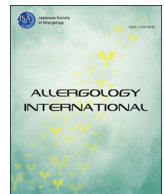




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Letter to the Editor

Identification of biomarkers for predicting the response to cyclosporine A therapy in patients with chronic spontaneous urticaria

Dear Editor,

Cyclosporine A (CsA) is recommended as a third-line treatment for patients with chronic urticaria (CU) in the guideline of the European Academy of Allergy and Clinical Immunology (EAACI), the Global Allergy and Asthma European Network (GA² LEN), the European Dermatology Forum (EDF), and the World Allergy Organization (WAO); i.e., CsA is recommended for use in patients with severe disease refractory to treatment with not only antihistamines, but also omalizumab.¹ However, no useful biomarkers for predicting the therapeutic response to CsA in patients with chronic spontaneous urticaria (CSU) presently exist. This letter describes efforts to identify biomarkers for predicting the response to CsA therapy in patients with CSU.

Thirty-four patients with CSU whose symptoms could not be controlled with second-generation H1-antihistamines at a dose of up to 2-fold of the recommended dose were treated with 3 mg/kg/day of CsA² for 4 weeks in the Department of Dermatology, Nihon University School of Medicine, Japan. Their median age was 47 years (range, 18–82 years), and the median duration of their CSU was 18 months (range, 2–480 months, Table 1). Subjects were excluded if steroids had been used within 3 weeks. Blood tests, which included the serum IgE level, basophil counts, IgG anti-nuclear Abs, IgG anti-thyroglobulin Abs, and IgG anti-thyroid microsomal Abs, were performed. IgG autoantibodies (AABs) to FcεRI α-chain (FcεRIα) and IgE were measured using ELISAs (see Supplementary Methods). The autologous serum skin test (ASST) was performed as described previously³ (see Supplementary Methods). The UAS7 is used to quantify the severity of pruritus and the number of hives appearing during a 1-week period.⁴ Five CSU health states were defined according to the UAS7: urticaria-free (UAS7 = 0); well-controlled urticaria (UAS7 = 1–6); mild urticaria (UAS7 = 7–15); moderate urticaria (UAS7 = 16–27); severe urticaria (UAS7 = 28–42).⁵ This study was approved by the Ethics Committee of the Nihon University School of Medicine, Japan. All the subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association. To identify variables associated with the response to CsA, we compared continuous variables between patients with a positive ASST and those with a negative ASST and between patients whose UAS7 was ≤6 and those whose UAS7 was >6 after treatment with CsA for 4 weeks using the Mann-Whitney-U test; to compare categorical variables, a 2-

sided Fisher's exact test was used with a pre-specified significance level of 0.05.

Although there is a lack of clinical studies comparing the efficacy of CsA in patients with positive and negative ASST, patients with a positive ASST often benefit from CsA.^{6–9} Thus, we compared the efficacy of CsA in patients with positive ASST results and in those with negative ASST results. Although the differences in the duration of illness; UAS7 before treatment; and clinical data including the serum IgE levels; basophil counts; positive rates of IgG AABs to the nucleus, thyroglobulin, and thyroid microsomal protein; concentrations of anti-FcεRIα and anti-IgE AABs; between patients with a negative ASST and those with a positive ASST were not statistically significant (Table 1), the therapeutic efficacy of CsA (percentage of patients whose UAS7 was ≤6 among all the patients receiving CsA treatment) in CSU patients with ASST-positive results was significantly higher than that in patients with ASST-negative results (Fig. 1A, $p = 0.0048$). Although the change in the UAS7 before and after treatment with CsA was significantly different in both patients with a negative ASST and those with a positive ASST ($p = 0.0088$ and $p < 0.0001$, Fig. 1B and C, respectively), the patients with a negative ASST, with the exception of one patient, exhibited a persistent severe or moderate level of urticaria after CsA treatment (Fig. 1B). We also compared the percentage of females ($p = 0.477$), age ($p = 0.558$), duration of illness ($p = 0.165$), UAS7 before treatment ($p = 0.081$), serum IgE level (Fig. 1D), basophil counts ($p = 0.385$), and IgG AAB levels to FcεRI α-chain (FcεRIα, Fig. 1E, $p = 0.506$) and IgE (Fig. 1F, $p = 0.986$) between patients whose UAS7 was 6 or less ($n = 13$) and those whose UAS7 was more than 6 after treatment with CsA ($n = 21$). This UAS7 was score of the last week of 4-week-treatment with CsA. The serum IgE level in the patients whose UAS7 was 6 or less was significantly lower than that in the patients whose UAS7 was more than 6 (Fig. 1D, $p = 0.0003$).

