Severe chronic spontaneous urticaria responding and not responding to omalizumab: analysis of the prognostic value of known and novel in-vitro variables

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Summary

Background. Chronic spontaneous urticaria (CSU) response to anti-IgE treatment can be rapid, late or absent. Recently, potential mechanisms of activation of mast cells alternative to FcεRI, including mas-related G protein-coupled receptor X2 (MRGPRX2), activation of coagulation cascade, and activation of eosinophils have been described. We measured several potential in-vitro markers, including well-known MRGPRX2 activators, in sera of patients CSU both responding and not responding to omalizumab.

Methods. D-dimer, substance P (SP), eosinophil cationic protein (ECP), soluble MRGPRX2, IgE anti-FcεRI, IgE anti-FcεRII, IgG anti-FcεRI and IgG anti-FcεRII were measured in 32 patients with

severe CSU at baseline and one week after the start of omalizumab therapy, and in 20 healthy controls.

Results. At baseline CSU patients showed significantly higher levels of D-dimer, IgE anti-FcεRI, IgG anti-FcεRI, and ECP ($p < 0.001$ in all cases), and significantly lower levels of soluble MRGPRX2 ($p = 0.009$) than controls. The two groups showed similar levels of IgG and IgE to FcεRII and SP. One week after the first omalizumab administration there was a significant drop of IgE anti-FcεRI ($p < 0.001$) and D-dimer ($p = 0.028$), in early responders. SP increased in all CSU patients ($p < 0.001$) irrespective of the final response to omalizumab. IgE anti-FcεRI response at one week was associated with the final response to omalizumab (OR:0.12 [95%CI 0.01-1.06]).

Conclusions. Severe CSU is associated with high plasma levels of several biomarkers including D-dimer, IgE anti-FcεRI, IgG anti-FcεRI and ECP and low levels of soluble MRGPRX2. IgE anti-FcεRI response at one week may predict the final response to omalizumab.

Key words

Chronic urticaria; Omalizumab; Biomarkers.

IMPACT STATEMENT

In severe CSU baseline D-dimer, IgE and IgG anti-FcεRI, and ECP are elevated while soluble MRGPRX2 is low. One week after omalizumab start IgE anti-FcεRI and D-dimer drop in early responders, while SP increases in all patients.

INTRODUCTION

The current international guidelines recommend second generation antihistamines at licensed dosage, at up to 4-fold the licensed dosage, and omalizumab as steps I-III in the treatment of chronic spontaneous urticaria (CSU) (1). Although a majority of patients gets complete or at least partial control of the disease by this stepwise treatment, about 10% of severe CSU patients seem totally unresponsive and have to be shifted to the treatment with cyclosporin. In a series of 296 antihistamine-resistant CSU patients treated with omalizumab at the Clinica San Carlo, 27 (9%) had to be shifted to cyclosporin treatment, always with excellent clinical responses (unpublished). Patients responding slowly or not responding to omalizumab are generally characterized by low total IgE levels (2), a feature that has been associated with autoimmune chronic spontaneous urticaria in the international PURIST study (3). Notably, in omalizumab-refractory patients IgE levels are lower than in partial/late responders to the drug (2). Anti-IgE therapy leads to a progressive downregulation of the high affinity IgE receptor that parallels the reduction in number of both free and membrane-bound IgE. Thus, if mast cell activation occurred always via the high affinity IgE receptor, one is tempted to speculate that in the minority of omalizumab-refractory patients the activation of effector cells might occur mainly by pathways that bypass the FcεRI which needs the presence of autoimmune IgE (4) or autoimmune IgG directed against membrane-bound IgE or FcεRI (5) to be activated. Recent studies have highlighted the potential relevance of MRGPRX2 receptor as an activation pathway in patients with CSU. MRGPRX2 is a G-coupled human mast cell receptor constitutively expressed by human skin mast cells which mediates non-immune adverse reactions without the involvement of antibody priming (6). MRGPRX2 is activated by several different substances, including neuropeptides such as Substance P (SP), as well as by mediators of eosinophils, namely eosinophil cationic protein, major basic protein and eosinophil peroxidase (7-8). Other mechanisms of mast cell activation potentially viable in CSU beyond the high affinity IgE receptor, including the coagulation cascade, the complement system, and the

platelet activating factor (PAF) vicious circle, have been recently reviewed in detail (9, 10). Interestingly, during the last years it was demonstrated that in CSU both eosinophils and endothelial cells are often activated and are potentially able to induce the histamine release from mast cells through different mechanisms, including the activation of the extrinsic pathway of the coagulation cascade by an hyperexpression of tissue factor (11-13). Starting from these observations, we measured several potential in-vitro markers, including well-known MRGPRX2 activators, in sera of patients CSU both responding and not responding to omalizumab.

