

# NLRP3-associated autoinflammatory diseases: Phenotypic and molecular characteristics of germline versus somatic mutations



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**Background:** *NLRP3*-associated autoinflammatory diseases (*NLRP3*-AIDs) include conditions of various severities, due to germline or somatic mosaic *NLRP3* mutations.

**Objective:** To identify mosaic- versus germline-specific *NLRP3* mutations' characteristics, we reinterpreted all the mutations reported in *NLRP3*-AIDs and performed an in-depth study of 3 novel patients.

**Methods:** The pathogenicity of all reported mosaic/germline mutations was reassessed according to international recommendations and their location on the *NLRP3* 3-dimensional structure. Deep-targeted sequencing and *NLRP3*-inflammasome-activation assays were used to identify the disease-causing mutation in 3 patients.

**Results:** We identified, in 3 patients, mosaic mutations affecting the same *NLRP3* amino acid (Glu569). This residue belongs to 1 of the 2 mosaic mutational hot spots that face each other in the core of the *NLRP3* ATPase domain. The review of the 90 *NLRP3*

mutations identified in 277 patients revealed that those hot spots account for 68.5% of patients (37 of 54) with mosaic mutations. Glu569 is affected in 22% of the patients (12 of 54) with mosaic mutations and in 0.4% of patients (1 of 223) with germline mutations. Only 8 of 90 mutations were found in mosaic and germinal states. All of the germline mutations were associated with a severe phenotype. These data suggest that mutations found only in mosaic state could be incompatible with life if present in germinal state. None of the 5 most frequent germline mutations was identified in mosaic state. Mutations found only in germinal state could, therefore, be asymptomatic in mosaic state.

**Conclusions:** The phenotypic spectrum of *NLRP3*-AIDs appears to be related to the germinal/mosaic status and localization of the underlying mutations. (*J Allergy Clin Immunol* 2020;145:1254-61.)

**Key words:** *NLRP3*-associated autoinflammatory diseases, *NLRP3*, somatic mosaic mutations, *NLRP3*-inflammasome activation, mutational hot spot

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Autoinflammatory diseases (AIDs) are associated with recurrent episodes of fever, sterile inflammation of joints and serosal membranes, skin rashes, lymphadenopathy, musculoskeletal symptoms, and biological inflammation. *NLRP3*-AIDs are a group of monogenic AIDs related to germline or somatic *NLRP3* mutations leading to *NLRP3*-inflammasome activation.<sup>1</sup> The *NLRP3* inflammasome is an intracellular multiprotein signaling complex that assembles around *NLRP3*, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and the procaspase 1 leading to autoproteolytic activation of caspase-1 and secretion of proinflammatory cytokines (mainly IL1 $\beta$ ).<sup>2</sup>

The *NLRP3* protein comprises an *N*-terminal pyrin domain, a central nucleotide-binding and oligomerization domain (NACHT, which stands for NAIP, CIITA, HET-E, and TP1), and *C*-terminal leucine-rich repeats (LRRs). NACHT is an ATPase domain including a nucleotide binding domain (NBD) that contains Walker A (ATP binding) and Walker B (Mg<sup>2+</sup> binding) catalytic motifs, the helical domain 1 (HD1), a winged HD, and the helical domain (HD2). Through the NBD, HD2, and LRR regions, *NLRP3* interacts with the Ser/Thr kinase NEK7 that mediates *NLRP3*-inflammasome activation.<sup>3</sup>

#### Abbreviations used

AID:	Autoinflammatory disease
ASC:	Apoptosis-associated speck-like protein containing a CARD
CAPS:	Cryopyrin-associated periodic syndrome
CINCA:	Chronic infantile neurological, cutaneous, and articular syndrome
CpG:	Cytosine-phosphate-guanine
EV:	Empty vector
FCAS:	Familial cold autoinflammatory syndrome
GFP:	Green fluorescent protein
HD1 (2):	Helical domain 1 (2)
LRR:	Leucine-rich repeat
MAP:	Mutated allele percentage
MWS:	Muckle-Wells syndrome
NACHT domain:	Nucleotide-binding and oligomerization domain (standing for NAIP, CIITA, HET-E, and TP1)
NBD:	Nucleotide binding domain
NGS:	Next-generation sequencing
NLRP3-AID:	NLRP3-associated autoinflammatory disease
OMIM:	Online Mendelian Inheritance in Man
WT:	Wild-type

*NLRP3*-AIDs, previously known as cryopyrin-associated periodic syndromes (CAPSs), include 3 overlapping entities with increasing severity: (1) the familial cold autoinflammatory syndrome (FCAS, Online Mendelian Inheritance in Man [OMIM] 120100) characterized by cold-induced inflammatory episodes of fever, arthralgia, and urticaria; (2) the Muckle-Wells syndrome (MWS, OMIM 191900) associating recurrent attacks of fever, urticaria, and arthritis with subsequent sensorineural hearing loss; and (3) the chronic infantile neurological, cutaneous, and articular syndrome (CINCA, OMIM 607115) characterized by central nervous system involvement, continuous urticarial rash, and deformative joint arthritis.

