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CONFLICT OF INTEREST

The authors state no conflicts of interest.

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Institutional review board approval was not required for this study as all the data were publicly available.

AUTHOR CONTRIBUTIONS

Conceptualization: AM; Data Curation: SD, CM; Formal Analysis: SD, PM; Investigation: SD; Methodology: AM, SD; Project Administration: AM; Resources: CM; Supervision: AM; Visualization: AM; Writing - Original Draft Preparation: SD; Writing - Review and Editing: SD, PM, KJL, SJL, CM, AM.

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MRGPRX2 Activation Causes Increased Skin Reactivity in Patients with Chronic Spontaneous Urticaria

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TO THE EDITOR

Previous studies showed that injection of basic secretagogues such as substance P, compound 48/80, and vasoactive intestinal peptide induced exaggerated responses in the skin of patients with chronic spontaneous urticaria (CSU) versus those of healthy controls through an unknown G protein-coupled receptor (Borici-Mazi et al., 1999; Bédard et al., 1986; Smith et al., 1992). The human receptor for these secretagogues has been identified as MRGPRX2, and in vitro work has shown that MRGPRX2 can activate mast cells (MCs) through a diverse range of drug ligands such as the neuromuscular blocking drug, atracurium, and a bradykinin B2 receptor

antagonist, icatibant (McNeil et al., 2015). Further in vitro studies have provided evidence of MRGPRX2 drug ligand activation in cell lines and animal models, but no human experiments have been performed (Che et al., 2018; Navinés-Ferrer et al., 2018; Roy et al., 2019). With the recent discovery that MRGPRX2 protein expression is increased in the MC taken from skin biopsies of patients with severe CSU (Fujisawa et al., 2014), we examined whether subjects with milder CSU would show a heightened in vivo functional skin response when tested with MRGPRX2 drug ligands compared with healthy controls. This study is a demonstration of an increased functional skin

response supporting MRGPRX2-increased protein expression in patients with CSU. We further demonstrate that drug-induced MC activation requires MRGPRX2 using knockout LAD2 cells and is not occurring through IgE pathways.

We conducted the study at the Johns Hopkins Asthma and Allergy Center (Baltimore, MD) after approval by the Institutional Review Board. After written informed consent, healthy subjects and subjects with mild CSU between the ages of 18 and 65 years underwent serial intradermal skin titration testing with two MRGPRX2 drug ligands, icatibant (0.01–100 µg/ml, 7×10^{-3} –76 µM, physiologic range 974 ± 280 ng/ml [Shire Pharmaceuticals, Lakewood, NJ]) and atracurium (0.001–10 µg/ml, 8×10^{-4} –8 µM, physiologic range ~100–3,000 ng/ml [Hospira, Lake Forest, IL]) and saline control (Hospira) as well as histamine (0.01–100 µg/ml,

