**Transcriptome analysis of severely active chronic
spontaneous urticaria shows an overall immunological skin
involvement**

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3 1 **Transcriptome analysis of severely active chronic spontaneous**
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5 2 **urticaria shows an overall immunological skin involvement**
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10 4 ***Short title: Transcriptome analysis in active chronic spontaneous urticaria***
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ABSTRACT

Background: The knowledge about chronic spontaneous urticaria (CSU) phenotypes is based on its clinical characteristics, associated comorbidities, course of the disease and its response to the available effective drugs. Genotype expression and its further correlation with CSU phenotypes are still unknown. We describe the cutaneous transcriptome of patients suffering a severely active CSU refractory to antihistamine treatment. **Methods:** Through the bioinformatic analysis of the whole Human Genome with Oligo Microarrays and Quantitative real-time polymerase chain reaction (qPCR), relevant genes expressed in non-lesional [NLS-CSU] and lesional skin [LS-CSU] and peripheral blood were identified in 20 patients suffering from severely active CSU and 10 healthy controls (HCs) **Results:** From 39 genes differentially expressed in NLS-CSU when compared with HCs, 31 (79.48%) were confirmed by qPCR corresponding to genes involved in epidermal homeostasis and dermal repair. From the analysis comparing LS-CSU with NLS-CSU, a selection of 142 genes was studied with qPCR, and 103 (72.53%) were confirmed. Differentially expressed genes in the phenomenon of wheal development are involved in a variety of biological functions as, epidermal differentiation, intracellular signal function, transcriptional factors cell cycle differentiation, inflammation or coagulation. Differentially expressed genes that uniformly increase or decrease along the skin worsening until the wheal appearance are shown. **Conclusion:** The skin of CSU patients with a severely active disease shows an overall immunological skin involvement showing a peculiar gene profile.

Key words: chronic spontaneous urticaria; genetics; microarray analysis; quantitative real-time PCR; RNA;transcriptome

Introduction

Chronic spontaneous urticaria (CSU), classically known as chronic idiopathic urticaria (CIU), is a common dermatosis with an estimated prevalence of 0.6 to 1% (1). CSU is defined by spontaneously occurring short-lived and itching wheals, angioedema, or both, for a minimum period of 6 weeks (2,3). An episode of CSU can last longer, even more than five years and more than one CSU episode can be suffered. CSU negatively affects the patient's quality of life and has a considerable impact on direct and indirect health-related costs (4).

The current knowledge about CSU phenotypes is based on its clinical characteristics, associated comorbidities, activity, course and response to the available effective drugs. The CSU phenotype showing the most active disease (highest Urticaria Activity Score [UAS]) with the worse prognosis commonly is refractory to high doses of antihistamines and associates angioedema, concomitant chronic inducible urticaria (CIndU) and/or serum autoreactivity (functional histamine-release autoantibodies) (4).

Mast cell activation and wheal kinetics are partially understood and explain the evanescent nature of hives. However, our knowledge about the biological characteristics of the apparently healthy skin during urticaria outbreak is unknown.

The genotype expression and its further correlation with CSU phenotypes are unknown (5). Functional analysis of gene expression from lesional and non-lesional CSU (LS-CSU and NLS-CSU, respectively) skin may provide new insights into the pathogenesis of hive. We describe the genetic signature of patients suffering severely active CSU refractory to antihistamine treatment (6–8). Our hypothesis is that the entire cutaneous integument provides a peculiar gene expression in severely active CSU.

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86 **Methods**

87 **Studied population: CSU patients and HCs**

88 Twenty patients suffering from severely active CSU and 10 HCs were
89 prospectively included in the study. Only patients showing severely active CSU
90 (Urticaria Activity Score (UAS) $7 \geq 16$) for at least 3 months were included (9). All
91 patients remained symptomatic despite a treatment with oral antihistamines. A
92 blanching period of previous drugs was guaranteed. HCs were selected from a
93 pool of volunteers without any active cutaneous or systemic disease. Ethical
94 approval by Institut Mar d'Investigacions Mèdiques-IMIM was obtained (EC-
95 2012/4913/I). The study was conducted in accordance with the Declaration of
96 Helsinki and European regulations. All participants provided written informed
97 consent to participate in the study; a signed consent was obtained for pictures
98 reproduction of the skin.

99

100 **Clinical assessment and interventions**

101 CSU patients were evaluated through a complete clinical history.
102 Complementary tests were performed according to an extended diagnostic
103 program (2) (Table S1).

104 Three cutaneous biopsies (punch of 4mm) were obtained from the skin: two
105 biopsy specimens from an active wheal or LS-CSU and one from NLS-CSU.
106 Samples were taken from LS-CSU (located on the trunk) with a minimum-
107 maximum evolution time of one-two hours. The NLS-CSU skin biopsy was
108 obtained from an area where neither a wheal nor other skin alterations (e.g.
109 desquamation or pigmentation) were presented within the previous 72 hours.

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3 110 Biopsies were prepared for routine pathology (hematoxinilin-eosin and Giemsa
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5 111 stains), mast-cell and basophil-specific immunohistochemistry (c-Kit and BB1
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7 112 expression) and transcriptome analysis (quick-freezing and cryopreserved
8
9 113 tissue stored at $-80^{\circ}\text{C}.$). Venous blood for gene array was also obtained. The
10
11 114 same procedures were applied to HCs.
12
13
14 115 Human total ribonucleic acid (RNA) was isolated from cryopreserved tissue
15
16 116 samples (50 vials) by using Trizol® (Sigma-Aldrich). Human total RNA was
17
18 117 isolated from blood samples (30 vials) stabilized in Tempus™ Blood RNA tubes
19
20
21 118 (Applied Biosystems) by using the Tempus™ Spin RNA Isolation Reagen Kit
22
23 119 (Applied Biosystems). RNA quality and integrity were determined with Agilent
24
25 120 RNA 6000 Nano Kit (Agilent 2100 Bioanalyzer, Agilent Technologies). RNA was
26
27 121 quantified by measuring A260nm on the ND-1000 Spectrophotometer
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29 122 (NanoDrop Technologies).
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124 **Gene array methodology**

125 Quality control of RNA samples was performed with the Bioanalyzer 2100
126 (Agilent Technologies, Palo Alto, CA, USA). Good quality samples with an RNA
127 Integrity Number (RIN) value >6 , and also some mildly degraded samples with a
128 RIN value between 4 and 6, were processed. For the partially degraded
129 samples, amplification and labeling quality control were performed during the
130 sample process in order to discard any effect on the microarray workflow. Gene
131 expression analysis of different human tissue samples was done using Agilent
132 Whole Human Genome Oligo Microarrays (one-color).
133 Samples were processed as follows. A linear T7-based amplification step with
134 100ng of each total ribonucleic acid (RNA) sample was performed. To produce
135 Cy3-labeled complementary RNA (cRNA), the RNA samples were amplified and

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3 136 labeled using the Agilent Low Input Quick Amp Labeling Kit (Agilent
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5 137 Technologies) following the manufacturer's protocol. Yields of cRNA and the
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7 138 dye-incorporation rate were measured with the ND-1000 Spectrophotometer
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9 139 (NanoDrop Technologies). For further processing, amplification/labeling as well
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12 140 as hybridization, the control B samples were randomly distributed. The
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14 141 hybridization procedure was performed according to the Agilent 60-mer oligo
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16 142 microarray processing protocol using the Agilent Gene Expression Hybridization
17
18 143 Kit (Agilent Technologies). Briefly, 600ng Cy3-labeled fragmented cRNA in
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20 144 hybridization buffer was hybridized overnight (17 hours, 65°C) to Agilent whole
21
22 145 human genome oligo microarrays 8x60K v2 using Agilent's recommended
23
24 146 hybridization chamber and oven. Finally, the microarrays were washed once
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26 147 with the Agilent gene expression wash buffer 1 for 1 min at room temperature
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28 148 followed by a second wash with preheated Agilent gene expression wash buffer
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30 149 2 (37 °C) for 1 min.
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36 151 **Bioinformatic analysis of the gene array**

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38 152 Blood data and tissue data were analyzed separately. Raw data were extracted
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40 153 file by file using R (v 2.15) and package Biobase. After quality control
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42 154 assessment, gene transcripts obtained were background corrected and
43
44 155 normalized between arrays with quantile normalization available in Limma
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46 156 package. The noise caused by non-expressed transcripts was avoided doing a
47
48 157 selection of those having a standard deviation (SD) above the 20% of SD
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50 158 distribution. A total of 46,973 gene identifiers were selected for both data sets.
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52 159 Linear models for Microarrays (Limma package) (10,11), with a false discovery
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54 160 rate (FDR) (12) multiple comparison correction, were used to detect differentially
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expressed transcripts between the conditions specified in Figure S1. Linear models were controlled by age. Variable sex was studied but no differences between groups were presented, so it was not included in the model. An adjusted P value of <0.05 and an absolute Logarithmic Fold Change (logFC) from 1 (that is, $\logFC > 1$ or < -1) were considered the significant thresholds to select final significant transcripts.

Functional analysis was performed as a first step with the Ingenuity Pathway Analysis (IPA) (<http://www.ingenuity.com>). All lists were loaded and a core analysis performed.

In order to study of tendencies among conditions (LS-CSU, NLS-CSU and HCs skin) a regression approach to find clusters of genes with similar expression profiles was performed (maSigPro package) (13).

In addition to our data, public data under accession number GSE57178 (14) (Gene Expression Omnibus [GEO]), based on the Affymetrix Human Gene 1.0 ST array, were downloaded and analyzed with the same methodology using the aroma.affymetrix (15) and Limma packages.

Complete gene expression profile

The complete microarray data obtained from the blood and the skin of the CSU patients and HCs is located in the GEO under the accession number GSE72542 (14).

Quantitative real-time polymerase chain reaction (qPCR)

Attending the results from the microarray comparative analysis, a set of genes was selected to be confirmed by qPCR. Gene selection was based on technical

186 aspects and also on a literature research gen by gen about their involvement in
187 mast cell biology, wheal pathogenesis, urticaria or allergy. These genes were
188 studied (blood and skin tissue), by microarray technique, and in an independent
189 set of 19 skin samples (3 HCs skin, 6 NLS-CSU and 10 LS-CSU). These
190 samples presented a RIN value <4 , consequently, were not suitable for
191 microarray analysis but were valid for qPCR. As a result, all patients' skin and
192 wheal paired samples were available for qPCR analysis.

193 For qPCR validation, the same RNA material used for microarrays was
194 processed. Therefore, RNA quality analysis for all the samples was performed
195 before qPCR analysis. Afterwards, complementary deoxyribonucleic acid
196 (cDNA) was reversely transcribed from 2000ng of total RNA using the
197 SuperScript VILO cDNA Synthesis Kit (Life Technologies), as described by the
198 manufacturer. TaqMan qPCR reactions were performed on cDNA samples
199 using TaqMan Universal PCR Master Mix II and on the Taqman Low Density
200 Array (TLDA) platform (Life Technologies), according to the manufacturer's
201 instructions. Fluorescence was detected in an ABI PRISM 7900 HT system (Life
202 Technologies).

203 The used qPCR platform was the TLDA in a 64-assay format (2 samples per
204 array were interrogated for the expression of 64 genes in triplicate). Four
205 different designs were performed according to the genes selected in each
206 comparison. Specifically, each TLDA design contained a maximum of 57
207 selected genes together with 7 housekeeping genes (18S, GUSB, B2M, TBP,
208 SDHA, RPLP0, PGK1) used as endogenous controls. This housekeeping genes
209 set was selected according to both our microarray results and the already
210 available published studies with qPCR done in skin samples (16–20).

qPCR data analysis was performed using the geNorm method for the selection of the best housekeeping genes (21), and the $2^{-\Delta\Delta Ct}$ method (22) was applied. Student's t-tests (paired and unpaired) were applied and genes were considered to be differentially expressed when P value was <0.05. The confirmed genes by qPCR of the tissue and the blood were studied according to the comparisons included in Figure S1.

RESULTS

Characteristics of the studied population

The clinical features for each patient are included in Table S2. Fourteen women and 6 men were included (mean age±SD of 51.5±15.7 years [range 20-81 years]). The HCs (N=10) ratio female:male was 1:1 and the mean age was 27.5±5.4 years (range 21-37 years). The disease mean duration was 4.9±9 SD years (range 3 months-40 years). Mean UAS7 was 33.6 (range 16-42). All the patients were symptomatic in spite of high doses of second generation H1-antihistamines (2) and were suitable to be treated with e.g. anti-immunoglobulin E (IgE) therapy or immunomodulation. 35% (7/20) of them were unresponsive to cyclosporine A. Attending other clinical characteristics as markers of a bad prognosis for CSU, 20% (4/20) of the patients suffered intermittent angioedema crisis (4). Twenty five per cent (5/20) showed concomitant inducible urticaria (symptomatic dermographism or delayed pressure urticaria) (4). Fifty five percent (11/20) of the patients showed a positive Autologous Serum Skin Test (ASST) and 55% (11/20) had an autoimmune disease associated (4). The autoimmune thyroid diseases were the most frequent concomitant conditions, affecting 25% (5/20) of the CSU patients (4). Other autoimmune diseases

reported included vitiligo, Sjögren syndrome and systemic lupus erythematosus. D-dimer serum level was high in 54% (7/13). The mean value of the total serum IgE was 277.5 ± 357.8 UI/ml (range 22-1470 UI/ml), slightly higher compared to the normal values detected in HCs (60.7 ± 54.4 UI/ml). Histopathological findings were compatible with a well-developed wheal. The epidermis always showed a normal appearance. Oedema was present in upper and mid dermis. A characteristic both superficial and deep perivascular/interstitial inflammatory infiltrate with lymphocytes, eosinophils and neutrophils without vascular damage, was observed in all cases (Figure S2). A mild to moderate increase of mast cell numbers was also observed. No morphological differences were found between the wheal samples studied in terms of edema intensity, location of the inflammatory infiltrate and predominant type of cells involved.

NLS-CSU transcriptome in CSU shows an abnormal mRNA expression of genes involved epidermal homeostasis

Based on the statistical criteria defined previously, 376 identifiers corresponding to 299 unique genes were differentially expressed (Figure 1A). Thirty-nine genes were selected for validation using qPCR; from those, 31 were confirmed (79.48%) (Figure 1B). Table 1 includes the genes confirmed by qPCR resulting from the comparison between the NLS-CSU patients and the skin from HCs. The up-regulated genes are involved in epidermal terminal differentiation, inflammation and dermal repair. A total of 19 out of the 31 genes identified, over and under expressed, codify epidermal proteins responsible for epidermal homeostasis. The analysis showed the overexpression of genes as SPINK5,

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261 FLG, ASPRV1, IL-37, FCGBP, or CD1A, previously reported to be
262 overexpressed in ichthyosis and atopic dermatitis (23). In NLS-CSU,
263 overexpression of genes involved in the dermal repair (such as arginase 1,
264 extracellular matrix protein 1 or matrillin) was observed. Matrillin encodes a
265 member of the von Willebrand factor A domain-containing protein family and
266 has been involved in wound healing. Genes codifying IL-37 and IL-36 were also
267 overexpressed. IL-37 is involved in the communication between innate and
268 adaptive immunity (elevated in atopic dermatitis) acting as anti-inflammatory
269 (24) whereas IL-36 is member of the IL-1 cytokine family and is secreted by
270 keratinocytes e.g. in contact dermatitis and is involved in epidermal hyperplasia.
271 CD1A gene codifies a protein shown by the Langerhans cells being involved in
272 the innate immunity and FCGBP gene codifies the Fc fragment of
273 immunoglobulin G (IgG) binding protein capable to bind e.g. IgE in atopic
274 patients (23).

