

## Report

# IFN- $\gamma$ /IL-6 and related cytokines in chronic spontaneous urticaria: evaluation of their pathogenetic role and changes during omalizumab therapy

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**Abstract**

**Background** Recent studies highlight that high levels of cytokines may precede the onset of many systemic autoimmune disorders and may also be related to chronic spontaneous urticaria (CSU) activity.

**Methods** Eight patients with CSU candidate to omalizumab therapy were enrolled. Four healthy controls were included with the purpose of comparing baseline cytokine levels. We evaluated serum levels of IFN- $\gamma$ , IL-2, 4, 6, 8, and 10, TNF- $\alpha$ , and GM-CSF. For the patient group, venous blood samples were drawn at T0, T1 (1 week after first drug administration), T2 (after 3 months), T3 (after 6 months), and in case of relapse. Cytokine levels were measured using the human cytokines 8-plex kit. Disease activity and effect of therapy were calculated by means of Urticaria Activity Score 7.

**Results** Higher levels of IL-6 and IFN- $\gamma$  were found in patients with CSU compared to those observed in the control group. Moreover, a common trend between these cytokines and the clinical history of disease could be hypothesized, with a decrease in levels of IFN- $\gamma$  and IL-6 following remission of CSU with omalizumab treatment. Levels of other tested cytokines were similar between patients and healthy subjects.

**Conclusion** IFN- $\gamma$  and IL-6 are proinflammatory cytokines that are strongly related to autoimmunity. Despite being limited by the small sample size, our data offer new insight into a better understanding of the pathogenesis of CSU and support the need for further investigations.

**Introduction**

Chronic spontaneous urticaria (CSU), also defined as idiopathic chronic urticaria (ICU), accounts for 75% of all types of urticaria. The etiology of the disease is still unknown. There is a strong female prevalence among Caucasian patients.<sup>1</sup> The diagnosis is generally late, specifically about 5 years from the onset of the first symptoms. The dermatological hallmarks of CSU are wheals and angioedema, sometimes both coexisting in the same patient.

According to current guidelines, the appropriate therapeutic approach consists in the administration of second-generation antihistamines as first-line drugs, which may be increased up to four times daily as second-line treatment.<sup>2</sup> Omalizumab, an anti-IgE monoclonal antibody, is recommended in cases of disease refractory to antihistamines.<sup>3</sup> Its exact mechanism of action is complex and still not completely understood. At the same time, the diverse set of the effects displayed by

omalizumab provides an experimental model for the study and the comprehension of the pathophysiology of CSU.

Our aim was to identify a specific cluster of cytokines in the serum of CSU patients treated with omalizumab, that could be related with the history of the disease, in terms of progression, remission, relapse, and physiopathology.

**Methods**

We performed a pilot study with longitudinal design and a single intervention arm (omalizumab therapy). A control group was included in order to allow a comparison between cytokine levels in CSU patients at baseline and in controls.

Eight patients were enrolled in this study according to the following inclusion criteria: subjects with a diagnosis of CSU, refractory to first and second lines of treatment with second-generation antihistamines, and candidates to treatment with omalizumab. The patient group was made up of six females

and two males with the following characteristics: age mean  $\pm$  SD [min;max] =  $57 \pm 4$  [51;64] years, duration of urticaria =  $25 \pm 19$  [6;48] months. The control group included four healthy female subjects with age =  $40 \pm 8$  [32;49] years.

Prior to enrollment, patients were thoroughly informed about the risks and benefits of treatment with omalizumab, and informed consent was obtained from all the subjects included in this study.

The group of CSU patients underwent a complete series of allergological tests, including skin prick tests and patch tests. Other laboratory investigations included complete blood cell count, ESR, CRP, total serum IgE, autoimmunity-related tests (ANA, BAT), thyroid function tests, D-dimer, and serum lipid profile. The severity of the disease was evaluated using Urticaria Activity Score summed over 7 days (UAS 7) and Urticaria Control Test (UCT).

Each of the eight subjects in the patient group was administered a treatment cycle of omalizumab 300 mg sc every 4 weeks for 6 months, which is standard prescribed dosing for CSU. The outcome of the therapy was subsequently evaluated, using UAS 7 and UCT scores. At the same time, the levels of relevant cytokines were measured as follows: blood sampling was carried out on the day of the start of therapy (T0), after 7 days (T1), after 3 months (T2), after 6 months (T3), and in case of relapse (R) observed in two patients. The levels of the following cytokines were evaluated: IL-2, IL-4, IL-6, IL-8, IL-10, TNF- $\alpha$ , IFN- $\gamma$ , and GM-CSF.

