Somatic *NLRP3* mosaicism in Muckle-Wells syndrome.

A genetic mechanism shared by different phenotypes of cryopyrin-associated periodic syndromes

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Abstract

Familial cold autoinflammatory syndrome, Muckle-Wells syndrome (MWS), and chronic infantile neurologic, cutaneous and articular (CINCA) syndrome are dominantly-inherited autoinflammatory diseases associated to *gain-of-function NLRP3* mutations and included in the cryopyrin-associated periodic syndromes (CAPS). A variable degree of somatic *NLRP3* mosaicism has been detected in ≈35% of patients with CINCA. However, no data are currently available regarding the relevance of this mechanism in other CAPS phenotypes.

Objective

To evaluate somatic *NLRP3* mosaicism as the disease-causing mechanism in patients with clinical CAPS phenotypes other than CINCA and *NLRP3* mutation-negative.

Methods

NLRP3 analyses were performed by Sanger's sequencing and by massively parallel sequencing. ASC-dependent NF-κB activation and transfection-induced THP-1 cell death assays determined the functional consequences of the detected variants.

Results

A variable degree (5.5-34.9%) of somatic *NLRP3* mosaicism was detected in 12.5% of enrolled patients, all of them with a MWS phenotype. Six different missense variants, three novel (p.D303A, p.K355T, and p.L411F), were identified. Bioinformatics and functional analyses confirmed that they were disease-causing, *gain-of-function NLRP3* mutations. All patients treated with anti-IL-1 drugs showed long-lasting positive responses.

Conclusions

We herein show somatic *NLRP3* mosaicism underlying MWS, probably representing a shared genetic mechanism in CAPS not restricted to CINCA syndrome. The data here described allowed definitive diagnoses of these patients, which had serious implications for gaining access to anti-IL-1 treatments under legal indication and for genetic counseling. The detection of somatic mosaicism is difficult when using conventional methods. Potential candidates should benefit from the use of modern genetic tools.

Key words.

Cryopyrin-associated periodic syndromes; CAPS; *NLRP3*; somatic mosaicism; massively parallel sequencing

Cryopyrin-associated periodic syndromes (CAPS) are a group of autoinflammatory diseases that include familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurologic, cutaneous and articular (CINCA) syndrome, also known as neonatal-onset multisystem inflammatory disease (NOMID) (1). Some clinical features are shared by almost all CAPS phenotypes (i.e. onset during childhood, an urticaria-like skin rash) whereas others are restricted to certain phenotypes (i.e. AA amyloidosis in MWS, destructive arthropathy in CINCA-NOMID) (1). CAPS are caused by dominantly-inherited or *de novo NLRP3* mutations (2-4). This gene encodes for cryopyrin, a component of one of the cytosolic complexes named inflammasomes that generates the active form of interleukin-1ß (IL-1ß) (5). Previous studies showed a *gain-of-function* behavior for those *NLRP3* mutations associated with CAPS because they provoke an uncontrolled IL-1ß overproduction, representing the basis from which to treat these patients with anti-IL-1 drugs (3, 6). Genetic heterogeneity was suggested in CINCA-NOMID because only ≈55% of patients were *NLRP3* mutation-positive (3-4). The use of novel genetic methods recently detected somatic *NLRP3* mosaicism in ≈35% of patients with CINCA-NOMID (7-8). However, no data are currently available about the role of this genetic mechanism in other CAPS phenotypes because genetic heterogeneity has hitherto been scarcely reported in previous studies.

We herein show the causal role of somatic *NLRP3* mosaicism in patients with MWS in whom previous studies did not detect *NLRP3* mutations, suggesting that this genetic mechanism is shared among the different CAPS phenotypes.

Patients & Methods

Patients.

For this study we enrolled patients with a clinical suspicious of CAPS, with a phenotype of Muckle-Wells syndrome and overlapping syndromes, and *NLRP3* mutation-negative in previous studies. The clinical inclusion criteria were the presence of an urticaria-like skin rash and at least one of the

following symptoms: recurrent fever, recurrent arthritis, recurrent aseptic meningitis, sensorineural deafness or AA amyloidosis (See supplementary Table S1 for details). All patients with a CINCA-NOMID phenotype were excluded. The patients' data were collected by direct interviews and chart reviews. Written-informed consent from patients (or patients' parents if younger than 18 years old) was obtained at each institution. The ethics committees of Hospital Clinic, Barcelona and the Graduate School of Medicine, Kyoto University approved this study, which was conducted in accordance with the Helsinki Declaration.

***NLRP3* analyses.**

These analyses were performed in the Graduate School of Medicine, Kyoto University or in the Hospital Clínic, Barcelona. Genomic DNA was obtained from whole peripheral blood using QIAmp DNA Blood Mini Kit (QIAgen, Germany). For Sanger's sequencing all exons of *NLRP3* gene were amplified by polymerase chain reaction (PCR) using the primers and conditions previously described (2). The PCR amplicons were purified with Illustra ExoStar 1-Step kit (GE Healthcare, USA), bidirectionally fluorescence sequencing using ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and run on an automated ABI 3730XL DNA analyzer. For massively parallel DNA sequencing, all exons of *NLRP3* gene were amplified as previously described (8). Library preparation and emulsion PCR were performed according to manufacturer's instructions. All sequencing runs were performed on the GS Junior 454 Sequencer using the GS Junior Titanium Sequencing kits (Roche, Switzerland). The obtained sequences were analyzed using the Amplicon Variant Analyzer software.

Bioinformatics analyses.

In silico sequence analyses were performed using two different algorithms. The Sorting Intolerant from Tolerant (SIFT) is a sequence homology-based tool that predicts whether the amino acid substitution is or is not probably damaging by reporting a score. The PolyPhen-2 is a tool for prediction of the possible impact of an amino acid substitution on the structure and function of a

protein, and qualitatively appraised as benign, possibly damaging or probably damaging (9-10).

Functional studies.

The functional consequences of the novel *NLRP3* variants were evaluated in two *in vitro* assays (11). Wild-type and mutant *NLRP3* cDNA, obtained by mutagenesis PCR, were subcloned into the expression vectors pEF-BOSEX and pcDNA5/TO (Invitrogen, USA). The ASC-dependent NF-κB activation was evaluated using a dual-luciferase reporter assay in HEK293FT cells transfected with *NLRP3*-pEF-BOSEX plasmids with an NF-κB reporter construct (pNF-kB-luc, BD Biosciences) and an internal control construct (pRLTK, Toyo Ink) in the presence or absence of ASC-expression plasmid. To evaluate the necrosis-like cell death, the THP-1 cell line was transfected with GFP-tagged *NLRP3*-pcDNA5/TO plasmids. After 4 hours, cells were stained with 7-aminoactinomycin D and cell death of GFP positive cell was analyzed by FACS Caliber (Becton-Dickinson).

Statistical analyses.

Continuous variables are presented as the mean ± SD or as the median and interquartile range, while categorical variables are presented as numbers, ratios and/or percentages. To detect potential differences among patients with germline mutations and with somatic mutations, the U-Mann Whitney test was used for continuous variables and Fisher's exact test was used for categorical variables.

