Acquired Cold Urticaria vs. Autoinflammatory Diseases, Genetic and Clinical Profile and Differential Diagnosis: Study of a Cohort of Patients in a Tertiary Reference Centre

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Acquired cold urticaria (ACU) is characterized by the development of itchy wheals after cold exposure. Generalized urticarial skin rashes triggered by cold exposure characterize certain monogenic autoinflammatory diseases (AIDs). The objective of this study is to investigate the presence of variants in genes causing AIDs that present with cold-induced urticarial skin rashes in patients clinically diagnosed with ACU, in order to look for susceptibility factors for the disease. Fifty patients with primary ACU were studied. Germline and post-zygotic variants on the *NLRP3*, *NLRP12*, *NLRC4* and *PLCG2* genes were investigated using next-generation sequencing technology. Seven patients (14%) carried 8 heterozygous germline variants in the following genes: *NLRP3* ($n = 1$), *NLRP12* ($n = 3$), *NLRC4* ($n = 1$), *PLCG2* ($n = 3$). No pathogenic or likely pathogenic variants were detected, and deep analyses of the sequences obtained did not identify any post-zygotic variant. In conclusion, ACU is not related to post-zygotic or germline pathogenic variants in the *NLRP3*, *NLRP12*, *NLRC4* and *PLCG2* genes.

Key words: autoinflammatory diseases; cold urticaria; genetic variant; urticaria; urticarial skin rash.

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SIGNIFICANCE

Acquired cold urticaria represents a subtype of inducible urticaria characterized by the development of itchy wheals after cold exposure. Generalized cold-induced urticarial rashes are also seen in certain monogenic autoinflammatory diseases. In the present study, we demonstrated that acquired cold urticaria is not related to the presence of germline and post-zygotic pathogenic variants on genes causing autoinflammatory diseases that present with cold-induced urticarial skin rashes (i.e. *NLRP3*, *NLRP12*, *NLRC4* and *PLCG2* genes). However, the presence of cold urticaria in addition to systemic manifestations, family history and/or laboratory abnormalities should alert physicians to the potential diagnosis of a monogenic autoinflammatory disease.

Cold urticaria is a physical urticaria characterized by the development of itchy wheals and/or angioedema after direct contact between the skin and cold air, liquids and/or objects (1–3). Its diagnosis is usually supported by cold-contact stimulation tests (CSTs), in which an immediate wheal is induced after the application of a cold stimulus to the skin (1). Cold urticaria syndromes are very heterogeneous and can be classified into acquired and familial disorders (3). Acquired cold urticaria (ACU) are further divided into 2 subgroups: (*i*) primary ("idiopathic") and (*ii*) secondary ACU, depending on the presence of an underlying disease or factor associated with the induction of the cold urticaria symptoms. A particular subtype of ACU, known as atypical ACU, is characterized by the negative responses after CSTs, and therefore, its diagnosis is established mainly on the basis of a detailed clinical history (3). ACU most frequently affects young adults, although up to 15–25% of patients may show an onset of symptoms before the age of 18 years (2, 4), with recent evidence suggesting that paediatric-onset patients might exhibit distinctive clinical features (2). Its symptoms are usually limited to cold-exposed skin areas and typically appear a few minutes after exposure to cold air, liquids and/or solids. Extensive cold contact may occasionally result in generalized symptoms, including headache, dyspnoea, hypotension or loss of consciousness (1, 5). It is known that ACU is caused by the release of histamine, leukotrienes, platelet activating factor and other proinflammatory mast-cell mediators (3–6). However, the complete pathophysiology of the disease, particularly the atypical ACU, remains undetermined (2).