## Laboratory tests

The evaluation of serum cytokine levels was performed by means of multiplexing technique with the human cytokines 8-plex Bio-Plex multiple assay, which is an immunoenzymatic assay allowing simultaneous sampling of eight different molecules collected in small samples within a single plate. Bio-Plex Manager™ software processes and reports output data in terms of median fluorescence intensity (MFI) and of concentration of analytes (pg/ml). The concentration of analytes in solution is directly proportional to the MFI of signal delivered.<sup>4</sup>

Serum levels of cytokines in the samples from CSU patients were compared throughout the course of therapy and to those from healthy controls at baseline. The outcomes of these measurements were then related to the clinical phenotype of the patients.

## Statistical analysis

The comparison between median serum levels of the cytokines in healthy controls and in CSU patients, at baseline, was carried out using the Mann-Whitney test, a nonparametric test with two independent samples. This type of test is performed when the two conditions to carry out a parametric test cannot be satisfied, namely when the distribution of the variable that is to be inferred is not normal and the sample size is small.

The comparison between levels of cytokines in patients at baseline versus each timepoint in the course therapy (T0 vs. T1, T2, and T3) was carried out using a paired T-test, a statistical analysis that is performed in order to assess a statistically significant mean difference between two measurements of a variable repeated in time on the same subject.

Spearman test was used to assess the correlation between mean serum levels of cytokines, measured in pg/ml, and time and between mean UAS7 score and time.

In all the statistical methods, a *P* value <0.05 was considered statistically significant.

## Results

The analysis, carried out with the multiplexing human 8-plex cytokine system, allowed the quantification of the following cytokines: IFN- $\gamma$ , IL-2, IL-4, IL-6, IL-8, IL-10, TNF- $\alpha$ , and GM-CSF.

Of the tested cytokines, IL-6 and IFN- $\gamma$  showed a significant elevation of serum levels in our study group at baseline compared to healthy subjects. In detail, median serum levels of IFN- $\gamma$  and IL-6 were higher in patients with active disease at time 0 (*P*-value < 0.05). Contrariwise, no increase in serum levels of cytokines was present in the control group. The results are shown in Figure 1a,b.

A significant decrease in the serum levels of IL-6 between T0 and T3 (mean difference = 5.18 pg/ml, *P* = 0.04) and in the serum levels of IFN- $\gamma$  between T0 and T2 (mean difference = 7.61, *P* = 0.03) was found in the CSU group of patients (Table 1).

Notably, our results highlight a decreasing trend between the values of IFN- $\gamma$  and IL-6 and time (for both variables, *P* = 0.2 and *r* = -0.8). The correlation between UAS7 and time result was significant (*P* < 0.001 *r* = -0.999). Figure 2a,b shows the trend of UAS7 score with that of IL-6 and IFN- $\gamma$ , respectively; even if the absolute values of the two cytokines are different between patients, their decrease precisely parallels remission of skin disease during therapy.

Relapse was observed in a subgroup of two patients, occurring 6 and 2 months after the end of a 6-month treatment cycle with omalizumab. The relapsing subjects were female patients of 54 and 64 years who had achieved complete response to omalizumab after the first and third dose of the drug, respectively. Because of the limited subsample size with two relapses, variations in the serum levels of cytokines in patients with relapse did not reach statistical significance.

The other cytokines, including IL-2 (Figure 3), IL-4, IL-8, IL-10, TNF- $\alpha$ , and GM-CSF, showed inconsistent variations throughout the course of our observation in CSU patients and no differences between groups.