PATIENTS AND METHODS

Patients

Thirty-two patients with severe CSU, i.e., urticaria activity score on 7 days (UAS7) ≥ 20, refractory to second generation antihistamines even at higher than licensed dosage, underwent subcutaneous omalizumab treatment at a dose of 300 mg every 4 weeks, following the indications of the European Academy of Allergy and Clinical Immunology (EAACI) guidelines [1]. Following the prescriptions by the Italian national regulatory agency (AIFA) that prohibit up-dosing and prevent the continuation of the treatment in the absence of a significant clinical response after 3 administrations, the patients were evaluated at baseline and then every 4 weeks for 12 weeks after the omalizumab initiation, and UAS7 was recorded at each time point. An early response was defined as the achievement of an UAS7 score ≤ 6 within 4 weeks after the first administration, a late response was defined as the achievement of the same reduction within 12 weeks after initiation, while a non-response was defined as the persistence of unchanged symptoms 4 weeks after the third administration (UAS7 ≥ 10). The patients were investigated at baseline and, due to funding shortage, only 1 week after omalizumab administration for the circulating levels of D-dimer, substance P (SP), eosinophil cationic protein (ECP), Mas-related G protein-coupled receptor X2

(MRGPRX2), IgE anti-FcεRI, IgE anti-FcεRII, IgG anti-FcεRI and IgG anti-FcεRII. Twenty healthy subjects, sex- and age-matched with patients, served as controls.

The study was conducted according to the ethical principles of the Declaration of Helsinki and the code of Good Clinical Practice. The patients and controls gave informed written consent to the use of their sera and relative data. The local review board approved the study.

Methods

D-dimer level were measured in Na-citrated plasma with a commercial ELISA method (Zymutest D-dimer, Hyphen Biomed, Neuville sur Oise, France). A highly purified monoclonal antibody specific for D-dimer is absorbed on wells of microplates. The analyte captured onto the solid phase is detected by a monoclonal antibody coupled to horse radish peroxidase. The intra- and inter-assay coefficients of variation (CV) were 10 and 15%, respectively. The lower detection limit was 0.22 µg/mL

Eosinophil cationic protein (ECP) plasma levels were measured by a commercial sandwich enzyme immunoassay (RNASE3) from Cloud-Clone Corp, Katy. TX, USA. The analyte is captured by a specific antibody adsorbed to the microtitration plate and detected by a second specific biotin-conjugated antibody. Intra- and inter-assay CV are 10% and 12%, respectively. The lower detection limit was 29 pg/mL.

Substance P (SP) plasma levels were measured by a commercial competitive inhibition enzyme immunoassay from Cloud-Clone Corp, Katy, TX, USA. A monoclonal antibody specific to SP has been pre-coated onto a microtitration plate. A competitive inhibition reaction is launched between biotin labeled SP and unlabeled SP (standards or samples) with the pre-coated antibody specific to SP. After washing, the biotin labeled SP is detected by avidin conjugated to horseradish peroxidase. Intra- and inter-assay CV are 10% and 12%, respectively. The lower detection limit was 4.99pg/mL

Mas-related G-protein coupled receptor member X2 (MRGPRX2) plasma levels were measured by a commercial sandwich ELISA kit from Assay Genie, Dublin, Ireland. Capture antibody is pre-coated onto the microtitration plate. The analyte is detected by a biotin conjugated specific antibody. Intra- and inter-assay CV are 8% and 10%, respectively. The lower detection limit was 0.938 ng/ml.