Autoinflammatory diseases (AIDs) are a group of inherited conditions of innate immunity characterized by seemingly unprovoked and recurrent episodes of sterile inflammation. The main subgroups among AIDs are the inflammasomopathies, which are characterized by a dysfunction of the inflammasome, a cytosolic multiprotein complex regulating the pyroptosis and the release of caspase-1 activation-dependent inflammatory cytokines (7). From a clinical point of view, patients with

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AIDs usually display fever and inflammation at different organs, including the skin, joints, central nervous system and gastrointestinal tract, and its clinical course may be occasionally complicated with AA-type amyloidosis as a consequence of the long-term systemic inflammation (7). In some conditions, cutaneous manifestations may represent the earliest and most prominent symptoms that often help clinicians to identify AIDs in their early stages (8, 9). This is the case of the generalized urticarial skin rash triggered and/or exacerbated by cold exposure, which is a characteristic feature of certain AIDs including cryopyrin-associated periodic syndromes (CAPS), NLRP12-associated familial cold-induced autoinflammatory syndrome (FCAS2), NLRC4-associated autoinflammatory syndrome and PLCγ2-associated antibody deficiency and immune dysregulation (PLAID) syndrome (8–11). These disorders are typically characterized by recurrent episodes of systemic inflammation, cold urticaria and multi-organ involvement with onset of symptoms during early infancy. However, atypical variants of the diseases may present with non-conventional clinical manifestations, disease onset during adulthood, absence of urticarial rash at the onset or throughout the course of the disease, or milder disease phenotypes with prominent and isolated cutaneous manifestations (12–14). It has been postulated that these atypical cases might be related to the presence of low-penetrance or post-zygotic mutations in known AIDs genes (12–15), and the diagnosis in such patients may be extremely difficult, often leading to a remarkable diagnostic delay.

Therefore, we hypothesized that the presence of variants on genes causing AIDs with urticarial skin rashes as the most prominent cutaneous feature (*NLRP3, NLRP12, NLRC4* and *PLCG2*) may constitute genetic susceptibility factors of the disease in patients clinically diagnosed with ACU, and may have relevant consequences regarding patients' definitive diagnosis and therapeutic approaches.

PATIENTS AND METHODS

Study design, participants and data collected

This prospective study included patients with ACU referred to the Urticaria Clinic of the Department of Dermatology of Hospital del Mar, Barcelona, Spain, during the period from November 2015 to June 2018 (**Fig. 1**). The diagnosis of ACU was based on a patient's clinical history of wheals, angioedema, or both, after cold exposure. Patients with positive evidence of an underlying condition associated with the induction of cold urticaria rashes (i.e. secondary ACU, such as cryoglobulinaemia, cold agglutinins, cryofibrinogenaemia, leukocytoclastic vasculitis, drugs or infectious diseases) were excluded. The clinical research ethics committee from Hospital del Mar granted ethical approval for the study (reference number: 2017/7469/I).

Following a systematized protocol, data regarding patients' epidemiological and clinical features were collected at their initial evaluation. Baseline blood tests from enrolled subjects included haemogram, liver and renal functions, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), levels of IgE, serum chemistry and serum electrophoresis. Disease severity was categorized into 3 types based on the classification suggested by Wanderer et al. (16): type I, localized urticaria and/or angioedema; type II, generalized urticaria and/or angioedema without hypotensive or respiratory symptoms; and type III, severe systemic reactions with ≥1 episodes suggestive of hypotension (i.e. dizziness, disorientation or shock) or respiratory distress (e.g. shortness of breath or wheezing). Other systemic manifestations occurring during the acute phase of the disease were also registered. Disease duration was defined as the time from symptom onset to the last follow-up visit, and disease control as an improvement of the signs and symptoms referred by the patient using the Urticaria Control Test (UCT) until they did not cause interferences in daily life. When the patient report outcome UCT scores >12 the disease is considered well controlled (1).