Results

Genetic analyses.

Fifty-six patients (23 Japanese and 33 Spanish) who fulfilled the inclusion criteria were enrolled. Sanger's sequencing of the *NLRP3* gene did not identify mutations in any patients. However, small peaks with reduced signal intensities compared with controls were detected in two patients: the A-to-C transversion at c.908 position in Patient 1, and the A-to-G transition at c.1000 position in

Patient 2, which encode for the p.Asp303Ala and p.Ile334Val cryopyrin variants, respectively (Figure 1-A and Table 1). Massively parallel DNA sequencing was performed in all patients and revealed somatic *NLRP3* mosaicism in seven patients (7/56; 12.5%). Six different nucleotide changes, all of them located in the exon 3, were detected, and their frequency varied notably among patients, ranging from 5.5% to 34.9% (Table 1). All *NLRP3* variants encode for nonsynonymous amino acid changes, being three of them novel (p.Asp303Ala, p.Lys355Thr, and p.Leu411Phe) and the remainder already described (p.Ile334Val, p.Phe523Leu and p.Glu567Lys) (Figure 1-B). In Patient 4 the frequency of the mutated *NLRP3* allele remained identical in blood samples obtained over an 8 year period (Table 1).

Bioinformatics and functional analyses.

All missense *NLRP3* variants were predicted to be possibly or probably damaging to cryopyrin structure and/or function according to at least one of the two algorithms employed, with the only exception of p.Glu567Lys variant (Table 1). Interestingly, this *NLRP3* variant was twice detected in the unrelated patients with somatic mosaicism, and has been also reported in other CAPS patients, reasonably supporting its pathogenic effect (7, 11). We did not find any of the detected *NLRP3* variants in two groups of ethnically-matched healthy individuals (Japanese controls n: 200 chromosomes; Spanish controls n: 500 chromosomes) nor in the database NCBI dbSNP Build 137 (Table 1), reasonably ruling out that they could be rare gene polymorphisms.

Finally we evaluated their functional consequences by two different *in vitro* assays. The results showed that all *NLRP3* variants induced both ASC-dependent NF-κB activation (Figure 1-C) and necrosis-like programmed cell death of THP-1 cell line (Figure 1-D) at similar or higher level than those induced by other well-known disease-causing mutations (p.R260W, p.D303N and p.Y570C). Altogether, these data clearly support a pathogenic effect for all *NLRP3* mutations detected as somatic mutations in the enrolled patients.

Clinical features of patients with somatic *NLRP3* mosaicism.

At the time of inclusion in the study, the clinical diagnosis of patients with somatic *NLRP3* mosaicism was compatible with MWS. Neither consanguinity nor familial history of the disease were reported in any of them. The inflammatory disease started during their infancy or childhood (median: 4 years; interquartile range: 1.3-9.0 years), with an urticaria-like skin rash and a marked inflammatory acute response as the main features at that time (See Table 2 for clinical details at the disease onset).

All patients referred to the chronic course of their disease, with variable disease evolution (median: 20 years; interquartile range: 12-26 years). During this time, recurrent arthritis (6/7; 85.7%), headache (5/7; 71.4%) and recurrent conjunctivitis (4/7; 57.1%) mainly added to those features detected at the disease onset. None of these patients developed AA amyloidosis, whereas 5 of them (71.4%) developed progressive bilateral sensorineural deafness. (See Table 3 for a detailed summary of clinical features detected during the course of the disease).

Outcome of anti-IL-1 blockade.

Five patients with somatic *NLRP3* mosaicism were treated with anti-IL-1 drugs. Patient 5 was only treated with anakinra (100 mg/24 hr s.c. during 20 months). Three patients only received canakinumab: Patient 2 (150 mg/8 weeks s.c. during 13 months), Patient 3 (2 mg/kg/8 weeks s.c. during 16 months), and Patient 6 (initial dose of 150 mg/4 weeks, subsequently increased up to 300 mg/4 weeks, during 14 months). Patient 7 was first treated with anakinra (1 mg/kg/24 hr s.c. during 24 months) and subsequently switched to canakinumab (150 mg/8 weeks s.c during 14 months). All patients showed a marked and sustained improvement while treated with anti-IL-1 drugs, with a complete remission of urticaria-like skin rash (5/5), fever (3/3), conjunctivitis (2/2) and aseptic meningitis (1/1), and marked benefits for arthritis (complete response in 75%) and headache (complete response in 75%, and marked improvement in 25%). Inversely, IL-1 blockade did not improve the sensorineural deafness (0/4). This clinical improvement was associated with sustained reductions of ESR and CRP level, and normalization of white blood cell, neutrophil, and platelets counts, and hemoglobin level (See Figure 2 for details).

Comparative phenotype analyses.

To identify potential clinical differences among patients with germline or with somatic *NLRP3* mutations two cohorts of MWS patients were compared. The group of MWS patients with somatic *NLRP3* mosaicism included the 7 patients here described whereas the cohort of MWS patients with germline mutations included 41 patients (13 Japanese and 28 Spanish) from our databases. In this last group the germline status was established by means of pedigree analyses and/or by massively parallel sequencing. As expected, the familial history of the disease was a significant variable between the two groups. No significant differences were detected among the main clinical features (fever, urticaria-like rash, joint, neurological and ocular involvements, and deafness) despite their variable frequency in each group (See Table 4 for details). However, patients with somatic *NLRP3* mosaicism seemed to have late onsets of the disease and of the neurosensorial deafness, an increased incidence of arthritis, and a reduced risk of developing AA amyloidosis when compared to patients with germline mutations.

Discussion

CINCA-NOMID syndrome represents the severest CAPS phenotype, and is usually a consequence of *de novo NLRP3* mutations. Recent works have established its genetic basis, with ≈55% of patients carrying germline *NLRP3* mutations and ≈35% carrying somatic *NLRP3* mosaicism (3-4, 7, 11-16). However, no studies addressing the presence of somatic *NLRP3* mosaicism have been undertaken in other CAPS phenotypes because genetic heterogeneity has been poorly described in them, with only five reported *NLRP3* mutation-negative MWS patients (17-19). This scenario prompted us to hypothesize that somatic *NLRP3* mosaicism might be an underlying genetic mechanism in patients with other CAPS phenotypes. For this proposal two ethnically different cohorts of candidates were screened, and 12.5% of them (7/56) carried variable degree of somatic *NLRP3* mosaicism in peripheral blood. Additional evidences, as shown here, definitively support that the detected *NLRP3* variants are pathogenic and include their absence in panels of ethnically

matched controls and in a database of genomic diversity, *in silico* analyses that predict their damaging effect for the function and/or structure of cryopyrin, and *in vitro* functional studies that clearly showed its *gain-of-function* behavior. Taken together these evidences support that somatic *NLRP3* mosaicism is a genetic mechanism shared by different CAPS phenotypes, and it is not restricted to CINCA-NOMID syndrome.