IgE and IgG anti- FCεRI and anti- FCεRII plasma levels were measured by home-made sandwich ELISAs. Recombinant human FCεRI (Sino Biological, Eschborn, Germany) or recombinant human FCεRII (Abcam, Cambridge, UK) were coated overnight onto microtitration plates at a concentration of 10 µg/mL. After washing, the residual binding sites were blocked with bovine serum albumin 1%. After further washes, serum dilutions (1:10) from patients and controls were added and incubated for 45 minutes at 37°C. For the determination of specific IgE, after washing, we added goat anti-human IgE (ε chain specific) (Sigma, St. Louis, Mo, USA). After a further incubation of 45 minutes and washing, donkey polyclonal anti-goat IgG peroxidase conjugated (Santa Cruz Biotechnology Inc, Dallas, Texas, USA) was added and incubated for 45 minutes. For the determination of specific IgG, we added a goat anti-human IgG (γ-chain specific) HRP conjugate (Sigma, St. Louis, Mo, USA) and incubated for 45 min. All the reactions were revealed by orthophenylenediamine (Sigma Chemical, St. Louis, Mo, USA). Both intra- and inter-assay CVs are less than 20%.

With the exception of D-dimer, all measurements were carried out in EDTA-anticoagulated plasma.

Statistics

The results are expressed as the medians and ranges (minimum-maximum). A Mann-Whitney U test was used to compare different groups and controls, whereas Wilcoxon-signed rank test was used to compare patients at different times. The correlations were assessed by means of Spearman's rho. P-values below 0.05, two-sided, were considered statistically significant. The results are expressed as

the medians and ranges (minimum-maximum). Univariate logistic models were fitted to identify prognostic factors on response including baseline and post therapy parameters: D-dimer, ECP, substance P, MRGPRX2 levels, IgE anti-FcεRI, IgE anti-FcεRII, IgG anti-FcεRII and IgG anti-FcεRII. Forest plot of univariate analysis was built. ROC curves were calculated to evaluate model performance. (IBM SPSS Statistics for Windows, version 29.0, IBM Corp., Armonk, NY, USA and Stata 18, StataCorp, College Station, TX, USA).

RESULTS

Patients at baseline

The demographics and clinical features of patients are summarized in Table 1. All 32 CSU patients showed a UAS-7 of 20 or more at baseline, and 17 of them had also angioedema. The most common comorbidities were atopy (9/32) and thyroiditis (5/32).

Patients showed a median age of 56 years (range 21-87), a M/F distribution of 11/21, and a median disease duration of 4 years (2-7). All patients showed an UAS-7 of 20 or more at baseline. Healthy controls showed a median age of 54 years (20-81) and a M/F ratio of 7/13 (p= NS)

Figure 1 shows the comparison between CSU patients at baseline and healthy controls for parameters potentially involved in CSU pathophysiology. At baseline, CSU patients showed significantly higher levels of D-dimer (median 473 ng/ml, [range 121-4530]) than healthy controls (147 ng/ml, [55-476] ng/ml) (p<0,001). We also found higher levels of IgE anti-FcεRI (562 OD, [283-1421] vs 151 OD, [73-455]) (p<0,001), IgG anti-FcεRI (315 OD [102-888] vs 160 OD [96-294]) (p< 0,001) and ECP (6595 pg/ml, [3960-9181] vs 3115 pg/ml, [1932-4252]) (p< 0,001). In contrast, MRGPRX2 levels were lower in CSU patients at baseline (16 ng/ml [10-76]) than in normal controls (26 ng/ml, [15-41]) (p=0.009). The two groups did not differ in the levels of IgG and IgE to the low affinity IgE receptor, FcεRII, as well as in SP levels.

Patients at baseline divided on the basis of the final response to omalizumab

Based on omalizumab response, 13 patients were considered early responders, 9 late responders and 10 non responders. The baseline values of the various parameters in the three subgroups are reported in Table 2. No significant difference was evident among the three subgroups for all the parameters tested at baseline (Tables 1 and 2). Concerning comorbidities, among the 13 early responders, 3 were atopic and 1 had thyroiditis; among the 9 late responders, 3 were atopic and 1 had thyroiditis; and among the 10 non-responders, 3 were atopic and 3 had thyroiditis. The duration of CSU was unrelated to omalizumab response.