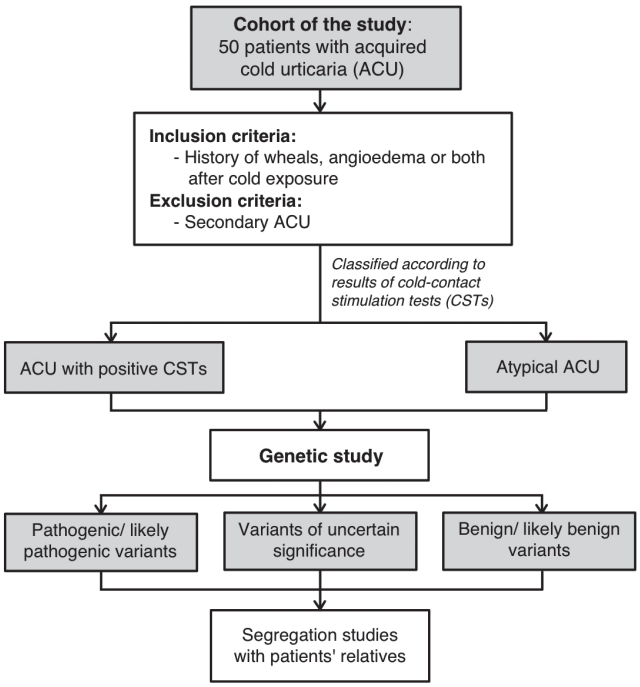

Fig. 1. Study design. CSTs: cold-contact stimulation tests.

According to current guidelines, the diagnosis of ACU was supported by the ice cube challenge test (1). The standard protocol consisted of the application of the cold stimulus over the patient's forearm for 5 min followed by 10 min of re-warming (1). The test was considered positive if a coalescent wheal was elicited over the application site. In addition, all patients underwent thresholds assessment using the TempTest® 3.0 at the baseline evaluation (1, 17). The cold stimulation time threshold and the critical temperature threshold were defined as the shortest time and the highest temperature at which a wheal appears through cold provocation, respectively. Subjects without wheal formation after the CSTs were labelled as atypical ACU.

In addition, whole peripheral blood was collected from enrolled subjects for genetic testing after obtaining written informed consent from patients (>18 years) or from their parents/guardians (<18 years). Genetic variants were classified according to the joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (18), and a genetic report of each enrolled patient was generated. Briefly, these recommendations classify the sequence variants into 5 categories (benign, likely benign, variant of uncertain significance (VUS), likely pathogenic, and pathogenic) according to the results of different criteria including:

(*i*) allele frequency in different public databases, (*ii*) previous reports of the variant in the medical literature, (*iii*) registration of the concrete sequence variant in phenotype-genotype registries (i.e. ClinVar, INFEVERS, Human Gene Mutation Database), (*iv*) structural features of the sequence variant, (*v*) results of functional studies, (*vi*) results of intrafamilial segregation analyses, (*vii*) *de novo* nature of the sequence variant, (*viii*) results of different bioinformatic analyses. In specific cases, genetic analysis of patient's relatives was requested in order to improve the clinical relevance and re-classification of detected variants.

Sequencing methods

Genomic DNA samples were prepared using a QIAmp DNA Blood Mini Kit (QIAgen, Germany). For simultaneous gene analyses, amplicons covering all coding exons and adjacent intronic boundaries of *NLRP3* (RefSeq NM_001243133.1), *NLRP12* (RefSeq NM_144687.2), *NLRC4* (RefSeq NM_001199139.1) and *PLCG2* (RefSeq NM_002661.4) genes were generated by in-house designed PCR amplification in an Access Array System 48.48 platform (Fluidigm, South San Francisco, CA, USA). Library preparation, control quality and quantification were performed according to the manufacturers' instructions. Emulsion PCR was performed on a One Touch2 platform, and sequencing was performed on an IonTorrent PGM platform using the IonTorrent PGM 400 bp Sequencing kit. Reads were mapped against the human reference genome build hg19 using the Burrows-Wheeler Aligner software, and bam and bai files were obtained. The obtained sequences were analysed using the Torrent Server and the Ion Reporter softwares (ThermoFisher Scientific, Waltham, MA, USA). High sequencing coverage was obtained for all genes (mean depth per amplicon was $\times > 2{,}000$), and thus allowed the detection of somatic mosaicism as low as 2%.

