Variants in autoinflammatory diseases-related genes in a family with cold-induced autoinflammatory syndrome

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Abstract

Objective

To characterise what immunogenetic alterations are present in a Spanish family having several members with a familial cold-induced autoinflammatory syndrome (FCAS), a kind of autoinflammatory disease (AID).

Methods

We present the case of two sisters (cases 1 and 2) with a similar clinical picture since their childhood. The symptoms start after exposure to cold and consist of recurrent fever, papules or urticaria, and oedema in hands and fingers. The mother had similar symptomatology as her daughters, which remitted after her first pregnancy, whereas the father is healthy. The patients and their parents were genotyped in a panel of 14 candidate genes using Next-Generation Sequencing (NGS). Real-time PCR was used to quantify IL1β mRNA levels from LPS-stimulated monocytes. ELISA was used to measure the IL1β and IL18 concentrations in supernatants and sCD25 levels in sera. IL1β, IL4, IL6, IL8, IL10, IL17A, IL18 and TNF-α serum levels were assessed using xMAP® Technology.

Results

All the genetic variants found in this family are benign with two exceptions: NLRC4 *p.Leu339Pro (present in both cases and their mother) and* PSTPIP1 *p.Gln219His (present in Case 1 and her father). The monocytes stimulated of the individuals with the NLRC4 variant produce higher levels of IL1β (protein and mRNA). Levels of TNF-α, IL4, and IL6 were higher in Case 1 than in the age-matched controls.*

Conclusion

The familial segregation and the clinical picture compatible with FCAS suggest that NLRC4 p.Leu339Pro causes the AIDs syndrome diagnosed in several family members.

Key words

autoinflammatory diseases, familial cold-induced autoinflammatory syndrome, next generation sequencing, NLRC4 variants, functional studies

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Introduction

Autoinflammatory diseases (AIDs) are monogenic hereditary entities with an innate immune system impaired function. This loss of control, by alteration of different pathways, results in Caspase-1 hyperactivation and the generation of cytokines. From a clinical perspective, they are disorders characterised by recurrent or persistent systemic or organ-specific unprovoked inflammation (1, 2). Usually, patients have recurrent fever episodes with other unspecific symptoms (abdominal, cutaneous, articular, etc.) that make difficult the clinical diagnosis (3). Diverse studies have provided evidence of the critical role that inflammasome-related genes play in the pathogenesis of AIDs (4). The inflammasomes are multimeric protein complexes that require two signals for their activation. The first is the induction of gene expression through the transcriptional factor NFκB and the second is the assembly of a complex that concludes in activation of caspase-1 and the IL1β/IL18 release and pyroptosis (5). Currently, various inflammasomes are known (6).

The introduction of next-generation sequencing (NGS) procedures has driven the molecular diagnosis development in these diseases (7). However, there is considerable incertitude about the clinical significance of multiple variants and diverse examples of a lack of correlation between the genotype and the clinical phenotype (8, 9). Variants in several genes are associated with the familial cold-induced autoinflammatory syndrome (FCAS), an AID with autosomal dominant inheritance. *NLRP3* is the best-known associated gene, although others, such as *NLRP12*, *NLRC4*, and *PLCG2*, are related to this disease (10-13). We present a genetic and functional study in a Spanish family with several members affected by FCAS.

Materials and methods

All the samples were obtained in the Hospital Universitario Virgen del Rocío, The Hospitales Universitarios Virgen Macarena and Virgen del Rocío ethical committee approved the study. All the participants provided written informed consent before their enrolment in the study.

Clinical description of the cases

They are two sisters with a similar clinical picture, although the symptoms are more pronounced in Case 1.