Among *NLRP3* mutations detected 50% (3/6) were novel, representing an unexpected high proportion for a small cohort. Taking into account their consequences on the cryopyrin function it is conceivable to hypothesize that, in germline status, they could be incompatible with life. We have also found a marked variability in the degree of somatic mosaicism among patients, which may have important consequences. For diagnostic purposes the level of somatic mosaicism could be the determining factor in achieving a definitive genetic diagnosis. Those patients with mosaicism around, or higher than, 15% will probably be detected in conventional studies using Sanger's method by means of careful analyses, as we have shown in the patients' chromatograms. However, those patients with frequencies of less than 15% are probably missed by Sanger's sequencing and will only be detected by using new technologies that are not currently widely available. The differences of disease severity observed among patients with somatic mosaicism, including those from this study and those from previous reports, could be explained by different and cumulative factors, which probably cannot be independently analyzed. These factors might include, at least, the type of amino acid exchange, its location in the cryopyrin, its functional consequence in the normal cryopyrin function, and the degree and tissue distribution of somatic mosaicism. We must also note that all known somatic *NLRP3* mutations seem to be located in some few amino acid residues (303, 355, 567) or in small regions of cryopyrin (303-307, 433-439 and 566-570), probably representing hot-spots for these types of mutations. Consequently these regions should be carefully analyzed when using Sanger's sequencing to identify potential carriers of somatic mosaicism.

All patients with somatic *NLRP3* mosaicism were sporadic patients, with no affected relatives, which is notably different from patients with germline mutations (positive familial history in 65.9%). Their main clinical features were compatible with a MWS phenotype and similar to those previously described in patients with germline mutations, with the potential exceptions of a reduced incidence of AA amyloidosis, an increased incidence of recurrent arthritis, and slightly older ages at the disease onset and also at onset of sensorineural deafness. It is interesting to note that most patients (4/7; 57.1%) were misdiagnosed as having juvenile idiopathic arthritis when the disease started, a similar misdiagnosis previously reported in different inherited autoinflammatory diseases (20-23). Despite the evidence shown here, the actual frequency of somatic *NLRP3* mosaicism is unknown and probably underestimated. In our study a potential bias in the selection of patients could exist because they were selected on the basis of the presence of an urticaria-like skin rash associated with other symptoms. Recent studies have described atypical CAPS presentations in patients with germline *NLRP3* mutations in whom urticaria-like skin rash was nearly absent (24-25). These data suggest that clinical diversity of CAPS is probably wider than previously described, and further studies are necessary to delineate the profile of potential candidates to carry somatic *NLRP3* mosaicism.

The evidence obtained may have serious implications for patients, especially with regard to treatment and genetic counseling. The outcome of IL-1 blockade in patients with somatic *NLRP3* mosaicism was nearly identical to those reported in patients with germline mutations (26-27). The only symptom that did not improve with IL-1 blockade was the sensorineural deafness. In this regard, apparently contradictory responses have been reported, with improvement or amelioration in some patients and no response in others (14, 17, 28-30). It has been suggested that the time of evolution of deafness previous to starting anti-IL-1 drugs could be a determining factor for the type of response, but probably additional and unknown factors could also play a role in this particular manifestation. We have also observed a notable delay in gaining access to anti-IL-1 drugs with respect to the disease onset (median: 20 years; interquartile range: 12-26 years), because these

treatments were administered under legal indication once the definitive CAPS diagnosis was established by means of the identification of somatic *NLRP3* mosaicism. Taking into account the excellent response observed to IL-1 blockade, it is reasonable to hypothesize that if this was started earlier it should have provoked the non-appearance of some severe complications such as deafness.

For an appropriate genetic counseling the scenario is extremely different in CAPS patients with germline or with somatic mutations. In the case of germline mutations, the risk of transmission to future pregnancies is 50%. Inversely, the prediction of the risk of transmission in cases of somatic mosaicism is more complex, because it may vary in the different tissues, it is not usually determined in gonadal tissues, and its detection probably requires new sensitive genetic methods that are not widely available. The vertical transmission of a somatic mutation is an extremely rare event, with only one case recently described in MWS (31). Consequently, this possibility should be considered during the genetic counseling of these patients, although one of the main messages to patients is that its probability remains low.

We show that somatic *NLRP3* mosaicism underlies MWS and is probably a shared genetic mechanism in different CAPS phenotypes, and not restricted to CINCA/NOMID syndrome. Its detection was achieved by using massively parallel sequencing, and functional studies confirmed the *gain-of-function* behavior of the detected variants. The detection of somatic mosaicism has had serious clinical implications for patients, including access to treatment under legal indication, adequate follow-up, and ensuring appropriate genetic counseling. Further studies are necessary to delineate the clinical phenotype of candidates to looking for somatic mosaicism, in which new sensitive genetic technologies should be used.

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Contributorship

K.N., T.H., J.Y., R.N. and J.I.A. designed research, discussed data and wrote the paper. E. G-R., E. R-O., F. R., E. I., T. Y., K. I., Tomoki Kawai, and O. O. performed genetic and functional investigations, discussed data and reviewed the manuscript. A. S., Toshinao Kawai, H. U., J. M. C., J. C., S. T., N. Kobayashi, J.L. C-R., N. O-C., J. A., S. J-T., C. V., J. F-M., I. C., J. H-R., M. M., M.T. D., M. B., S. B., M. Y., T. Kubota, R. K., N. A., K. S., N. I., M. K. S, and N. Kambe. Provided clinical data and blood samples, discussed data and reviewed the manuscript.

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31. Jiménez-Treviño S, González-Roca E, Ruiz-Ortiz E, et al. First report of vertical transmission of a somatic *NLRP3* mutation in cryopyrin-associated periodic syndromes. Ann Rheum Dis 2013; 72: 1109-1110.

Figure legends.

Figure 1.

A. Sense (upper rows) and antisense (bottom rows) chromatograms from four patients with somatic *NLRP3* mosaicism and controls obtained by Sanger's sequencing using genomic DNA extracted from whole blood. The black arrows show the *NLRP3* positions where the somatic mutations were detected. The percentage in the upper panels represents the frequency of the mosaicism obtained by massively parallel DNA sequencing in each patient. The red arrow indicates the c.1231 C>T *NLRP3* polymorphism (rs#148478875). B. Structural organization of cryopyrin. Above the protein structure are indicated all missense cryopyrin variants that have been detected as somatic mutations in CINCA-NOMID patients in previous reports, and those below the protein structure are the missense variants detected as somatic mutations in the present study. C and D. ASC-dependent NF-κB activation (C) and necrotic THP-1 cell death (D) induced by the detected *NLRP3* mutations. Values are the mean ± SD of triplicate experiments, and data are representative of two independent experiments. Abbreviations: S, sense; AS, anti-sense; Pt, patient; C, control; None, nothing transfected; mock, vector without *NLRP3*; WT, wild-type *NLRP3*.

Figure 2.