Primarily related to germline heterozygous *NLRP3* mutations, *NLRP3*-AIDs were described in familial forms of AIDs transmitted as autosomal dominant traits,<sup>4</sup> as well as in sporadic cases due to *de novo* *NLRP3* mutations.<sup>5,6</sup> The identification of somatic mosaic *NLRP3* mutations has further broadened the clinical spectrum of *NLRP3*-AIDs<sup>7,8</sup> with the reports of late onset diseases<sup>9-11</sup> and paucisymptomatic forms.<sup>12</sup> The advances in next-generation sequencing (NGS) technologies have led to the identification of an increasing number of *NLRP3* sequence variations in AID patients, raising the question of the pathogenicity of the identified sequence variations.

In this study, in an attempt to identify possible germline- and mosaic-specific features of *NLRP3* mutations, we reassessed all previously reported *NLRP3* variations identified in *NLRP3*-AID patients. To evaluate the functional impact of the mutations, we studied their molecular characteristics such as their location on the 3-dimensional structure of the *NLRP3* protein and the amino acid change, as well as their associated phenotypic severity. In this study, we also took into account 3 unrelated *NLRP3*-AID patients with mosaic variations involving the same amino acid. One of those patients also carried a novel germline variation. We assessed the pathogenicity of the identified variants through intrafamilial segregation for the germline one and by performing inflammasome-activation studies.

## METHODS

### Patients

Clinical features were collected through a standardized form. The study conforms to the Helsinki declaration regarding ethical principles for medical research. Written informed consent was obtained from each patient or their parents.

### Study of the *NLRP3* gene

Genomic DNA was extracted from peripheral blood leukocytes of patients and parents from proband III using standard procedures. PBMCs were isolated from peripheral blood using Lymphocyte Separating Medium, Pancoll human (PAN-Biotech, Aidenbach, Germany). PBMCs were separated by positive selection using immunomagnetic microbeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD14 (monocytes) followed by anti-CD15 (neutrophils), anti-CD19 (B cells), and anti-CD3 (T cells). DNA was then extracted from monocytes, neutrophils, B cells, T cells, urine, and buccal cells using proteinase K digestion followed by a phenol-chloroform extraction protocol.

**Sanger sequencing.** *NLRP3* exon 3 and its intronic junctions were amplified by PCR and sequenced using the Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, Calif) on an ABI 3130XL automated capillary DNA sequencer (Applied Biosystems). Sequences were analyzed against the reference sequence (NM\_004895).

**Next generation sequencing.** Next-generation sequencing (NGS) was performed using a custom sequence capture (Nimblegen SeqCap EZ Choice system; Roche Sequencing, Pleasanton, Calif) of the exons and the flanking intronic sequences of the main AID-causing genes: *ADA2*, *CARD14*, *IL1RN*, *IL36RN*, *LACC2*, *LPIN2*, *MEFV*, *MVK*, *NLR4*, *NLRP12*, *NLRP3*, *NOD2*, *PLCG2*, *PSMB8*, *PSTPIP1*, *RBCK1*, *TMEM173*, *TNFAIP3*, *TNFRSF11A*, and *TNFRSF1A*. Sequencing was performed on MiSeq or Nextseq500 (Illumina, San Diego, Calif) platforms according to the manufacturer's instructions. The conventional bioinformatics pipeline, previously described,<sup>13</sup> was used to study germline mutations. For the mosaic-dedicated bioinformatics pipeline, sequence reads in fastq format were aligned to the reference human genome (hg19) with Bowtie2 (Johns Hopkins University, Maryland). Variant calling was performed with VarScan (Washington University School of Medicine, Missouri) with a threshold for the minimum mutated allele percentage (MAP) of 1%. Variant calls in virtual contact file (.vcf) format were then annotated through ANNOVAR (University of Pennsylvania). All results obtained by NGS were confirmed by Sanger sequencing.

### Mutagenesis, cell culture, and functional tests

Site-directed mutagenesis of the *NLRP3* wild-type (WT) expression vector (pNLRP3-WT) was performed to generate the plasmids carrying the missense variations identified in the patients: pNLRP3-Glu569Gly, pNLRP3-Glu569Lys, and pNLRP3-Gly769Ser.

Human embryonic kidney HEK293T cells stably expressing green fluorescent protein (GFP)-tagged-ASC and FLAG-tagged procaspase1 (designated ASC-GFP\_C1-FLAG) were transfected with 375 ng of mentioned plasmids using FUGENE HD (Promega, Madison, Wis). After 24 hours, the transfected cells were observed using a Nikon Eclipse TS100 inverted fluorescent microscope (Nikon Instruments, Melville, NY). Manual counting of cells containing ASC-GFP specks was then performed in 5 randomly selected fields at 20 $\times$  magnification. The percentage of speck-positive cells was calculated as the number of specks divided by the total number of counted cells.