## Discussion

To date, two main different models of autoimmunity have been described for the pathophysiology of CSU. The first model

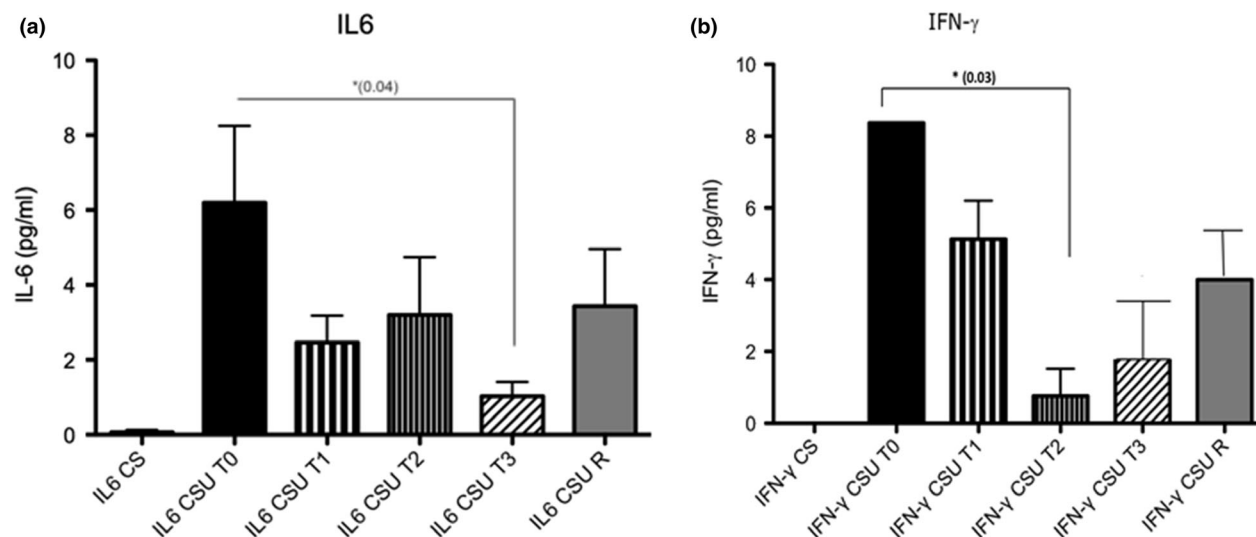


**Figure 1** Mean serum values of IL-6 and IFN- $\gamma$ . Mean serum values, expressed in pg/ml, of IL-6 (a) and IFN- $\gamma$  (b) in healthy controls (CS) and in CSU patients at T0, after 1 week (T1), after 3 months (T2), after 6 months (T3) from the start of treatment with omalizumab, and following disease relapse in two patients (R)

**Table 1** Cytokine levels in CSU patients

|           | IFN- $\gamma$ | IL-6 | IL-2 | IL-4 | IL-8 | IL-10 | GM-CSF | TNF- $\alpha$ |
|-----------|---------------|------|------|------|------|-------|--------|---------------|
| T0 (mean) | 8.37          | 6.2  | 4.8  | 0.04 | 16.8 | 11.01 | 7      | 9.11          |
| T1 (mean) | 5.1           | 2.46 | 5.5  | 0.07 | 11.7 | 9.03  | 5      | 6.94          |
| $P^a$     | ns            | ns   | ns   | ns   | ns   | ns    | ns     | ns            |
| T2 (mean) | 0.76          | 3.2  | 4.44 | 0.05 | 12.6 | 6.84  | 7.6    | 10.5          |
| $P^a$     | 0.03          | ns   | ns   | ns   | ns   | ns    | ns     | ns            |
| T3 (mean) | 1.8           | 1.02 | 4.4  | 0.03 | 5.7  | 6.23  | 5.7    | 7.51          |
| $P^a$     | ns            | 0.04 | ns   | ns   | ns   | ns    | ns     | ns            |
| R (mean)  | 4             | 3.43 | 4.2  | 0.4  | 3    | 8.075 | 3      | 7.75          |
| $P^a$     | ns            | ns   | ns   | ns   | ns   | ns    | ns     | ns            |

Mean levels of cytokines in the group of CSU patients at T0, after 1 week (T1), after 3 months (T2), after 6 months (T3) from the start of treatment with omalizumab, and following disease relapse (R, observed in two patients) are reported. A statistically significant decrease from baseline was observed in the serum levels of IL-6 at T3 ( $P = 0.04$ ), and in the serum levels of IFN- $\gamma$  at T2 ( $P = 0.03$ ). Values are expressed in pg/ml.

<sup>a</sup> $P$  values of paired T-test comparing IFN- $\gamma$  and IL-6 levels to baseline (T0) are reported. Ns, nonsignificant.

suggests a type 2 hypersensitivity reaction underlying CSU pathogenesis that would be mediated by IgG autoimmune antibodies (IgGAbs) against the high-affinity Fc $\epsilon$ RI receptor, located on the surface of basophils and mast cells. The second model involves type 1 hypersensitivity mediating the pathogenic reactions of CSU.