Abbreviations: CSU, chronic spontaneous urticaria; MC, mast cell

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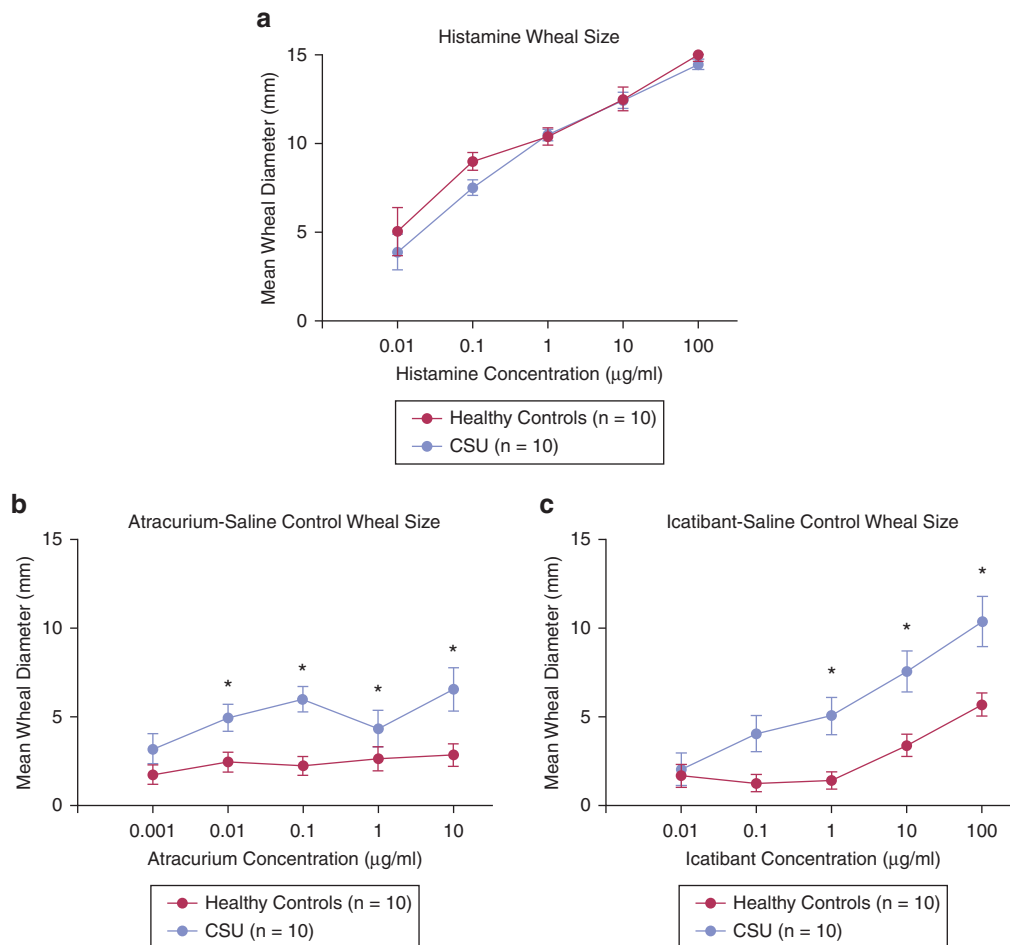


Figure 1. Skin test responses to MRGPRX2 ligands in healthy subjects and subjects with CSU. Skin test responses to (a) histamine, (b) atracurium, and (c) icatibant. The mean saline wheal in subjects with CSU was $1.5 \text{ mm} \pm 2.59$ versus $5.53 \text{ mm} \pm 2.74$ in healthy controls, $P < 0.05$. The results represent the arithmetic mean $\pm$ SEM of the wheal size at each concentration. AUC was calculated for each dose-response curve (Supplementary Table S3). Asterisks represent significant dose comparisons ($P < 0.05$). AUC, area under the curve; CSU, chronic spontaneous urticaria.

9×10^{-2} – $900 \mu\text{M}$ [HollisterStier, Spokane, WA]). The drugs chosen were based on the in vitro evidence of the presence of MRGPRX2 activation (icatibant and atracurium) (McNeil et al., 2015) and either Food and Drug

Administration approval for subcutaneous injection (icatibant) or a history of safe use in skin testing (atracurium) (Leysen et al., 2014). A single, unblinded investigator did all the skin testing with the exception of two subjects. By design,

all subjects with CSU recruited had a history of pruritus and urticaria for >6 consecutive weeks but were able to withdraw all antihistamines at 7 days before the study for skin testing; therefore, all subjects had a mild phenotype with an

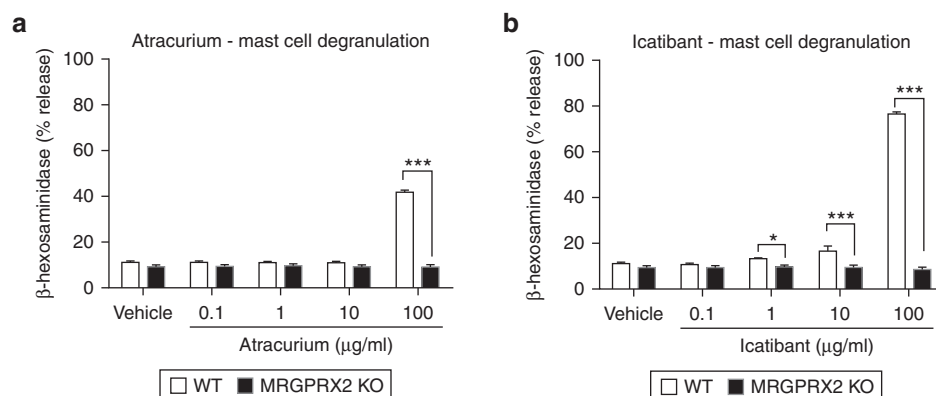


Figure 2. MRGPRX2 ligand-induced degranulation of human WT and MRGPRX2-deficient LAD2 MCs. (a) Atracurium and (b) icatibant degranulation was measured by β -hexosaminidase release. Data represent the mean $\pm$ SEM of the percentage release at each concentration. * $P < 0.05$; *** $P < 0.001$. The experiments were independently repeated three times. KO, knockout; MC, mast cell; WT, wild type.