Human monocytic THP1 cells were initially primed with 100 ng/mL phorbol 12-myristate 13-acetate for 3 hours, then transfected with 500 ng of the plasmids using the FF-100 program in the 4D-Nucleofector, Amaxa (Lonza Group, Basel, Switzerland). Transfected THP1 cells were treated directly with 100 ng/mL LPS at 37°C. After 24 hours, cytokine measurements by ELISA assays were performed following manufacturer's instructions (R&D Systems, Minneapolis, Minn).

**TABLE I.** Clinical and molecular characteristics of 3 *NLRP3*-AID patients

	Proband I	Proband II	Proband III
Phenotype	MWS	MWS	CINCA
Age at onset	Birth	3 months	Birth
Fever-urticaria-arthralgia	+	+	+
Adenopathy	+	+	+
Hearing loss	+	+	–
Papilledema	+	–	+
Facial dysmorphism (frontal bossing)	–	–	+
Aseptic meningitis	–	–	+
Other signs	Clubbing fingers, splenomegaly, monoclonal IgGκ	Nausea, diarrhea, clubbing fingers	Mild growth retardation, diarrhea, conjunctivitis, developmental delay, autism spectrum disorder, orchitis
<i>NLRP3</i> variations (MAP)	c.1705G>A, p.(Glu569Lys) (7%)	c.1705G>A, p.(Glu569Lys) (13%)	c.2305G>A, p.(Gly769Ser) (50%) c.1706A>G p.(Glu569Gly) (10%)

**TABLE II.** *NLRP3* DNA sequencing data in different cell types of the 3 *NLRP3*-AID patients

	Proband I MAP (mutated reads/to- total reads)	Proband II MAP (mutated reads/to- total reads)	Proband III MAP (mutated reads/to- total reads)
Mosaic mutation	c.1705G>A p.(Glu569Lys)	c.1705G>A p.(Glu569Lys)	c.1706A>G p.(Glu569Gly)
Peripheral blood	7 (70/951)	13 (75/581)	10 (15/148)
Monocytes	19 (200/1046)	15 (99/667)	9 (81/931)
Neutrophils	15 (165/1103)	14 (113/826)	9 (81/946)
B cells	17 (190/1110)	13 (104/773)	7 (52/758)
T cells	7 (96/1282)	11 (90/795)	10 (53/505)
Buccal cells	5 (69/1451)	ND	ND

ND, Not determined.

Values are percentages (*n/n*).

MAP is given by the ratio of the mutated reads divided by the total number of reads determined by NGS.

## Methodology for literature review of *NLRP3* mutations

Literature review of *NLRP3* mutations was performed in PubMed using the terms “*NLRP3*” and “mutation”. Based on the classification of the American College of Medical Genetics,<sup>14</sup> only pathogenic and likely pathogenic mutations were taken into account.

## *NLRP3* crystal structure analysis

*NLRP3* crystal structure analysis was performed using the human *NLRP3* crystal (Protein Data Bank accession number 6NPY) by Sharif et al.<sup>3</sup> The 3-dimensional structure was visualized with the PyMOL software (Schrödinger, New York, NY).

## Statistical analyses

Differences were analyzed using the unpaired Student *t* test and were plotted with the Prism 5 (GraphPad Software, San Diego, Calif). A *P* value of less than .05 was considered statistically significant.

## RESULTS

### Clinical phenotype of *NLRP3*-AID patients

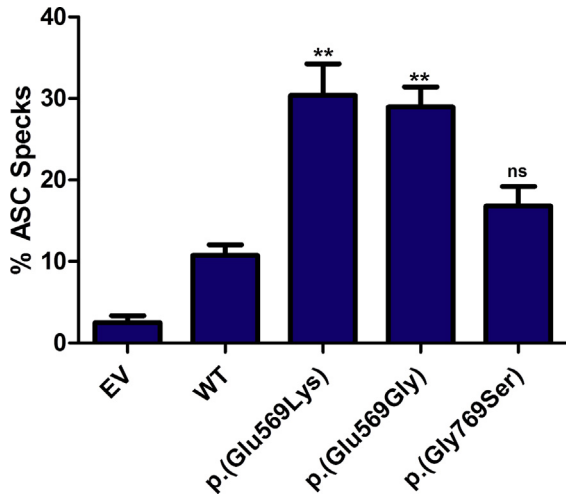
We studied 3 unrelated patients presenting with early onset of fever, urticaria, arthralgia, and inflammatory biological profile. Probands I and II, both 50 years old, also developed sensorineural hearing loss around the age of 30 and were, therefore, suggestive

of MWS. Proband III, a 4-year-old boy, displayed a clinical phenotype evocative of CINCA shortly after birth (Table I, and see Data E1 in this article’s Online Repository at [www.jacionline.org](http://www.jacionline.org)). None of the patients had a family history of AID.

To identify the molecular defect responsible for the patients’ phenotype, we followed an NGS approach based on the targeted sequencing of the main genes so far involved in AIDs. For probands I and II, no mutation was detected by a previous *NLRP3* analysis by Sanger sequencing.