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276 **LS-CSU transcriptome in CSU shows an abnormal mRNA expression of a**
277 **plethora of genes showing large inflammatory reaction**

278 An unpaired analysis of all available samples was performed, detecting 713
279 differentially expressed identifiers corresponding to 435 unique genes. A
280 second analysis was performed on the 9-paired samples, obtaining a total of
281 700 differentially expressed identifiers corresponding to 433 unique genes
282 (Figure 2A). From these, 549 identifiers (337 unique genes) were coincident
283 (78%) to the first list. A group of 142 genes differentially expressed from the
284 paired analysis was select to be confirmed by qPCR. The selection was done
285 on the basis of the methodological criteria and a careful assessment of their

286 potential involvement in mast cell biology and urticaria. A total of 103 genes
287 were confirmed by qPCR (72.53%) (Figure 2B).

288 Genes differentially expressed confirmed by qPCR resulting from the paired
289 comparison between the LS-CSU and the NLS-CSU and their main biological
290 functions are included in Table 2. These genes codify proteins involved in a
291 variety of biological functions as, e.g., barrier function, intracellular signal
292 function, transcriptional factors cell cycle differentiation, inflammation or
293 coagulation (Figure S3).

294 A functional analysis of the genes differentially expressed was performed with
295 IPA. Seven different functional pathways were identified (comprising genes
296 involved in cellular movement, inflammatory response, hematological system,
297 connective tissue disorders, cellular growth and differentiation, dermatological
298 disease and conditions, cell-to-cell signaling and deoxyribonucleic acid [DNA]
299 replication) (Table S4).

300 The functional networks relating differentially expressed genes are built around
301 IL-1a, IL-6, CCL2 and FOSB. IL-1a and IL-6 are related with genes responsible
302 for proteins involved in the cellular movement, inflammatory response or
303 hematological system. CCL2 and FOSB gene is being related with other genes
304 involved in connective tissue disorders, inflammatory disease and skeletal and
305 muscular disorder (Figure 3).

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307 **Time course analysis of differentially expressed genes from HCs to NLS-** 308 **CSU and LS-CSU**

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310 A total of 3,039 genes were differentially expressed between the skin of HCs
311 and LS-CSU. This list included genes that were confirmed as differentially

expressed in previous comparisons. In order to investigate the behavior of these genes, a time course analysis was conducted using the MaSigPro methodology (13). From the 204 Agilent probes collected at the 3 conditions (healthy skin from HCs, NLS-CSU and LS-CSU), 4 different clusters were considered: 2 with a clear increasing pattern and 2 with a decreasing pattern (Figure 4). These results show that progression of differentially expressed genes is uniformly increased or decreased along the skin worsening until the wheal appearance.

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CSU patients' blood gene expression compared with HCs

There were no differences in blood gene expression between groups, according to the established statistical criteria.

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DISCUSSION

Our study was focused in the genetic signature of a particular subset of patients suffering from severely active CSU. In our series the mean UAS7 was 33.6 (range 16-42) (25–28).

Patients suffering from CSU continuously generate oedematous and evanescent lesions developing all over the skin of the entire body secondary to different triggers systemically driven. Apparently normal or healthy skin in severe CSU patients is genetically different from normal skin in HCs; according with our results an overexpression of genes mainly involved in epidermal terminal differentiation, barrier function, inflammation and dermal repair is shown.

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3 336 Despite CSU is not an a priori epidermal disorder we observed an
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5 337 overexpression of genes involved in terminal epidermal differentiation and
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7 338 barrier function both in LS-CSU versus NL-CSU (21 genes) and NLS-CSU
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9 339 versus skin from HCs (19 genes) comparisons. A differentially profile of genes
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11 340 involved in epidermal homeostasis was observed between both comparisons.
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13 341 E.g. LS-CSU versus NLS-CSU showed involucrin and aquaporin3
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15 342 overexpression, whereas NLS-CSU versus skin from HCs disclosed filaggrin
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17 343 overexpression (Table S5). The influence of dermal inflammation in epidermal
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19 344 homeostasis has been demonstrated in several cutaneous inflammatory
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21 345 disorders, such as atopic dermatitis or psoriasis, but the relevance of this
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23 346 phenomenon in CSU has not been defined. In a recent study conducted in 16
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25 347 Korean patients with CSU/CIU, an increased expression of the gene that
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27 348 codifies the filaggrin was observed (by qPCR and immunohistochemistry).
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29 349 Additionally, a potential relationship between filaggrin gene expression and
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31 350 urticaria activity was detected. (29) The authors found an increased amount of
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33 351 cis-urocanic acid (filaggrin metabolite) in the CSU epidermis and speculate
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35 352 about its pathogenic potential role. Although this study cannot be
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37 353 methodologically compared with our study (e.g. the urticaria activity was based
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39 354 in a different score) (9,30), these results also support an activation of genes
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41 355 involved in epidermal differentiation and barrier function in patients with
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43 356 CSU/CIU. Other genes that we found to be overexpressed in the NLS-CSU
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45 357 codified proteins involved in dermal repair (arginase 1, extracellular matrix
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47 358 protein 1 and matrillin 4), and cytokines that commonly are described in innate
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49 359 and acquired immunity for atopy and contact dermatitis (IL-37, IL-36, CD1a,
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51 360 FCGB) but also involved in epidermal hyperplasia (IL-36) and as anti-
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361 inflammatory (IL-37). NLS-CSU shows as, like in psoriasis and atopic
362 dermatitis, that CSU shows an overall immunological skin involvement.

363 The wheal defines urticaria as the consequence of the activation of the skin
364 mast cells, the activity of the released mediators and the recruited cells. LS-
365 CSU group showed a plethora of genetic information resulting in a very active
366 inflammatory process. The role of some of the proteins codified by these genes
367 is well known. This is the case of genes that codify adhesion molecules (ICAM
368 1, selectin) (31,32), chemokines (CCL18, CCL8, CXCL1) (33), receptors of
369 released mediators [platelet-activating factor receptor (34), IL-4R (35), TNFR
370 (36)], immunoglobulin receptors (Fc fragment of IgG, high affinity I-b, receptor–
371 CD64 and low affinity III-a receptor CD16a) (37), certain interleukins [IL-1B
372 (38,39), IL-6 or TNF alpha-induced protein 6 (40)], the metabolism of lipids
373 (Phospholipase A2, prostaglandin-endoperoxidase synthase 2) (41,42), some
374 dermal repair molecules (Hyaluronan synthase 3) (43), or intracellular
375 transcriptional factors (FOSB) (44). Some of them are a therapeutic target for
376 CSU (e.g. as IL-1B, with relevance in auto inflammatory syndromes).

377 Genes regulating intracellular signals as the regulators of G-protein signalling
378 (RGS) appear interesting. RGS 16 and 1 were overexpressed whereas RGS5
379 (expressed by neutrophils) was under expressed. It has been described the
380 regulation of FcεRI in mast cells through agonist-induced phosphorylation of G
381 protein-coupled receptors by GPCR kinases promoting their desensitization and
382 internalization (45).

383 The overexpression of the genes codifying FcεRI in LS-CSU and NLS-CSU and
384 its further down regulation using omalizumab has been described by expression
385 microarrays considering just the p-value without qPCR confirmation (46). We

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3 386 have also detected an overexpression of genes that codify the FcεRI (FCER1A
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5 387 and FCER1G) in both LS-CSU and NLS-CSU compared to HCs considering the
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7 388 p value (not the adjusted p value), being the log FC good, over 1,2. As it was
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9 389 expected for methodological reasons, the confirmation of FCER1A and
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11 390 FCER1G overexpression by qPCR was not achieved (Table S3).

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14 391 Other differentially expressed genes in the LS-CSU with a pathogenic interest
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16 392 were related to intracellular transcriptional factors (ATF3, EGR1, FOSL1, MYC,
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18 393 NR4A2, NR4A3, CSRNP1), intracellular signals (AKR1B10, DDX, FPR1, MT2A,
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20 394 NAMPT, SLC25A25, STEAP4, TUBB2A), lipid metabolism (LDLR, CH25H),
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22 395 innate immunity (LILRB3, LILRB4, TLR2), metalloproteinase (ADAMTS4), T
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24 396 lymphocytes activation (CD69), growth factor (AREG-amphiregulin, CSF3R) or
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26 397 other chemokynes (CCL2, CCL4, CXCL2).

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29 398 Methodological differences aside, the current results go in agreement with the
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31 399 study of Patel et al. (47). Some common genes were found in their and our
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33 400 study including: SELE, CH25H, CCL2, MT2A, CD69, SRGN, ADAMTS,
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35 401 STEAP4, S100A8, THBS1 and TNFAIP6. Also, some common functional
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37 402 pathways involved in the disease, such as cellular movement, inflammatory
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39 403 response, cell-to-cell signaling, tissue development or the hematologic systems
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41 404 are described.

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44 405 The study of tendencies among conditions (LS-CSU, NLS-CSU and HCs skin)
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46 406 showed a uniform increased or decreased gene progression along the skin
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48 407 worsening from the HCs to the LS-CSU. Conversely, from the genetic point of
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50 408 view no significant differences in gene expression were observed between the
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52 409 blood of the CSU patients and that of HCs.

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3 410 The genes studied accomplished both the experimental defined conditions and
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5 411 the analytical procedures according to high standards. Therefore current
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7 412 genetic profile results provided are robust and and consistent to be considered
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9 413 as a useful tool within the research in CSU. (14) It should be pointed out that
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11 414 the gene signature described here corresponds to patients with severely active
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13 415 CSU, which is based on patients with the worst prognosis and the most difficult
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15 416 management. In such cases, the overall skin acts as a unique immunologic
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17 417 organ. In order to establish phenotype differences based in the CSU activity a
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19 418 comparative study of the transcriptome pattern observed in NLS and LS-CSU
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21 419 between patients with severely and mild active CSU would be useful. In this
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23 420 sense, severely active CSU shows an overall integument involvement.
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25 421 According with this genetic behaviour severely active CSU can be addressed as
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27 422 a disease that involve simultaneously all the body surface area addressed by
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29 423 dynamic trigger factors.
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36 425 **Author contributions**

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38 426 A. Giménez-Arnau: Study design and coordination, inclusion of patients,
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40 427 sampling, analysis and interpretation of the clinical and genetic results,
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42 428 manuscript responsible
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44 429 L. Curto-Barredo: inclusion of patients, sampling, and analysis of the clinical
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46 430 results, manuscript supervision
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48 431 L. Nonell: Bioinformatic analysis, manuscript responsible
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50 432 E. Puigdecanet: Studies by qPCR, manuscript responsible
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52 433 J. Yelamos: Immunologic assessment manuscript supervision
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54 434 R. Gimeno: Immunologic assessment, manuscript supervision
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435 S. Rüberg: Microarray study, manuscript supervision

436 L. Santamaria-Babi: Advisor of the project, manuscript supervision

437 R.M.Pujol: Advisor of the project, manuscript supervision

438

439 **Conflict of interest**

440 There is no conflict of interest to declare by the authors Giménez-Arnau A,

441 Curto-Barredo L, Nonell L, Puigdecanet E, Yelamos J, Gimeno R, Rüberg S,

442 Santamaria-Babi L, Pujol RM.

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Table 1. Genes confirmed by qPCR resulting from the comparison between the NLS-CSU and the skin from the HCs and its main biological function (ordered by logFC)

Gene	Complete name of the gen	logFC	P.Value	adj P Value	Function
SPRR2E	Small proline-rich protein 2E	4,41	0,00009	0,01698	Epidermal differentiation
LCE2D	Late cornified envelope 2D	4,25	0,00002	0,00748	Epidermal differentiation
LCE2C	Late cornified envelope 2C	4,19	0	0,00595	Epidermal differentiation
LCE2A	Late cornified envelope 2A	4,06	0	0,00595	Epidermal differentiation
CDSN	Corneodesmosin	4,05	0,00001	0,00677	Epidermal differentiation
ASPRV1	Skin Aspartic protease	3,86	0,00003	0,0104	Epidermal differentiation
LCE1B	Late cornified envelope 1B	3,54	0,00008	0,01571	Epidermal differentiation
MATN4	Matrilin 4	3,34	0,00002	0,00861	Dermal repair
FLG2	Filaggrin family member 2	3,24	0,00002	0,0086	Epidermal differentiation
NFE2	Nuclear factor (erythroid-derived 2),	3	0	0,00008	Transcriptional factor
SLURP1	Secreted LY6/PLAUR domain containing 1	2,91	0,00017	0,02471	Epidermal differentiation
KRTDAP	Keratinocyte differentiation-associated protein	2,81	0,00004	0,01097	Epidermal differentiation
DSC1	Desmocollin 1	2,77	0,00011	0,01877	Epidermal differentiation
GJB4	Gap junction protein, beta 4, 30.3kDa	2,71	0,00006	0,01341	Epidermal differentiation
ARG1	Arginase 1	2,61	0,00007	0,01536	Epidermal differentiation
THEM5	Thioesterase superfamily member 5	2,6	0,00001	0,00652	Intracellular signal function
CD1A	CD1a molecule	2,5	0,00001	0,00652	Innate immunity
IL37	Interleukin 37	2,41	0,00075	0,04759	Cytokines
FCGBP	Fc fragment of IgG binding protein	2,27	0	0,00193	Immune function /FC receptor
CSTA	Cystatin A (stefin A)	2,1	0,00004	0,01076	Epidermal differentiation
LCE4A	Late cornified envelope 4A	1,85	0,00005	0,01305	Epidermal differentiation
IL36G	Interleukin 36, gamma	1,81	0,00015	0,02354	Cytokines
SPINK5	Serine peptidase inhibitor, Kazal type 5	1,71	0,00001	0,00652	Epidermal differentiation
KRT10	Keratin 10	1,63	0,00014	0,02265	Epidermal differentiation
EPPK1	Epiplakin 1	1,6	0,00072	0,04655	Epidermal differentiation
RDH12	Retinol dehydrogenase 12 (all-trans/9-cis/11-cis)	1,59	0,00026	0,0291	Intracellular signal function
ECM1	Extracellular matrix protein 1	1,47	0,00025	0,02906	Dermal repair
FLG;	Filaggrin	1,43	0,00156	0,06262	Epidermal differentiation
RPTN	Repetin	1,26	0,00002	0,00791	Epidermal differentiation
CD55	CD55 molecule, decay accelerating factor for complement	1,12	0,00051	0,03948	Complement
CISH	Cytokine-inducible SH2-containing protein	-2,63	0	0,00595	Cytokines