Case 1 is a woman, 18-year-old at diagnosis. She presents from birth: recurrent fever (37.5º – 38ºC), papules or urticaria with or without pruritus in limbs and nose, occasionally with oedema in hands and fingers. The symptoms begin 1-4 hours after exposure to cold or temperature changes and resolve 12-24 hours later, with 1–3 attacks during the cold season (December-January). The clinical picture disappears or improves with heat, but it does not respond to corticosteroid or antihistamine therapy. Analytical values in asymptomatic periods were in a normal range. Tests included the haemogram, biochemical (including acute phase reactants), coagulation parameters, serum immunoglobulins, complement components, and serum tryptase levels. Antinuclear and antithyroid autoantibodies and the ice cube test performed twice were negative. Antinuclear and antithyroid autoantibodies and the ice cube test performed twice were negative.

Case 2 is a woman, 23 years old at diagnosis. She has a history of mild persistent allergic rhinitis due to sensitisation to mites and pollen. Since the age of 8, she has had fever episodes (37.5ºC) associated with general malaise, headache and dizziness, papules and urticaria (usually not pruritic) in limbs, oedema in fingers, and polyarthralgia. Like her sister, the symptoms begin 1–4 hours after exposure to changes in temperature or cold, lasting up to 24 hours with a similar frequency. Her laboratory test results were analogous to her sibling, and the ice cube test result was always negative. This patient had been treated unsuccess with corticosteroids and antihistamines. She started anti-IL1R therapy (100 mg/0.67 ml per day) in cold seasons, remaining asymptomatic from then. None of the patients reports an allergy to drugs or food.

Concerning the family, the mother had similar symptomatology as her daughters, although remitted after her first

pregnancy, when she was 26, whereas the father is healthy. Regarding the maternal family, the grandfather had symptoms similar to those of his daughter and granddaughters. A maternal uncle has articular symptomatology that is not related to cold.

Next-generation and Sanger sequencing study

To obtain genomic DNA QIamp DNA mini kit (Qiagen, Barcelona, Spain) was used according to the manufacturer's instructions with 200 μl of peripheral blood of each individual as starting material. DNA preparations were quantified in a Qubit® 3.0 fluorometer and diluted to 0.67 ng/µl. A custom-designed NGS gene panel (AmpliSeq™ software, Ion Torrent, Thermo Fisher Scientific, Waltham, MA) targeting all the coding regions and flanking intronic sequences of 14 AIDs-related genes was used in this study: *CECR1* (ADA2), *IL1RN* (NM_173842.2, DIRA), *LPIN2* (*NM_014646*.2, Majeed Syndrome), *IL36RN* (NM_173170.1, DITRA), *MEF*V (NM_000243.2, FMF), *MVK* (NM_000431.3, HIDS), *NLRC4* NM_021209.4 AIFEC, FCAS4), *NLRP3* (NM_001243133.1, CAPS, FCAS1), *NLRP12* (NM_144687.2, Guadalupe Syndrome, FCAS2), *NOD2 (*NM_022162.2, CD/BS/EOS), *PLCG2* (NM_002661.5, *APLAID, FCAS3), PSTPIP1* (NM_003978.3, PAPA), *TMEM173* (NM_198282.3, SAVI), *TNFRSF1A* (NM_001065.3, TRAPS). The 14 gene panel generated 236 amplicons with 44,024 base pairs. Concerning quality, there were more than 20 readings for 99.15% of the amplicons and more than 100 for 98.31%. Variant calling was performed with Ion Reporter™ Software v. 5.0, using a mutation detection and quantification pipeline implemented in the software. The human genome, hg19, was used as a reference to align the readings. Variants reported by Ion Reporter were verified and filtered by visual inspection on Integrative Genomics Viewer IGV v. 2.8.13, Broad Institute, to check the missense mutation presence and to perform a confirmation of the reading alignments.

To verify the presence of new variants and to investigate the presence of the *NLRC4* variant in the patients' uncle, performed a Sanger sequencing with BigDye™ Direct Cycle Sequencing Kit according to the manufacturer's instructions using primers generated by Thermo Fisher NGS Sanger confirmation software on Ion Reporter.