average urticaria activity score of 0.5 and typically required a single daily antihistamine (Supplementary Table S1). Subjects who had taken steroids in the last 4 weeks, tricyclics in the last 2 weeks, or immunomodulators were excluded. The healthy controls lacked any atopic or urticaria history (Supplementary Table S2).

For each drug concentration, a fixed volume of 0.03 ml was injected on the volar aspect of the forearm. Individual wheals were recorded at 15 minutes, and the total area under the curve of wheal diameters was calculated. The wheal diameter for each concentration of the MRGPRX2 ligand was determined for each subject and corrected by the subjects' saline wheal size to account for possible dermatographism. Comparisons between the subjects with CSU and healthy controls were performed using the student's *t*-test using Stata (StataCorp, College Station, TX). For LAD2 studies, similar concentrations of icatibant and atracurium were incubated with wild-type or MRGPRX2-knockout cells for 30 minutes at 37 °C, and supernatants were tested for β -hexosaminidase release (Supplementary Materials and Methods). In selected subjects, basophil activation test was performed in whole blood. Cells were stimulated for 30 minutes with anti-IgE, icatibant, atracurium (at concentrations used for skin testing), or N-formyl-L-methionyl-L-leucyl-phenylalanine (10^{-6} M) at 37 °C. Cells were then stained with CD63 phycoerythrin antibody (Beckman Coulter, Brea, CA), lyse-fixed, and run on FACS Calibur flow cytometer (BD, Franklin Lakes, NJ) for analysis (Supplementary Materials and Methods).

Histamine skin test responses were similar in both groups (Figure 1a). Skin responses to the two MRGPRX2 drug ligands, atracurium and icatibant, revealed differences between the subject groups. Overall, subjects with CSU showed significantly greater wheal responses to atracurium at all but the lowest tested doses, with a mean area under the curve of 21.50 ± 3.54 for subjects with CSU versus area under the curve of 9.65 ± 2.32 for control subjects ($P = 0.01$) (Figure 1b and Supplementary Table S3). Subjects with CSU also showed a significantly greater wheal response to icatibant at

concentrations of 1 μ g/ml, 10 μ g/ml, and 100 μ g/ml ($P < 0.05$) and overall larger area under the curve of 23.16 ± 4.06 than that of the healthy controls (9.79 ± 1.76 , $P = 0.01$) (Figure 1c and Supplementary Table S3). In vitro testing with similar drug concentrations with wild-type and MRGPRX2-knockout LAD2 cells revealed a similar pattern as skin reactivity with a more robust β -hexosaminidase release with icatibant (Figure 2). To further establish that these drug ligands were activating skin MCs through non-IgE pathways, we performed the basophil activation testing with the same concentrations used for skin testing in three subjects with evidence of skin test reactivity to both atracurium and icatibant (Supplementary Figure S1). We failed to observe significant basophil activation at any of the drug concentrations tested but did observe expected basophil activation with anti-IgE stimulation, supporting that an IgE-independent pathway is involved in skin MC activation for both atracurium and icatibant. Whereas a recent report demonstrated MRGPRX2 presence and activation by ciprofloxacin but not PMX-53 on basophils (Wedi et al., 2020), others have not found basophil activation with ligands such as 48/80, and this remains an area of active research (Ishizaka et al., 1985; Lawrence et al., 1987; Van Gasse et al., 2019).

Our results support differentiation in skin reactivity to both drug ligands at almost every dose in subjects with mild CSU compared with healthy controls. The LAD2 knockout results confirm that these two ligands stimulate mediator release through the MRGPRX2 receptor, with icatibant appearing to be a stronger ligand on the basis of both the in vivo and in vitro studies. Whereas the results of increased MRGPRX2 ligand skin reactivity in subjects with CSU need to be replicated, they carry implications as a potential disease target in CSU as well as various other MC-mediated disorders.