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9 HCs, healthy controls; logFC, Logarithmic Fold Change; NLS-CSU, non-lesional skin-chronic spontaneous urticaria; qPCR,
10 quantitative real-time polymerase chain reaction.
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12 *logFC from 1 (that is, >1 or <-1).
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14 **adj P value: adjusted P value.
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Table 2. Genes confirmed by qPCR resulting from the paired comparison between the LS-CSU and the NLS-CSU and its main biological function (ordered by logFC)

Gen	Complete name of the gen	LogF C	P value	adj P values	Function
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	3,93	0,00005	0,01486	Intracellular Transcriptional factor
S100A9	S100 calcium binding protein A9	3,38	0,00001	0,01411	Cell cycle and differentiation.
ADAMTS4	ADAM metalloproteinase with thrombospondin type 1 motif, 4	3,26	0,00003	0,01411	Inflammation -metalloproteinases
IL6	Interleukin 6 (interferon, beta 2)	3,23	0,00005	0,01486	Cytokines
S100A8	S100 calcium binding protein A8	3,17	0,00001	0,01411	Cell cycle and differentiation.
SOCS3	Suppressor of cytokine signaling 3	3,07	0	0,00996	Cytokines-suppressor
SPRR2D	Small proline-rich protein 2D	2,76	0,00011	0,01486	Epidermal differentiation
IL1B	Interleukin 1, beta	2,76	0,00005	0,01486	Cytokines
S100A7	S100 calcium binding protein A7	2,67	0,00234	0,02665	Cell cycle and differentiation.
SPRR2A	Small proline-rich protein 2A	2,63	0,00009	0,01486	Epidermal differentiation
KRT16	Keratin 16	2,56	0,00006	0,01486	Epidermal differentiation
KRT86;KRT81	Keratin 86	2,56	0,00161	0,02265	Epidermal differentiation
CCL2	Chemokine (C-C motif) ligand 2	2,47	0,00001	0,01411	Chemokines
CXCL2	Chemokine (C-X-C motif) ligand 2	2,46	0,00001	0,01411	Chemokines
FPR1	Formyl peptide receptor 1	2,46	0,00014	0,01486	Intracellular signal function
SELE	Selectin	2,43	0,00007	0,01486	Cell adhesion
SERPINB3	Serpin peptidase inhibitor, clade B (ovalbumin), member	2,38	0,00092	0,01857	Coagulation /Inflammation
CCL4L1;CCL4;CCL4L2	Chemokine (C-C motif) ligand 4	2,37	0,00001	0,01411	Chemokines
KRTAP3-1	Keratin associated protein 3-1	2,29	0,00145	0,02188	Epidermal differentiation
ATF3	Activating transcription factor 3 (2 times)	2,29	0,00044	0,01642	Intracellular Transcriptional factor
SPRR2F	Small proline-rich protein 2F	2,24	0,00021	0,0149	Epidermal differentiation
CYR61	Cysteine-rich, angiogenic inducer, 61	2,24	0,00015	0,01486	Cell adhesion
SPRR2C	Small proline-rich protein 2C	2,21	0,00003	0,01411	Epidermal differentiation
CCL18	Chemokine (C-C motif) ligand 18	2,2	0,0051	0,03802	Chemokines
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	2,19	0,00001	0,01411	Chemokines
AREG	Amphiregulin	2,18	0,00027	0,01516	Growth factor
NR4A3	Nuclear receptor subfamily 4, group A, member 3	2,18	0,00094	0,01875	Intracellular Transcriptional factor
FOSL1	FOS-like antigen 1	2,17	0,00004	0,01486	Intracellular Transcriptional factor
ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)	2,15	0	0,01411	Intracellular signal function
KRTAP1-5	Keratin associated protein 1-5	2,1	0,005	0,03762	Epidermal differentiation
PI3	Peptidase inhibitor 3, skin-derived	2,09	0,00018	0,0149	Epidermal differentiation - Antimicrobial peptide

EGR1	Early growth response 1	2,09	0,00009	0,01486	Intracellular Transcriptional factor
IVL	Involucrin	2,08	0,00008	0,01486	Epidermal differentiation
KRTAP1-1	Keratin associated protein 1-1	2,04	0,00403	0,03379	Epidermal differentiation
SPRR1B	Small proline-rich protein 1B	2,04	0,00018	0,0149	Epidermal differentiation
SPRR2G	Small proline-rich protein 2G	2,04	0,00141	0,02154	Epidermal differentiation
HAS3	Hyaluronan synthase 3	2,03	0,00017	0,0149	Dermal repair
SPRR1A	Small proline-rich protein 1A	2	0,00006	0,01486	Epidermal differentiation
SPRR2B	Small proline-rich protein 2B	2	0,00182	0,02402	Epidermal differentiation
KRT6C	Keratin 6C	1,93	0,00032	0,01535	Epidermal differentiation
CD69	CD69 molecule	1,91	0,00002	0,01411	Inflammation - T lymphocytes activation
KRTAP9-4	Keratin associated protein 9-4	1,88	0,00305	0,02969	Epidermal differentiation
FGFBP1	Fibroblast growth factor binding protein 1	1,86	0	0,01411	Dermal repair
TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	1,73	0,00011	0,01486	Cytokines - TNF
KRTAP4-1	Keratin associated protein 4-1	1,72	0,00149	0,02204	Epidermal differentiation
SLC25A25	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25	1,7	0,00014	0,01486	Intracellular signal function
CH25H	Cholesterol 25-hydroxylase hyaluronan synthase 1	1,69	0,00003	0,01411	Lipid metabolism
KRTAP26-1	Keratin associated protein 26-1	1,68	0,00314	0,03016	Epidermal differentiation
CSRNP1	Cysteine-serine-rich nuclear protein 1	1,65	0,00011	0,01486	Intracellular Transcriptional factor
LDLR	Low density lipoprotein receptor	1,64	0,00006	0,01486	Lipid metabolism
ADAMTS8	ADAM metalloproteinase with thrombospondin type 1 motif, 8	1,59	0,00292	0,0291	Inflammation - metalloproteinases
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1,58	0,00003	0,01411	Inflammation -lipids
NAMPT	Nicotinamide phosphoribosyltransferase	1,58	0,00003	0,01411	Intracellular signal function
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1,58	0,00002	0,01411	Intracellular Transcriptional factor
AKR1B10	Aldo-keto reductase family 1, member B10 (aldose reductase)	1,56	0,00002	0,01411	Intracellular signal function
FCGR1C;FCGR1B;58FCGR1A	Fc fragment of IgG, high affinity Ib, receptor (CD64)	1,55	0,00114	0,02	Immune function /FC receptor
NR4A2	Nuclear receptor subfamily 4, group A, member 2	1,51	0,0025	0,02747	Intracellular Transcriptional factor
MT2A	Metallothionein 2A	1,48	0,00249	0,02746	Intracellular signal function
SRGN	Serglycin	1,45	0,00672	0,0439	Intracellular signal function
KRTAP4-7	Keratin associated protein 4-7	1,43	0,00334	0,03088	Epidermal differentiation
DUSP1	Dual specificity phosphatase 1	1,42	0,00082	0,01801	Inflammation /Oxidative stress
C10orf99	Chromosome 10 open reading frame 99	1,4	0,00281	0,02878	Cytokine
TUBB2A	Tubulin, beta 2A class IIa	1,39	0,00008	0,01486	Intracellular signal function
THBS1	Thrombospondin 1	1,38	0,00436	0,03505	Coagulation
SOD2	Superoxide dismutase 2, mitochondrial	1,35	0,00143	0,02173	Inflammation /Oxidative stress
RGS16	Regulator of G-protein signaling 16 (2 times)	1,32	0,00006	0,01486	Intracellular signal function

RGS1	Regulator of G-protein signaling 1	1,32	0,00006	0,01486	Intracellular signal function
CCL8	Chemokine (C-C motif) ligand 8	1,31	0,00022	0,0149	Chemokines
VNN2	Vanin 2	1,3	0,00681	0,04427	Cell adhesion
ICAM1	Intercellular adhesion molecule 1	1,29	0,00039	0,01597	Cell adhesion
SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	1,28	0,00031	0,01525	Growth inhibitor
STEAP4	STEAP family member 4	1,28	0,00018	0,0149	Intracellular signal function
SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	1,27	0,00205	0,02521	Coagulation
TNFRSF10C	Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	1,26	0,00392	0,03343	Cytokines-TNF
PNP	Purine nucleoside phosphorylase	1,26	0,00006	0,01486	Inflammation - T lymphocytes activation
S100A2	S100 calcium binding protein A2	1,24	0,00002	0,01411	Cell cycle and differentiation.
CSF3R	Colony stimulating factor 3 receptor (granulocyte	1,24	0,00208	0,02537	Growth factor
ADAMTS1	ADAM metalloproteinase with thrombospondin type 1 motif, 1	1,24	0,00013	0,01486	Inflammation -metalloproteinases
DDX21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	1,23	0,00033	0,01548	Intracellular signal function
AQP3	Aquaporin 3 (Gill blood group)	1,22	0,00007	0,01486	Epidermal differentiation
CXCR2	Chemokine (C-X-C motif) receptor 2	1,21	0,00035	0,01554	Chemokines
LILRB3	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	1,18	0,00503	0,03773	Innate immunity
PLA2G3	Phospholipase A2, group III	1,17	0,00013	0,01486	Inflammation -lipids
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	1,16	0,00663	0,04357	Cytokines -TNF
PLAUR	Plasminogen activator, urokinase receptor	1,14	0,0013	0,02099	Coagulation
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1)	1,14	0,00037	0,0158	Coagulation
FFAR2	Free fatty acid receptor 2	1,14	0,00115	0,02003	Inflammation -lipids
FCGR3B;FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	1,12	0,00312	0,03003	Immune function /FC receptor
LILRB4	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4	1,12	0,00551	0,03959	Innate immunity
C5AR1	Complement component 5a receptor 1	1,07	0,00171	0,02337	Complement
PTAFR	Platelet-activating factor receptor	1,06	0,0017	0,02324	Coagulation
IRF1	Interferon regulatory factor 1	1	0,002	0,02496	Intracellular Transcriptional factor
IL4R	Interleukin 4 receptor	0,96	0,00092	0,01863	Cytokines
TLR2	Toll-like receptor 2	0,88	0,00299	0,02948	Innate immunity
RGS5	Regulator of G-protein signaling 5	-1,08	0,00211	0,02555	Intracellular signal function
VWF	Von Willebrand factor	-1,17	0,00065	0,0171	Coagulation
LMOD1	Leiomodin 1 (smooth muscle)	-1,17	0,00064	0,0171	Thyroid associated disease
CXCL12	Chemokine (C-X-C motif) ligand 12	-1,18	0,00622	0,04215	Chemokines

PDE5A	Phosphodiesterase 5A, cGMP-specific	-1,18	0,00091	0,01855	Intracellular signal function
VWF	Von Willebrand factor	-1,25	0,00056	0,01689	Coagulation
TIMP3	TIMP metalloproteinase inhibitor 3	-1,33	0,00137	0,02136	Inflammation -metalloproteinases
RHPN2	Rhopilin, Rho GTPase binding protein 2	-1,34	0,01176	0,06032	Intracellular signal function
CCL15-CCL14;CCL14	Chemokine (C-C motif) ligand 14	-1,43	0,00009	0,01486	Chemokines

LogFC, Logarithmic Fold Change; LS-CSU, lesional skin- chronic spontaneous urticaria; NLS-CSU, non-lesional skin- chronic spontaneous urticaria; qPCR, quantitative real-time polymerase chain reaction.

*LogFC from 1 (that is, >1 or <-1).

**adj P value: adjusted P value.

Figure legends

Figure 1. Heat map tissue illustrating (A) gene array of the NLS-CSU patients versus the skin of HCs (376 genes), and (B) qPCR of the NLS-CSU patients confirmed genes versus the skin of HCs (31/39 studied genes, 80%). Sample titled A#-S corresponds to NLS-CSU; sample titled B# corresponds to HCs skin. *Confirmed genes are listed and biological functions represented in different colors.*

Figure 2. Heat map tissue illustrating (A) gene array of the paired samples of LS-CSU patients versus the NLS-CSU (700 genes paired analysis), and (B) qPCR of the LS-CSU patients confirmed genes versus NLS-CSU (103/142 studied genes, 73%). Sample titled A#-S corresponds to NLS-CSU; sample titled A#-W corresponds to LS-CSU. *Confirmed genes are listed and biological functions represented in different colors.*

Figure 3. Functional network according to Ingenuity Pathway Analysis (IPA). IL-1a and IL-6 (A) and CCL2 and FOSB (B) organize the functional networks that relate more differentially expressed genes. (A) Network of genes involved in cellular movement, inflammatory response, hematological system development and function. (B) Network involving connective tissue disorders, inflammatory disease and skeletal and muscular disorder.

Figure 4. Behaviour of the genes differentially expressed from the skin of the HCs to the LS-CSU. From the 204 Agilent probes collected at the 3 conditions

(healthy skin from HCs, NLS-CSU and LS-CSU), 4 different clusters were considered: 2 with a clear increasing pattern and 2 with a decreasing pattern. The progression of differentially expressed genes is uniformly increased or decreased along the skin worsening until the wheal appearance.

For Peer Review

Transcriptome analysis of severely active chronic spontaneous urticaria shows an overall immunological skin involvement

Short title: Transcriptome analysis in active chronic spontaneous urticaria

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ABSTRACT

Background: The knowledge about chronic spontaneous urticaria (CSU) phenotypes is based on its clinical characteristics, associated comorbidities, course of the disease and its response to the available effective drugs. Genotype expression and its further correlation with CSU phenotypes are still unknown. We describe the cutaneous transcriptome of patients suffering a severely active CSU refractory to antihistamine treatment. **Methods:** Through the bioinformatic analysis of the whole Human Genome with Oligo Microarrays and Quantitative real-time polymerase chain reaction (qPCR), relevant genes expressed in non-lesional [NLS-CSU] and lesional skin [LS-CSU] and peripheral blood were identified in 20 patients suffering from severely active CSU and 10 healthy controls (HCs) **Results:** From 39 genes differentially expressed in NLS-CSU when compared with HCs, 31 (79.48%) were confirmed by qPCR corresponding to genes involved in epidermal homeostasis and dermal repair. From the analysis comparing LS-CSU with NLS-CSU, a selection of 142 genes was studied with qPCR, and 103 (72.53%) were confirmed. Differentially expressed genes in the phenomenon of wheal development are involved in a variety of biological functions as, epidermal differentiation, intracellular signal function, transcriptional factors cell cycle differentiation, inflammation or coagulation. Differentially expressed genes that uniformly increase or decrease along the skin worsening until the wheal appearance are shown. **Conclusion:** The skin of CSU patients with a severely active disease shows an overall immunological skin involvement showing a peculiar gene profile.