All detected candidate gene variants were re-sequenced using Sanger method, in which concrete gene regions were amplified by in-house designed PCR, purified with Illustra ExoStar 1-Step kit (GE Healthcare, Chicago, IL, USA), bidirectional fluorescence sequenced using ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and run on an automated ABI 3730XL DNA analyser (Applied Biosystems, USA).

RESULTS

A total of 50 patients fulfilling the inclusion criteria were enrolled in the study. Clinical and demographic characteristics of such patients are summarized in **Table I**. The median (range) age at disease onset was 27 (1–74) years, and 34 (68.0%) patients were female. None of the 50 patients referred family history of similar symptoms (urticarial skin rashes after cold exposure). Thirty-nine (78.0%) patients experienced generalized urticaria after cold exposure (types II and III reactions), with 12 (24.0%) showing severe reactions with ≥ 1 episode suggestive of hypotension or respiratory distress. Other reported systemic symptoms during urticarial flares include arthralgias ($n = 5$), abdominal pain ($n = 2$) and myalgias ($n = 1$). Fourteen (28.0%) patients were diagnosed with atypical ACU due to the negative results of the CSTs. Regarding response to treatment, which was evaluated after performing the respective genetic analyses, 38 (76.0%) and 12 (24.0%) patients showed a satisfactory and a poor response to non-sedating H1-antihistamines (doses ranging from 1 to 4 times the recommended dose depending on the patient's symptoms response), respectively. From these 12 non-responders to antihistamines,

Table I. Clinical and demographic characteristics of the study population

	All patients ($n = 50$)	ACU patients with positive CSTs ($n = 36$)	Atypical ACU patients ($n = 14$)
Patients carrying germline variants on AIDs genes, *n* (%)	7 (14.0)	2 (5.6)	5 (35.7)
Female sex, *n* (%)	34 (68.0)	27 (75.0)	7 (50.0)
Age, years, median (range)	39 (5–79)	39 (9–79)	38 (5–70)
Age at disease onset, years, median (range)	27 (1–74)	27 (1–74)	26 (1–60)
Atopy, *n* (%)	11 (22.0)	10 (27.8)	1 (7.1)
Family history of cold urticaria symptoms, *n* (%)	0 (0)	0 (0)	0 (0)
Angioedema, *n* (%)	8 (16.0)	6 (16.7)	2 (14.3)
Recurrent sinopulmonary infections, *n* (%)	5 (10.0)	4 (11.1)	1 (7.1)
Associated autoimmune conditions, *n* (%)[a]	2 (4)	0 (0)	2 (14.3)
Positive antinuclear antibodies, *n* (%)[b]	8 (16.7)	5 (14.3)	3 (23.1)
Time from cold exposure to symptoms onset, min, median (range)	5 (0.5–180)	5 (0.5–30)	10 (1–180)
Disease severity, *n* (%)			
I	11 (22.0)	7 (19.4)	4 (28.6)
II	27 (54.0)	19 (52.8)	8 (57.1)
III	12 (24.0)	10 (27.8)	2 (14.3)
Fever or other systemic symptoms, n (%)[c]	8 (16.0)	5 (13.9)	3 (21.4)
Cold triggers, *n* (%)			
Water	46 (92.0)	33 (91.7)	13 (92.9)
Solids	24 (48.0)	23 (63.9)	1 (7.1)
Air	41 (82.0)	30 (83.3)	11 (78.6)
Critical stimulation time threshold, min, median (range)	3 (1–5)	3 (1–5)	–
Critical temperature threshold, ºC, median (range)	14 (4–26)	14 (4–26)	–
Total serum IgE, kU/l, median (range)	69.5 (5–4,700)	71 (5–4,700)	61 (5–1,689)
Elevated inflammatory markers during active disease, *n* (%)[d]	7 (14.0)	6 (16.7)	1 (7.1)
Normal pattern of serum electrophoresis, *n* (%)	48 (96.0)	35 (97.2)	13 (92.9)
Disease control, *n* (%)[e]	45 (90.0)	32 (88.9)	13 (92.9)
Disease duration, years, median (range)	7 (1–26)	8 (1–26)	4 (1–15)

