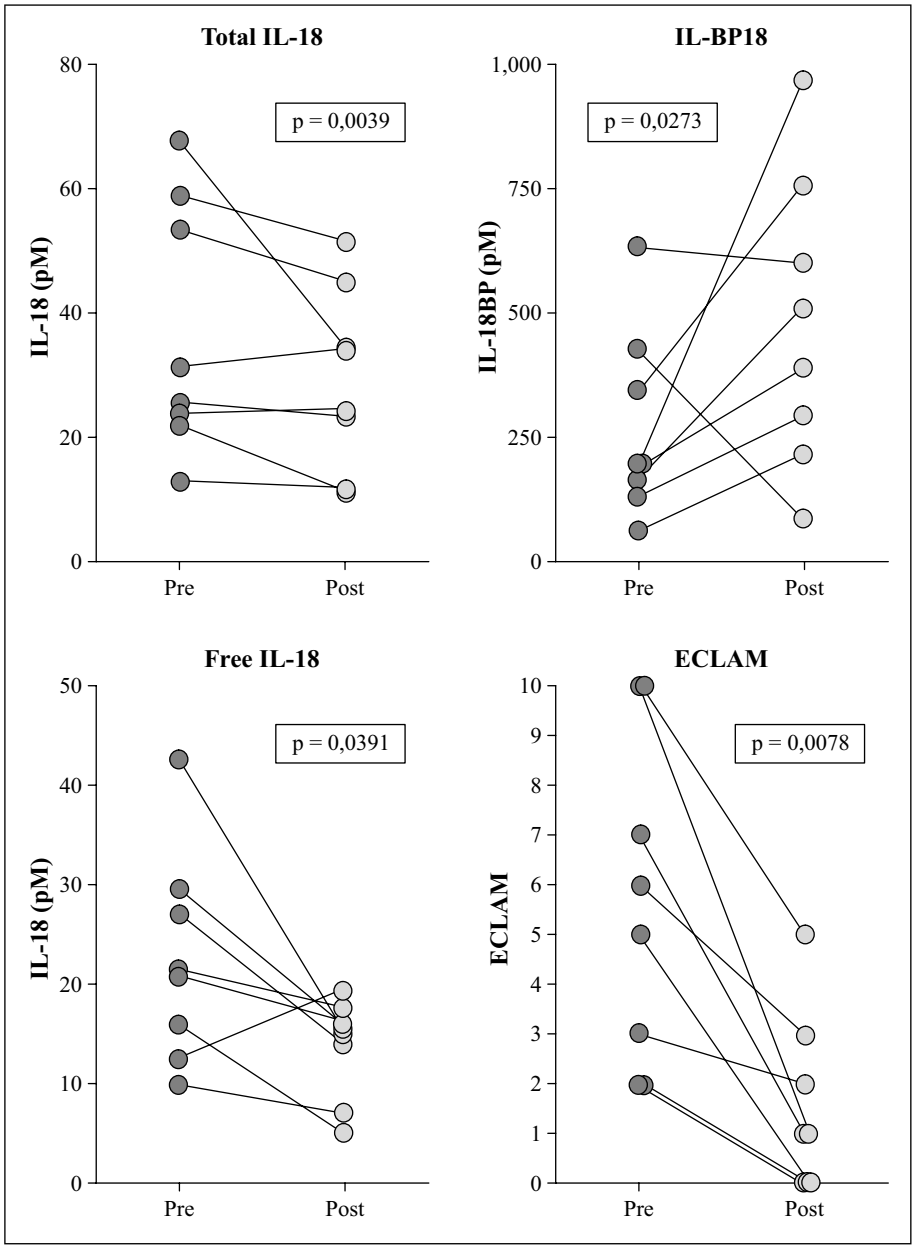

Figure 4

Serum levels of IL-18 and IL-18BP in follow-up.
In eight patients assessed pre- and post-treatment, the variations in the levels of total IL-18 (upper left), IL-18BP (upper right), and total IL-18 (lower left) were evaluated in comparison with any decrease in the ECLAM score (lower right).

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None of the authors has any conflict of interest to disclose.

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