Key words: chronic spontaneous urticaria; genetics; microarray analysis; quantitative real-time PCR; RNA;transcriptome

Introduction

Chronic spontaneous urticaria (CSU), classically known as chronic idiopathic urticaria (CIU), is a common dermatosis with an estimated prevalence of 0.6 to 1% (1). CSU is defined by spontaneously occurring short-lived and itching wheals, angioedema, or both, for a minimum period of 6 weeks (2,3). An episode of CSU can last longer, even more than five years and more than one CSU episode can be suffered. CSU negatively affects the patient's quality of life and has a considerable impact on direct and indirect health-related costs (4).

The current knowledge about CSU phenotypes is based on its clinical characteristics, associated comorbidities, activity, course and response to the available effective drugs. The CSU phenotype showing the most active disease (highest Urticaria Activity Score [UAS]) with the worse prognosis commonly is refractory to high doses of antihistamines and associates angioedema, concomitant chronic inducible urticaria (CIndU) and/or serum autoreactivity (functional histamine-release autoantibodies) (4).

Mast cell activation and wheal kinetics are partially understood and explain the evanescent nature of hives. However, our knowledge about the biological characteristics of the apparently healthy skin during urticaria outbreak is unknown.

The genotype expression and its further correlation with CSU phenotypes are unknown (5). Functional analysis of gene expression from lesional and non-lesional CSU (LS-CSU and NLS-CSU, respectively) skin may provide new insights into the pathogenesis of hive. We describe the genetic signature of patients suffering severely active CSU refractory to antihistamine treatment (6–8). Our hypothesis is that the entire cutaneous integument provides a peculiar gene expression in severely active CSU.

Methods

Studied population: CSU patients and HCs

Twenty patients suffering from severely active CSU and 10 HCs were prospectively included in the study. Only patients showing severely active CSU (Urticaria Activity Score (UAS) $7 \geq 16$) for at least 3 months were included (9). All patients remained symptomatic despite a treatment with oral antihistamines. A blanching period of previous drugs was guaranteed. HCs were selected from a pool of volunteers without any active cutaneous or systemic disease. Ethical approval by Institut Mar d'Investigacions Mèdiques-IMIM was obtained (EC-2012/4913/I). The study was conducted in accordance with the Declaration of Helsinki and European regulations. All participants provided written informed consent to participate in the study; a signed consent was obtained for pictures reproduction of the skin.

Clinical assessment and interventions

CSU patients were evaluated through a complete clinical history. Complementary tests were performed according to an extended diagnostic program (2) (Table S1).

Three cutaneous biopsies (punch of 4mm) were obtained from the skin: two biopsy specimens from an active wheal or LS-CSU and one from NLS-CSU. Samples were taken from LS-CSU (located on the trunk) with a minimum-maximum evolution time of one-two hours. The NLS-CSU skin biopsy was obtained from an area where neither a wheal nor other skin alterations (e.g. desquamation or pigmentation) were presented within the previous 72 hours.

Biopsies were prepared for routine pathology (hematoxylin-eosin and Giemsa stains), mast-cell and basophil-specific immunohistochemistry (c-Kit and BB1 expression) and transcriptome analysis (quick-freezing and cryopreserved tissue stored at $-80^{\circ}\text{C}.$). Venous blood for gene array was also obtained. The same procedures were applied to HCs.

Human total ribonucleic acid (RNA) was isolated from cryopreserved tissue samples (50 vials) by using Trizol® (Sigma-Aldrich). Human total RNA was isolated from blood samples (30 vials) stabilized in Tempus™ Blood RNA tubes (Applied Biosystems) by using the Tempus™ Spin RNA Isolation Reagen Kit (Applied Biosystems). RNA quality and integrity were determined with Agilent RNA 6000 Nano Kit (Agilent 2100 Bioanalyzer, Agilent Technologies). RNA was quantified by measuring A260nm on the ND-1000 Spectrophotometer (NanoDrop Technologies).

Gene array methodology

Quality control of RNA samples was performed with the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Good quality samples with an RNA Integrity Number (RIN) value >6 , and also some mildly degraded samples with a RIN value between 4 and 6, were processed. For the partially degraded samples, amplification and labeling quality control were performed during the sample process in order to discard any effect on the microarray workflow. Gene expression analysis of different human tissue samples was done using Agilent Whole Human Genome Oligo Microarrays (one-color).

Samples were processed as follows. A linear T7-based amplification step with 100ng of each total ribonucleic acid (RNA) sample was performed. To produce Cy3-labeled complementary RNA (cRNA), the RNA samples were amplified and

labeled using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies) following the manufacturer's protocol. Yields of cRNA and the dye-incorporation rate were measured with the ND-1000 Spectrophotometer (NanoDrop Technologies). For further processing, amplification/labeling as well as hybridization, the control B samples were randomly distributed. The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). Briefly, 600ng Cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight (17 hours, 65°C) to Agilent whole human genome oligo microarrays 8x60K v2 using Agilent's recommended hybridization chamber and oven. Finally, the microarrays were washed once with the Agilent gene expression wash buffer 1 for 1 min at room temperature followed by a second wash with preheated Agilent gene expression wash buffer 2 (37 °C) for 1 min.

Bioinformatic analysis of the gene array

Blood data and tissue data were analyzed separately. Raw data were extracted file by file using R (v 2.15) and package Biobase. After quality control assessment, gene transcripts obtained were background corrected and normalized between arrays with quantile normalization available in Limma package. The noise caused by non-expressed transcripts was avoided doing a selection of those having a standard deviation (SD) above the 20% of SD distribution. A total of 46,973 gene identifiers were selected for both data sets. Linear models for Microarrays (Limma package) (10,11), with a false discovery rate (FDR) (12) multiple comparison correction, were used to detect differentially

expressed transcripts between the conditions specified in Figure S1. Linear models were controlled by age. Variable sex was studied but no differences between groups were presented, so it was not included in the model. An adjusted P value of <0.05 and an absolute Logarithmic Fold Change (logFC) from 1 (that is, $\log FC > 1$ or < -1) were considered the significant thresholds to select final significant transcripts.

Functional analysis was performed as a first step with the Ingenuity Pathway Analysis (IPA) (<http://www.ingenuity.com>). All lists were loaded and a core analysis performed.

In order to study of tendencies among conditions (LS-CSU, NLS-CSU and HCs skin) a regression approach to find clusters of genes with similar expression profiles was performed (maSigPro package) (13).

In addition to our data, public data under accession number GSE57178 (14) (Gene Expression Omnibus [GEO]), based on the Affymetrix Human Gene 1.0 ST array, were downloaded and analyzed with the same methodology using the aroma.affymetrix (15) and Limma packages.

Complete gene expression profile

The complete microarray data obtained from the blood and the skin of the CSU patients and HCs is located in the GEO under the accession number GSE72542 (14).

Quantitative real-time polymerase chain reaction (qPCR)

Attending the results from the microarray comparative analysis, a set of genes was selected to be confirmed by qPCR. Gene selection was based on technical

aspects and also on a literature research gen by gen about their involvement in mast cell biology, wheal pathogenesis, urticaria or allergy. These genes were studied (blood and skin tissue), by microarray technique, and in an independent set of 19 skin samples (3 HCs skin, 6 NLS-CSU and 10 LS-CSU). These samples presented a RIN value <4 , consequently, were not suitable for microarray analysis but were valid for qPCR. As a result, all patients' skin and wheal paired samples were available for qPCR analysis.

For qPCR validation, the same RNA material used for microarrays was processed. Therefore, RNA quality analysis for all the samples was performed before qPCR analysis. Afterwards, complementary deoxyribonucleic acid (cDNA) was reversely transcribed from 2000ng of total RNA using the SuperScript VILO cDNA Synthesis Kit (Life Technologies), as described by the manufacturer. TaqMan qPCR reactions were performed on cDNA samples using TaqMan Universal PCR Master Mix II and on the Taqman Low Density Array (TLDA) platform (Life Technologies), according to the manufacturer's instructions. Fluorescence was detected in an ABI PRISM 7900 HT system (Life Technologies).

The used qPCR platform was the TLDA in a 64-assay format (2 samples per array were interrogated for the expression of 64 genes in triplicate). Four different designs were performed according to the genes selected in each comparison. Specifically, each TLDA design contained a maximum of 57 selected genes together with 7 housekeeping genes (18S, GUSB, B2M, TBP, SDHA, RPLP0, PGK1) used as endogenous controls. This housekeeping genes set was selected according to both our microarray results and the already available published studies with qPCR done in skin samples (16–20).

qPCR data analysis was performed using the geNorm method for the selection of the best housekeeping genes (21), and the $2^{-\Delta\Delta Ct}$ method (22) was applied. Student's t-tests (paired and unpaired) were applied and genes were considered to be differentially expressed when P value was <0.05. The confirmed genes by qPCR of the tissue and the blood were studied according to the comparisons included in Figure S1.

RESULTS

Characteristics of the studied population

The clinical features for each patient are included in Table S2. Fourteen women and 6 men were included (mean age±SD of 51.5±15.7 years [range 20-81 years]). The HCs (N=10) ratio female:male was 1:1 and the mean age was 27.5±5.4 years (range 21-37 years). The disease mean duration was 4.9±9 SD years (range 3 months-40 years). Mean UAS7 was 33.6 (range 16-42). All the patients were symptomatic in spite of high doses of second generation H1-antihistamines (2) and were suitable to be treated with e.g. anti-immunoglobulin E (IgE) therapy or immunomodulation. 35% (7/20) of them were unresponsive to cyclosporine A. Attending other clinical characteristics as markers of a bad prognosis for CSU, 20% (4/20) of the patients suffered intermittent angioedema crisis (4). Twenty five per cent (5/20) showed concomitant inducible urticaria (symptomatic dermographism or delayed pressure urticaria) (4). Fifty five percent (11/20) of the patients showed a positive Autologous Serum Skin Test (ASST) and 55% (11/20) had an autoimmune disease associated (4). The autoimmune thyroid diseases were the most frequent concomitant conditions, affecting 25% (5/20) of the CSU patients (4). Other autoimmune diseases

reported included vitiligo, Sjögren syndrome and systemic lupus erythematosus. D-dimer serum level was high in 54% (7/13). The mean value of the total serum IgE was 277.5 ± 357.8 UI/ml (range 22-1470 UI/ml), slightly higher compared to the normal values detected in HCs (60.7 ± 54.4 UI/ml).

Histopathological findings were compatible with a well-developed wheal. The epidermis always showed a normal appearance. Oedema was present in upper and mid dermis. A characteristic both superficial and deep perivascular/interstitial inflammatory infiltrate with lymphocytes, eosinophils and neutrophils without vascular damage, was observed in all cases (Figure S2). A mild to moderate increase of mast cell numbers was also observed. No morphological differences were found between the wheal samples studied in terms of edema intensity, location of the inflammatory infiltrate and predominant type of cells involved.

NLS-CSU transcriptome in CSU shows an abnormal mRNA expression of genes involved epidermal homeostasis

Based on the statistical criteria defined previously, 376 identifiers corresponding to 299 unique genes were differentially expressed (Figure 1A). Thirty-nine genes were selected for validation using qPCR; from those, 31 were confirmed (79.48%) (Figure 1B). Table 1 includes the genes confirmed by qPCR resulting from the comparison between the NLS-CSU patients and the skin from HCs. The up-regulated genes are involved in epidermal terminal differentiation, inflammation and dermal repair. A total of 19 out of the 31 genes identified, over and under expressed, codify epidermal proteins responsible for epidermal homeostasis. The analysis showed the overexpression of genes as SPINK5,

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FLG, ASPRV1, IL-37, FCGBP, or CD1A, previously reported to be overexpressed in ichthyosis and atopic dermatitis (23). In NLS-CSU, overexpression of genes involved in the dermal repair (such as arginase 1, extracellular matrix protein 1 or matrillin) was observed. Matrillin encodes a member of the von Willebrand factor A domain-containing protein family and has been involved in wound healing. Genes codifying IL-37 and IL-36 were also overexpressed. IL-37 is involved in the communication between innate and adaptive immunity (elevated in atopic dermatitis) acting as anti-inflammatory (24) whereas IL-36 is member of the IL-1 cytokine family and is secreted by keratinocytes e.g. in contact dermatitis and is involved in epidermal hyperplasia. CD1A gene codifies a protein shown by the Langerhans cells being involved in the innate immunity and FCGBP gene codifies the Fc fragment of immunoglobulin G (IgG) binding protein capable to bind e.g. IgE in atopic patients (23).

LS-CSU transcriptome in CSU shows an abnormal mRNA expression of a plethora of genes showing large inflammatory reaction

An unpaired analysis of all available samples was performed, detecting 713 differentially expressed identifiers corresponding to 435 unique genes. A second analysis was performed on the 9-paired samples, obtaining a total of 700 differentially expressed identifiers corresponding to 433 unique genes (Figure 2A). From these, 549 identifiers (337 unique genes) were coincident (78%) to the first list. A group of 142 genes differentially expressed from the paired analysis was select to be confirmed by qPCR. The selection was done on the basis of the methodological criteria and a careful assessment of their

potential involvement in mast cell biology and urticaria. A total of 103 genes were confirmed by qPCR (72.53%) (Figure 2B).

Genes differentially expressed confirmed by qPCR resulting from the paired comparison between the LS-CSU and the NLS-CSU and their main biological functions are included in Table 2. These genes codify proteins involved in a variety of biological functions as, e.g., barrier function, intracellular signal function, transcriptional factors cell cycle differentiation, inflammation or coagulation (Figure S3).

A functional analysis of the genes differentially expressed was performed with IPA. Seven different functional pathways were identified (comprising genes involved in cellular movement, inflammatory response, hematological system, connective tissue disorders, cellular growth and differentiation, dermatological disease and conditions, cell-to-cell signaling and deoxyribonucleic acid [DNA] replication) (Table S4).

The functional networks relating differentially expressed genes are built around IL-1a, IL-6, CCL2 and FOSB. IL-1a and IL-6 are related with genes responsible for proteins involved in the cellular movement, inflammatory response or hematological system. CCL2 and FOSB gene is being related with other genes involved in connective tissue disorders, inflammatory disease and skeletal and muscular disorder (Figure 3).

Time course analysis of differentially expressed genes from HCs to NLS-CSU and LS-CSU

A total of 3,039 genes were differentially expressed between the skin of HCs and LS-CSU. This list included genes that were confirmed as differentially

expressed in previous comparisons. In order to investigate the behavior of these genes, a time course analysis was conducted using the MaSigPro methodology (13). From the 204 Agilent probes collected at the 3 conditions (healthy skin from HCs, NLS-CSU and LS-CSU), 4 different clusters were considered: 2 with a clear increasing pattern and 2 with a decreasing pattern (Figure 4). These results show that progression of differentially expressed genes is uniformly increased or decreased along the skin worsening until the wheal appearance.

CSU patients' blood gene expression compared with HCs

There were no differences in blood gene expression between groups, according to the established statistical criteria.