Laboratory values obtained in the five patients treated with different anti-IL-1 drugs. Patient's graphics were ordered as follows: First, those graphics from the patient who only received treatment with anakinra (Pt 5), followed by those from patients who only received treatment with canakinumab (Pt 2, 3 and 6) and finally those from the patient who received both treatments (Pt 7). Vertical bars represent the mean ± SD of values obtained during treatment periods. Horizontal discontinued lines represent the upper limit of the normal range, with the only exception of hemoglobin box, in which this line represents the lower limit of the normal range. Abbreviations: ESR, Erythrocyte Sedimentation Rate; CRP, C-reactive protein; WBC, White Blood cell Count; PMN, Polymorphonuclears.

Table legends.

Table 1.

Summary of genetic data of patients with somatic *NLRP3* mosaicism. [1]NCBI Reference Sequence NM_001243133.1. [2]Blood sample collected in 2002. [3]Blood sample collected in 2009. [4]Mean of two independent experiments. [5]Mean of four independent experiments. [6]Data of population genetics obtained from NCBI dbSNP Build 137. [7]Analyses performed by Sanger's sequencing. Abbreviations: Pt, patient; MWS, Muckle-Wells syndrome; SIFT, Sorting Intolerant from Tolerant; n.d., not done.

Table 2.

Summary of clinical features of patients with somatic *NLRP3* mosaicism at the onset of the disease. [1]Defined by increased values of white blood cells (normal range 4.00-11.00x10^3/dL), circulating neutrophils (normal range 45-75%), platelets (normal range 130-400x10^3/dL), C-reactive protein (normal range <1 mg/dL) and/or Erythrocyte Sedimentation Rate (normal <10 mm/hr). [2]Low-grade fever. Abbreviations: Pt, Patient; CNS, Central Nervous System; n.a., not available; JIA, Juvenile Idiopathic Arthritis; So-JIA, Systemic-onset Juvenile Idiopathic Arthritis; TRAPS, TNF Receptor-Associated Periodic Syndrome; -, absent.

Table 3.

Summary of clinical manifestations detected in patients with somatic *NLRP3* mosaicism during the course of the disease. [1]Occasionally. [2]Always. Abbreviations: Pt, Patient; CNS, Central Nervous System; M, male; F, female; -, No or absent.

Table 4.

Comparison of main clinical data of patients carrying germline versus somatic *NLRP3* mutations. Patients with germline mutations were carriers of one of the next *NLRP3* mutations: p.R170S (c.508 C>A), p.R260W (c.778 C>T), p.V262A (c.785 T>C), p.D303N (c.907 G>A), p.H312P (c.935

A>C), p.T348M (c.1043 C>T), p.A439T (c.1315 G>A), p.A439V (c.1316 C>T), p.F443L (c.1329 C>G), p.E567A (c.1700 A>C) and p.Y859C (c.2576 A>G). Abbreviations: n.s., not significant differences.

Legend of Supplemental Table.

Supplemental Table S1. Summary of clinical manifestations detected in patients enrolled in this study with no somatic *NLRP3* mosaicism. Abbreviations: CNS, Central Nervous System; M, male; F, female; y, years; mo, months; -, negative or absent; n.a., not available.

Table 1. Summary of genetic data of patients with somatic *NLRP3* mosaicism. [1]NCBI Reference Sequence NM_001243133.1. [2]Blood sample collected in 2002. [3]Blood sample collected in 2009. [4]Mean of two independent experiments. [5]Mean of four independent experiments. [6]Data of population genetics obtained from NCBI dbSNP Build 137. [7]Analyses performed by Sanger's sequencing. Abbreviations: Pt, patient; MWS, Muckle-Wells syndrome; SIFT, Sorting Intolerant from Tolerant; n.d., not done.

Pt (Country)	Phenotype	Nucleotide Exchange[1]	Amino acid exchange	Massively parallel DNA sequencing		Bioinformatics analyses			Reference	Analyzed relatives	
				Mutated allele frequency	Coverage	SIFT	PolyPhen-2	Population Genetics[6]		Kinship	Results
1 (Spain)	MWS	c.908 A>C	p.D303A	31.3%[4]	622x[4]	Damaging	Probably damaging	Absent	Present Study	n.d.	n.d.
2 (Japan)	MWS	c.1000 A>G	p.I334V	34.9%[4]	1060x[4]	Damaging	Benign	Absent	12	Father	Negative[7]
										Mother	Negative[7]
3 (Japan)	MWS	c.1064 A>C	p.K355T	20.2%[4]	100x[4]	Tolerated	Probably damaging	Absent	Present Study	n.d.	n.d.
4[2] (Spain)	MWS	c.[1231 C>T; 1233 G>T]	p.L411F	14.4%[4]	590x[4]	Tolerated	Possibly damaging	Absent	Present Study	Mother	Negative[7]
4[3] (Spain)	MWS	c.[1231 C>T; 1233 G>T]	p.L411F	15.6%[4]	870x[4]	Tolerated	Possibly damaging	Absent	Present Study	Mother	Negative[7]
5 (Spain)	MWS	c.1569 C>A	p.F523L	8.7%[5]	569x[5]	Tolerated	Possibly damaging	Absent	3	Daughter	Negative[7]
6 (Japan)	MWS	c.1699 G>A	p.E567K	5.6%[4]	1211x[4]	Tolerated	Benign	Absent	11	n.d.	n.d.
7 (Japan)	MWS	c.1699 G>A	p.E567K	5.5%[4]	724x[4]	Tolerated	Benign	Absent	11	n.d.	n.d.

Table 2. Summary of clinical features of patients with somatic *NLRP3* mosaicism at the onset of the disease. [1]Defined by increased values of white blood cells (normal range 4.00-11.00x10^3/dL), circulating neutrophils (normal range 45-75%), platelets (normal range 130-400x10^3/dL), C-reactive protein (normal range <1 mg/dL) and/or Erythrocyte Sedimentation Rate (normal <10 mm/hr). [2]Low-grade fever. Abbreviatures: Pt, Patient; CNS, Central Nervous System; n.a., not available; JIA, Juvenile Idiopathic Arthritis; So-JIA, Systemic-onset Juvenile Idiopathic Arthritis; TRAPS, TNF Receptor-Associated Periodic Syndrome; -, absent.