The present study had a few limitations. The study sample number was relatively small, and since this was a hypothesis-generating study, the findings need to be confirmed in a different population(s). To identify the cutoff value of IgE, we examined the dose–response relationship between the “linear values of IgE” and the “frequency of remission of CSU in response to CsA” using a receiver operating characteristic (ROC) curve. However, we could not estimate a definite cutoff value for IgE because of the small number of patients and the paucity of previous reports suitable for verifying the validity of the cutoff value. Using the ROC curve analysis, the tentative cutoff value for IgE was 88.5 IU/mL, which yielded a sensitivity of 81.0% and a specificity of 69.2% (odds ratio, 9.56; area under the curve, 0.843). We plan to perform a cohort study in the future with an enrollment of more than 100 individuals to identify a definite cutoff value.

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Table 1
Comparison of patient characteristics between CSU patients with a negative ASST and those with a positive ASST.

	All	ASST		p value*
		Negative	Positive	
Number	34	13	21	
Female sex (%)	59	62	57	>0.999 [†]
Age (years), median (range)	47 (18–82)	47 (18–82)	46 (32–82)	0.965 [‡]
Durations of illness (months), median (range)	18 (2–480)	30 (2–300)	13 (2–480)	0.396 [‡]
Urticaria Activity Score 7, mean (range)	33 (20–42)	34 (21–42)	32 (20–42)	0.424 [‡]
Serum IgE level (IU/mL, mean ± SD)	258 ± 384	231 ± 230	275 ± 459	0.576 [‡]
Blood basophils (/mm ³ , mean ± SD)	33.1 ± 26.4	34.2 ± 29.2	32.5 ± 25.3	0.979 [‡]
Anti-nuclear Abs positive (%)	12	15	9.5	0.627 [‡]
Anti-thyroglobulin Abs positive (%)	8.8	7.7	9.5	>0.999 [†]
Anti-thyroid microsomal Abs positive (%)	8.8	0	14	0.270 [‡]
Anti-FcεRIα AAbs (μg/mL, mean ± SD)	1.83 ± 1.95	1.21 ± 1.29	2.20 ± 2.20	0.129 [‡]
Anti-IgE AAbs (μg/mL, mean ± SD)	1.22 ± 1.35	0.83 ± 0.39	1.47 ± 1.67	0.353 [‡]

SD, standard deviation; AAbs, autoantibodies.

*p value; ASST negative vs ASST positive.

[†] Fisher exact test.[‡] Mann Whitney U test.

Next, we compared the change in the UAS7 before and after CsA treatment in patients with a negative ASST and low serum IgE levels ($\leq$ the cutoff value, 88.5 IU/mL), those with a positive ASST and low serum IgE levels, those with a negative ASST and high serum IgE levels (>88.5 IU/mL), and those with a positive ASST and high serum IgE levels (Fig. 2A, B, C and D, respectively). A significant difference in the change in the UAS7 before and after CsA treatment was observed in patients with a positive ASST and low serum IgE levels and those with a positive ASST and high serum IgE levels ($p = 0.0039$ and $p = 0.0005$, respectively). The percentage of patients whose UAS7 was ≤ 6 among all the patients receiving CsA treatment was

significantly higher in patients with low IgE levels than in those with high IgE levels ($p = 0.009$, Fig. 2E), but the percentage of patients with a positive ASST in patients with low IgE levels was not significantly different from those with high IgE levels (Fig. 2F), suggesting that the ASST results and IgE levels are independent biomarkers for predicting the response to CsA therapy.

The ASST is a nonspecific screening test that evaluates the presence of serum histamine-releasing factors of any type, not just histamine releasing autoantibodies.¹ In our study, the concentrations of anti-FcεRIα and anti-IgE AAbs were not significantly different between patients with a negative ASST and those with a positive ASST,