[a]Registered autoimmune conditions included 2 patients diagnosed with autoimmune thyroid disease. [b]Antinuclear antibodies were tested in 48 patients with acquired cold urticaria (ACU): 35 with positive cold-contact stimulation tests (CSTs) and 13 atypical ACU patients. [c]Systemic manifestations during urticarial flares different from hypotension or respiratory symptoms. [d]The evaluated inflammatory markers were the C-reactive protein and the erythrocyte sedimentation rate. [e]Disease control with conventional treatments (counselling + antihistamines and/or omalizumab).

7 were treated with omalizumab, achieving a well-controlled disease (UCT ≥ 12), and the remaining 5 patients have just initiated third-line therapies (omalizumab [*n* = 4] and cyclosporine [*n* = 1]) and are pending to assess the clinical evolution.

Regarding genetic studies, a total of 7 patients (14.0%) carried 8 heterozygous germline variants with allelic frequencies lower than 1% in public databases (1000 Genomes Project, ExAC and gnomAD) in the following genes: *NLRP3* (*n* = 1), *NLRP12* (*n* = 3), *NLRC4* (*n* = 1), *PLCG2* (*n* = 3). According to the ACMG recommendations, all of them were classified as "likely benign" except for 3, which were classified as VUS and were included one in each of the following genes: *NLRP3*, *NLRP12* and *PLCG2* (**Tables II** and **III**). Sanger sequencing confirmed all 8 heterozygous germline variants. No pathogenic or likely pathogenic variants were detected in any of the patients included in the study. Furthermore, deep analysis of the obtained sequences did not identify any post-zygotic gene variant.

Genetic characteristics of the 7 patients carrying heterozygous germline variants with allelic frequencies lower than 1% in public databases are shown in Table II. All these rare variants were missense, except for one, which was synonymous. Three variants (42.8%) were classified as VUS, being all of them previously described in public databases. Notably, these 3 VUS have higher predicted scores by Combined Annotation-Dependent Depletion than those variants classified as probably benign. Regarding the genetic investigations performed in the patients' relatives, genotyping of both patient's parents was possible in 4 families (patients 1, 4, 5 and 7). In all these cases, the concrete gene variant detected in the proband was also detected in at least one of their healthy relatives. The participation of a single relative (mother) was possible for patient number 6, and no missense variant was detected. Relatives' participation for patients 2 and 3 was not possible.

Clinical features of the 7 patients carrying the aforementioned germline variants are summarized in Table III. Regarding the 3 patients carrying VUS (patients 2, 3 and 6), 2 (66.7%) exhibit an adult-onset ACU and 2 (66.7%) showed negative responses to CSTs. All 3 patients referred symptoms triggered by the exposure of cold water and

Table II. Summary of genetic characteristics from patients carrying germline variants with allelic frequencies lower than 1% in public databases (1000 Genomes Project, ExAC and gnomAD)