DISCUSSION

Our study was focused in the genetic signature of a particular subset of patients suffering from severely active CSU. In our series the mean UAS7 was 33.6 (range 16-42) (25–28).

Patients suffering from CSU continuously generate oedematous and evanescent lesions developing all over the skin of the entire body secondary to different triggers systemically driven. Apparently normal or healthy skin in severe CSU patients is genetically different from normal skin in HCs; according with our results an overexpression of genes mainly involved in epidermal terminal differentiation, barrier function, inflammation and dermal repair is shown.

Despite CSU is not an a priori epidermal disorder we observed an overexpression of genes involved in terminal epidermal differentiation and barrier function both in LS-CSU versus NL-CSU (21 genes) and NLS-CSU versus skin from HCs (19 genes) comparisons. A differentially profile of genes involved in epidermal homeostasis was observed between both comparisons. E.g. LS-CSU versus NLS-CSU showed involucrin and aquaporin3 overexpression, whereas NLS-CSU versus skin from HCs disclosed filaggrin overexpression (Table S5). The influence of dermal inflammation in epidermal homeostasis has been demonstrated in several cutaneous inflammatory disorders, such as atopic dermatitis or psoriasis, but the relevance of this phenomenon in CSU has not been defined. In a recent study conducted in 16 Korean patients with CSU/CIU, an increased expression of the gene that codifies the filaggrin was observed (by qPCR and immunohistochemistry). Additionally, a potential relationship between filaggrin gene expression and urticaria activity was detected. (29) The authors found an increased amount of cis-urocanic acid (filaggrin metabolite) in the CSU epidermis and speculate about its pathogenic potential role. Although this study cannot be methodologically compared with our study (e.g. the urticaria activity was based in a different score) (9,30), these results also support an activation of genes involved in epidermal differentiation and barrier function in patients with CSU/CIU. Other genes that we found to be overexpressed in the NLS-CSU codified proteins involved in dermal repair (arginase 1, extracellular matrix protein 1 and matrillin 4), and cytokines that commonly are described in innate and acquired immunity for atopy and contact dermatitis (IL-37, IL-36, CD1a, FCGB) but also involved in epidermal hyperplasia (IL-36) and as anti-

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inflammatory (IL-37). NLS-CSU shows as, like in psoriasis and atopic dermatitis, that CSU shows an overall immunological skin involvement.

The wheal defines urticaria as the consequence of the activation of the skin mast cells, the activity of the released mediators and the recruited cells. LS-CSU group showed a plethora of genetic information resulting in a very active inflammatory process. The role of some of the proteins codified by these genes is well known. This is the case of genes that codify adhesion molecules (ICAM 1, selectin) (31,32), chemokines (CCL18, CCL8, CXCL1) (33), receptors of released mediators [platelet-activating factor receptor (34), IL-4R (35), TNFR (36)], immunoglobulin receptors (Fc fragment of IgG, high affinity I-b, receptor-CD64 and low affinity III-a receptor CD16a) (37), certain interleukins [IL-1B (38,39), IL-6 or TNF alpha-induced protein 6 (40)], the metabolism of lipids (Phospholipase A2, prostaglandin-endoperoxidase synthase 2) (41,42), some dermal repair molecules (Hyaluronan synthase 3) (43), or intracellular transcriptional factors (FOSB) (44). Some of them are a therapeutic target for CSU (e.g. as IL-1B, with relevance in auto inflammatory syndromes).

Genes regulating intracellular signals as the regulators of G-protein signalling (RGS) appear interesting. RGS 16 and 1 were overexpressed whereas RGS5 (expressed by neutrophils) was under expressed. It has been described the regulation of FcεRI in mast cells through agonist-induced phosphorylation of G protein-coupled receptors by GPCR kinases promoting their desensitization and internalization (45).

The overexpression of the genes codifying FcεRI in LS-CSU and NLS-CSU and its further down regulation using omalizumab has been described by expression microarrays considering just the p-value without qPCR confirmation (46). We

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3 have also detected an overexpression of genes that codify the FcεRI (FCER1A
4 and FCER1G) in both LS-CSU and NLS-CSU compared to HCs considering the
5 p value (not the adjusted p value), being the log FC good, over 1,2. As it was
6 expected for methodological reasons, the confirmation of FCER1A and
7 FCER1G overexpression by qPCR was not achieved (Table S3).
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12 Other differentially expressed genes in the LS-CSU with a pathogenic interest
13 were related to intracellular transcriptional factors (ATF3, EGR1, FOSL1, MYC,
14 NR4A2, NR4A3, CSRNP1), intracellular signals (AKR1B10, DDX, FPR1, MT2A,
15 NAMPT, SLC25A25, STEAP4, TUBB2A), lipid metabolism (LDLR, CH25H),
16 innate immunity (LILRB3, LILRB4, TLR2), metalloproteinase (ADAMTS4), T
17 lymphocytes activation (CD69), growth factor (AREG-amphiregulin, CSF3R) or
18 other chemokynes (CCL2, CCL4, CXCL2).
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22 Methodological differences aside, the current results go in agreement with the
23 study of Patel et al. (47). Some common genes were found in their and our
24 study including: SELE, CH25H, CCL2, MT2A, CD69, SRGN, ADAMTS,
25 STEAP4, S100A8, THBS1 and TNFAIP6. Also, some common functional
26 pathways involved in the disease, such as cellular movement, inflammatory
27 response, cell-to-cell signaling, tissue development or the hematologic systems
28 are described.
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32 The study of tendencies among conditions (LS-CSU, NLS-CSU and HCs skin)
33 showed a uniform increased or decreased gene progression along the skin
34 worsening from the HCs to the LS-CSU. Conversely, from the genetic point of
35 view no significant differences in gene expression were observed between the
36 blood of the CSU patients and that of HCs.
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The genes studied accomplished both the experimental defined conditions and the analytical procedures according to high standards. Therefore current genetic profile results provided are robust and and consistent to be considered as a useful tool within the research in CSU. (14) It should be pointed out that the gene signature described here corresponds to patients with severely active CSU, which is based on patients with the worst prognosis and the most difficult management. In such cases, the overall skin acts as a unique immunologic organ. In order to phenotype the patients according with the CSU activity a comparative study of the transcriptome pattern in NLS and LS-CSU with patients suffering mild CSU would be useful. In this sense, severely active CSU shows an overall integument involvement. According with this genetic behaviour severely active CSU can be addressed as a disease that involve simultaneously all the body surface area addressed by dynamic trigger factors.

Author contributions

- A. Giménez-Arnau: Study design and coordination, inclusion of patients, sampling, analysis and interpretation of the clinical and genetic results, manuscript responsible
- L. Curto-Barredo: inclusion of patients, sampling, and analysis of the clinical results, manuscript supervision
- L. Nonell: Bioinformatic analysis, manuscript responsible
- E. Puigdecanet: Studies by qPCR, manuscript responsible
- J. Yelamos: Immunologic assessment manuscript supervision
- R. Gimeno: Immunologic assessment, manuscript supervision
- S. Rüberg: Microarray study, manuscript supervision

L. Santamaria-Babi: Advisor of the project, manuscript supervision

R.M.Pujol: Advisor of the project, manuscript supervision

Conflict of interest

There is no conflict of interest to declare by the authors Giménez-Arnau A, Curto-Barredo L, Nonell L, Puigdecenet E, Yelamos J, Gimeno R, Rüberg S, Santamaria-Babi L, Pujol RM.

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Table 1. Genes confirmed by qPCR resulting from the comparison between the NLS-CSU and the skin from the HCs and its main biological function (ordered by logFC)

Gene	Complete name of the gen	logFC	P.Value	adj P Value	Function
SPRR2E	Small proline-rich protein 2E	4,41	0,00009	0,01698	Epidermal differentiation
LCE2D	Late cornified envelope 2D	4,25	0,00002	0,00748	Epidermal differentiation
LCE2C	Late cornified envelope 2C	4,19	0	0,00595	Epidermal differentiation
LCE2A	Late cornified envelope 2A	4,06	0	0,00595	Epidermal differentiation
CDSN	Corneodesmosin	4,05	0,00001	0,00677	Epidermal differentiation
ASPRV1	Skin Aspartic protease	3,86	0,00003	0,0104	Epidermal differentiation
LCE1B	Late cornified envelope 1B	3,54	0,00008	0,01571	Epidermal differentiation
MATN4	Matrilin 4	3,34	0,00002	0,00861	Dermal repair
FLG2	Filaggrin family member 2	3,24	0,00002	0,0086	Epidermal differentiation
NFE2	Nuclear factor (erythroid-derived 2),	3	0	0,00008	Transcriptional factor
SLURP1	Secreted LY6/PLAUR domain containing 1	2,91	0,00017	0,02471	Epidermal differentiation
KRTDAP	Keratinocyte differentiation-associated protein	2,81	0,00004	0,01097	Epidermal differentiation
DSC1	Desmocollin 1	2,77	0,00011	0,01877	Epidermal differentiation
GJB4	Gap junction protein, beta 4, 30.3kDa	2,71	0,00006	0,01341	Epidermal differentiation
ARG1	Arginase 1	2,61	0,00007	0,01536	Epidermal differentiation
THEM5	Thioesterase superfamily member 5	2,6	0,00001	0,00652	Intracellular signal function
CD1A	CD1a molecule	2,5	0,00001	0,00652	Innate immunity
IL37	Interleukin 37	2,41	0,00075	0,04759	Cytokines
FCGBP	Fc fragment of IgG binding protein	2,27	0	0,00193	Immune function /FC receptor
CSTA	Cystatin A (stefin A)	2,1	0,00004	0,01076	Epidermal differentiation
LCE4A	Late cornified envelope 4A	1,85	0,00005	0,01305	Epidermal differentiation
IL36G	Interleukin 36, gamma	1,81	0,00015	0,02354	Cytokines
SPINK5	Serine peptidase inhibitor, Kazal type 5	1,71	0,00001	0,00652	Epidermal differentiation
KRT10	Keratin 10	1,63	0,00014	0,02265	Epidermal differentiation
EPPK1	Epiplakin 1	1,6	0,00072	0,04655	Epidermal differentiation
RDH12	Retinol dehydrogenase 12 (all-trans/9-cis/11-cis)	1,59	0,00026	0,0291	Intracellular signal function
ECM1	Extracellular matrix protein 1	1,47	0,00025	0,02906	Dermal repair
FLG;	Filaggrin	1,43	0,00156	0,06262	Epidermal differentiation
RPTN	Repetin	1,26	0,00002	0,00791	Epidermal differentiation
CD55	CD55 molecule, decay accelerating factor for complement	1,12	0,00051	0,03948	Complement
CISH	Cytokine-inducible SH2-containing protein	-2,63	0	0,00595	Cytokines

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9 HCs, healthy controls; logFC, Logarithmic Fold Change; NLS-CSU, non-lesional skin-chronic spontaneous urticaria; qPCR,
10 quantitative real-time polymerase chain reaction.
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12 *logFC from 1 (that is, >1 or <-1).
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14 **adj P value: adjusted P value.
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Table 2. Genes confirmed by qPCR resulting from the paired comparison between the LS-CSU and the NLS-CSU and its main biological function (ordered by logFC)

Gen	Complete name of the gen	LogF C	P value	adj P values	Function
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	3,93	0,00005	0,01486	Intracellular Transcriptional factor
S100A9	S100 calcium binding protein A9	3,38	0,00001	0,01411	Cell cycle and differentiation.
ADAMTS4	ADAM metalloproteinase with thrombospondin type 1 motif, 4	3,26	0,00003	0,01411	Inflammation -metalloproteinases
IL6	Interleukin 6 (interferon, beta 2)	3,23	0,00005	0,01486	Cytokines
S100A8	S100 calcium binding protein A8	3,17	0,00001	0,01411	Cell cycle and differentiation.
SOCS3	Suppressor of cytokine signaling 3	3,07	0	0,00996	Cytokines-suppressor
SPRR2D	Small proline-rich protein 2D	2,76	0,00011	0,01486	Epidermal differentiation
IL1B	Interleukin 1, beta	2,76	0,00005	0,01486	Cytokines
S100A7	S100 calcium binding protein A7	2,67	0,00234	0,02665	Cell cycle and differentiation.
SPRR2A	Small proline-rich protein 2A	2,63	0,00009	0,01486	Epidermal differentiation
KRT16	Keratin 16	2,56	0,00006	0,01486	Epidermal differentiation
KRT86;KRT81	Keratin 86	2,56	0,00161	0,02265	Epidermal differentiation
CCL2	Chemokine (C-C motif) ligand 2	2,47	0,00001	0,01411	Chemokines
CXCL2	Chemokine (C-X-C motif) ligand 2	2,46	0,00001	0,01411	Chemokines
FPR1	Formyl peptide receptor 1	2,46	0,00014	0,01486	Intracellular signal function
SELE	Selectin	2,43	0,00007	0,01486	Cell adhesion
SERPINB3	Serpin peptidase inhibitor, clade B (ovalbumin), member	2,38	0,00092	0,01857	Coagulation /Inflammation
CCL4L1;CCL4;CCL4L2	Chemokine (C-C motif) ligand 4	2,37	0,00001	0,01411	Chemokines
KRTAP3-1	Keratin associated protein 3-1	2,29	0,00145	0,02188	Epidermal differentiation
ATF3	Activating transcription factor 3 (2 times)	2,29	0,00044	0,01642	Intracellular Transcriptional factor
SPRR2F	Small proline-rich protein 2F	2,24	0,00021	0,0149	Epidermal differentiation
CYR61	Cysteine-rich, angiogenic inducer, 61	2,24	0,00015	0,01486	Cell adhesion
SPRR2C	Small proline-rich protein 2C	2,21	0,00003	0,01411	Epidermal differentiation
CCL18	Chemokine (C-C motif) ligand 18	2,2	0,0051	0,03802	Chemokines
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	2,19	0,00001	0,01411	Chemokines
AREG	Amphiregulin	2,18	0,00027	0,01516	Growth factor
NR4A3	Nuclear receptor subfamily 4, group A, member 3	2,18	0,00094	0,01875	Intracellular Transcriptional factor
FOSL1	FOS-like antigen 1	2,17	0,00004	0,01486	Intracellular Transcriptional factor
ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)	2,15	0	0,01411	Intracellular signal function
KRTAP1-5	Keratin associated protein 1-5	2,1	0,005	0,03762	Epidermal differentiation
PI3	Peptidase inhibitor 3, skin-derived	2,09	0,00018	0,0149	Epidermal differentiation - Antimicrobial peptide