Limitations of this study include small sample size and the variability of intradermal skin testing. Although MRGPRX2 antagonists have been reported (Azimi et al., 2016; Ogasawara et al., 2019), there are no Food and Drug Administration–approved MRGPRX2 antagonists currently available to confirm that the skin test

response is fully mediated through MRGPRX2. However, we were able to exclude IgE pathway utilization in the subjects and demonstrate the dependence of MC activation by these drugs to be selective for MRGPRX2. We present a heightened skin sensitivity to MRGPRX2 ligands in subjects with CSU, which conveys a functional consequence to the past report of heightened protein expression of MRGPRX2 in CSU skin MCs.

Data availability statement

All the data for this study are displayed in the manuscript.

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CONFLICT OF INTEREST

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Wild-type LAD2 cells were obtained from the lab of Dr. Dean Metcalfe at NIAID/NIH.

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Conceptualization: MS, SSS; Formal Analysis: MS, NL, SSS; Funding Acquisition: XD, SSS; Investigation: MS, NL, ETO, KC, XD, SSS; Methodology: MS, NL, LG, XD, SSS; Project Administration: XD, SSS; Resources: XD, SSS; Supervision: XD, SSS; Validation: MS, NL, SSS; Visualization: MS, NL, SSS; Writing - Original Draft Preparation: MS, SSS; Writing - Review and Editing: MS, NL, ETO, KC, LG, XD, SSS

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2020.06.030>.

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CD23 Levels on B Cells Determine Long-Term Therapeutic Response in Patients with Atopic Eczema Treated with Selective IgE Immune Apheresis

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TO THE EDITOR

The complex pathogenesis of atopic eczema involves an impaired epidermal barrier, dysbiosis, and aberrant type 2 immunity (Eyerich and Eyerich, 2018; Werfel et al., 2015) depending on cytokines such as IL-4, IL-5, IL-9, IL-13, and IL-31 as well as eosinophils, basophils, mast cells, and IgE (Eyerich and Eyerich, 2018). This is underpinned by the clinical efficacy of novel therapeutic targets interacting with type 2 immunity such as the IL-4Ra antibody dupilumab (Blauvelt et al., 2017).

The role of IgE in the pathogenesis of atopic eczema is discussed controversially because an intrinsic type of atopic eczema with normal IgE levels exists.

However, IgE levels usually correlate well with atopic eczema severity (Aral et al., 2006), and anti-IgE therapies, for example, with omalizumab, a humanized anti-IgE antibody, or selective IgE immune apheresis (IA) are efficient at least in a subgroup of patients. It was shown that cytokine levels in the skin but not circulating IgE correlate with long-term clinical response (Reich et al., 2019). However, the underlying mechanisms are still poorly understood. Therefore, the aim of this study was to identify the mode of action of IA and to define the biomarkers predicting therapeutic outcome.

Experiments were approved by the Paul Ehrlich Institute and by the ethics

committee of the Faculty of Medicine of Technical University of Munich, and written informed patients' consent was obtained. A total of 10 patients with highly elevated serum IgE levels (15.032 ± 10.226 IU/ml) were treated with 10 sessions of IA (Figure 1a and Supplementary Table S1), with a follow-up after 4 weeks. Circulating IgE levels were efficiently reduced after each IA session but were fully restored at follow-up in all the patients (Figure 1b). Nevertheless, some patients showed sustained clinical improvements at follow-up. Patients were clustered into responders and non-responders by means of minimum value of the root mean square distance and were defined by scoring atopic dermatitis (SCORAD) 40 responses (Figure 1b and e). Circulating IgE levels tended to correlate with the SCORAD before IA ($r = 0.619$, $P = 0.056$) and at follow-up; responders



Abbreviations: IA, immune apheresis; SCORAD, scoring atopic dermatitis

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SUPPLEMENTARY MATERIAL AND METHODS