Pt	Age at disease onset	Cold-exposure trigger	Urticaria-like skin rash	Fever	Joint involvement	CNS involvement	Acute inflammatory response[1]	First Diagnoses
1	18 years	-	Yes	Yes	Arthralgias	-	Yes	
2	2 years	-	Yes	-	Arthralgias	-	Yes	JIA
3	1 week	-	Yes	-	-	-	Yes	Chronic urticaria, So-JIA
4	14 years	-	Yes	Yes	-	-	Yes	Erythema nodosa
5	4 years	Yes	Yes	Yes	Arthralgias	-	Yes	
6	4 years	Yes	Yes	Yes[2]	Oligoarthritis	-	Yes	Oligo-JIA
7	7 months	-	Yes	Yes	Oligoarthritis	-	n.a.	So-JIA, TRAPS

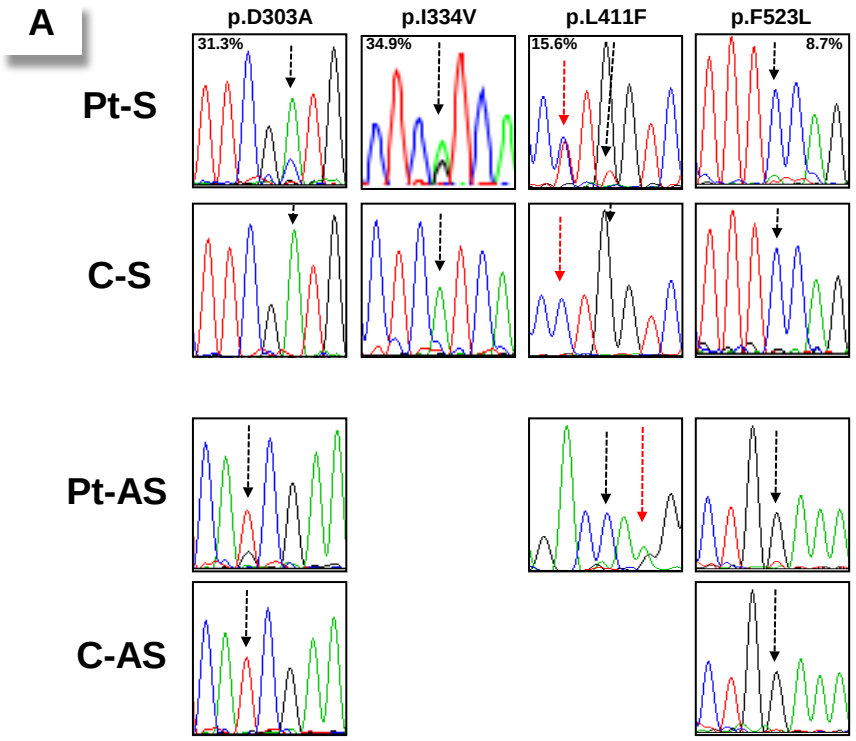
Table 3. Summary of clinical manifestations detected in patients with somatic *NLRP3* mosaicism during the course of the disease. [1]Occasionally. [2]Always. Abbreviations: Pt, Patient; CNS, Central Nervous System; M, male; F, female; -, No or absent.

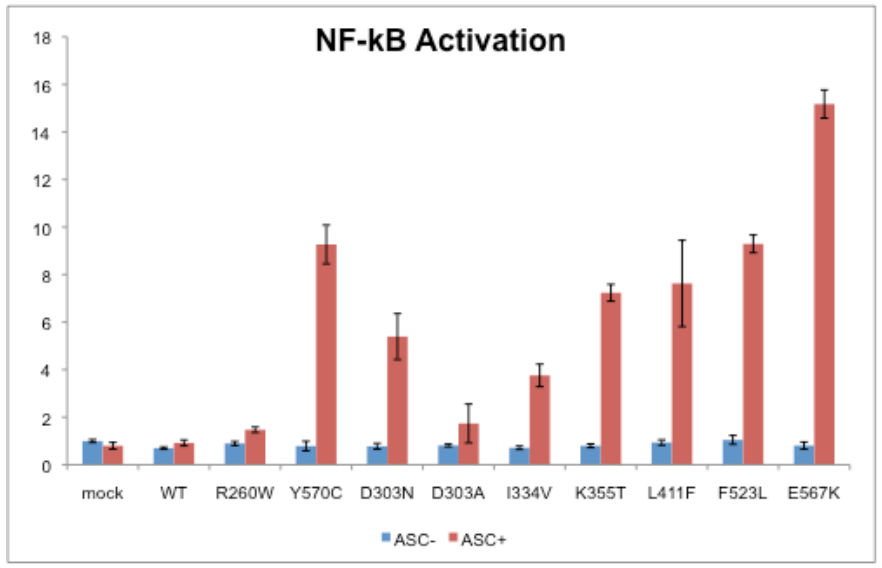
Pt	Sex (Age)	Cold-exposure trigger	Urticaria-like skin rash	Fever	Joint involvement					CNS involvement			Deafness (age at onset)	Ocular involvement	AA amyloidosis
					Type of arthritis	Involved joints	Symmetric	Erosive	Arthropathy	Headache	Aseptic meningitis	Papilledema			
1	M (39y)	-	Yes	Yes	Polyarthritis	Large and small	-	-	-	-	-	-	Yes (38 years)	Conjunctivitis	-
2	M (14y)	-	Yes	-	-	-	-	-	-	Yes	Yes	-	Yes (7 years)	-	-
3	F (12y)	-	Yes	-	Monoarthritis	Large	-	-	-	Yes	-	-	Yes (6 years)	-	-
4	F (41y)	-	Yes	Yes	Polyarthritis	Small	-	-	-	Yes	-	-	-	Conjunctivitis	-
5	M (64y)	Yes[2]	Yes	Yes[1]	Polyarthritis	Large and small	-	-	-	-	-	-	Yes (45 years)	-	-
6	F (16y)	Yes[1]	Yes	Yes	Oligoarthritis	Large	-	-	-	Yes	-	-	-	Conjunctivitis	-
7	M (16y)	-	Yes	Yes	Oligoarthritis	Large	-	-	-	Yes	-	-	Yes (13 years)	Conjunctivitis	-

Table 4. Comparison of main clinical data of patients carrying germline versus somatic *NLRP3* mutations. Patients with germline mutations were carriers of one of the next *NLRP3* mutations: p.R170S (c.508 C>A), p.R260W (c.778 C>T), p.V262A (c.785 T>C), p.D303N (c.907 G>A), p.H312P (c.935 A>C), p.T348M (c.1043 C>T), p.A439T (c.1315 G>A), p.A439V (c.1316 C>T), p.F443L (c.1329 C>G), p.E567A (c.1700 A>C) and p.Y859C (c.2576 A>G). Abbreviations: n.s., not significant differences.

Clinical features		Patients with germline *NLRP3* mutations (n:41)	Patients with somatic *NLRP3* mutations (n:7)	p
Age at disease onset (years) – median (interquartile range)		0.5 (0.0-4.4)	4.0 (1.3-9.0)	n.s. (p=0.223)
Delay of diagnosis (years) – median (interquartile range)		33.0 (10-49)	20 (12-26)	n.s. (p=0.416)
Presence of familial history of the disease (%)		65.9	0	P=0.002
Cold exposure as disease triggering factor (%)		36.6	28.6	n.s. (p=1.000)
Fever (%)		63.4	71.4	n.s. (p=1.000)
Urticaria-like skin rash (%)		87.8	100	n.s. (p=1.000)
Joint involvement	Arthralgias (%)	80.5	85.7	n.s. (p=1.000)
	Arthritis (%)	53.7	85.7	n.s. (p=0.214)
Neurological involvement	Headache (%)	56.1	71.4	n.s. (p=0.683)
	Aseptic meningitis (%)	29.3	14.3	n.s. (p=0.656)
	Papiledema (%)	12.2	0	n.s. (p=1.000)
Ocular involvement	Conjunctivitis (%)	61.0	57.1	n.s. (p=1.000)
	Uveitis (%)	17.1	0	n.s. (p=0.573)
Sensorineural deafness (%)		68.3	71.4	n.s. (p=1.000)
Age at onset of deafness (years) – median (interquartile range)		7.0 (5.5-11)	13.0 (7-38)	n.s. (p=0.210)
AA amiloidosis (%)		17.1	0	n.s. (p=0.573)