The c.1705G>A, p.(Glu569Lys) missense variation with a MAP of 7% and 13% was identified in peripheral blood DNA of probands I and II, respectively (Table II, and see Fig E1 in this article’s Online Repository at [www.jacionline.org](http://www.jacionline.org)). This variation, which is absent from the Genome Aggregation Database (gnomAD; Broad Institute, Cambridge, Mass), has already been reported in several *NLRP3*-AID patients.<sup>11,12,15-18</sup>

In proband III, the NGS analysis revealed a novel heterozygous *NLRP3* variation, the c.2305G>A, p.(Gly769Ser), which is not described in gnomAD and, according to the MaxEntScan software (Massachusetts Institute of Technology, Cambridge, Mass), is not predicted to affect splicing. In the 3-dimensional structure of *NLRP3*, Gly769 is located on an external part of an LRR motif (see Fig E2 in this article’s Online Repository at [www.jacionline.org](http://www.jacionline.org)), outside the region that interacts with NEK7. Secondly, the access to parents’ DNA samples revealed that p.(Gly769Ser) was inherited from the asymptomatic father,



**FIG 1.** Impact of *NLRP3* mutations on ASC-speck formation. HEK (ASC-GFP\_C1-FLAG) cells were transfected with 375 ng of the expression plasmids encoding the WT *NLRP3* or the *NLRP3* protein carrying the missense variations p.(Glu569Gly), p.(Glu569Lys), and p.(Gly769Ser) or the EV. The percentage of ASC specks was calculated as described in the methods. Results represent the means + SDs from 3 independent experiments. *P* values were calculated using unpaired Student *t* test as compared to the WT. \*\**P* < .01; ns = nonsignificant.

which argues against its involvement in the AID phenotype. The NGS data were therefore subsequently reanalyzed using a mosaic-specific pipeline and the c.1706A>G, p.(Glu569Gly) variation with a MAP of 9% in peripheral blood DNA was detected. This variation, absent from gnomAD, has never been reported in *NLRP3*-AID patients.

The cellular distribution of the identified mosaic variations was then determined by NGS and Sanger sequencing of DNA isolated from cells of different embryonic origins. In proband I, who carries the p.(Glu569Lys) variation, a wide cellular distribution of the mutated allele was found with MAP ranging from 5% to 19%. It ranged from 11% to 15% in proband II who also carries the p.(Glu569Lys) variation. It is worth noting that proband III, who had the most severe phenotype (CINCA) and carries the p.(Glu569Gly) missense variation, displayed the lowest rate of the mutated allele (ranging from 7% to 10%, depending on the analyzed cell types) (Table II, and Fig E1).

### Functional consequences of identified *NLRP3* variations

To assess the pathogenicity of the identified *NLRP3* variations, we studied ASC-speck formation, a common readout of inflammasome activation.<sup>19</sup> In agreement with the known role of *NLRP3* in inflammasome assembly and ASC-speck formation, cells transfected with pNLRP3-WT displayed a significantly higher percentage of ASC specks as compared to cells transfected with the empty vector (EV) alone. However, no significant difference was observed between the cells transfected with the constructs carrying either the germline variant (pNLRP3-Gly769Ser) or the WT sequence. In contrast, cells transfected with pNLRP3-Glu569Gly or with pNLRP3-Glu569Lys (used as a positive control of inflammasome activation) showed a significantly higher percentage of ASC specks as compared to cells transfected with pNLRP3-WT. Therefore, a gain-of-function effect on inflammasome activation