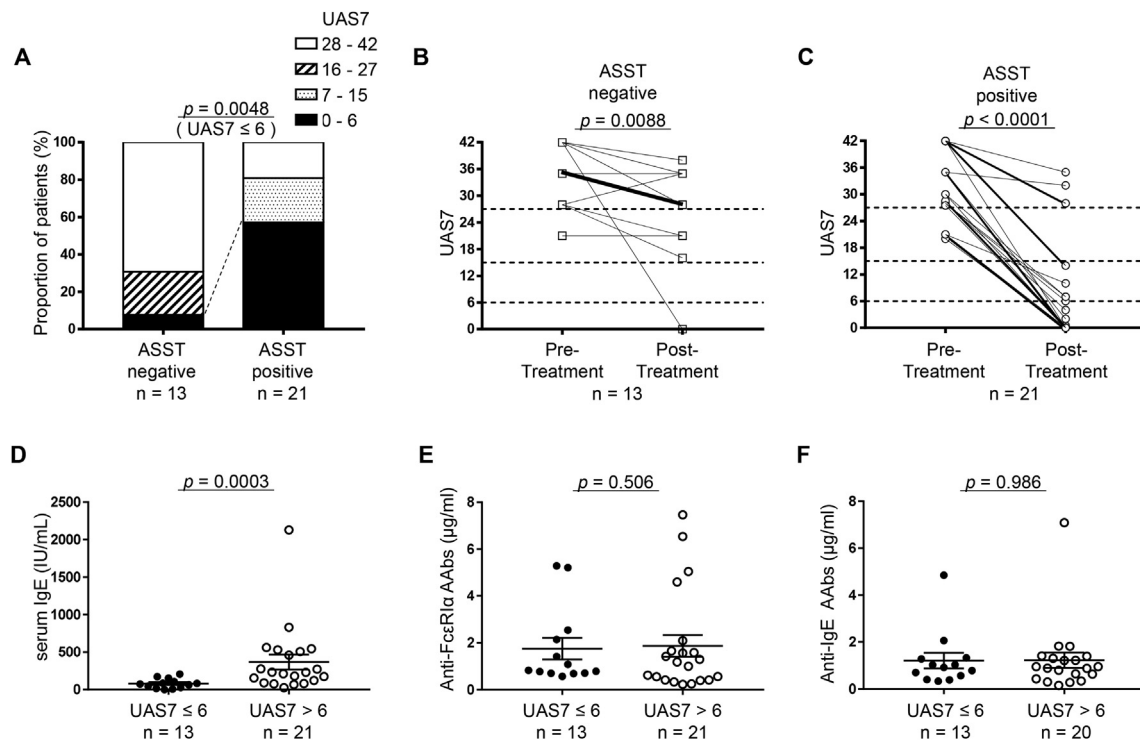


Fig. 1. (A) Comparison of percentages of CSU health states among all the patients receiving CsA treatment between CSU patients with a negative ASST and those with a positive ASST. Closed, dotted, hatched and open bars indicate urticaria free plus well-controlled urticaria, mild urticaria, moderate urticaria, and severe urticaria, respectively. $p = 0.048$: comparison of percentages of patients whose UAS7 was 6 or less between CSU patients with a negative ASST and those with a positive ASST. (B, C) Changes in UAS7 before and after CsA treatment in patients with a negative ASST (B) and those with a positive ASST (C). A bold line in (B) indicates that four patients showed the same response. Bold lines in (C) indicate that two patients showed the same response. The dotted lines indicate the borderlines of the CSU health states. (D–F) Comparison of the serum IgE level (D), and IgG AAb levels to FcεRIα (E) and IgE (F) between patients whose UAS7 was 6 or less and those whose UAS7 was more than 6. The median and inter-quartile range are also shown.