According to this so-called “autoallergy” model, the main role in the pathology is played by “cytokinergic IgE antibodies”. Cytokinergic IgEs are a subpopulation of IgE antibodies that are different from the classically described IgEs—which are also

known as “reagins”—and mediate anaphylaxis. Cytokinergic IgEs are termed cytokinergic “antigen-independent” antibodies, reflecting the notion that they are released in the serum at constant rate and have weak immunogenic activity, but they can bind the Fc $\epsilon$ RI even in the absence of antigen.<sup>5–7</sup> Specifically, they induce the activation of intracellular pattern recognition receptors (PRRs), toll-like receptors (TLR) 7, and TLR 9, which form homodimers upon engagement. The signaling cascade of TLRs involves transduction of the signal via MyD88/IRAK/TRAF intermediates, which ultimately lead to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B). This master regulator of transcription mediates the expression of chief cytokines in the inflammatory response, such as IL-6 and IFN- $\gamma$ .<sup>7</sup>

By releasing these potent mediators of acute response, cytokinergic IgEs are able to elicit violent inflammatory reactions.<sup>7</sup> Omalizumab, binding the C $\epsilon$ 3 domain of free IgE, prevents its binding to high-affinity IgE receptor (Fc $\epsilon$ RI) and downregulates the expression of Fc $\epsilon$ RI on mast cells and basophils.<sup>8</sup> This activity of omalizumab inhibits the formation of large aggregates of Fc $\epsilon$ RI (that are typical of highly cytokinergic IgEs), avoiding the activation of mast cells and the consequent release of these proinflammatory cytokines. All these findings justify the reduction of serum levels of IL-6 and IFN- $\gamma$ , observed in our CSU patients, during treatment with omalizumab.

Interferon (IFN) plays a pivotal role in the pathogenesis of autoimmune diseases and, in particular, type 1 IFNs—A and B classes of IFNs—contribute to shaping the innate and adaptive immune responses in autoimmune processes.<sup>9</sup> The role of type 2 IFN- $\gamma$  in the pathogenesis and maintenance of autoimmune diseases, including systemic lupus erythematosus (SLE), is less

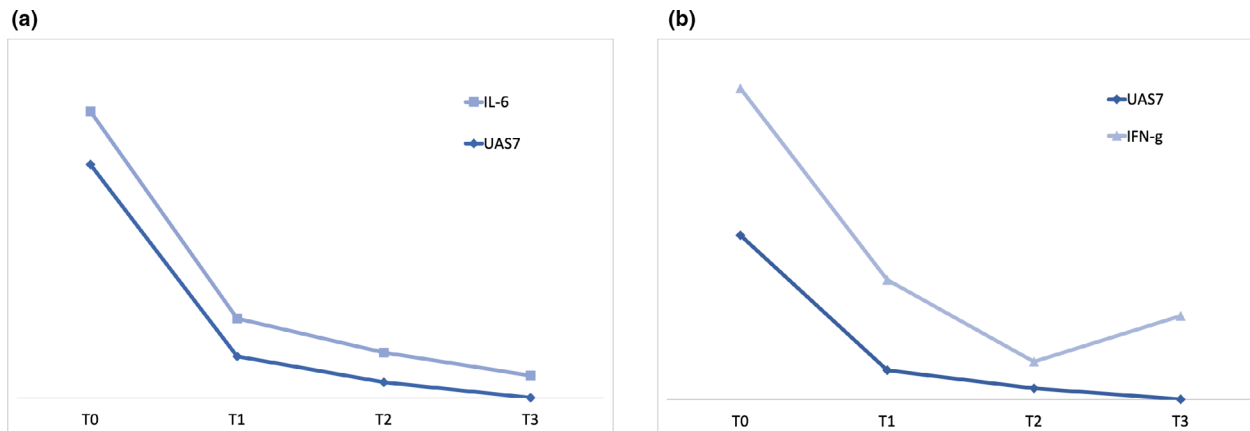


**Figure 2** Decreasing trend between cytokine values and time. (a) Shows the trend between mean serum levels of IL-6 measured in pg/ml and time ( $P = 0.2$  and  $r = -0.8$ ) and the correlation between UAS7 score and time ( $P < 0.001$   $r = -0.999$ ), using Spearman test. (b) Shows the trend between mean serum levels of IFN- $\gamma$  measured in pg/ml and time ( $P = 0.2$  and  $r = -0.8$ ) and the correlation between UAS7 score and time ( $P < 0.001$   $r = -0.999$ ), using Spearman test