Patients one-week after the first omalizumab administration divided on the basis of final response

The values of the various parameters one week after the first injection of omalizumab are reported in Table 2. In early responders, already one week after the first omalizumab administration, the levels of D-dimer dropped significantly (244 ng/ml [83-722]) from baseline (393 ng/ml [121-2623]) ($p=0.028$) and were significantly lower than in non-responders (378 ng/ml, [191-1781]) ($p=0.05$). D-dimer level was not able to discriminate between late and non-responders one week after the start of treatment. A similar behavior in early responders was observed for the levels of IgE to FcεRI, which significantly decreased from 612 OD (370-1421) to 314 OD (255-622) after one week and were significantly lower than in non-responders (391 OD, [261-1137]) ($p=0.045$) (Table 1 and Figure 2). Again, no statistically significant difference was observed between late- and non-responders one week after the start of the treatment. Levels of substance P significantly increased in all CSU patients one week after omalizumab administration from 140 [20-1114] to 395 [51-1586] ($p<0.001$), without any difference among the three subgroups. Finally, the three subgroups did not show significant differences in the levels of ECP, MRGPRX2, IgE-anti-FcεRII, IgG-anti-FcεRI and IgG-anti-FcεRII one week after the start of anti-IgE treatment.

Prediction of the final response to omalizumab by different factors at baseline and after one-week therapy.

Logistic regression analysis showed no association of the parameters at baseline with the final response to omalizumab. In contrast, one week after the start of biological therapy, the reduction of IgE anti-FcεRI (figure 3) was significantly associated with a final good response to omalizumab (Figure 4), with an odds ratio (OR) of 0.12 (95% CI: 0.01-1.06). Considering again the IgE anti-FcεRI response after one week of omalizumab therapy, the ROC (receiver operating characteristic) curve showed a good capacity to discriminate between responders and non-responders after three months of therapy as indicated by an area under the curve (AUC) of 0.66 (95% CI: 0.45-0.88) (Figure 5)

DISCUSSION

In the present work we studied several potential mast cell activators in patients with severe CSU with the aim to detect whether differences existed between patients and normal control, between different patients' subgroups, and whether omalizumab-refractory patients could be identified before the start of the treatment.

Concerning D-dimer plasma levels, we confirmed our previous findings that D-dimer represents an excellent marker of severity of CSU, as its levels were significantly more elevated in our patients than in normal controls irrespective of the final clinical response to omalizumab (14). At the same time, we confirmed also that D-dimer level is an excellent marker of disease activity as it strictly parallels the clinical response to CSU treatment (15,16). What was still unknown is the rapid drop of elevated D-dimer in response to anti-IgE treatment, that was already significant as short as one week after the first omalizumab administration. This demonstrates that in CSU the activation of the coagulation cascade is an exquisitely inflammatory process that stops suddenly as soon as the mast cell activation ceases.

In this study, we did not evaluate other potential predictors of response to omalizumab, such as anti-TPO autoantibodies and total IgE, which we had assessed in a previous study (17). That study concluded that thyroid autoimmunity alone cannot serve as a clinical predictor of response to omalizumab, whereas total IgE levels remain the most reliable prognostic marker for omalizumab response in patients with severe CSU (17). Other authors found that the IgG anti-TPO/total IgE ratio may be a good predictor of omalizumab response (18,19). Similarly, the autologous serum skin test (ASST) was not carried out in the patients included in the present work; however, a recent prospective study performed by one of us demonstrated that a positive ASST predicts a slow response to Omalizumab (20).

The analysis of the levels of FcεRI IgE in plasma confirms that type I autoimmunity is largely prevalent in CSU patients, irrespective on their response to omalizumab (21,22), although IgE levels gradually decreased from early omalizumab responders to late and non-responders. Omalizumab response is probably largely influenced by the co-occurrence of autoimmune IgG to the high affinity IgE receptor (21,22). In effect, recent studies showed that autoimmune CSU is in most cases associated with auto-allergic immune reactivity, but not vice-versa (23). Interestingly, but not surprisingly, the levels dropped as soon as one week after omalizumab administration, which indirectly confirms the good quality of our data and the rapidity of action of the drug.

