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***NLRP3*-associated autoinflammatory diseases: phenotypic and molecular characteristics of germline *versus* somatic mutations**

Camille Louvrier<sup>1,2</sup> PharmD, Eman Assrawi<sup>1</sup> MD PhD, Elma El Khouri<sup>1</sup> PhD, Isabelle Melki<sup>3</sup> MD PhD, Bruno Copin<sup>2</sup>, Emmanuelle Bourrat<sup>3</sup> MD, Noémie Lachaume<sup>3</sup> MD, Bérengère Cador-Rousseau<sup>4</sup> MD, Philippe Duquesnoy<sup>1</sup>, William Piterboth<sup>2</sup>, Fawaz Awad<sup>1§</sup> MD PhD, Claire Jumeau<sup>1</sup> PhD, Marie Legendre<sup>1,2</sup> PharmD PhD, Gilles Grateau<sup>1,5</sup> MD, Sophie Georgin-Lavialle<sup>1,5</sup> MD PhD, Sonia A. Karabina<sup>1</sup> PhD, Serge Amselem<sup>1,2\*</sup> MD PhD, Irina Giurgea<sup>1,2\*</sup> MD PhD

\*Correspondence to: Irina Giurgea and Serge Amselem, INSERM UMR\_S933, Hôpital Armand Trousseau, 26 avenue du Dr. Arnold Netter, 75012 Paris, France; Tel.: +33 1 44 73 52 39; Fax: +33 1 44 73 52 19; Email: irina.giurgea@inserm.fr, serge.amselem@inserm.fr

**1)** Sorbonne Université, INSERM, UMRS\_933 «Maladies génétiques d'expression pédiatrique», Hôpital Armand Trousseau, Paris, 75012 France

**2)** Département de Génétique médicale, Hôpital Armand Trousseau, Paris, 75012, France

**3)** Service de Pédiatrie générale, maladies infectieuses et médecine interne pédiatrique, Centre de référence Rhumatismes et Auto-Immunité Systémique de l'Enfant (RAISE), Assistance Publique-Hôpitaux de Paris, Hôpital Robert Debré, Paris, 75019, France

**4)** Service de Médecine interne, Centre Hospitalier Universitaire Pontchaillou, Rennes, 35033, France

**5)** Service de Médecine interne, et Centre de référence des maladies autoinflammatoires et des amyloses inflammatoires (CEREMAIA), Assistance Publique-Hôpitaux de Paris, Hôpital Tenon, Paris, 75012, France

**§** Present address: Al-Quds University, Faculty of Medicine, Biochemistry and Molecular Biology Department, Abu Deis, Jerusalem, Palestine.

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## ABSTRACT

### Background

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

### Objective

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

### Methods

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

### Results

We identified, in three patients, mosaic mutations affecting the same *NLRP3* aminoacid (Glu569). This residue belongs to one of the two mosaic mutational hotspots 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 hotspots account for 68.5% (37/54) of patients with mosaic mutations. Glu569 is affected in 22% (12/54) of the patients with mosaic mutations and in 0.4% (1/223) of those with germline mutations. Only 8/90 mutations were found in mosaic and germinal state. 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 five most frequent germline mutations was identified in mosaic state. Mutations found only in germinal state could, therefore, be asymptomatic in mosaic state.

### Conclusion

The phenotypic spectrum of *NLRP3*-AIDs appears to be related to the germinal/mosaic status and localization of the underlying mutations.

## KEY MESSAGES

- This study, which reviews and reinterprets all previously reported *NLRP3* variations, shows on the human *NLRP3* cryo-electron 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 both in germinal and in somatic mosaic state. Noteworthy, 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 aminoacid change, could account for the occurrence of mutations in mosaic or in germinal state.

## CAPSULE SUMMARY

The mosaic/germinal status and localization of the underlying mutation participate in the phenotypic spectrum of *NLRP3*-AID patients. The identification of novel germline variations should not hamper the search for mosaic mutations. Functional studies are mandatory.

## KEY WORDS

*NLRP3*-associated autoinflammatory diseases, *NLRP3*, somatic mosaic mutations, *NLRP3*-inflammasome activation, mutational hotspot.