Figure 1


A
p.D303A
p.I334V
p.L411F
p.F523L
31.3%
34.9%
15.6%
8.7%
Pt-S
C-S
Pt-AS
C-AS


C
NF-kB Activation
18
16
14
12
10
8
6
4
2
0
mock
WT
R260W
Y570C
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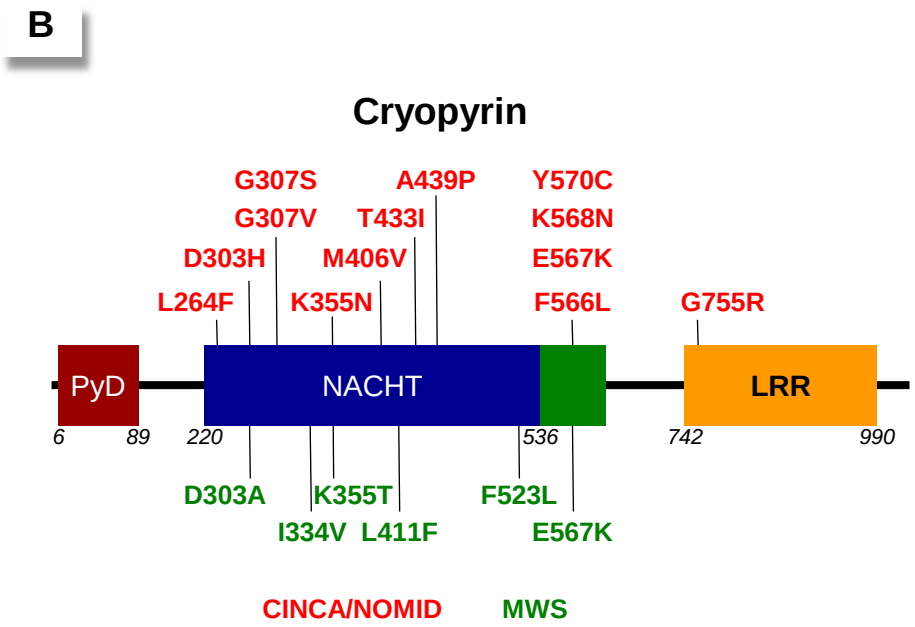
K355T
L411F
F523L
E567K
ASC-
ASC+


B
Cryopyrin
G307S
G307V
D303H
L264F
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Y570C
K568N
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F566L
G755R
PyD
NACHT
LRR
6
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220
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742
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F523L
E567K
CINCA/NOMID
MWS

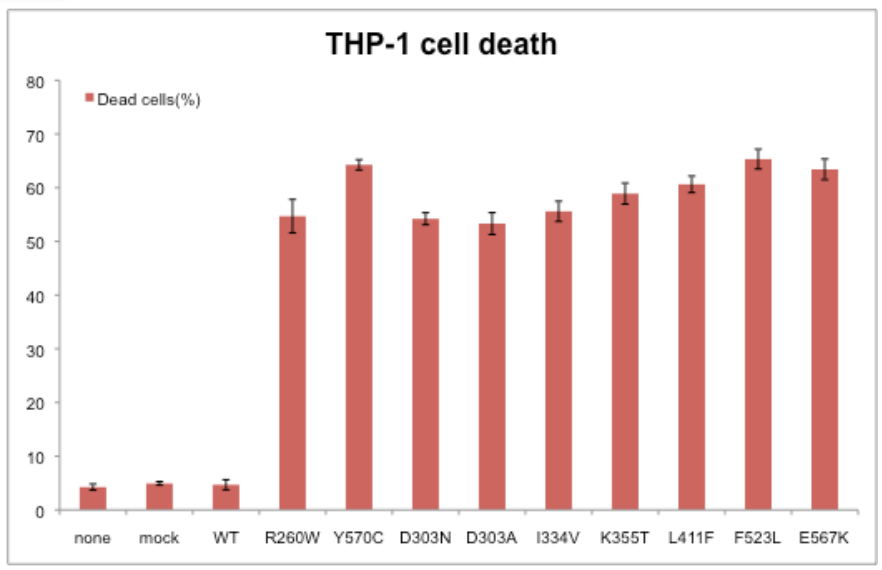
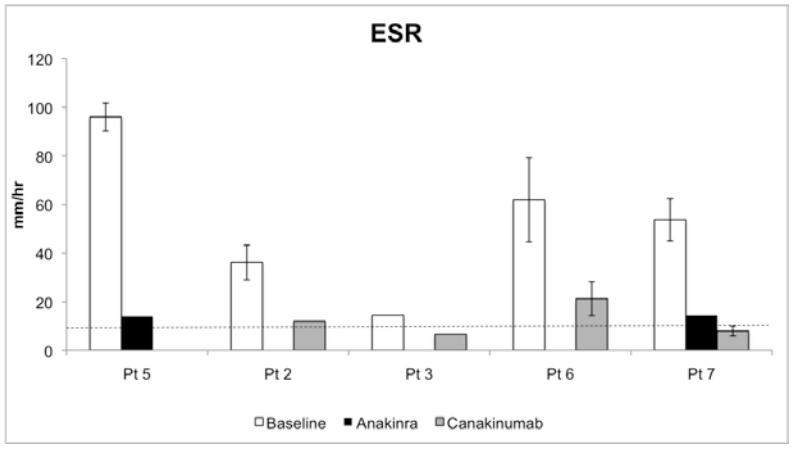
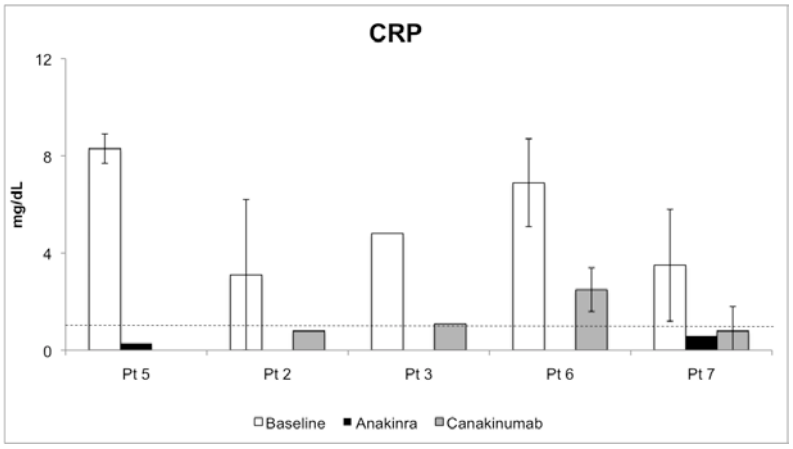
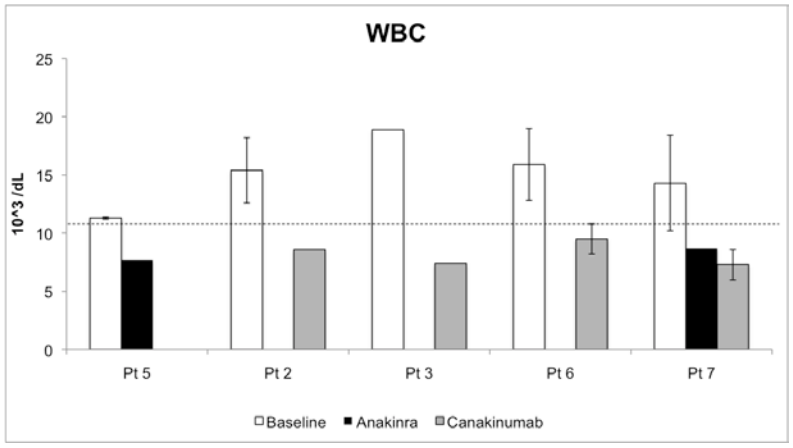
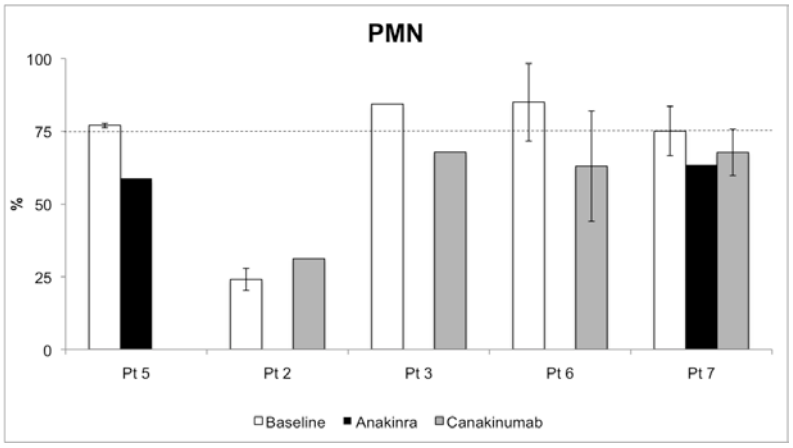