According to Infevers (*https://infevers.umai-montpellier.fr/web/*) and the American College of Medical Genetics and Genomics (ACMG) criteria (using Varsome, https://varsome.com/) variants were catalogued as Benign (B), Likely Benign (LB), Variant of Uncertain Significance (VUS), likely pathogenic (LP), and pathogenic (P).

Monocytes isolation and culture conditions

Monocytes were isolated from peripheral blood of the individuals with Dynabeads® FlowComp™ Human CD14 (Invitrogen) when asymptomatic and without treatment. Cells were resuspended to a concentration of $5x10^7$/mL in RPMI1640, supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 20 ng/mL of gentamycin, and 5 ng/mL of GM-CSF. For each sample, into two wells of a 96-well U plate, 200 µL of the cell suspension (10^4 monocytes) were dispensed, one of the wells treated with phosphate buffer saline (PBS) and the other with 0.1 ng/mL lipopolysaccharide (LPS). The cultures were incubated for 19 h with 5% CO2 at 37ºC. Plates were centrifugated at 3,000 r.p.m, 10s to separate supernatants and cell pellets, using the supernatants to quantify IL1β and IL18, and cell pellets to isolate RNA.

Quantification of IL-1β mRNA levels

The RNA was isolated from the cell pellets with the PicoPure RNA isolation kit (Arcturus). Each RNA was used in a retrotranscription to obtain cDNA with SuperScript IV VILO. IL-1β mRNA expression assay was carried out with TaqMan probes (Hs01555410_m1) using β2-microglobulin as a housekeeping gene (Hs00187842_m1 assay). To calculate the relative mRNA levels used the 2-ΔΔCt method with the values of the corresponding PBS-wells as calibrators.

Quantification of IL1β and IL18 in supernatants

The quantification of the protein levels in the supernatants was assessed in the triplicated samples with the following assays: Human IL1β ELISA Kit (Invitrogen. Assay range 3.9-250 pg/ml) and MBL Human IL18 ELISA Kit (Medical and Biological laboratories Co Ltd, Japan. Sensitivity 12.5 pg/ml).

Quantification of cytokines and CD25s in sera

Sera levels of IL1β, IL4, IL6, IL8, IL10, IL17A, and TNF-α in all the members of the family and 20 healthy controls matched in age (older than 18-years) were quantified using a cytokine array with xMAP technology (MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel, Merck KgaA, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 25 µl of serum of each individual were mixed with colour-code microspheres coated with specific capture antibodies. After overnight incubation at 4ºC and the subsequent addition of the second antibody and the fluorescent tag (streptavidin-phycoerythrin), the results are read in a LABscan 3D and interpreted with the xPONENT software. Concentrations (pg/mL) of each cytokine are calculated by extrapolating data in a standard curve. For quantification of the IL18 in serum used the same technology and procedure but an independent assay (MILLIPLEX MAP Human IL-18 Singleplex Magnetic Bead Kit, Merck). Cytokines levels in healthy controls were used to establish the normal range. The levels of sCD25 were investigated in the sera of the four family members using the Human IL2sRα ELISA Set (Becton Dickinson Pharmingen, San Diego, CA, USA. Sensitivity 7.8 pg/ml).

Results

Table I displays the genetic variants found in each family member in the 14 genes studied, and it shows the clinical significance of each variant according to Infevers (https://infevers.umai-montpellier.fr/web/) and the ACMG criteria. All the variants found in this family are benign with two exceptions:

Table I. Genetics variants found in the 14 genes studied in this family and their classification.