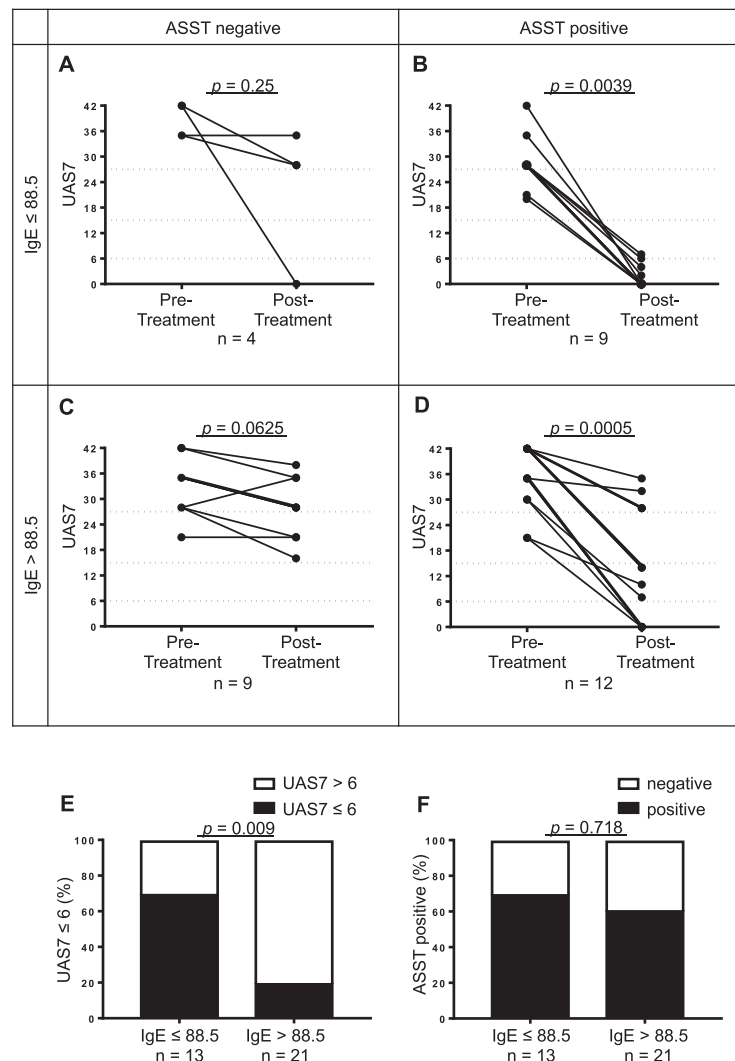


Fig. 2. (A–D) Comparison of the change in UAS7 before and after treatment with CsA in patients with a negative ASST and low serum IgE levels (A), those with a positive ASST and low serum IgE levels (B), those with a negative ASST and high serum IgE levels (C), and those with a positive ASST and high serum IgE levels (D). The dotted lines indicate the borderlines of the CSU health states. Bold lines in (B) and (D) indicate that two patients showed the same response. A bold line in (C) indicates that three patients showed the same response. (E–F) Comparisons of the percentages of patients whose UAS7 was ≤6 among all the patients after treatment with CsA (E) and the percentages of patients with a positive ASST (F) between patients with low serum IgE levels and those with high serum IgE levels.

suggesting that these AAbs might not be major factors inducing serum-mediated vascular leakage^{10,11} or that the abilities of the AAbs to induce FcεRI-crosslinking might be important factors. Further studies are needed to clarify this point. Nonresponders of CSU patients to omalizumab therapy reportedly had significantly lower serum IgE levels than responders.¹² ASST-positive omalizumab responders were reportedly 5.5 times more likely to have a slow response to treatment compared with ASST-negative responders.¹³ Therefore, positive ASST results and low serum IgE levels would be useful biomarkers for predicting the response to CsA therapy in patients with CSU.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2018.09.006>.

Conflict of interest

The authors have no conflict of interest to declare.

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Supplementary Methods

Ethical considerations

This study was approved by the Ethics Committees of the Nihon University School of Medicine. All the subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association.

Definitions and study protocol

The diagnosis of CSU was made according to the classification of the European Academy of Allergy and Clinical Immunology (EAACI), the Global Allergy and Asthma European Network (GA²LEN), the European Dermatology Forum (EDF), and the World Allergy Organization (WAO).¹ According to these criteria, chronic inducible urticaria was defined as the spontaneous appearance of wheals, angioedema, or both over a period of 6 weeks because of known causes such as infectious diseases, type I allergy, autoimmune diseases, or drug allergy.^{1, 2} Subjects were excluded if steroids or immunosuppressive drugs had been used within 3 weeks. The UAS7 is a patient-reported scoring system that captures the severity of pruritus and the number of hives appearing during 1 week.^{3, 4} The intensity of pruritus (range, 0 [none] to 3 [severe]) and the number of hives (range, 0 [none] to 3 [>50 hives]) were recorded daily

(maximum, 6 points per day). The scores were then summed for 1 week to represent the UAS7 (scale, 0–42).

Autologous serum skin test (ASST)

The ASST was performed using 50 μ L of the patient's own serum intradermally injected into the flexor aspect of the forearm; 50 μ L of saline was injected 3 to 5 cm away as a control. The results were measured after 30 min. If the serum-injected site manifested a wheal with a diameter of 1.5 mm greater than that of the saline-injected site, the result was considered positive.⁵ The sera used for the ASST were also used for measurement of concentrations of the AAbs.

Isolation of IgG fraction from serum

IgG was purified from sera of patients with CSU and NC subjects using Ab-Rapid SPiN EX (ProteNova, Kagawa, Japan) according to the manufacturer's protocol. The purified IgG was replaced with PBS using Amicon centrifugal filter units (Merck Millipore, Darmstadt, HE, Germany). The final concentrations of the purified IgG were diluted to 1/6 of the serum IgG concentrations. Recovery rates of IgG fraction using

Ab-Rapid SPiN EX were $95.9 \pm 2.3\%$ under condition of 1:100000 dilution of serum (n = 3).