EGR1	Early growth response 1	2,09	0,00009	0,01486	Intracellular Transcriptional factor
IVL	Involucrin	2,08	0,00008	0,01486	Epidermal differentiation
KRTAP1-1	Keratin associated protein 1-1	2,04	0,00403	0,03379	Epidermal differentiation
SPRR1B	Small proline-rich protein 1B	2,04	0,00018	0,0149	Epidermal differentiation
SPRR2G	Small proline-rich protein 2G	2,04	0,00141	0,02154	Epidermal differentiation
HAS3	Hyaluronan synthase 3	2,03	0,00017	0,0149	Dermal repair
SPRR1A	Small proline-rich protein 1A	2	0,00006	0,01486	Epidermal differentiation
SPRR2B	Small proline-rich protein 2B	2	0,00182	0,02402	Epidermal differentiation
KRT6C	Keratin 6C	1,93	0,00032	0,01535	Epidermal differentiation
CD69	CD69 molecule	1,91	0,00002	0,01411	Inflammation - T lymphocytes activation
KRTAP9-4	Keratin associated protein 9-4	1,88	0,00305	0,02969	Epidermal differentiation
FGFBP1	Fibroblast growth factor binding protein 1	1,86	0	0,01411	Dermal repair
TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	1,73	0,00011	0,01486	Cytokines - TNF
KRTAP4-1	Keratin associated protein 4-1	1,72	0,00149	0,02204	Epidermal differentiation
SLC25A25	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25	1,7	0,00014	0,01486	Intracellular signal function
CH25H	Cholesterol 25-hydroxylase hyaluronan synthase 1	1,69	0,00003	0,01411	Lipid metabolism
KRTAP26-1	Keratin associated protein 26-1	1,68	0,00314	0,03016	Epidermal differentiation
CSRNP1	Cysteine-serine-rich nuclear protein 1	1,65	0,00011	0,01486	Intracellular Transcriptional factor
LDLR	Low density lipoprotein receptor	1,64	0,00006	0,01486	Lipid metabolism
ADAMTS8	ADAM metalloproteinase with thrombospondin type 1 motif, 8	1,59	0,00292	0,0291	Inflammation - metalloproteinases
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1,58	0,00003	0,01411	Inflammation -lipids
NAMPT	Nicotinamide phosphoribosyltransferase	1,58	0,00003	0,01411	Intracellular signal function
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1,58	0,00002	0,01411	Intracellular Transcriptional factor
AKR1B10	Aldo-keto reductase family 1, member B10 (aldose reductase)	1,56	0,00002	0,01411	Intracellular signal function
FCGR1C;FCGR1B;58FCGR1A	Fc fragment of IgG, high affinity 1b, receptor (CD64)	1,55	0,00114	0,02	Immune function /FC receptor
NR4A2	Nuclear receptor subfamily 4, group A, member 2	1,51	0,0025	0,02747	Intracellular Transcriptional factor
MT2A	Metallothionein 2A	1,48	0,00249	0,02746	Intracellular signal function
SRGN	Serpin	1,45	0,00672	0,0439	Intracellular signal function
KRTAP4-7	Keratin associated protein 4-7	1,43	0,00334	0,03088	Epidermal differentiation
DUSP1	Dual specificity phosphatase 1	1,42	0,00082	0,01801	Inflammation /Oxidative stress
C10orf99	Chromosome 10 open reading frame 99	1,4	0,00281	0,02878	Cytokine
TUBB2A	Tubulin, beta 2A class IIa	1,39	0,00008	0,01486	Intracellular signal function
THBS1	Thrombospondin 1	1,38	0,00436	0,03505	Coagulation
SOD2	Superoxide dismutase 2, mitochondrial	1,35	0,00143	0,02173	Inflammation /Oxidative stress
RGS16	Regulator of G-protein signaling 16 (2 times)	1,32	0,00006	0,01486	Intracellular signal function

RGS1	Regulator of G-protein signaling 1	1,32	0,00006	0,01486	Intracellular signal function
CCL8	Chemokine (C-C motif) ligand 8	1,31	0,00022	0,0149	Chemokines
VNN2	Vanin 2	1,3	0,00681	0,04427	Cell adhesion
ICAM1	Intercellular adhesion molecule 1	1,29	0,00039	0,01597	Cell adhesion
SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	1,28	0,00031	0,01525	Growth inhibitor
STEAP4	STEAP family member 4	1,28	0,00018	0,0149	Intracellular signal function
SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	1,27	0,00205	0,02521	Coagulation
TNFRSF10C	Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	1,26	0,00392	0,03343	Cytokines-TNF
PNP	Purine nucleoside phosphorylase	1,26	0,00006	0,01486	Inflammation - T lymphocytes activation
S100A2	S100 calcium binding protein A2	1,24	0,00002	0,01411	Cell cycle and differentiation.
CSF3R	Colony stimulating factor 3 receptor (granulocyte	1,24	0,00208	0,02537	Growth factor
ADAMTS1	ADAM metalloproteinase with thrombospondin type 1 motif, 1	1,24	0,00013	0,01486	Inflammation -metalloproteinases
DDX21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	1,23	0,00033	0,01548	Intracellular signal function
AQP3	Aquaporin 3 (Gill blood group)	1,22	0,00007	0,01486	Epidermal differentiation
CXCR2	Chemokine (C-X-C motif) receptor 2	1,21	0,00035	0,01554	Chemokines
LILRB3	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	1,18	0,00503	0,03773	Innate immunity
PLA2G3	Phospholipase A2, group III	1,17	0,00013	0,01486	Inflammation -lipids
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	1,16	0,00663	0,04357	Cytokines -TNF
PLAUR	Plasminogen activator, urokinase receptor	1,14	0,0013	0,02099	Coagulation
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1)	1,14	0,00037	0,0158	Coagulation
FFAR2	Free fatty acid receptor 2	1,14	0,00115	0,02003	Inflammation -lipids
FCGR3B;FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	1,12	0,00312	0,03003	Immune function /FC receptor
LILRB4	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4	1,12	0,00551	0,03959	Innate immunity
C5AR1	Complement component 5a receptor 1	1,07	0,00171	0,02337	Complement
PTAFR	Platelet-activating factor receptor	1,06	0,0017	0,02324	Coagulation
IRF1	Interferon regulatory factor 1	1	0,002	0,02496	Intracellular Transcriptional factor
IL4R	Interleukin 4 receptor	0,96	0,00092	0,01863	Cytokines
TLR2	Toll-like receptor 2	0,88	0,00299	0,02948	Innate immunity
RGS5	Regulator of G-protein signaling 5	-1,08	0,00211	0,02555	Intracellular signal function
VWF	Von Willebrand factor	-1,17	0,00065	0,0171	Coagulation
LMOD1	Leiomodin 1 (smooth muscle)	-1,17	0,00064	0,0171	Thyroid associated disease
CXCL12	Chemokine (C-X-C motif) ligand 12	-1,18	0,00622	0,04215	Chemokines

PDE5A	Phosphodiesterase 5A, cGMP-specific	-1,18	0,00091	0,01855	Intracellular signal function
VWF	Von Willebrand factor	-1,25	0,00056	0,01689	Coagulation
TIMP3	TIMP metalloproteinase inhibitor 3	-1,33	0,00137	0,02136	Inflammation -metalloproteinases
RHPN2	Rhopilin, Rho GTPase binding protein 2	-1,34	0,01176	0,06032	Intracellular signal function
CCL15-CCL14;CCL14	Chemokine (C-C motif) ligand 14	-1,43	0,00009	0,01486	Chemokines

LogFC, Logarithmic Fold Change; LS-CSU, lesional skin- chronic spontaneous urticaria; NLS-CSU, non-lesional skin- chronic spontaneous urticaria; qPCR, quantitative real-time polymerase chain reaction.

*LogFC from 1 (that is, >1 or <-1).

**adj P value: adjusted P value.

Figure legends

Figure 1. Heat map tissue illustrating (A) gene array of the NLS-CSU patients versus the skin of HCs (376 genes), and (B) qPCR of the NLS-CSU patients confirmed genes versus the skin of HCs (31/39 studied genes, 80%). Sample titled A#-S corresponds to NLS-CSU; sample titled B# corresponds to HCs skin. *Confirmed genes are listed and biological functions represented in different colors.*

Figure 2. Heat map tissue illustrating (A) gene array of the paired samples of LS-CSU patients versus the NLS-CSU (700 genes paired analysis), and (B) qPCR of the LS-CSU patients confirmed genes versus NLS-CSU (103/142 studied genes, 73%). Sample titled A#-S corresponds to NLS-CSU; sample titled A#-W corresponds to LS-CSU. *Confirmed genes are listed and biological functions represented in different colors.*

Figure 3. Functional network according to Ingenuity Pathway Analysis (IPA). IL-1a and IL-6 (A) and CCL2 and FOSB (B) organize the functional networks that relate more differentially expressed genes. (A) Network of genes involved in cellular movement, inflammatory response, hematological system development and function. (B) Network involving connective tissue disorders, inflammatory disease and skeletal and muscular disorder.

Figure 4. Behaviour of the genes differentially expressed from the skin of the HCs to the LS-CSU. From the 204 Agilent probes collected at the 3 conditions

(healthy skin from HCs, NLS-CSU and LS-CSU), 4 different clusters were considered: 2 with a clear increasing pattern and 2 with a decreasing pattern. The progression of differentially expressed genes is uniformly increased or decreased along the skin worsening until the wheal appearance.

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396x204mm (150 x 150 DPI)

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399x193mm (150 x 150 DPI)

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255x139mm (150 x 150 DPI)

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177x177mm (96 x 96 DPI)

Supplementary material

(Five supplementary Tables and three supplementary figures are provided)

Tables

Table S1. Clinical, laboratory, pathological and treatment parameters analyzed

Clinical parameters	Laboratory parameters	Treatment parameters	Pathological parameters
<ul style="list-style-type: none">• Age• Gender• Disease duration• Previous episodes of CSU• Angioedema associated• UAS• UAS7• Atopy• Thyroid dysfunction• Autoimmune diseases• Inducible Urticaria• Dermographic urticaria• Delayed Pressure urticaria• ASST• Wheal and erythema diameter of ASST• APST• Wheal and erythema diameter of APST	<ul style="list-style-type: none">• CPR• ESR• IgE• D-Dimer• Anti-thyroid antibodies• Platelet count• PDW• MPV• P-LCR• Eosinophil count• Basophil count• CD63 expression assay• Helicobacter pylori breath test	<ul style="list-style-type: none">• Refractory to antihistamines at licensed doses• Refractory to antihistamines up to 4 times the licensed dose• Refractory to cyclosporine• Corticosteroids use	<ul style="list-style-type: none">• Edema¹• Type of inflammatory infiltrate¹• Eosinophil and degranulated eosinophil count^{1,4}• Interstitial and intravascular neutrophil count^{1,4}• Mast Cell count^{2,4}• Basophil count^{3,4}

APST, Autologous Plasma Skin Test; ASST, Autologous Plasma Skin Test; CRP, C-Reactive Protein; CSU, chronic spontaneous urticaria; ESR, Erythrocyte Sedimentation Rate 1h; IgE, immunoglobulin E; MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet large cell ratio; UAS, urticaria activity score. ¹ Hematoxylin-eosin, ² Giemsa – cKIT, ³ BB1- Basogranulin, ⁴ Average in 3 fields of 100x.

Table S2. Clinical features of the patients included in the study, one by one

Case	Sex	Age (years)	Duration (months)	Angio edema	Autoimmunity	Thyroid impairment	ASST	CIndU	UAS7	IgE	DD	AntiH1 x1-R	AntiH1 x4-R	CsA-R
A1	F	75	36	-	Autoimmune hypo-thyroidism	Hypo-thyroidism	-	DU	16	119	199	+	-	-
A2	M	34	12	-	-	-	-	-	36	190	NA	+	-	NA
A3	M	29	36	+	-	-	-	-	42	NA	597	+	+	+
A4	M	75	3	-	-	-	-	-	42	171	NA	+	+	-
A5	F	38	60	+	-	-	-	DPU	30	909	1016	+	+	+
A7	M	37	132	-	-	-	-	-	21	251	NA	+	+	-
A8	F	44	12	-	-	-	+	DPU	34	114	316	+	+	-
A10	M	67	6	-	-	-	-	-	28	168	NA	+	+	-
A12	F	58	12	-	Autoimmune hypo-thyroidism and vitiligo	Hypo-thyroidism	+	-	42	22	1050	+	-	-
A13	F	59	12	-	-	-	-	DU	27	231	417	+	-	-
A14	F	81	3	-	-	Antibodies against TPO	+	-	42	72.7	NA	+	-	-
A15	M	49	24	+	-	-	+	-	42	268	NA	+	+	-
A16	F	66	480	+	-	-	+	-	28	220	199	+	-	-
A17	F	33	6	-	-	-	+	-	42	245	109	+	-	-
A18	F	58	6	-	Sjögren syndrome	Antibodies against TG	+	-	35	97.8	300	+	-	-
A19	F	49	156	-	Autoimmune hypo-thyroidism	Hypo-thyroidism	+	-	28	62.7	NA	+	+	-
A20	F	36	60	-	-	-	+	-	25	373	199	+	-	-

A21	F	60	3	+	Systemic lupus erythematosus	-	+	DPU	42	10.8	NA	+	-	-
A22	F	43	12	-	Multiple sclerosis	-	+	DU + DPU	28	55.9	180	+	-	-
A23 XX	F	40	108	+	-	-	-	DPU	42	1470	1051	+	-	+

AntiH1x1-R, Refractory to antihistamines at licenced dose; AntiH1 x4-R, Refractory to antihistamines up to 4 fold dose; ASST, Autologous Serum Skin Test; CIndU, Chronic Inducible Urticaria; CsA-R, Refractory to Cyclosporine A; DD, D-Dimer (Normal values: 0-250); DU, Dermographic Urticaria; DPU, Delayed pressure urticaria; F, female; IgE, immunoglobuline E (total serum levels); M: male; NA, Not available; TG, thyroglobulin; TPO, thyroid peroxidase; UAS7 : Urticaria Activity Score 7.

Table S3. Gene expression and qPCR study of FCER1A and FCER1G, genes that codify the IgE receptor FcεRI

Gene expression of FCER1A and FCER1G						
Gene	LogFC	P Value	*adj P Value	LogFC	P Value	*adj P Value
	NLS-CSU vs HCs			LS-CSU vs HCs		
FCER1A	1.40	0.00395	0.08923	1.21	0.00735	0.03220
FCER1A	1.30	0.00485	0.09771	1.28	0.00811	0.03457
FCER1A	1.31	0.00579	0.10561	1.23	0.00929	0.03832
FCER1A	1.27	0.00620	0.10832	1.20	0.00951	0.03895
FCER1A	1.29	0.00623	0.10850	1.19	0.00961	0.03925
FCER1A	1.27	0.00706	0.11452	1.14	0.01177	0.04570
FCER1A	1.30	0.00771	0.11988	1.22	0.01188	0.04602
FCER1A	1.31	0.00789	0.12065	1.15	0.01267	0.04830
FCER1A	1.26	0.00813	0.12229	1.15	0.01357	0.05096
FCER1A	1.21	0.01117	0.14057	1.13	0.01535	0.05583
FCER1G	1.20	0.09199	0.36751	1.90	0.00910	0.03771
FCER1G	-0.05	0.79531	0.92560	0.16	0.37453	0.56663
qPCR study of FCER1A and FCER1G						
Gene	DDC t	RQ	P Value	Significance	Comparison	
FCER1A	0.0122	0.9916	0.9755	NO	LS-CSU vs HCs	
FCER1G	0.7666	0.5878	0.0684	NO	LS-CSU vs HCs	
FCER1A	-0.5235	1.4378	0.1363	NO	NLS-CSU vs HCs	
FCER1G	-0.3477	1.2725	0.3454	NO	NLS-CSU vs HCs	
FCER1A	-0.5113	1.4253	0.1923	NO	NLS-CSU vs LS-CSU	
FCER1G	0.4189	0.7480	0.3389	NO	NLS-CSU vs LS-CSU	

FCER1A & FCER1G, genes that codify the FcεRI; DDC t, is Delta Delta Ct ; HCs, healthy controls; logFC, *Logarithmic* Fold Change; LS-CSU, lesional skin-chronic spontaneous urticarial; NLS-CSU, non-lesional skin-chronic spontaneous urticaria; qPCR, Quantitative real-time polymerase chain reaction; RQ, Relative Quantification of the calibrator is 1; . *adj P value: adjusted P value.