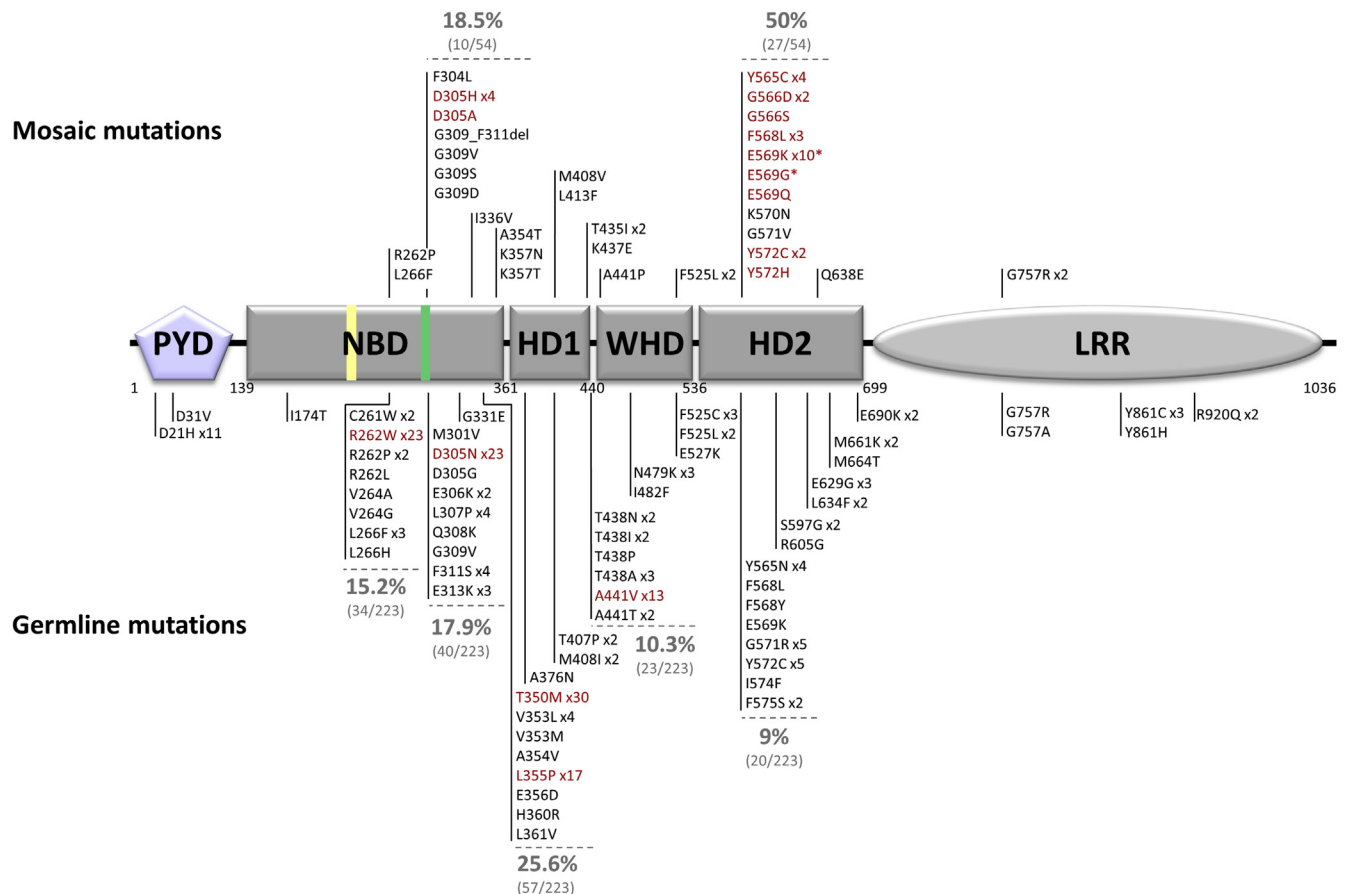
was observed for p.(Glu569Gly), leading us to classify this variation as pathogenic (Fig 1).

To further assess the functional consequences of the p.(Gly769Ser) variation, we tested its impact on cytokine secretion. To this end, THP1 cells were transfected with the EV, pNLRP3-WT, pNLRP3-Glu569Lys (used as positive control) or pNLRP3-Gly769Ser and treated with LPS, a well-known activator of the *NLRP3* inflammasome. IL1 $\beta$  secretion was measured in the cell culture supernatants, and significantly higher levels were found in the supernatant of cells transfected with pNLRP3-Glu569Lys as compared to cells transfected either with pNLRP3-WT or with the EV. When compared with WT-*NLRP3*, similar amounts of secreted IL1 $\beta$  were measured for the p.(Gly769Ser) variant (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Taken together, these results led us to classify the p.(Gly769Ser) variant as probably benign.

### Different *NLRP3* mutations occur in somatic mosaic and germinal states

In an attempt to identify possible germline- and mosaic-specific characteristics of *NLRP3* mutations, we reinterpreted all previously reported *NLRP3* variations according to both the associated disease severity and their localization in the protein domains. The 3 unrelated *NLRP3*-AID patients with mutations involving the same residue but associated with different phenotypic severity were included in this study. Based on the American College of Medical Genetics classification of sequence variations,<sup>14</sup> we classified as “likely pathogenic” or “pathogenic” 90 distinct *NLRP3* mutations identified in 277 patients: 33 somatic mosaic mutations in 54 unrelated patients and 65 germline mutations reported in 223 unrelated patients (Fig 2, and see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). It is worth noting that half of the reported cases (27 of 54) with mosaic mutations carry mutations located in the HD2 domain of *NLRP3*, including the p.(Gly569Lys) mutation, whereas only 9% of the patients (20 of 223) with germline mutations carry mutations located in this region (Fig 2). A second mosaic mutational hot spot (10 of 54, 18.5%) involves Phe304 to Gly309 amino acids that overlap the Walker B motif of the NBD. Among all reported mutations, only 8 of them have been found in both a mosaic and a germinal state (Fig 3). These 8 mutations were identified in a germinal state in 16 patients (of 223, 7.2%), and it is noteworthy that all these patients presented with the severe CINCA phenotype. The same mutations were identified in mosaic state in 22 patients (of 54, 40.7%) who displayed moderate (MWS) to severe (CINCA) phenotypes (Fig 3). On the other hand, the most frequent germline mutations, such as p.(Thr350Met), p.(Arg262Trp), p.(Asp305Asn), p.(Leu355Pro), and p.(Ala441Val), have never been reported in a mosaic state (Fig 4, A), whereas some of these mutations involve amino acids that are changed in other substitutions in mosaic state—p.(Arg262Pro), p.(Asp305His), p.(Asp305Ala), and p.(Ala441Pro) (Fig 2).