					Population genetics		Bioinformatics				
Pat. No.	Gene	Nucleotide exchange[a]	Amino acid exchange	dbSNP	1000 Genomes Project (%; hom)	gnomAD (%; hom)	Polyphen-2 (HumDiv score)	SIFT(score)	Mutation taster (score)	CADD	Pathogenicity classification[b]
1	*NLRC4*	c.2785G>T	p.Ala929Ser	rs61754192	0.30; 0	0.76; 9	Benign (0)	Tolerated (0.78)	Polymorphism (99)	5.770	Likely benign
2	*NLRP3*	c.2336G>A	p.Gly779Asp	rs768252357	0; 0	0.002; 0	Possibly damaging (0.623)	Tolerated (0.65)	Polymorphism (94)	21.3	VUS
	PLCG2	c.3125G>C	p.Ser1042Thr	rs114262189	0.82; 1	0.30; 14	Benign (0.033)	Tolerated (0.4)	Polymorphism (58)	15.82	Likely benign
3	*NLRP12*	c.857C>T	p.Pro286Leu	rs201940393	0; 0	0.014; 0	Possibly damaging (0.877)	Deleterious (0)	Polymorphism (98)	19.76	VUS
4	*NLRP12*	c.910C>T	p.His304Tyr	rs141245482	0.22; 0	0.45; 4	Probably damaging (0.958)	Deleterious (0.01)	Polymorphism (83)	22.9	Likely benign
5	*NLRP12*	c.2830C>A	p.Arg944=	rs104895570	0.28; 0	0.34; 3	–	–	Polymorphism	0.613	Likely benign
6	*PLCG2*	c.1274T>G	p.Phe425Cys	rs1166280990	0; 0	0.0008; 0	Possibly damaging (0.641)	Deleterious (0)	Disease causing (205)	22.9	VUS
7	*PLCG2*	c.1565C>G	p.Pro522Arg	rs72824905	0.28; 0	0.51; 8	Benign (0)	Tolerated (0.21)	Polymorphism (103)	17.37	Likely benign

[a]NCBI RefSeq: *NLRC4*, NM_001199139.1; *NLRP3*, NM_001243133.1; *NLRP12*, NM_144687.2; *PLCG2*, NM_002661.4. [b]Classification of pathogenicity of sequence variants based on the joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology: (*i*) benign, (*ii*) likely benign, (*iii*) variant of uncertain significance, (*iv*) likely pathogenic and (*v*) pathogenic.
CADD: combined annotation-dependent depletion; dbSNP: database for single nucleotide polymorphisms; gnomAD: genome aggregation database; hom: homocigotos; SIFT: sorting intolerant from tolerant; VUS: variant of uncertain significance.

Table III. Clinical features of patients carrying germline variants with allelic frequencies lower than 1% in public databases (1000 Genomes Project, ExAC and gnomAD)

Patient number/sex	Gene variant	Age at disease onset (years)	Recurrent sino-pulmonary infections	Time from cold exposure to symptoms onset (min)	Disease severity	Fever or other systemic symptoms	Cold urticaria triggers	Response to CSTs	Elevated inflammatory markers?[a]	Disease control	Disease duration (years)
1/ F	*NLRC4*	5	Yes	5	Type-II	Arthralgias	Water and air	Negative	No	Antihistamines	6
2/ M	*NLRP3, PLCG2*	35	No	5	Type-II	Abdominal pain	Water and air	Negative	No	Antihistamines	12
3/ F	*NLRP12*	54	No	10	Type-II	–	Water and air	Positive	No	Antihistamines	13
4/ F	*NLRP12*	44	No	10	Type-III	Arthralgias	Water and air	Negative	No	Omalizumab	7
5/ F	*NLRP12*	10	No	5	Type-II	–	Water, air and objects	Negative	No	Antihistamines	4
6/ M	*PLCG2*	5	No	10	Type-II	–	Water and air	Negative	No	Antihistamines	4
7/ F	*PLCG2*	14	No	5	Type-II	–	Water, air and objects	Positive	No	Antihistamines	25

[a]The evaluated inflammatory markers during active disease were the C-reactive protein and the erythrocyte sedimentation rate.
CSTs: cold-contact stimulation tests.

cold air, but not to cold objects, and they also experienced generalized cold urticaria, with one subject (33.3%) also referring abdominal pain during urticaria flares. The time from cold exposure to onset of symptoms was less than 10 min in all cases. None of these patients had associated autoimmune conditions or history of granulomatous skin disease or sinopulmonary infections during childhood. In addition, blood tests (including haemogram, CRP, ESR and serum electrophoresis) were within normal range during active disease. Regarding response to treatment, these 3 patients carrying VUS showed a satisfactory clinical response to antihistamines.