Expression of recombinant human (rh) soluble form of FcεRIα ectodomain (soluble FcεRIα) and purification of secreted rh soluble FcεRIα

The truncated human FcεRIα ectodomain (1-172 amino acids) was secreted from transfected Chinese hamster ovary (CHO) cells as described.^{6, 7} Secreted rh soluble FcεRIα was purified from the culture supernatant of the cells using mouse anti-FcεRIα (clone CRA2).⁸ rh soluble FcεRIα was eluted using an acid buffer (0.1 M glycine/0.2 M NaCl, pH2.7), immediately neutralized using a basic buffer (1 M Tris-HCl, pH9), quantified using Bradford method, and applied to a gradient gel for SDS-PAGE, as described previously.^{6, 7}

Generation of humanized anti-human FcεRIα mAb, clone CRA2

The anti-human FcεRIα mAb, clone CRA2,⁸ was humanized by complementarity-determining region grafting onto human V region frameworks encoded by human germline V and J genes, as described previously.⁹ Appropriate light and heavy chain expression vectors were co-transfected into COS7 cells using

LipofectAMINE reagent (Life Technologies, Grand Island, NY), as described previously.⁹ The purification of recombinant mAb was performed as described.⁸

Measurement of concentrations of anti-FcεRIα AAbs

An enzyme-linked immuno-sorbent assay (ELISA) for detecting anti-human FcεRIα AAbs was performed as previously described¹⁰⁻¹³ with the exception that a standard curve for IgG anti-FcεRIα was generated using humanized anti-FcεRIα mAb (clone CRA2, isotype IgG1), which was generated by Takai et al.⁹ Briefly, 1 µg/mL of purified recombinant soluble FcεRIα proteins⁷ in sodium carbonate, pH9.6, were used to coat polystyrene microwells of an ELISA plate (Maxisorp; Nunc, Roskilde, Denmark) in duplicates and were incubated overnight at 4°C. The plate was washed 4 times with TBS containing 0.1% Tween 20 (TBST) and then blocked with 10% FBS in PBS for 1 h at room temperature. After washing, the purified IgG from the sera of NC subjects and patients with CSU were added at a 1:10 dilution with PBS to each well, then incubated for 2 h at room temperature. After washing, HRP-conjugated anti-human IgG mAb (clone G18-145; BD Pharmingen, Franklin, NJ), HRP-conjugated anti-human IgG1 mAb (clone HP6069; ThermoFisher Scientific, Waltham, MA), or HRP-conjugated anti-human IgG4 mAb (clone HP6025; ThermoFisher Scientific) was

added to each well at a 1:10,000 dilution for measuring its concentration of total IgG, IgG1 or IgG4, respectively, then incubated for 1 h at room temperature. The reactions in the wells were developed with hydrogen peroxide plus 3,3',5,5'-tetramethylbenzidine in 100 mM citrate-phosphate buffer, pH5.0 (KPL, Gaithersburg, MD), and their absorbances were monitored at 450 nm and 570 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Yokohama, Japan) after the addition of 100 μ L of 2 N sulfuric acid. A standard curve for IgG anti-Fc ϵ RI α was generated using humanized anti-Fc ϵ RI α mAb (clone CRA2).⁹ The linear region of the standard curve was used to quantify the anti-Fc ϵ RI α in the purified IgG.

Measurement of concentrations of anti-IgE AAbs

An ELISA was used to detect anti-IgE AAbs, as previously described.¹⁴⁻¹⁷ Briefly, the polystyrene microwells of an ELISA plate (Maxisorp; Nunc) were coated with human myeloma IgE (1 μ g/mL in PBS, Calbiochem, San Diego, CA) and incubated overnight at 4°C. Unbound protein was removed by four washes with TBST. Free binding sites were blocked by incubation with 10% FBS in PBS for 1 h at room temperature. After washing, the purified IgG from the sera of NC subjects and patients with CSU were added at a 1:10 dilution with PBS to each well, which were then incubated for 2 h at room temperature. After washing, HRP-conjugated anti-human IgG

mAb (clone G18-145; BD Pharmingen) was added to each well at a 1:10,000 dilution, which was then incubated for 1 h at room temperature. The reactions in the wells were developed with hydrogen peroxide plus 3,3',5,5'-tetramethylbenzidine in 100 mM citrate-phosphate buffer, pH5.0 (KPL), and their absorbances were monitored at 450 nm and 570 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific) after the addition of 100 μ L of 2 N sulfuric acid. The quantity of reactive IgG was calculated using standard amounts of purified human IgG (Jackson Immuno Research Laboratories, West Grove, PA) ranging from 0.775 ng/mL to 50 ng/mL bound directly to the plates. To normalize the data among the plates, one specific donor's anti-IgE level was measured repeatedly on all of the plates.

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