With the aim to further assess the functional impact of the mutations, we located on the crystal structure of *NLRP3*<sup>3</sup> the 5 amino acids involved in the most frequently reported mutations in mosaic and germinal states. Noteworthy, the mosaic mutations are located in the core of the NACHT domain close to the ATPase domain, while the germline mutations are scattered throughout the NACHT domain (Fig 4, B).



**FIG 2.** Somatic mosaic and germline *NLRP3* mutations on a domain organization model of the protein. *Top*, All mutations reported in a mosaic state. *Bottom*, All mutations reported in a germinal state. The 5 most frequently reported mutated amino acids are in red. The asterisks denote mutations identified in this study. The Walker A and Walker B motifs are in yellow and green, respectively. PYD, pyrin domain.

## DISCUSSION

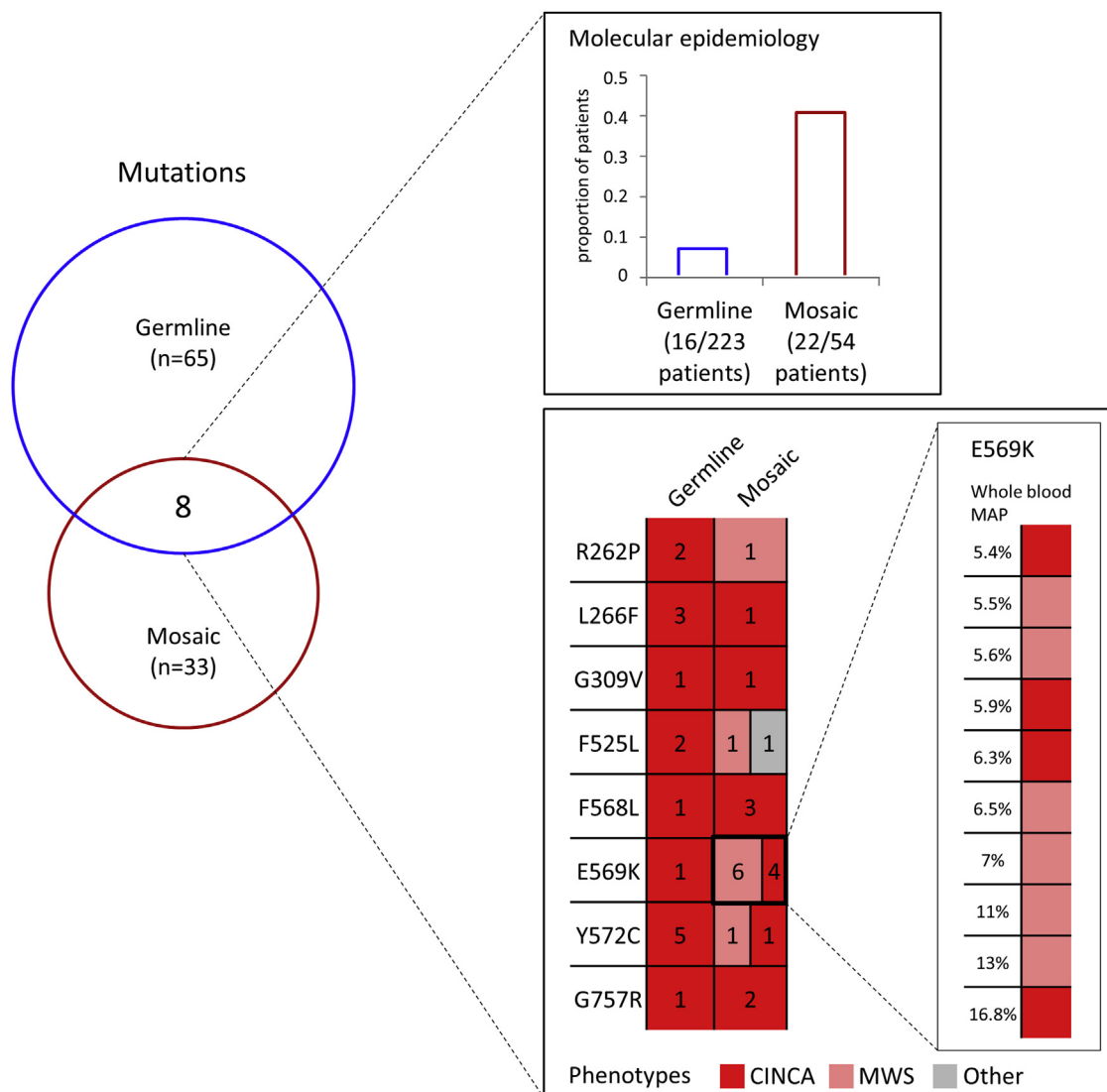
For an optimal medical care of patients with *NLRP3*-AID, an early molecular *NLRP3* diagnosis is essential. Indeed, only 1 of the 3 patients identified in the current study received an effective anti-IL1 treatment prior to the identification of a *NLRP3* mutation. Although the use of NGS-based technologies increases the sensitivity of detection of somatic mutations usually undetectable by Sanger sequencing, 2 critical points are to be followed for an accurate molecular diagnosis of *NLRP3*-AIDs: (1) high-depth sequencing and specific bioinformatics pipelines are necessary to detect low level mosaicism; (2) intrafamilial segregation studies of germline variations and functional assays of all newly identified variations are mandatory, because, as shown here, a benign sequence variation can hide the pathogenic one.