DISCUSSION

AIDs may present with a myriad of cutaneous lesions, including urticarial and maculopapular eruptions, pustules, ulcerative lesions, and granulomatous and erysipelas-like lesions (19). Urticarial rash, usually triggered and/or exacerbated by generalized cold exposure, represents the most prominent cutaneous feature in patients with CAPS, FCAS2, PLAID and NLRC4-inflammasomopathies (8–11, 20). In addition to skin lesions, patients with such conditions may have a great variety of systemic symptoms. Thus, patients with CAPS usually have arthralgias, myalgias, recurrent fever, headache or conjunctivitis (20), with the severest phenotypes also developing sensorineural hearing loss, central nervous symptoms and/or deforming arthropathy (20). The phenotype of NLRP12 and NLRC4-associated familial cold-induced autoinflammatory syndrome (FCAS) may resemble a mild form of CAPS (10, 21). Patients with PLAID exhibit long-standing cold urticaria, recurrent sinopulmonary infections, hypogammaglobulinaemia, autoimmunity and granulomatous skin disease (11, 22). Physicians should consider these diagnoses in patients presenting with a combination of the aforementioned symptoms in addition to further hints, including a positive family history, laboratory abnormalities (e.g. elevated inflammation markers during active disease) and poor response to conventional therapies. A selected gene mutation analysis will confirm the diagnosis, and a prompt and early treatment with specific drugs can dramatically improve the clinical manifestations (10, 23, 24).

The cold urticarial rash observed in patients with the aforementioned AIDs may be indistinguishable from that of ACU. However, it is convenient to remark that there are some features that may point to AIDs and help to distinguish them from ACU (8, 11, 22, 25, 26). In AIDs, cold-induced urticarial rash frequently starts during infancy or childhood and is lifelong (25, 26). These symptoms are usually triggered by generalized exposure to cold stimuli, such as cold air or cold water, rather than by contact with cold objects (11, 22). Their development is often delayed (> 1–2 h) after cold exposure, and is typically associated with negative results on conventional CSTs (8, 9, 11, 22, 25, 26). Clinically, the cold urticarial rash in AIDs has a broader spectrum of lesions, occasionally with urticarial features, but more commonly with erythematous and oedematous papules and plaques. This rash is rather symmetrically distributed on the trunk and/or extremities, usually sparing the head, rarely pruritic, and often described as tender and/or eliciting a burning sensation (8, 9). In contrast, ACU is characterized by the typical itchy wheal-and-flare-type skin reactions that are asymmetrically distributed on the body (1, 2, 5). The duration of single lesions, on average, is also longer in AIDs (up to 24 h) compared with ACU (minutes to hours) (25, 26). Finally, patients with AIDs do not respond to conventional urticaria therapies, such as antihistamines, leukotriene antagonists and/or omalizumab (8).

The pathophysiology of cold urticaria is yet to be fully elucidated, but the activation and degranulation of tissue-resident mast cells and circulating basophils with the subsequent release of inflammatory mediators have been shown to play key roles (6, 27). As yet, it is unclear exactly what causes such activation and degranulation. The most supported hypothesis is that different autoantigens, the expression of which might be induced by the appropriate environmental triggers, bind to IgE on the surface of mast cells and basophils through the high-affinity IgE receptor (FcεRI), resulting in the activation of these cells and the release of the inflammatory mediators (6, 27). In this sense, the FcεRI expression on effector cells has been found to be significantly upregulated in patients with ACU (28). In this complex process, nevertheless, no genetic susceptibility factors have yet been identified. Thus, to the best of our knowledge, this is the first study to investigate the presence of both germline and post-zygotic variants on genes previously described as associated with AIDs in a large cohort of patients with ACU.