D
THP-1 cell death
Dead cells(%)
80
70
60
50
40
30
20
10
0
none
mock
WT
R260W
Y570C
D303N
D303A
I334V
K355T
L411F
F523L
E567K

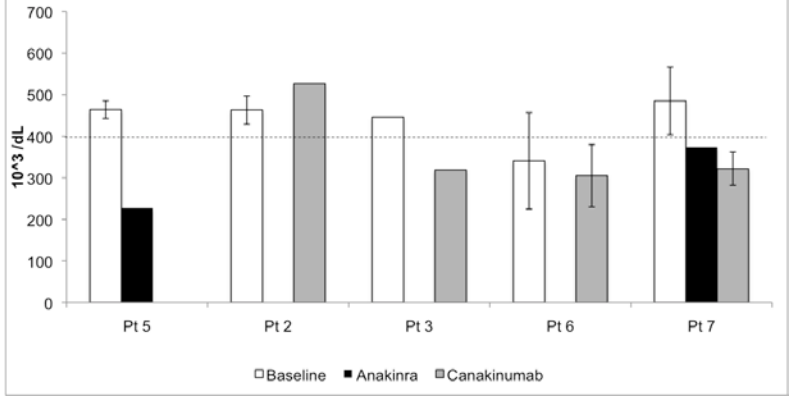
Figure 2

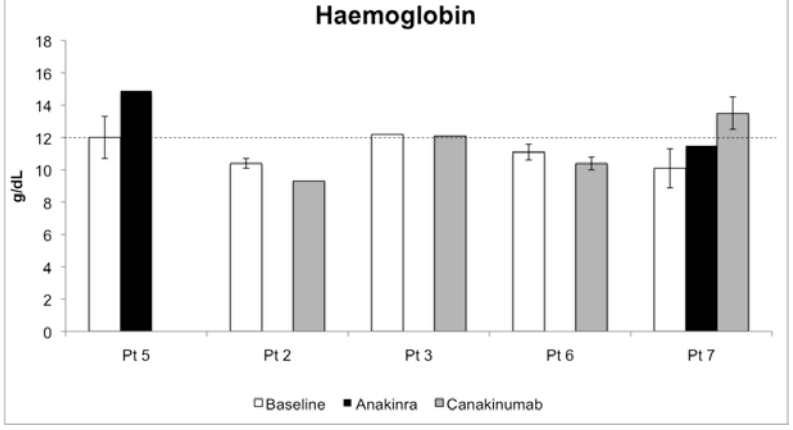

ESR
mm/hr
120
100
80
60
40
20
0
Pt 5
Pt 2
Pt 3
Pt 6
Pt 7
Baseline
Anakinra
Canakinumab


CRP
mg/dL
12
8
4
0
Pt 5
Pt 2
Pt 3
Pt 6
Pt 7
Baseline
Anakinra
Canakinumab


WBC
10^3 /dL
25
20
15
10
5
0
Pt 5
Pt 2
Pt 3
Pt 6
Pt 7
Baseline
Anakinra
Canakinumab


PMN
%
100
75
50
25
0
Pt 5
Pt 2
Pt 3
Pt 6
Pt 7
Baseline
Anakinra
Canakinumab


Platelets
10^3 /dL
700
600
500
400
300
200
100
0
Pt 5
Pt 2
Pt 3
Pt 6
Pt 7
Baseline
Anakinra
Canakinumab


Haemoglobin
g/dL
18
16
14
12
10
8
6
4
2
0
Pt 5
Pt 2
Pt 3
Pt 6
Pt 7
Baseline
Anakinra
Canakinumab

Supplemental Table 1. Summary of clinical manifestations detected in patients enrolled in this study with no somatic *NLRP3* mosaicism. Abbreviations: CNS, Central Nervous System; M, male; F, female; y, years; mo, months; -, negative or absent; n.a., not available.