Genes	Father	Mother	Case 1	Case 2	ID	Infevers	ACMG	MAF EUR
ADA2	H335R	H335R	H335R	H335R	rs2231495[1]	V-B	B	0.33
IL1RN	No variant	No variant	No variant	No variant				
IL36RN	No variant	No variant	No variant	No variant				
LPIN2	No variant	No variant	No variant	No variant				
MEFV	R202Q	No variant	R202Q	R202Q	rs224222[1]	V-B	B	0.28
MVK	No variant	S52N	No variant	S52N	rs7957619[1]	V-B	B	0.15
NLRP3	No variant	No variant	No variant	No variant				
NLRC4	No variant	L339P	L339P	L339P		NV-LP	LP	Unkown
NLRP12	No variant	No variant	No variant	No variant				
NOD2	No variant	P268S R702W	P268S	P268S R702W	rs2066842[1] rs2066844	V-LB V-LB	B B	0.24 0.05
PLCG2	No variant	No variant	No variant	No variant				
PSTPIP1	Q219H	No variant	Q219H	No variant	rs139362350	NC	VUS	0.0002
STING1	H232R R293Q/G230A/R71H	H232R	H232R	H232R	rs1131769[1] rs7380824[1,2]/rs 78233829[1,2]/rs 11554776[1,2]	NR NR/NR/NR	B B/B/B	0.85 0.15/0.15/0.15
TNFRSF1A	No variant	No variant	No variant	No variant				

[1]MAF in EUR >0.05
[2]Polymorphisms in linkage disequilibrium (D=1.0).
All the variants were in heterozygosis with one exception, NOD2 P268S, homozygous in the mother.
B: benign; V-B: validated benign; V-LB: validated-likely benign; VUS: variant of uncertain significance; NV-LP: not validated-likely pathogenic; LP: likely pathogenic; NC: not classified; NR: not reported.

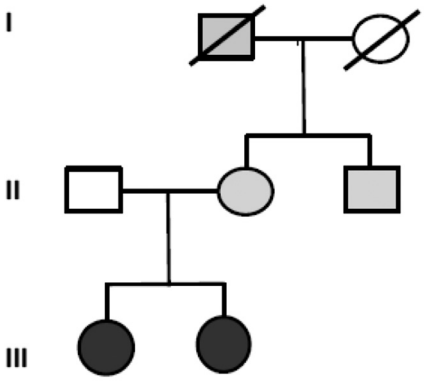

Fig. 1. Familial pedigree.
I. Maternal grandparents. The grandfather had symptoms FCAS compatible, but he could not be included in the study.
II. Parents. Father is healthy, and he has the variant *PSTPIP1* p.Gln219His. Mother had similar symptomatology as her daughters until her first pregnancy, and she has the variant *NLRC4* p.Leu339Pro. The mother's brother has the same variant and some articular symptomatology but not related to cold.
III. Patients. They are both females with a similar clinical picture compatible with FCAS. Both have the variant *NLRC4* p.Leu339Pro. Only Case 1 has the variant *PSTPIP1* p.Gln219His.

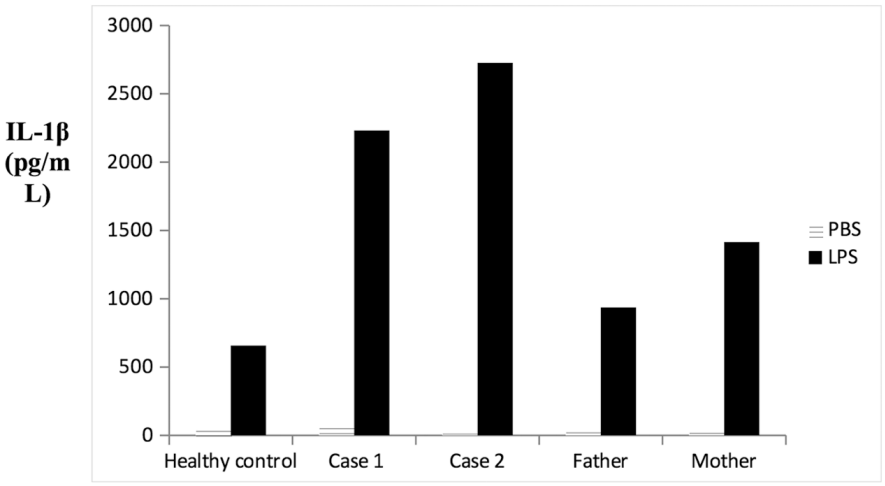

Fig. 2. IL1β levels in monocyte supernatants.
IL1β levels in supernatants of monocyte cultures before and after stimulation with LPS.
The study includes all the members of the family and an unrelated healthy individual as negative control.