By studying the level of mosaicism of the identified mosaic mutations in different cell types, we observed a wide distribution, indicating that the mutational event arose early during embryogenesis. However, in proband I, a higher percentage of the mutated allele was observed in myeloid cells and B lymphocytes. A similar pattern was already observed in adult patients with a late onset of the disease.<sup>11</sup> We did not observe any correlation between the level of mosaicism and the severity of the disease phenotype, neither among the 3 patients, nor in the

previously reported ones (Fig 3), thereby suggesting that additional genetic and/or environmental factors might modulate the expressivity of *NLRP3*-AIDs.

The amino acid Glu569 of *NLRP3* is the most frequently affected residue involved in mosaic mutations.<sup>7</sup> The c.1705G>A p.(Glu569Lys) mutation has been reported (including the current study) in 10 patients.<sup>11,12,15-18</sup> Of note, this G>A transition is located on a cytosine-phosphate-guanine (CpG) dimer that favors this type of mutational event.<sup>20</sup> Two other mosaic mutations—c.1705G>C p.(Glu569Gln)<sup>11</sup> and c.1706A>G p.(Glu569Gly) (proband III)—implicate the same amino acid without involving a CpG dimer. Overall, Glu569 is affected in 22% of patients (12 of 54) carrying a mosaic mutation and in only 0.4% of patients (1 of 223) with a germline mutation. The patients carrying a mosaic mutation involving Glu569 had either a moderate (n = 7) or a severe phenotype (n = 5).<sup>11,12,15-18</sup> It is worth noting that the single patient with a germline mutation involving this residue had a very severe neonatal CINCA syndrome,<sup>21</sup> thereby suggesting that mutations found only in mosaic state could be incompatible with life if present in germinal state.

While taking into account all reported *NLRP3* mutations (n = 90) identified in 277 patients, further observations can be made. First, we noticed that in half of the patients with a mosaic mutation (27 of 54), the affected amino acid lies on a short segment



**FIG 3.** Characteristics of the *NLRP3* mutations reported in both mosaic and germline states. *Left*, Venn diagram of mosaic and somatic *NLRP3* mutations is presented. *Right*, The 8 common mutations carried by 38 patients in either a mosaic or a germinal state and the phenotypes of the corresponding patients are detailed. For the p.(Glu569Lys) mutation (E569K), the levels of mosaicism are also given.

(Tyr565 to Tyr572) of the HD2 subdomain of the NACHT domain, whereas only 9% of the patients (20 of 223) with a germline mutation carry a mutation in that region. In an attempt to explain this striking observation, we highlighted the mutated amino acids on the crystal structure of NLRP3. The region spanning Tyr565 to Tyr572 is located in the core of the NACHT domain facing the Walker B motif, a sequence involved in ATP hydrolysis and essential for NLRP3-inflammasome activation.<sup>22</sup> Interestingly, the region of the Walker B motif includes the second hot spot (Phe304 to Gly309) for mosaic mutations (18.5%, 10 of 54 patients). The 2 mutational hot spots account for 68.5% of the patients (37 of 54) with mosaic mutations, but only 26.9% of the patients (60 of 223) with germline mutations. Second, only a minority of the reported *NLRP3* mutations (8 of 90) were identified in both somatic mosaic and germinal states. In germinal state, these 8 mutations concern 16 unrelated patients (of 223, 7.1%) who are all sporadic cases presenting with the severe CINCA phenotype.<sup>5,21-28</sup> Third,