Patient (Country)	Sex (Age)	Age at onset	Cold-exposure trigger	Urticaria-like skin rash	Fever	Joint involvement		CNS involvement			Deafness	Ocular involvement	AA amyloidosis	Gene analyses performed
						Arthritis	Arthropathy	Headache	Aseptic meningitis	Papilledema				
1 (Japan)	M (9 y)	9 y	-	Yes	-	Yes	-	-	-	n.a.	n.a.	n.a.	-	*NLRP3* negative
2 (Japan)	F (4 y)	3 y	-	Yes	Yes	Yes	-	-	-	n.a.	-	n.a.	-	*TNFRSF1A* negative *NLRP3* negative *NOD2* negative *NLRP12* negative
3 (Japan)	F (14 y)	3 y	Yes	Yes	Yes	Yes	-	Yes	-	n.a.	n.a.	n.a.	-	*NLRP3* negative
4 (Japan)	F (1 y)	0 y	-	Yes	Yes	Yes	-	-	-	n.a.	-	n.a.	-	*NLRP3* negative
5 (Japan)	F (3 y)	3 y	-	Yes	Yes	Yes	-	-	-	-	n.a.	-	-	*MEFV* p.E148Q / wt *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative
6 (Japan)	F (3 y)	0 y	-	Yes	Yes	-	-	Yes	-	-	-	-	-	*NLRP3* negative
7 (Japan)	F (16 y)	7 y	-	Yes	Yes	-	-	Yes	-	-	Yes	-	-	*NLRP3* negative *NLRP12* negative
8 (Japan)	M (44 y)	20s y	Yes	Yes	Yes	-	-	-	-	n.a.	-	n.a.	-	*NLRP3* negative
9 (Japan)	F (28 y)	0 y	-	Yes	Yes	Yes	-	-	-	n.a.	-	n.a.	-	*NLRP3* negative
10 (Japan)	F (1 y)	1 y	-	Yes	Yes	-	-	-	-	-	-	-	-	*TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative
11 (Japan)	F (2 y)	8 mo	-	Yes	Yes	-	-	-	-	-	-	Glaucoma	-	*MEFV* p.E148Q / wt *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative *PSTPIP1* negative
12 (Japan)	F (10 mo)	9 mo	-	Yes	Yes	-	-	-	-	n.a.	n.a.	n.a.	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative

														NLRP3 negative *NOD2* p.R435M / wt
13 (Japan)	M (66 y)	53 y	Yes	Yes	Yes	Yes	-	Yes	Yes	-	-	-	-	*NLRP3* negative *NLRP12* negative
14 (Japan)	F (59 y)	7 y	-	Yes	Yes	-	-	Yes	-	-	n.a.	Cotton-wool patch	-	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative *NOD2* negative
15 (Japan)	M (24 y)	18 y	-	Yes	Yes	-	-	Yes	-	n.a.	n.a.	n.a.	-	*NLRP3* negative
16 (Japan)	M (5 y)	4 y	-	Yes	Yes	-	-	Yes	-	-	-	-	-	*NLRP3* negative
17 (Japan)	F (75 y)	12 y	-	Yes	Yes	-	-	-	-	n.a.	Yes	n.a.	-	*NLRP3* negative
18 (Japan)	M (5 y)	5 y	-	Yes	Yes	Yes	-	-	-	-	-	-	-	*NLRP3* negative
19 (Japan)	M (9 y)	7 y	-	Yes	Yes	Yes	-	Yes	-	-	Yes	-	-	*NLRP3* negative
20 (Spain)	M (10 y)	7 mo	-	Yes	Yes	-	-	-	-	-	Yes	-	Yes	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative
21 (Spain)	F (7 y)	6 mo	-	Yes	Yes	Yes	-	-	-	-	-	Conjunctivitis	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative
22 (Spain)	F (7 y)	3 mo	-	Yes	Yes	-	-	Yes	Yes	-	-	-	-	*MVK* negative *NLRP3* negative
23 (Spain)	F (18 y)	9 y	-	Yes	Yes	Yes	-	Yes	Yes	-	-	Conjunctivitis	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative
24 (Spain)	F (10 y)	1.5 y	-	Yes	Yes	Yes	-	-	-	-	-	-	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative
25 (Spain)	M (40 y)	3 y	-	Yes	Yes	-	-	Yes	-	-	Yes	-	-	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative
26 (Spain)	M (11 y)	2 mo	-	Yes	Yes	Yes	-	-	-	-	-	-	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative

27 (Spain)	M (26 y)	5 y	-	Yes	Yes	-	-	Yes	Yes	-	Yes	-	-	*TNFRSF1A* negative *NLRP3* negative
28 (Spain)	F (24 y)	1.5 y	-	Yes	Yes	Yes	-	Yes	-	-	Yes	-	Yes	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative
29 (Spain)	F (26 y)	8 mo	-	Yes	Yes	Yes	-	Yes	-	-	-	-	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative
30 (Spain)	F (40 y)	Infancy	-	Yes	-	Yes	-	-	-	-	Yes	-	Yes	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative
31 (Spain)	F (25 y)	1 mo	-	Yes	Yes	Yes	-	Yes	Yes	-	-	Conjunctivitis	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative
32 (Spain)	M (23 y)	2 y	-	Yes	Yes	Yes	-	Yes	-	-	-	Conjunctivitis	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative
33 (Spain)	M (28 y)	3 y	-	Yes	Yes	-	-	-	-	-	Yes	Conjunctivitis	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative
34 (Spain)	M (72 y)	Infancy	-	Yes	Yes	Yes	-	Yes	-	-	-	-	Yes	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative
35 (Spain)	F (27 y)	17 y	-	Yes	Yes	Yes	-	Yes	-	-	-	Uveitis	-	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative
36 (Spain)	F (34 y)	30 y	-	Yes	Yes	Yes	-	Yes	-	-	-	-	-	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative
37 (Spain)	F (34 y)	14 y	-	Yes	Yes	-	-	Yes	-	-	Yes	-	-	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative
38 (Spain)	M (22 y)	1.5 y	-	Yes	Yes	Yes	-	Yes	-	-	-	-	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* p.N289S / wt
39 (Spain)	M (7 y)	4 y	-	Yes	Yes	-	-	-	-	-	-	Conjunctivitis	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative
40 (Spain)	F (30 y)	22 y	-	Yes	Yes	Yes	-	-	-	-	-	-	-	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative

41 (Spain)	M (12 y)	10 y	-	Yes	Yes	Yes	-	-	-	-	-	-	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative
42 (Spain)	F (7 y)	3 y	-	Yes	Yes	-	-	-	-	-	-	Uveitis	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative
43 (Spain)	M (67 y)	36 y	-	Yes	Yes	-	-	Yes	-	-	Yes	-	-	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative
44 (Spain)	F (24 y)	1.5 y	-	Yes	Yes	Yes	-	-	-	-	Yes	-	Yes	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative *NOD2* negative
45 (Spain)	F (17 y)	0 y	Yes	Yes	Yes	Yes	-	Yes	-	-	-	-	-	*NLRP3* negative
46 (Spain)	M (11 y)	5 y	Yes	Yes	Yes	-	-	Yes	-	-	-	-	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative
47 (Spain)	M (4 y)	8 mo	-	Yes	Yes	Yes	-	-	-	-	-	Conjunctivitis	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative
48 (Spain)	M (63 y)	37 y	-	Yes	Yes	-	-	-	-	-	-	-	Yes	*MEFV* negative *TNFRSF1A* negative *NLRP3* negative
49 (Spain)	F (45 y)	24 y	-	Yes	Yes	-	-	-	-	-	Yes	Conjunctivitis	-	*MEFV* negative *TNFRSF1A* negative *MVK* negative *NLRP3* negative