NLRC4 p.Leu339Pro and *PSTPIP1* p.Gln219His. *NLRC4* p.Leu339Pro detected in Case 1, Case 2 and their mother is LP according to the Infevers and ACMG criteria. Concerning *PSTPIP1* p.Gln219His carried by Case 1 and her father, it is not classified in Infevers and considered VUS with the ACMG criteria. Figure 1 displays the familiar segregation data of the genetic variants and clinical features.

Regarding the rest of the studies, no differences in concentration of IL1β among unstimulated (PBS) supernatants were detected (HC: 60.5 pg/mL, Case 1: 79.3 pg/mL; Case 2: 39.15 pg/mL, father: 49.1 pg/mL, mother: 45.8 pg/mL). After stimulation with LPS, levels of IL1β augmented in all the individuals, although the lowest values correspond to the father (HC: 657.3 pg/mL, Case 1: 2231.6 pg/mL; Case 2: 2726.7 pg/mL; father: 936.4 pg/mL and mother: 1415.3 pg/mL) (Fig.

2). Regarding the levels of expression of mRNA of IL1β, the relative quantity of mRNA in cultures with LPS was 10 fold-times in case 1: 1067.5, case 2: 734.2, and mother: 648.1 than in father: 80.4. (Fig. 3). Concerning IL18, the observed values were similar in all the samples after LPS stimulation (Case 1: 298.8 pg/ml, Case 2: 154.6 pg/ml, father: 351.1 pg/ml, and mother: 187.9 pg/ml) (Fig. 4). Regarding the cytokine serum values, Case 1 presented increased levels of TNF-α, (14.14 pg/mL), IL4 (270.22 pg/mL), and IL6 (44.73 pg/mL) (upper than the mean in controls+2SD. Healthy control values: TNF-α, 7.18±2.38 pg// mL, IL4 30.33±22.58 pg/mL, and IL6 6.08±4.75 pg/mL). The rest of the cytokine values in all the family members are comparable to those obtained in the healthy controls. The concentration of CD25s in serum was similar in all the family members (Case 1: 2.98 ng/ml, Case 2: 2.12 ng/ml, father: 2.54 ng/ml, and mother: 2.18 ng/ml) and, all of them were in a normal range (up to 7.5 ng/mL).

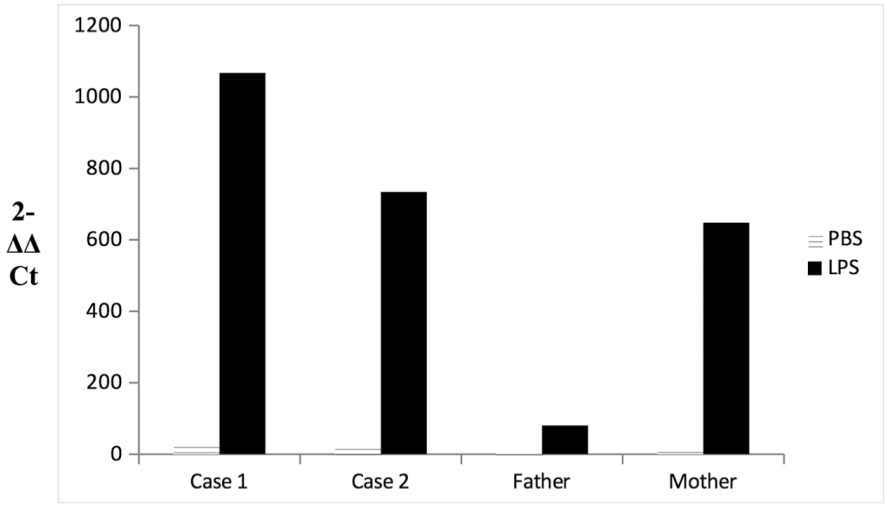

Fig. 3. Relative mRNA IL1β expression in the members of the family.
IL1β mRNA expression in monocyte cultures before and after stimulation with LPS. The study includes all the members of the family.
The relative mRNA levels were calculated with the 2-ΔΔCt method using the values of the corresponding PBS-wells as calibrators.