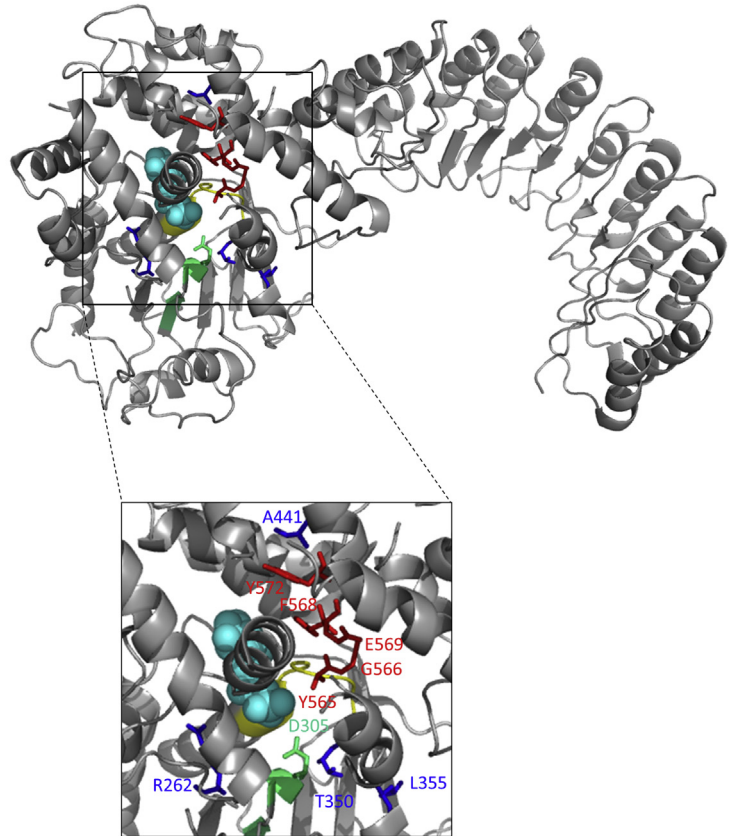
the most frequent germline mutations have never been reported in *NLRP3*-AID patients in a mosaic state despite their location on CpG dimers for 4 of them (Figs 2 and 4, A). It is tempting to speculate that those mutations reported in a germinal state only may display a very mild phenotype (or even be asymptomatic) if present in a mosaic state and could, therefore, escape diagnosis. In keeping with this hypothesis, the single report of the p.(Thr350Met) mutation in a mosaic state concerns an asymptomatic mother who transmitted the mutation to her child who presented with an MWS phenotype.<sup>29</sup> Conversely, somatic mosaic mutations could be responsible for severe phenotypes or be incompatible with life if present in a germinal state.<sup>18</sup> A negative selection of gametes carrying the most activating mutations is another attractive hypothesis. Indeed, recent studies have shown a prominent NLRP3 staining of peritubular cells of testis from patients with impaired spermatogenesis.<sup>30,31</sup> Moreover, 2 different studies suggested hypofertility in *NLRP3*-AID patients.<sup>32,33</sup>

A Most frequent *NLRP3* mutations

Germline mutations	CpG dimer	Patients' phenotype	Reported in mosaic state
T350M	Yes	MWS – CINCA (N=30)	No
R262W	Yes	FCAS – MWS (N=23)	No
D305N	Yes	FCAS/MWS – MWS – CINCA (N=23)	No
L355P	No	FCAS (N=17)	No
A441V	Yes	FCAS – MWS – CINCA (N=13)	No

Mosaic mutations	CpG dimer	Patients' phenotype	Reported in germinal state
E569K	Yes	MWS – CINCA (N=10)	CINCA (N=1)
E569G	No	CINCA (N=1)	No
E569Q	No	MWS (N=1)	No
Y565C	No	MWS – CINCA (N=4)	No
D305H	No	CINCA (N=4)	No
D305A	No	MWS (N=1)	No
F568L	No	CINCA (N=3)	CINCA (N=1)
G566D	No	MWS (N=2)	No
G566S	No	CINCA (N=1)	No
Y572C	No	MWS – CINCA (N=2)	CINCA (N=5)
Y572H	No	CINCA (N=1)	No

## B



**FIG 4.** Patients' phenotypes and localization in the 3-dimensional structure of NLRP3 of the most frequently reported mutations. **A**, Characteristics associated with the most frequent mosaic and germline mutations. **B**, The 5 most frequently mutated amino acids in mosaic state (in red), in germline state (in blue), and in both mosaic and germinal states (in green) are highlighted. Cyan, adenosine diphosphate; yellow, Walker A motif; green, Walker B motif.

From a general viewpoint, it is therefore important to underline that the phenotypes observed in *NLRP3*-AID patients (from FCAS to CINCA) represent only a part of the phenotypic spectrum associated with a mutation in *NLRP3*. Indeed, on the basis of the epidemiological data presented in this study, it appears that the *NLRP3* mutations cover a phenotypic spectrum larger than that revealed by the patients' phenotype: some mutations could be lethal or asymptomatic (and therefore not reported) depending on their molecular characteristics (ie, germinal/mosaic status, localization in the protein, and amino acid change).

In conclusion, as illustrated here, the early detection of an *NLRP3* mosaicism is of key importance to establish the diagnosis of *NLRP3*-AID and to initiate anti-IL1 treatment. Our study also sheds light on a diagnostic pitfall due to the identification of several *NLRP3* sequence variations in the same patient, thereby underlining the importance of functional and segregation studies to assess the pathogenicity of the identified sequence variations. The 2 hot spots for mosaic mutations are located around the Walker B motif, essential for NLRP3-inflammasome activation. The phenotypic spectrum of *NLRP3*-AIDs appears to be related to the germinal/mosaic status and localization of the underlying mutations.

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## Key messages

- This study, which reviews and reinterprets all previously reported *NLRP3* variations, shows on the human NLRP3 cryoelectron microscopy structure that the mosaic mutations are mainly situated in the core of the NLRP3-inflammasome activating domain, while germline mutations are scattered throughout this domain.
- Only a minority of *NLRP3* mutations is found in both germinal and somatic mosaic states. It is noteworthy that in the germinal state, those mutations were all found in sporadic cases presenting with the most severe AID phenotype. The mutation localization in the NLRP3 protein but also the amino acid change could account for the occurrence of mutations in mosaic or germinal states.

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