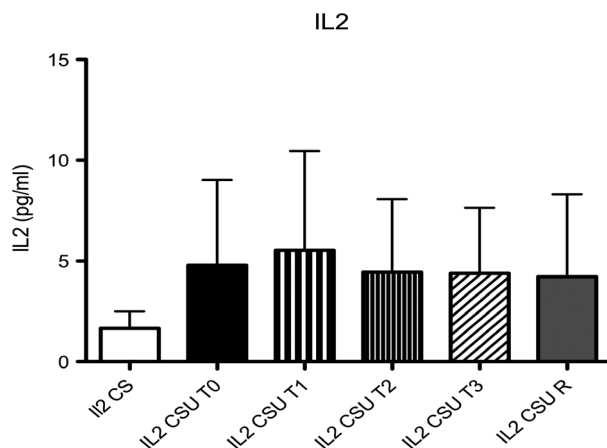


**Figure 3** Mean serum levels of IL-2. No statistically significant difference could be observed comparing the mean serum values, expressed in pg/ml, of IL-2 in healthy controls (CS) and in CSU patients at T0, after 1 week (T1), after 3 months (T2), after 6 months (T3) from the start of treatment with omalizumab, and following disease relapse (R). IL-4, IL-8, IL-10, GM-CSF, and TNF- $\alpha$  displayed similar behavior, with nonsignificant results

understood, although nowadays IFN- $\gamma$  is recognized as a central factor in autoimmune disorders.<sup>10</sup>

IL-6, the other interleukin related to the trend of CSU in our study, is known to be a fundamental factor for the expression of other proinflammatory cytokines in the inflammatory cascade, such as TNF- $\alpha$  and IL1- $\beta$ , as well as for the production of antibodies. It is notable indeed that the release of IL-6 and IFN- $\gamma$  precedes the appearance of antibodies in the serum.<sup>11</sup> Besides, it is known that patients with SLE presenting with manifest disease show a significant increase in the serum levels of IFN- $\gamma$  and that IL-6 and IFN- $\gamma$  show an interdependent relation<sup>12</sup> that is similar to the one revealed in CSU by our results.

Consequently, these two high-potency pro-inflammatory cytokines (IL-6 and IFN- $\gamma$ ) could be the relevant effectors in the autoimmune pathogenesis underlying CSU. With regard to this hypothesis, recently, a wide set of self-antigens that are recognized by specific IgE autoantibodies, defined as “autoallergic targets”, has been identified in patients with CSU.<sup>5</sup>

Indeed, while in allergic disorders the titer of IgE specific for one allergen directly correlates with the entity of the allergic response, the same cannot be said for CSU, where the pathogenesis is mediated by autocytokinergic IgEs (IgEAABs). Accordingly, even small amounts of these autoantibodies, often not detectable with modern laboratory measurements, are able to elicit a powerful inflammatory response. This latter mechanism may explain the efficacy of omalizumab in patients with total serum IgE within the normal range; this is partly because of the fact that the routinely performed IgE quantification is not sensitive for autocytokinergic IgEAABs.<sup>13,14</sup>

Finally, according to the literature, omalizumab, by binding circulating IgE, prevents subsequent IgE-driven responses, such as the release of inflammatory mediators histamine and leukotrienes, by basophils and mast cells.<sup>15</sup> To this regard, Roth *et al.*<sup>16</sup> found that omalizumab significantly reduces RNA and protein levels of IL-6, IFN- $\gamma$ , IL-8, TNF $\alpha$ , and IL-4 (in a dose-dependent manner in IgE-stimulated human airway smooth muscle cells isolated from biopsy specimens of patients with asthma and chronic obstructive pulmonary disease) by inducing B-cell death, as also confirmed by Chan *et al.*<sup>15</sup> Accordingly, the reduction of IL-6 and IFN- $\gamma$  that we have detected in our study may likely be explained by this last theory and pathway.

In conclusion, high levels of IL-6 and IFN- $\gamma$  may precede the onset of many systemic autoimmune disorders and may also be related to CSU activity, as shown by our data. The decrease in levels of IFN- $\gamma$  and IL-6 in CSU patients during remission

following treatment with omalizumab and the increase during the phases of relapse support our hypothesis.

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