The levels of autoimmune IgG to the high affinity IgE receptor (IgG-anti-FcεRI), that are considered responsible for histamine release in patients with autoimmune CSU (3), increased gradually from early omalizumab responders up to late and non-responders. These auto-antibodies were missing in normal controls. Not surprisingly the levels of circulating IgG autoantibodies to the high affinity IgE receptor did not change one week after the first administration of the anti-IgE mAb.

IgG-anti-FcεRII autoantibodies have been considered as part of the type IIb autoimmune process in CSU as activators of eosinophils (24). Interestingly they were detected mainly in late and non-responders to omalizumab although at low levels (table 2).

ECP, a marker of eosinophil activation, was elevated in CSU patients irrespective of the final omalizumab response. This finding confirms our previous observations about eosinophil activation as a turning point in the expression of both tissue factor, that causes the activation of the coagulation cascade by the extrinsic pathway, and of vasoactive substances such as VEGF (12,25,26). Another important point to keep in mind is that ECP is able to activate mast cells via the MRGPRX2 receptor.

Substance P, which was investigated as one of the several substances able to activate mast cells by membrane MRGPRX2 receptor, was not elevated in our patients irrespective on their response to omalizumab. This finding fully confirms the result of an old study by our group (27), although other groups got different results (28). To our surprise, the plasma levels of substance P increased dramatically in the same patients as short as one week after the first administration of omalizumab, irrespectively of the final clinical response to the drug. A similar trend of substance P was previously observed in CSU at 3 and 6 months of therapy in two Turkish studies (29, 30). One could speculate that, since substance P is an activator of mast cells via the MRGPRX2 receptor, the down-regulation of mast cell function may eventually lead to a release of substance P from the receptors they are bound to.

Soluble MRGPRX2 showed uniformly low levels in all CSU subsets, and such levels were even lower than those detected in normal controls. This finding is quite surprising, in view of previous studies reporting a hyper-expression of Mas-related gene X in skin mast cells (31), and the detection of elevated MRGPRX2 serum levels in patients with severe CSU positively correlated with UAS7 (32). Our finding might theoretically suggest that the plasma levels of the soluble form of this receptor are inversely related to the degree of activation of the mast cell or, in other words, that

strongly activated mast cells retain the MRGPRX2 on their surface as a highly expressed membrane receptor. Such levels did not increase one week after omalizumab administration, irrespective of the final response to the drug. It would be interesting to re-measure MRGPRX2 levels in CSU patients stably in clinical remission

In summary, our study confirms the relevance of D-dimer as a non-specific marker of severity and of acute inflammation in a proportion of severely affected patients. It also shows the rapid drop in circulating IgE after omalizumab administration, which may explain the nearly immediate clinical response observed in some CSU patients. On the other hand, the low levels of circulating MRGPRX2 and of SP at baseline were unexpected; particularly impressive was the increase in SP levels as short as 7 days after the first omalizumab administration. Following-up our in-vitro variables for longer that one week would have been ideal, but due to funding shortage this was not possible. IgE anti-FcεRI response at one week might be proposed as one further marker to predict the response to omalizumab. However, this assay is not present in most settings, while other in vivo and in vitro assays such as the autologous serum skin test, total IgE levels, or thyroid autoimmunity are much more commonly available. Although we were not able to detect novel markers of response to omalizumab and larger studies are needed to confirm our findings, we believe that this study, albeit with its evident limitations, may open new pathways in the understanding of the complex immune-pathogenesis of CSU.

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Table 1. Demographic and clinical features of chronic spontaneous urticaria (CSU) patients.

	CSU patients (n = 32)	Healthy controls (n = 20)
Age - Median (range)	56 (21-87)	54 (20-81)
Male/Female	11/21	7/13
UAS-7 - Median (range)	31 (20-42)	
Duration of disease – median years (range)	4 (2-7)	
Angioedema (n / total)	17 / 32	
Atopy (n / total)	9 / 32	
Thyroiditis (n / total)	5 / 32	
Eosinophils n/μL– Median (range)	100 (40-800)	100 (30 -350)

Table 2. Values of parameters involved in the pathogenesis of CSU at baseline and after one-week therapy with omalizumab in 32 CSU patients divided according to the final response to omalizumab.