Table S4. Functional pathways including the differentially expressed genes confirmed by qPCR

Functional pathways	Differentially expressed genes
Cellular Movement, Inflammatory Response, Hematological System Development and Function	BCR (complex), C5AR1, CD69, FGFBP1, Fibrinogen, Focal adhesion kinase, ICAM1, IgG, IL6, IL12 (family), IL1B, IVL, JINK1/2, LDLR, LILRB4, lymphotoxin-alpha1-beta2, Mapk, N-cor, PDE5A, PI3K p85, Pkc(s), PLA2G3, PTAFR, PTGS2, Ras homolog, S100A8, S100A9, SAA, SERPINA1, SERPINA3, SPRR2C, SRGN, THBS1, TLR2, TNFRSF12A
Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders	AKR1B10, CCL2, CCL4, CCL8, Cdk, CSF3R, CXCL1, CXCL2, CXCR2, DUSP1, Fcer1, FCGR1A, FCGR3A /FCGR3B, FOSB, Gm-csf, lfn gamma, IL1, IL12 (complex), IL4R, Immunoglobulin, IRF1, Jnk, LDL, NAMPT, NFkB (complex), Nr1h, NR4A3, PDGF BB, PI3K (family), PNP, S100A7, SOCS3, TIMP3, Tlr, ZFP36
Cellular Movement, Cellular Development, Cellular Growth and Proliferation	26s Proteasome, Akt, Ap1, AREG, ATF3, CD3, Cg, Creb, CXCL12, CYR61, DDX21, EGR1, ERK, ERK1/2, estrogen receptor, FOSL1, FPR1, Gsk3, HAS3, Hdac, Hsp27, MAP2K1/2, Mek, MT2A, MYC, NR4A2, P38 MAPK, PI3K (complex), PLAUR, Rac, SELE, SERPINE1, STAT5a/b, TCR, Vegf
Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders	ADAMTS4, ADORA2B, CCL11, CCL14, CCL18, CD163, Cpla2, CSRNP1, CXCL2, Fcgr2, FCGR1A, FCG R1B, FGB, HAMP, HAS1, IgG, IL4, IL17RB, IL4R, KHSRP, MAPK13, MMP10, NGF, RGS1, SELE, SERPIN B3, SLC25A25, TLR6, TNF, TNFAIP6, TNFSF15, TNIP3, TOB1, TREM1, TUBB2A
Cellular Development, Embryonic Development, Hair and Skin Development and Function	ACPP, ADAMTS1, AQP3, AR, ARPC4, CASP4, CH25H, CYFIP2, EHF, ELF3, EREG, FBLN1, FFAR2, FOS L2, FSH, IL6R, ITGB5, KRT6C, Lh, LMOD1, MGEA5, PIK3CD, RANGAP1, RGS5, RGS16, S100A2, SERPI NE2, SMARCA4, SPRR1B, SPRR2A, SPRR2G, STK17A, THBS1, VEGFA, VEGFC
Dermatological Diseases and Conditions, Cell-To-Cell	CD69, COL18A1, CXCL2, DDB2, DIO1, FOSL2, GDF15, Growth hormone, Histone h3, Histone h4, HNRNPK, HNRNPUL1, HOXA9, HPSE, HSD11B2, IGFBP1, IL1A, KRT16, LDLR, LEP, NFKBIZ, PI3, P

Signaling and Interaction, Cellular Movement	PRC1,RNA polymerase II,SELE,SMYD3,SOD2,SP3,SPRR2B,SPRR2D,SPRR2F,SUV39H1,TNFRSF10C,TNFSF10,VWF
Molecular Transport, DNA Replication, Recombination, and Repair, Tissue Morphology	STEAP4, XRN2

Functional analysis was performed by the Ingenuity Pathway Analysis (IPA).

Table S5. Genes over expressed that codify epidermal proteins involved in terminal skin differentiation and barrier function found in both comparison LS-CSU versus NLS-CSU and also between the NLS-CSU and the healthy skin of HCs (ordered by LogFC)

Gene	Complete name of the gene	LogFC
NLS-CSU and the healthy skin of HCs		
SPRR2E	Small proline-rich protein 2E	4.41
LCE2D	Late cornified envelope 2D	4.25
LCE2C	Late cornified 2C	4.19
LCE2A	Late cornified envelope 2A	4.06
CDSN	Corneodesmosin	4.05
ASPRV1	Skin Aspartic protease	3.86
LCE1B	Late cornified envelope 1B	3.54
FLG2	Filaggrin family member 2	3.24
SLURP1	Secreted LY6/PLAUR domain containing 1	2.91
KRTDAP	Keratinocyte differentiation-associated protein	2.81
DSC1	Desmocollin 1	2.77
GJB4	Gap junction protein. beta 4. 30.3kDa	2.71
CSTA	Cystatin A (stefin A)	2.1
LCE4A	Late cornified envelope 4A	1.85
SPINK5	Serine peptidase inhibitor. Kazal type 5	1.71
KRT10	Keratin 10	1.63
EPPK1	Epiplakin 1	1.6
FLG	Filaggrin	1.43
RPTN	Repetin	1.26
LS-CSU versus NLS-CSU		
SPRR2D	Small proline -rich protein 2D	2.76
SPRR2A	Small proline -rich protein 2A	2.63
KRT16	Keratin 16	2.56
KRT86; KRT81	Keratin 86	2.56
KRTAP3-1	Keratin associated protein 3-1	2.29
SPRR2F	Small proline -rich protein 2F	2.24
SPRR2C	Small proline -rich protein 2C	2.21
KRTAP1-5	Keratin associated protein 1-5	2.1
PI3	Peptidase inhibitor 3, skin-derived	2.09
IVL	Involucrin	2.08
KRTAP1-1	Keratin associated protein 1-1	2.04
SPRR1B	Small proline -rich protein 1B	2.04
SPRR2G	Small proline -rich protein 2G	2.04
SPRR1A	Small proline -rich protein 1A	2
SPRR2B	Small proline -rich protein 2B	2
KRT6C	Keratin 6C	1.93
KRTAP9-4	Keratin associated protein 9-4	1.88
KRTAP4-1	Keratin associated protein 4-1	1.72

KRTAP26-1	Keratin associated protein 26-1	1.68
KRTAP4-7	Keratin associated protein 4-7	1.43
AQP3	Aquaporin 3 (Gill blood group)	1.22

HCs, healthy controls; logFC, Logarithmic Fold Change; LS-CSU, lesional skin-chronic spontaneous urticarial; NLS-CSU, non-lesional skin-chronic spontaneous urticaria

For Peer Review

Figures

Figure S1. Comparisons addressed during bioinformatics study with the samples obtained from CSU patients and HCs

Figure S2. Pathology of the wheal

(A) Wheal biopsied no more than 1 hour after its appearance. (B) Normal epidermis. Oedema of the upper/mid dermis with (C) a mixed inflammatory perivascular infiltrate (neutrophils and/or eosinophils, without vessel-wall necrosis). (D) Increased number of degranulated mast cells observed with Giemsa staining. (E) Increased number of positive c-Kit cells corresponding to dermal mast cells.

Figure S3. Biological functions of genes in LS-CSU versus NLS-CSU

(*) Functions and Genes under expressed:

RGS5 (Regulator of G-protein signalling 5), intracellular signal function
PDE5A (Phosphodiesterase 5A. cGMP-specific), intracellular signal function
RHPN (Rhopilin. Rho GTPase binding protein 2) as intracellular signal
LMOD1 (Leiomodin 1), thyroid associated disease
CXCL12 (C-X-C motif), ligand 12 as chemokine
CCL15-CCL14 (C-C motif) ligand 14, as chemokine
VWF (Von Willebrand factor) involved in coagulation.
TIMP3 (TIMP metalloproteinase inhibitor 3) ,metalloproteinases inhibitor.

Supplementary material

(Five supplementary Tables and three supplementary figures are provided)

Tables

Table S1. Clinical, laboratory, pathological and treatment parameters analyzed

Clinical parameters	Laboratory parameters	Treatment parameters	Pathological parameters
<ul style="list-style-type: none"> • Age • Gender • Disease duration • Previous episodes of CSU • Angioedema associated • UAS • UAS7 • Atopy • Thyroid dysfunction • Autoimmune diseases • Inducible Urticaria • Dermographic urticaria • Delayed Pressure urticaria • ASST • Wheal and erythema diameter of ASST • APST • Wheal and erythema diameter of APST 	<ul style="list-style-type: none"> • CPR • ESR • IgE • D-Dimer • Anti-thyroid antibodies • Platelet count • PDW • MPV • P-LCR • Eosinophil count • Basophil count • CD63 expression assay • Helicobacter pylori breath test 	<ul style="list-style-type: none"> • Refractory to antihistamines at licensed doses • Refractory to antihistamines up to 4 times the licensed dose • Refractory to cyclosporine • Corticosteroids use 	<ul style="list-style-type: none"> • Edema¹ • Type of inflammatory infiltrate¹ • Eosinophil and degranulated eosinophil count^{1,4} • Interstitial and intravascular neutrophil count^{1,4} • Mast Cell count^{2,4} • Basophil count^{3,4}

APST, Autologous Plasma Skin Test; ASST, Autologous Plasma Skin Test; CRP, C-Reactive Protein; CSU, chronic spontaneous urticaria; ESR, Erythrocyte Sedimentation Rate 1h; IgE, immunoglobulin E; MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet large cell ratio; UAS, urticaria activity score. ¹ Hematoxylin-eosin, ² Giemsa – cKIT, ³ BB1- Basogranulin, ⁴ Average in 3 fields of 100x.

Table S2. Clinical features of the patients included in the study, one by one

Case	Sex	Age (years)	Duration (months)	Angio edema	Autoimmunity	Thyroid impairment	ASST	CIndU	UAS7	IgE	DD	AntiH1 x1-R	AntiH1 x4-R	CsA-R
A1	F	75	36	-	Autoimmune hypo-thyroidism	Hypo-thyroidism	-	DU	16	119	199	+	-	-
A2	M	34	12	-	-	-	-	-	36	190	NA	+	-	NA
A3	M	29	36	+	-	-	-	-	42	NA	597	+	+	+
A4	M	75	3	-	-	-	-	-	42	171	NA	+	+	-
A5	F	38	60	+	-	-	-	DPU	30	909	1016	+	+	+
A7	M	37	132	-	-	-	-	-	21	251	NA	+	+	-
A8	F	44	12	-	-	-	+	DPU	34	114	316	+	+	-
A10	M	67	6	-	-	-	-	-	28	168	NA	+	+	-
A12	F	58	12	-	Autoimmune hypo-thyroidism and vitiligo	Hypo-thyroidism	+	-	42	22	1050	+	-	-
A13	F	59	12	-	-	-	-	DU	27	231	417	+	-	-
A14	F	81	3	-	-	Antibodies against TPO	+	-	42	72.7	NA	+	-	-
A15	M	49	24	+	-	-	+	-	42	268	NA	+	+	-
A16	F	66	480	+	-	-	+	-	28	220	199	+	-	-
A17	F	33	6	-	-	-	+	-	42	245	109	+	-	-
A18	F	58	6	-	Sjögren syndrome	Antibodies against TG	+	-	35	97.8	300	+	-	-
A19	F	49	156	-	Autoimmune hypo-thyroidism	Hypo-thyroidism	+	-	28	62.7	NA	+	+	-
A20	F	36	60	-	-	-	+	-	25	373	199	+	-	-

A21	F	60	3	+	Systemic lupus erythematosus	-	+	DPU	42	10.8	NA	+	-	-
A22	F	43	12	-	Multiple sclerosis	-	+	DU + DPU	28	55.9	180	+	-	-
A23 XX	F	40	108	+	-	-	-	DPU	42	1470	1051	+	-	+

AntiH1x1-R, Refractory to antihistamines at licenced dose; AntiH1 x4-R, Refractory to antihistamines up to 4 fold dose; ASST, Autologous Serum Skin Test; CIndU, Chronic Inducible Urticaria; CsA-R, Refractory to Cyclosporine A; DD, D-Dimer (Normal values: 0-250); DU, Dermographic Urticaria; DPU, Delayed pressure urticaria; F, female; IgE, immunoglobuline E (total serum levels); M: male; NA, Not available; TG, thyroglobulin; TPO, thyroid peroxidase; UAS7 : Urticaria Activity Score 7.

Table S3. Gene expression and qPCR study of FCER1A and FCER1G, genes that codify the IgE receptor FcεRI

Gene expression of FCER1A and FCER1G						
Gene	LogFC	P Value	*adj P Value	LogFC	P Value	*adj P Value
	NLS-CSU vs HCs			LS-CSU vs HCs		
FCER1A	1.40	0.00395	0.08923	1.21	0.00735	0.03220
FCER1A	1.30	0.00485	0.09771	1.28	0.00811	0.03457
FCER1A	1.31	0.00579	0.10561	1.23	0.00929	0.03832
FCER1A	1.27	0.00620	0.10832	1.20	0.00951	0.03895
FCER1A	1.29	0.00623	0.10850	1.19	0.00961	0.03925
FCER1A	1.27	0.00706	0.11452	1.14	0.01177	0.04570
FCER1A	1.30	0.00771	0.11988	1.22	0.01188	0.04602
FCER1A	1.31	0.00789	0.12065	1.15	0.01267	0.04830
FCER1A	1.26	0.00813	0.12229	1.15	0.01357	0.05096
FCER1A	1.21	0.01117	0.14057	1.13	0.01535	0.05583
FCER1G	1.20	0.09199	0.36751	1.90	0.00910	0.03771
FCER1G	-0.05	0.79531	0.92560	0.16	0.37453	0.56663
qPCR study of FCER1A and FCER1G						
Gene	DDC t	RQ	P Value	Significance	Comparison	
FCER1A	0.0122	0.9916	0.9755	NO	LS-CSU vs HCs	
FCER1G	0.7666	0.5878	0.0684	NO	LS-CSU vs HCs	
FCER1A	-0.5235	1.4378	0.1363	NO	NLS-CSU vs HCs	
FCER1G	-0.3477	1.2725	0.3454	NO	NLS-CSU vs HCs	
FCER1A	-0.5113	1.4253	0.1923	NO	NLS-CSU vs LS-CSU	
FCER1G	0.4189	0.7480	0.3389	NO	NLS-CSU vs LS-CSU	

FCER1A & FCER1G, genes that codify the FcεRI; DDC t, is Delta Delta Ct ; HCs, healthy controls; logFC, *Logarithmic* Fold Change; LS-CSU, lesional skin-chronic spontaneous urticarial; NLS-CSU, non-lesional skin-chronic spontaneous urticaria; qPCR, Quantitative real-time polymerase chain reaction; RQ, Relative Quantification of the calibrator is 1; . *adj P value: adjusted P value.