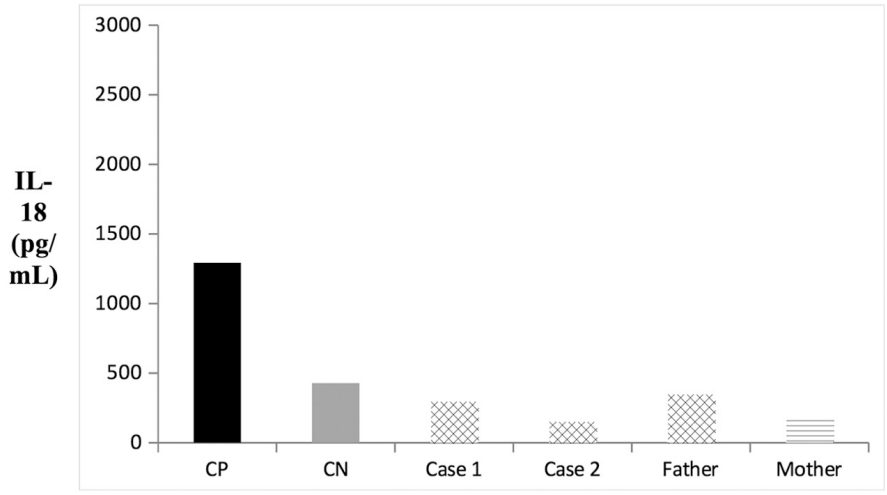

Fig. 4. IL18 levels in monocyte supernatants after LPS stimulation.
IL18 levels in supernatants of monocyte cultures after stimulation with LPS.
The study includes all the members of the family, and two unrelated healthy individual as negative (CN) and positive controls (CP).

Discussion

The most relevant result in this study is the description of two new missense and rare variants, *NLRC4* p.Leu339Pro and *PSTPIP1* p.Gln219His, in this Spanish family with two members having an FCAS-like phenotype. These two new variants, not previously reported to Infevers or ClinVar databases, are not present in any of the more than 1,000 individuals sequenced in our laboratory. The clinical picture of the AID found in this family was compatible with FCAS, a disease with an autosomal dominant pattern. The family branch from which the disease trait comes seems to be the maternal one. The mother had similar symptoms until the first pregnancy, and the maternal grandfather suffered from a similar disease (family self-reporting), but the father is healthy. This maternal inheritance pattern suggests *NLRC4* p.Leu339Pro as the causal variant, although the maternal uncle with rheumatological clinical symptoms FCAS-unrelated also bears this variant. Although *NLRC4* p.Leu339Pro is LP according to Infevers and the ACMG Criteria, the biological significance of this variant has to be validated because there is no evidence of association with pathology demonstrated by functional assays or by family segregation in other studies.

The genetic analysis of the members of the present family discarded variants in *NLRP3* (FCAS1), the best known of the genes involved in FCAS, *NLRP12* (FCAS2) and *PLCG2* (FCAS3). *NLRC4* gene, previously related to FCAS, encodes a cytoplasmic NOD-like receptor. Its stimulation recruits and proteolytically activates Caspase-1 within the inflammasome, particularly in response to microbial flagellins. This molecule has an N-terminal domain that is the caspase activation and recruitment domain (CARD); a nucleotide-binding and oligomerisation domain (NOD) that comprises a nucleotide-binding domain (NBD), a helicoidal-domain 1 (HD1), and a winged helicoidal-domain (WD). The last two domains are HD2 and the C-terminal domain c (LRR) (14). The ADP-mediated interaction between the NBD and the WD is critical for stabilising the closed conformation of