	BASELINE			ONE-WEEK THERAPY		
	Early responders	**Late responders**	**Non responders**	**Early responders**	**Late responders**	**Non responders**
D-Dimer - ng/ml	393 (121-2623)	370 (157-4530)	378 (191-1781)	244 (83-722) *$	350 (179-3784)	378 (191-1781)
ECP - pg/ml	6392 (3987-8394)	7323 (4766-8797)	6595 (3960-9181)	5918 (3262-7832)	6900 (3461-8202)	5613 (3839-7860)
Substance P - pg/ml	171 (52-581)	235 (46-1114)	119 (20-321)	319 (51-909) $$	400 (70-1586) $$	591 (134-1060) $$
MRGPRX2 - ng/ml	15.8 (10.4-53.7)	16.0 (11.7-76.4)	18.0 (11.2-40.3)	15.1 (6.5-23.6)	17.9 (11.1-69.8)	18.5 (10.0-36.9)
IgE anti-FceRI - OD	612 (370-1421)	557 (283-1043)	475 (400-1399)	314 (255-622) **$$	398 (230-566)	391 (261-1137)
IgE anti-FceRII - OD	497 (353-688)	560 (201-667)	521 (207-1306)	521 (344-651)	575 (192-700)	572 (218-1287)
IgG anti-FceRI - OD	282 (102-583)	304 (121-888)	417 (161-882)	268 (78-565)	263 (100-738)	484 (93-890)
IgG anti-FceRII - OD	282 (154-668)	356 (175-754)	377 (144-1618)	279 (137-676)	372 (168-709)	234 (153-1391)

FIGURE LEGENDS

Figure 1. Plasma levels of D-dimer, eosinophil cationic protein (ECP), substance P, soluble mas-related G-protein coupled receptor member X2 (MRGPRX2), IgE and IgG anti-FCεRI and anti-FCεRII in 32 patients with severe chronic spontaneous urticaria (CSU) at baseline and 20 healthy controls. Results are expressed as median values, interquartile ranges (boxes), and 5th and 95th percentiles (whiskers).