Somatic gene mosaicism has been described as an important disease-causing mechanism in many AIDs (29–33). In particular, somatic *NLRP3* mosaicism has been shown to play an important role in the pathogenesis of patients with a clinical diagnosis of CAPS, but mutation negative by conventional genetic studies (29, 30). Some recent studies support the role of somatic mosaicisms in milder and/or later-onset of the disease (15, 34). The novel next-generation sequencing (NGS) technologies have been crucial to identify low-level somatic mosaicism and allowed achieving the definitive diagnosis and starting the appropriate anti-inflammatory treatment. In the present study, a deep search for somatic mosaicism using highly-sensitive NGS technology in the 4 candidate genes did not identify any candidate variant.

On the other hand, germline variants on the *NLRP3*, *NLRP12*, *NLRC4* and *PLCG2* genes with low allelic frequencies (<1%) in public databases were found in 7 of our 50 patients with ACU. Nevertheless, no pathogenic

or likely pathogenic variants were detected, and 4 out of 7 subjects carried variants classified as probably benign according to ACMG recommendations. By contrast, VUS were detected in the remaining 3 patients. Despite the fact that these patients exhibited certain clinical features that could resemble the cold urticaria symptoms associated with AIDs (developing a generalized urticaria (no patient was classified as type-I severity) triggered mostly by the exposure to cold water and/or cold air rather than cold objects, with a high proportion of atypical ACU), the exact causal role of such genetic variants is unknown. VUS have been described both in patients with recurrent inflammatory attacks and in healthy subjects, raising the question of whether these variants are silent polymorphisms or low-penetrance disease-associated mutations (35–37). It has been also postulated that these genetic variations might function as susceptibility alleles to inflammation rather than disease-associated mutations, causing an inflammatory phenotype in concomitance with other eventual environmental and/or genetic factors (12). However, there are several reasons that suggest that the genetic variants found in our study do not play a relevant role in these particular patients. First, all patients achieved control of the disease manifestations with conventional urticaria therapies (antihistamines and omalizumab), which support the involvement of mast cells and basophils in the pathophysiology of the disease, in contrast to AIDs in which there is no evidence for histamine release (25). In addition, the delayed cutaneous response of hours after cold exposure, which is a typical feature of AIDs, was not seen in any of these patients. The normal values of acute phase reactants during active disease and the absence of family history are also not characteristic of patients with AIDs. Finally, the presence of the same genetic variant in the – otherwise healthy – relatives of the analysed patients supports the non-pathogenicity of the findings. Taken together, these observations strongly support the fact that the detected variants in genes causing AIDs with cold-induced urticarial rash, particularly the *NLRP3, NLRP12, NLRC4* and *PLCG2* genes, are not responsible for the non-familial ACU cases reported here, and may represent incidental findings of the genetic analysis performed.

In summary, a careful and comprehensive medical history and physical examination should be performed in patients of any age presenting with cold-induced urticarial skin reactions. The presence of cold urticaria in addition to systemic manifestations, family history, laboratory abnormalities (e.g. elevated inflammation markers) and/or poor response to conventional therapies should alert physicians to the potential diagnosis of a monogenic AID. According to our results, ACU is not related to post-zygotic or germline pathogenic variants on the *NLRP3, NLRP12, NLRC4* and *PLCG2* genes. The present study also highlights the importance of being careful in the interpretation of the results of complex genetic studies in subjects without enough evidence to suspect an AID, in order to avoid false-positive diagnoses and the consequent overtreatment, given the high frequency of healthy carriers. Further studies focusing on the investigation of genetic susceptibility factors in patients with chronic urticaria would help a better understanding of the complex pathogenesis of the disease.

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