Generation of MRGPRX2 knockout LAD2 cells

Two single-guide RNAs with 2'-O-methyl 3'-phosphorothioate-modified nucleotides at three terminal positions at both the 5' and 3' ends targeting the beginning of exon 2 of *MRGPRX2* gene were purchased from Synthego (Menlo Park, CA). The single-guide RNA sequences are 5'-UAGUUCAU-GACGGCCGGAGG-3' and 5'-UGGAGAAGCCGAAAAACCAG-3'. Single-guide RNAs were complexed with *Streptococcus pyogenes* Cas9 nuclease (Synthego) to form ribonucleoprotein. Ribonucleoprotein complexes were transfected into the LAD2 cells using Lipofectamine CRISPRMAX transfection reagent (Thermo Fisher, Waltham, MA). After 2 weeks of transfection, cells were stained with MRGPRX2 antibody (BioLegend, San

Diego, CA) and subjected to FACS for selection of populations that lack the expression of MRGPRX2. Sorted cells were expanded, and their deficiency in MRGPRX2 expression was confirmed by DNA sequencing, flow cytometry, and nonresponse to C48/80 and substance P.

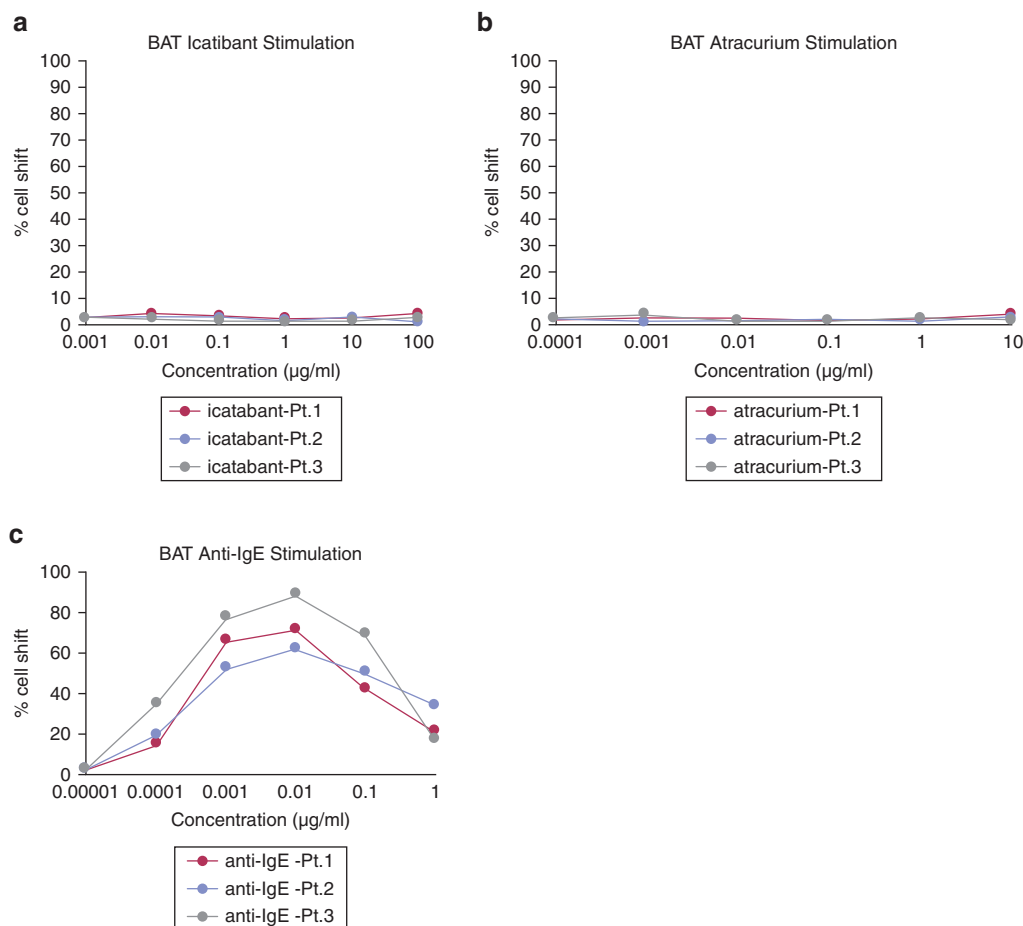
β -hexosaminidase release assay

LAD2 cells were harvested and resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer containing 0.4% BSA. Then 20,000 cells were seeded per well and stimulated with icatibant, atracurium, or other known MRGPRX2 agonists (C48/80, substance P, cortistatin-14, or proadrenomedullin N-terminal 20 peptide 8–20) at the indicated concentrations for 30 minutes at 37 °C and 5% carbon dioxide. After a 30-minute incubation, the supernatant was collected, and cells were lysed using 0.1% Triton X-100. The supernatant and cell lysates were