NLRC4. One of the control mechanisms of the activation of this inflammasome is the molecule structure itself so that the gain of function variants of the gene are associated with AIDs. The NOD region forms an ADP-binding pocket which maintains the molecule in its inactive conformation. Variants in the NOD area will cause spontaneous activation of the inflammasome if they alter the molecular structure (14, 15). The variant NLRC4 p.Leu339Pro is located in HD1.

Different studies report a relationship between *NLCR4* variants and several entities with different severity. For instance, there are variants in this gene associated with enterocolitis and autoinflammation (AIFEC) and macrophage activation (MAS) syndromes. A deep spectrum of clinical symptoms and severity have been found even in members of the same family bearing identical variants (16-23). Several studies have reported the association between *NLCR4* mutations and clinical features of FCAS: Kitamura *et al*. described a variant in WD (p.His443Pro) in a Japanese family with several members affected (13). Wolker-Touw *et al*. reported another variant in WD (p.Ser445Pro) in a large pedigree with phenotype variable but, in general, similar to CAPS (24). Karacan *et al*. found a variant also in HD1 (Arg310Stop) in a patient with a provisional diagnosis of CAPS/TRAPS (8). At present, the patients with compatible clinical features and *NLCR4* mutations, which is the case of this family, are classified as Familial Cold Autoinflammatory Syndrome 4 (FCAS4).
(https://www.malacards.org/card/familial_cold_autoinflammatory_syndrome_4).

Regarding the rest of the genetic variants found in the family, they are all well-known polymorphisms or variants classified as benign/likely benign. Nevertheless, we cannot discard the contribution of other variants to the susceptibility or modification of the course of the disease. Only in Case 1, the family member with the most severe symptoms and the only one with altered levels of some inflammatory cytokines, *NLRC4* p.Leu339Pro coexists with the other new missense-rare variant described in this family, *PSTPIP1* p.Gln219His, classified as VUS according to ACMG criteria. Variants in *PSTPIP1* are related to pyogenic sterile arthritis, pyoderma gangrenosum, and acne and hyperzincemia syndromes (PAPA) (25). Case 1 and her father (who bears the variant in *PSTPIP1*) did not have clinical features compatible with this disease.

Regarding the functional study, levels of IL1 in the supernatant of monocytes stimulated with LPS correlated with the variant p.Leu339Pro in *NLRC4*. The father (with neither the variant nor symptoms) had the lowest levels of IL1. The mother (with the variant but asymptomatic) had intermediate values between the father and the daughters (with the variant and symptoms). These results suggest a spontaneous activation of the inflammasome without a second signal in the cases and their mother. We cannot explain the spontaneous clinical remission in the mother, but she could have developed additional regulatory mechanisms during her first pregnancy (post-translational modifications: phosphorylation, ubiquitination, etc.) (25). These changes could determine a drop in the spontaneous production of IL1 below the clinical symptom threshold. LPS and IL1 provide the first signal for mRNA and pro-IL1 production. Therefore, the undetectable mRNA levels in the father would correspond to an early degradation (4 h) after stimulation only with LPS (26). Nevertheless, in the cases and their mother, the IL1β mRNA levels may be maintained by autocrine stimulation. Although variants in *NLRC4* have been associated with high concentrations of IL18 and sCD25, we found similar levels of these molecules in patients and controls, possibly this relationship is specific to MAS, which is not the clinical condition found in this family (18, 19, 21, 24).

NGS permits us to screen several genes simultaneously and increase our knowledge about the genetic bases of different pathologies quickly helping to diagnose even in cases with clinical features matching with several syndromes (27).

In conclusion, our results suggest a new variant, p.Leu339Pro, in *NLRC4* causes the AIDs syndrome diagnosed in several members of this family.

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