Table S4. Functional pathways including the differentially expressed genes confirmed by qPCR

Functional pathways	Differentially expressed genes
Cellular Movement, Inflammatory Response, Hematological System Development and Function	BCR (complex), C5AR1, CD69, FGFBP1, Fibrinogen, Focal adhesion kinase, ICAM1, IgG, IL6, IL12 (family), IL1B, IVL, JINK1/2, LDLR, LILRB4, lymphotoxin-alpha1-beta2, Mapk, N-cor, PDE5A, PI3K p85, Pkc(s), PLA2G3, PTAFR, PTGS2, Ras homolog, S100A8, S100A9, SAA, SERPINA1, SERPINA3, SPRR2C, SRGN, THBS1, TLR2, TNFRSF12A
Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders	AKR1B10, CCL2, CCL4, CCL8, Cdk, CSF3R, CXCL1, CXCL2, CXCR2, DUSP1, Fcer1, FCGR1A, FCGR3A /FCGR3B, FOSB, Gm-csf, lfn gamma, IL1, IL12 (complex), IL4R, Immunoglobulin, IRF1, Jnk, LDL, NAMPT, NFkB (complex), Nr1h, NR4A3, PDGF BB, PI3K (family), PNP, S100A7, SOCS3, TIMP3, Tlr, ZFP36
Cellular Movement, Cellular Development, Cellular Growth and Proliferation	26s Proteasome, Akt, Ap1, AREG, ATF3, CD3, Cg, Creb, CXCL12, CYR61, DDX21, EGR1, ERK, ERK1/2, estrogen receptor, FOSL1, FPR1, Gsk3, HAS3, Hdac, Hsp27, MAP2K1/2, Mek, MT2A, MYC, NR4A2, P38 MAPK, PI3K (complex), PLAUR, Rac, SELE, SERPINE1, STAT5a/b, TCR, Vegf
Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders	ADAMTS4, ADORA2B, CCL11, CCL14, CCL18, CD163, Cpla2, CSRNP1, CXCL2, Fcgr2, FCGR1A, FCG R1B, FGB, HAMP, HAS1, IgG, IL4, IL17RB, IL4R, KHSRP, MAPK13, MMP10, NGF, RGS1, SELE, SERPIN B3, SLC25A25, TLR6, TNF, TNFAIP6, TNFSF15, TNIP3, TOB1, TREM1, TUBB2A
Cellular Development, Embryonic Development, Hair and Skin Development and Function	ACPP, ADAMTS1, AQP3, AR, ARPC4, CASP4, CH25H, CYFIP2, EHF, ELF3, EREG, FBLN1, FFAR2, FOS L2, FSH, IL6R, ITGB5, KRT6C, Lh, LMOD1, MGEA5, PIK3CD, RANGAP1, RGS5, RGS16, S100A2, SERPI NE2, SMARCA4, SPRR1B, SPRR2A, SPRR2G, STK17A, THBS1, VEGFA, VEGFC
Dermatological Diseases and Conditions, Cell-To-Cell	CD69, COL18A1, CXCL2, DDB2, DIO1, FOSL2, GDF15, Growth hormone, Histone h3, Histone h4, HNRNPK, HNRNPUL1, HOXA9, HPSE, HSD11B2, IGFBP1, IL1A, KRT16, LDLR, LEP, NFKBIZ, PI3, P

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Signaling and Interaction, Cellular Movement	PRC1,RNA polymerase II,SELE,SMYD3,SOD2,SP3,SPRR2B,SPRR2D,SPRR2F,SUV39H1,TNFRSF10C,TNFSF10,VWF
Molecular Transport, DNA Replication, Recombination, and Repair, Tissue Morphology	STEAP4, XRN2

Functional analysis was performed by the Ingenuity Pathway Analysis (IPA).

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Table S5. Genes over expressed that codify epidermal proteins involved in terminal skin differentiation and barrier function found in both comparison LS-CSU versus NLS-CSU and also between the NLS-CSU and the healthy skin of HCs (ordered by LogFC)

Gene	Complete name of the gene	LogFC
NLS-CSU and the healthy skin of HCs		
SPRR2E	Small proline-rich protein 2E	4.41
LCE2D	Late cornified envelope 2D	4.25
LCE2C	Late cornified 2C	4.19
LCE2A	Late cornified envelope 2A	4.06
CDSN	Corneodesmosin	4.05
ASPRV1	Skin Aspartic protease	3.86
LCE1B	Late cornified envelope 1B	3.54
FLG2	Filaggrin family member 2	3.24
SLURP1	Secreted LY6/PLAUR domain containing 1	2.91
KRTDAP	Keratinocyte differentiation-associated protein	2.81
DSC1	Desmocollin 1	2.77
GJB4	Gap junction protein. beta 4. 30.3kDa	2.71
CSTA	Cystatin A (stefin A)	2.1
LCE4A	Late cornified envelope 4A	1.85
SPINK5	Serine peptidase inhibitor. Kazal type 5	1.71
KRT10	Keratin 10	1.63
EPPK1	Epiplakin 1	1.6
FLG	Filaggrin	1.43
RPTN	Repetin	1.26
LS-CSU versus NLS-CSU		
SPRR2D	Small proline -rich protein 2D	2.76
SPRR2A	Small proline -rich protein 2A	2.63
KRT16	Keratin 16	2.56
KRT86; KRT81	Keratin 86	2.56
KRTAP3-1	Keratin associated protein 3-1	2.29
SPRR2F	Small proline -rich protein 2F	2.24
SPRR2C	Small proline -rich protein 2C	2.21
KRTAP1-5	Keratin associated protein 1-5	2.1
PI3	Peptidase inhibitor 3, skin-derived	2.09
IVL	Involucrin	2.08
KRTAP1-1	Keratin associated protein 1-1	2.04
SPRR1B	Small proline -rich protein 1B	2.04
SPRR2G	Small proline -rich protein 2G	2.04
SPRR1A	Small proline -rich protein 1A	2
SPRR2B	Small proline -rich protein 2B	2
KRT6C	Keratin 6C	1.93
KRTAP9-4	Keratin associated protein 9-4	1.88
KRTAP4-1	Keratin associated protein 4-1	1.72

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KRTAP26-1	Keratin associated protein 26-1	1.68
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HCs, healthy controls; logFC, Logarithmic Fold Change; LS-CSU, lesional skin-chronic spontaneous urticarial; NLS-CSU, non-lesional skin-chronic spontaneous urticaria

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Figures

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Figure S3. Biological functions of genes in LS-CSU versus NLS-CSU

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 TIMP3 (TIMP metalloproteinase inhibitor 3), metalloproteinases inhibitor.

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Professor Werner Aberer

Dear Prof Werner Aberer,

Thank you for the revision of the manuscript ALL-2017-00095. Please find enclosed the revision to the manuscript entitled **Transcriptome analysis of severely active chronic spontaneous urticaria shows an overall immunological skin involvement, which** we submit for consideration for publication in *Allergy*. We proceed to answer the reviewer requests.

Answer to Reviewer: 1

1. Although multiple factors have been determined in pathogenesis of CSU, the current knowledge and understanding of urticarial processes are still not satisfying. Analysis of gene expression profiles, based on microarray technologies allows for better recognition of pathogenesis of this condition, including its molecular pathways, and finally development of more efficient therapies. In addition, new markers based on gene expression might be correlated with CSU phenotypes and used to individualize therapies.

Also, despite the fact that CSU is not an a priori epidermal disorder, the authors observed interesting overexpression of genes involved in terminal epidermal differentiation and barrier function in both, LS-CSU versus NL-CSU and NLS-CSU versus healthy controls.

Moreover, the results presents support the hypothesis of overall immunological-inflammatory skin involvement in CSU patients, at least in individuals showing severe symptoms. This is in line with the concept of CSU as a chronic inflammatory disease.

Bringing some novel, important information and determining new directions for future studies, the study delivered is a valuable contribution to recognition of the urticarial processes.

We extremely thank these comments, which accurately collect the message of the manuscript

2. Information should be included to define limitations of the study and/or indicate the need for correlation with UAS7, which seems particularly important when comparing patients with mild CSU symptoms (best if evaluated after withdrawal of antihistamine drugs) to those with severe CSU. This would certainly bring some vital information on CSU phenotypes.

We agree with this observation and comment. The manuscript shows the transcriptome analysis of patients suffering severely active chronic spontaneous urticaria. The inclusion criteria of the study define the patient's characteristics. An Urticaria Activity Score (UAS) 7 ≥16 for at least 3 months was necessary. Also a blanching period of any previous drugs or treatment was requested and guaranteed before the inclusion of the patients in the study. It is not the objective of this study to compare the gene expression of severely active CSU patients with the gene expression of patients suffering of mild CSU. We agree that this comparison would be interesting if we try to establish differences between phenotypes based on the activity of the disease. But this was not the main objective of this present approach.

As the reviewer suggest, we've included in the discussion the following sentence addressing this limitation and the interest of this comparison. (Line 417- 419)

"In order to establish phenotype differences based in the CSU activity a comparative study of the transcriptome pattern observed in NLS and LS-CSU between patients with severely and mild active CSU would be useful."

Answer to Reviewer: 2

1. The paper is, to my knowledge, the first transcriptome analysis in chronic urticaria. The panel of identified involved genes could build a base for further research in this still incomplete understood disease.

We thank very much this comment.

2. page 3 line 32: I suggest to include an and/or instead of or (as all combinations are possible)

According with the suggested comment "and/or instead of or" in the following sentence, page 3 line72

"The CSU phenotype showing the most active disease (highest Urticaria Activity Score [UAS]) with the worse prognosis commonly is refractory to high doses of antihistamines and associates angioedema, concomitant chronic inducible urticaria (CIndU) and/or serum autoreactivity (functional histamine-release autoantibodies) (4)."

3. Results section: I think there is no "a" or "the" needed in front of number %.

According with the suggested comment "a" or "the" in front the number% has been eliminated in the following paragraph.

Characteristics of the studied population (line 2192-39)

The clinical features for each patient are included in Table S2. Fourteen women and 6 men were included (mean age $\pm$ SD of 51.5 $\pm$ 15.7 years [range 20-81 years]). The HCs (N=10) ratio female:male was 1:1 and the mean age was 27.5 $\pm$ 5.4 years (range 21-37 years). The disease mean duration was 4.9 $\pm$ 9 SD years (range 3 months-40 years). Mean UAS7 was 33.6 (range 16-42). All the patients were symptomatic in spite of high doses of second generation H1-antihistamines (2) and were suitable to be treated with e.g. anti-immunoglobulin E (IgE) therapy or immunomodulation. 35% (7/20) of them were unresponsive to cyclosporine A. Attending other clinical characteristics as markers of a bad prognosis for CSU, 20% (4/20) of the patients suffered intermittent angioedema crisis (4). Twenty five per cent (5/20) showed concomitant inducible urticaria (symptomatic dermographism or delayed pressure urticaria) (4). Fifty five percent (11/20) of the patients showed a positive Autologous Serum Skin Test (ASST) and 55% (11/20) had an autoimmune disease associated (4). The autoimmune thyroid diseases were the most frequent concomitant conditions, affecting 25% (5/20) of the CSU patients (4). Other autoimmune diseases reported included vitiligo, Sjögren syndrome and systemic lupus erythematosus. D-dimer serum level was high in 54% (7/13). The mean value of the total serum IgE was 277.5 $\pm$ SD357.8 UI/ml (range 22-1470 UI/ml), slightly higher compared to the normal values detected in HCs (60.7 $\pm$ SD 54.4 UI/ml).

4. Are there any histological pictures/descriptions of the NON-LS-Skin?

Thank you for this comment. There is no pathological description from the NLS- CSU because we did not obtain samples for pathology of non-lesional skin as we've obtained from the wheal. The biopsies from the wheal studied with Hematoxylin- Eosin, Giemsa and c_KIT, were obtained from the hives with the objective to confirm the diagnosis and rule out differential diagnosis. The main objective of the study was to compare the gene expression of lesional and non-lesional skin in patients with severely active CSU and with the skin of healthy controls.

The NLS-CSU skin biopsy for gene array study was obtained from an area where neither a wheal nor other skin alterations (e.g. desquamation or pigmentation) were presented within the previous 72 hours. This fact should guarantee a no lesional skin. The gene differences between NLS- CSU and the gene expression of the skin from healthy controls are new findings and we agree that future studies in order to demonstrate by immunohistochemistry some of the changes or other genetic studies should include pathology samples from the NLS- CSU.

5. Was any subgroup analysis done (Angioedema + vs - ; co-inducible Urticaria + vs -; etc...)

Thank you for this comment, we agree with the reviewer on the interest of this point.

The table S2 “Clinical features of the patients” included in the study, one by one, registers the clinical parameters of the 20 patients. There are indeed some clinical differences in the little group regarding e.g the concomitant presence of inducible urticaria or angioedema but no subanalysis was performed comparing the groups due to the limited number of patients per subgroup (4 with angioedema, 2 with concomitant delayed pressure urticaria and 2 with concomitant symptomatic dermographism).

However, a study of correlation between all the confirmed genes obtained, over or under expressed in the different comparisons, and the quantitative and qualitative clinical parameter assessed in this group of patients was done and it is now in the analysis phase. This type of analysis shows information that needs to be accurately evaluated looking for its clinical relevance. The observations will need further confirmation studies.

Editorial Office Request

1. In the marked version of your revised manuscript, please use the line numbering function.
A numbering function manuscript is included
2. Please additionally provide an unmarked version of your revised manuscript.
An unmarked version manuscript is included
3. Please cross-check your manuscript according to our guidelines to authors.
The manuscript was cross-checked according with Allergy guidelines.

This study has unique characteristics; the results provide new insights into chronic spontaneous urticaria knowledge. In this manuscript, the genetic signature of patients suffering severely active chronic spontaneous urticaria (CSU) identifying the relevant genes expressed in tissue is described. The results provide interesting information that supports CSU as a systemic disease with the overall integument involved. The current genetic profile results are robust and consistent enough to be considered as a useful tool within the research in CSU.

The documents of this submission contain original research, which has not been previously published and has not been submitted for publication elsewhere while under consideration. None of the authors have any conflicts of interest to disclose in the work described.

We thank you in advance for taking our comments and revision into consideration and we look forward to hearing from you.

Yours sincerely,

Ana M Giménez-Arnau MD

For Peer Review

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