incubated with p-nitrophenyl-N-acetyl- β -D glucosamine in 0.04 M citrate buffer (pH 4.5) for 90 minutes at 37 °C. The reactions were developed by adding 0.4 M glycine (pH 10.7). Absorbance was measured at 405 nm and 570 nm as the reference using FlexStation 3 plate reader (Molecular Devices, San Jose, CA). The β -hexosaminidase release into cell supernatant was reported as a percent of total β -hexosaminidase content.

Basophil activation test

In select subjects, basophil activation test was performed in whole blood. Cells were stimulated for 30 minutes with anti-IgE (1×10^{-4} – $1 \mu\text{g/ml}$), icatibant, atracurium (at concentrations mentioned above), or N-formyl-L-methionyl-L-leucyl-phenylalanine (10^{-6} M) at 37 °C. Cells were gated by scatter and CD123⁺/CCR3⁺ as well as CD63 antibody, lyse-fixed, and run on FACS-Calibur flow cytometer (BD, Franklin Lakes, NJ) for analysis.



Supplementary Figure S1. BAT with icatibant, atracurium, and anti-IgE in three healthy control subjects with skin test reactivity to icatibant and atracurium. BAT, basophil activation test; Pt., patient.

Supplementary Table S1. Characteristics of Chronic Urticaria Subjects

Patient ID	Age, y	Sex	Race	Duration of Illness, y	UAS Score ¹	Treatment	Atopic History
1	53	F	Cau	1	1	Cetirizine	SAR, AD
2	20	M	AA	2	0	Cetirizine	None
3	25	F	Cau	2	2	Fexofenadine + cetirizine	SAR, AD, AS
4	54	M	Cau	3	0	Cetirizine	SAR, AD, AS
5	63	F	AA	6	1	Fexofenadine	SAR
6	35	M	Cau	10	0	Fexofenadine	SAR, AD
7	37	F	Cau	7	0	Loratadine	AS
8	51	F	AA	10	1	Fexofenadine	SAR
9	64	F	Cau	4	0	Levocetirizine	None
10	23	M	Cau	5	0	Cetirizine	SAR, FA, AS

Abbreviations: AA, African American; AD, atopic dermatitis; AS, asthma; Cau, Caucasian; F, female; FA, food allergy; ID, identifier number; M, male; SAR, seasonal allergic rhinitis; UAS, urticaria activity score.

¹Sum of itch 0–3 and 0–3 for hives, with 0 = no hive, 1 = 1–6 hives, 2 = 7–12, and 3 = > 12 hives.

Supplementary Table S2. Characteristics of Nonatopic Control Subjects

Patient ID	Age, y	Sex	Race	Other Disease	Atopic History
1	45	F	Cau	Hypothyroidism	No
2	35	M	Cau	HTN	No
3	46	F	Cau	HTN and/or CKD	No
4	28	F	Cau	None	No
5	44	F	Cau	Migraines	No
6	37	M	AA	None	No
7	51	F	Cau	Depression and/or HTN and/or rosacea	No
8	27	F	Cau	None	No
9	38	M	Asian	None	No
10	19	F	Cau	None	No

Abbreviations: AA, African American; Cau, Caucasian; CKD, chronic kidney disease; F, female; HTN, hypertension; ID, identifier number; M, male.

Supplementary Table S3. Comparison of the AUC between Healthy Subjects and Subjects with CSU

Reagents	Healthy (n = 10)	CSU (n = 10)	P-value
Histamine	40.9 ± 1.52	39.67 ± 1.70	0.59
Atracurium-saline control	9.65 ± 2.32	21.50 ± 3.54	0.01
Icatibant-saline control	9.79 ± 1.76	23.1 ± 4.06	0.01

Abbreviations: AUC, area under the curve; CSU, chronic spontaneous urticaria.

Atracurium and icatibant are saline corrected. Mean is reported with SEM.