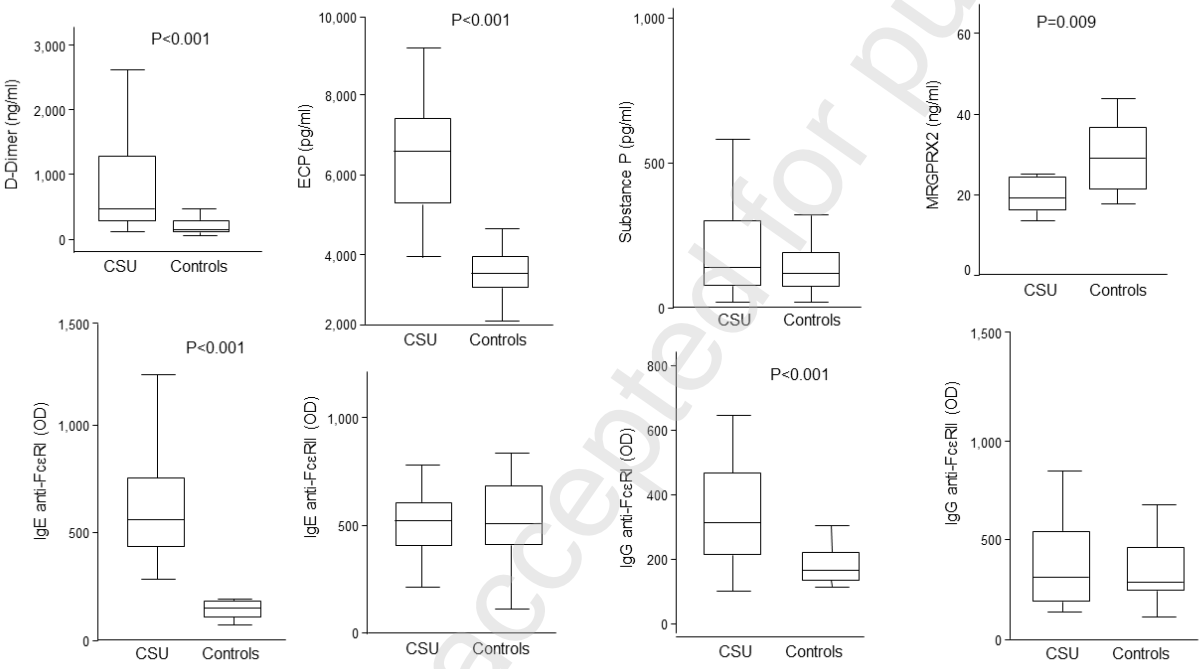

Figure 2. Upper part: plasma levels of IgE anti-FCεRI and D-dimer in patients with severe chronic spontaneous urticaria at baseline and one-week after the first omalizumab administration divided on the basis of final response. Lower part: clinical response to omalizumab, expressed as urticaria activity score on 7 days (UAS7).

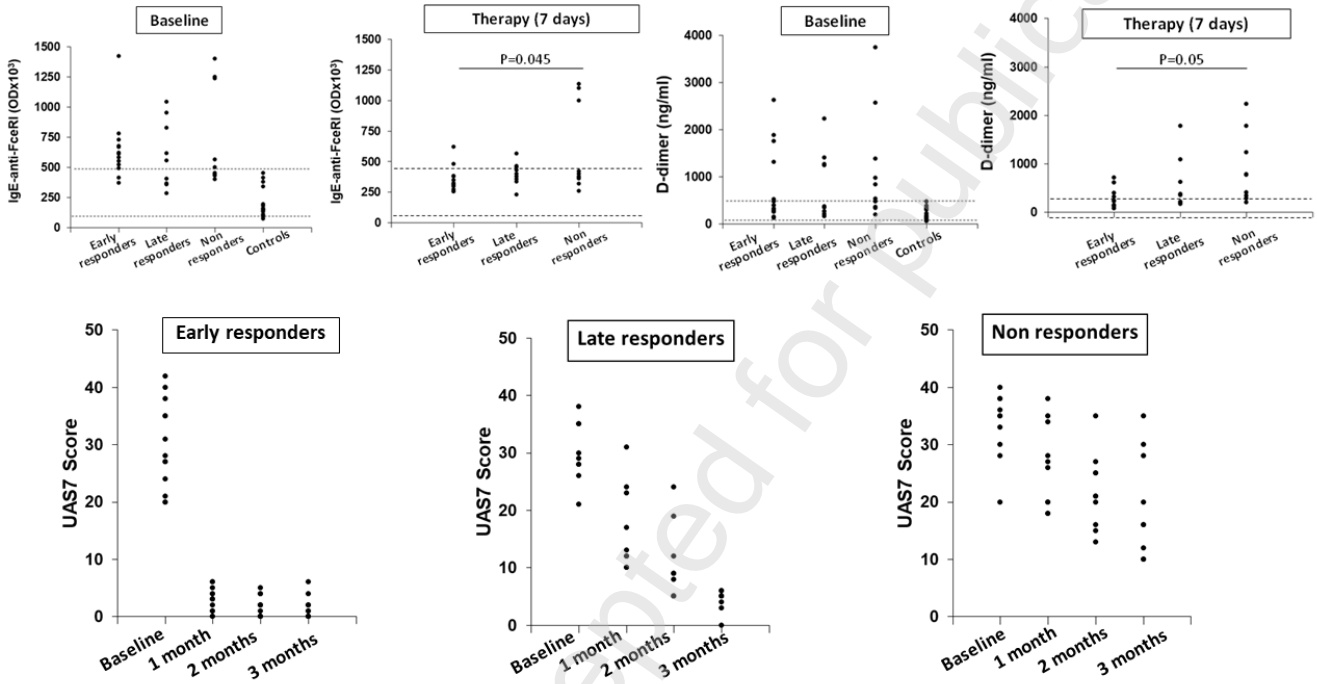

Figure 3. Plasma levels of IgE anti-FcεRI at baseline and after one week from the first administration of omalizumab in patients with severe chronic spontaneous urticaria divided on the basis of final response to the drug.

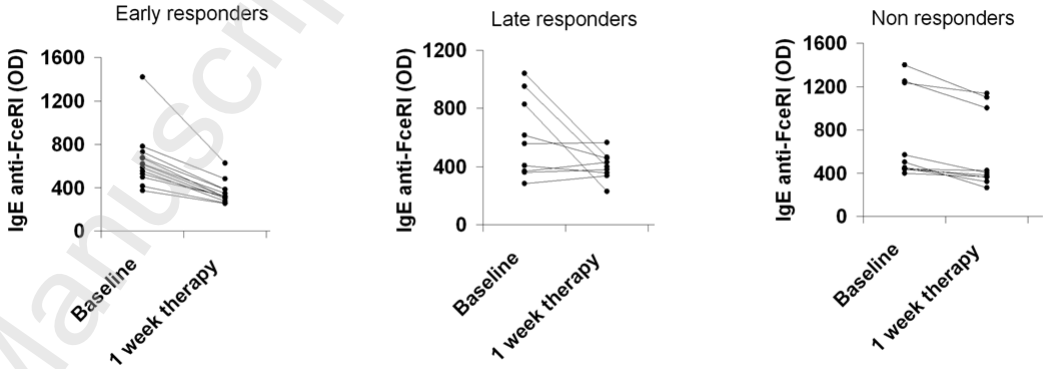

Figure 4. Evaluation of the association between the response of the various parameters after one week from the first administration of omalizumab and the final clinical response to the drug. Logistic regression analysis shows that IgE anti-FcεRI levels after one week of therapy were significantly associated with a final good response to omalizumab, with an odds ratio (OR) of 0.12 (95% CI: 0.01-1.06).

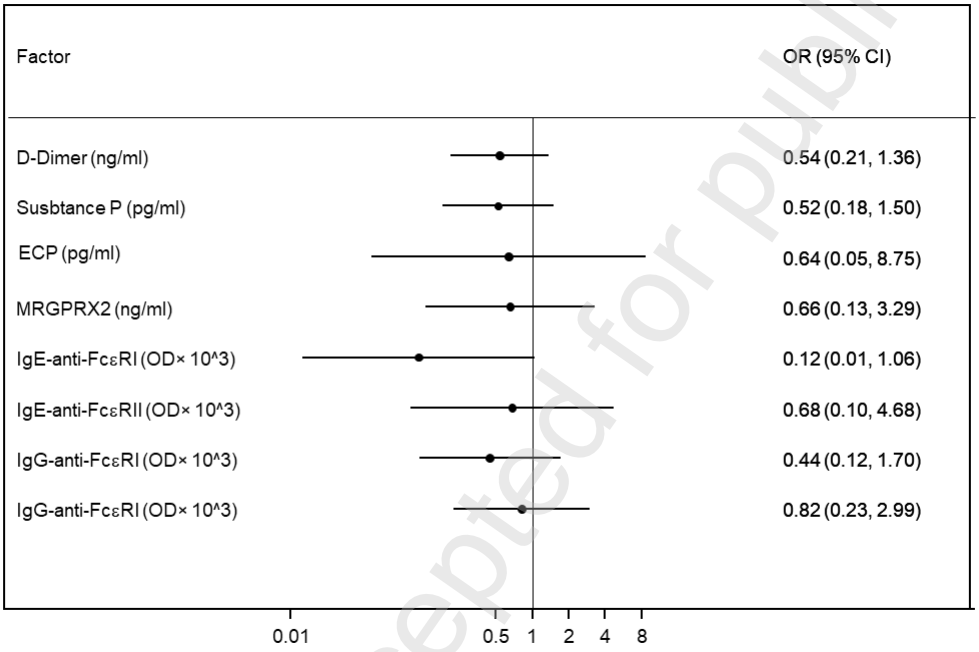

Figure 5. Receiver operating characteristic (ROC) curve relative to the IgE anti-FcεRI response after one week of omalizumab therapy, showing a good capacity to discriminate between responders and non-responders as indicated by an area under the curve (AUC) of 0.66 (95% CI: 0.45-0.88).