## ABBREVIATIONS

ACMG: American College of Medical Genetics

AID: autoinflammatory disease

ASC: apoptosis-associated speck-like protein containing a CARD

ATP: adenosine triphosphate

92 CAPS: cryopyrin-associated periodic syndrome  
 93 CINCA: chronic infantile neurological, cutaneous and articular syndrome  
 94 CRP: C-reactive protein  
 95 EV: empty vector  
 96 FCAS: familial cold autoinflammatory syndrome  
 97 HD1: helical domain 1  
 98 HD2: helical domain 2  
 99 HEK293T: human embryonic kidney cell line  
 100 IL1 $\beta$ : interleukin 1 $\beta$   
 101 LPS: lipopolysaccharide  
 102 LRR: leucine-rich repeat  
 103 MAP: mutated allele percentage  
 104 MWS: Muckle-Wells syndrome  
 105 NACHT domain: nucleotide-binding and oligomerization domain (standing for NAIP, CIITA,  
 106 HET-E and TP1)  
 107 NBD: nucleotide binding domain  
 108 NEK7: NIMA related kinase 7  
 109 NGS: next generation sequencing  
 110 NLRP3: nucleotide-binding domain, leucine-rich repeat, pyrin domain-containing protein 3  
 111 NLRP3-AID: NLRP3-associated autoinflammatory disease  
 112 PBMC: peripheral blood mononuclear cell  
 113 PCR: polymerase chain reaction  
 114 PYD: pyrin domain  
 115 THP1 cells: human monocytic cell line  
 116 WHD: winged helix domain  
 117 WT: wild-type  
 118

## INTRODUCTION

Autoinflammatory disorders (AIDs) associate 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 ASC (apoptosis-associated speck-like protein containing a CARD) and the pro-caspase 1 leading to autoproteolytic activation of caspase-1 and secretion of proinflammatory cytokines (mainly IL1 $\beta$ )<sup>2</sup>.

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

*NLRP3*-AIDs, previously known as cryopyrin-associated periodic syndromes (CAPSs), include three overlapping entities with increasing severity: (i) the familial cold autoinflammatory syndrome (FCAS, OMIM#120100) characterized by cold-induced inflammatory episodes of fever, arthralgia and urticaria; (ii) the Muckle-Wells syndrome (MWS, OMIM#191900) associating recurrent attacks of fever, urticaria and arthritis with subsequent sensorineural hearing loss; and (iii) 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 an autosomal dominant trait<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.

153           In this study, in an attempt to identify possible germline-specific and mosaic-specific  
154 features of *NLRP3* mutations, we re-assessed all previously reported *NLRP3* variations  
155 identified in *NLRP3*-AID patients. To evaluate the functional impact of the mutations, we  
156 studied their molecular characteristics such as their location on the 3D structure of the NLRP3  
157 protein and the aminoacid change, as well as their associated phenotypic severity. In this  
158 study, we also took into account three unrelated *NLRP3*-AID patients with mosaic variations  
159 involving the same aminoacid. One of those patients also carried a novel germline variation.  
160 We assessed the pathogenicity of the identified variants through intra-familial segregation for  
161 the germline one, and by performing inflammasome-activation studies.

## PATIENTS AND 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 consents were 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. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using Pancoll density gradient (PAN-Biotech). PBMCs were separated by positive selection using immunomagnetic microbeads (MACS, Miltenyi Biotec), 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 polymerase chain reaction (PCR) and sequenced using the Big Dye Terminator sequencing kit (Applied Biosystems) on an ABI 3130XL automated capillary DNA sequencer (Applied Biosystems). Sequences were analyzed against the reference sequence (NM\_004895).

Next generation sequencing (NGS): NGS was performed using a custom sequence capture (Nimblegen SeqCap EZ Choice system, Roche) of the exons and the flanking intronic sequences of the main AID-causing genes: *ADA2*, *CARD14*, *IL1RN*, *IL36RN*, *LACC2*, *LPIN2*, *MEFV*, *MVK*, *NLRC4*, *NLRP12*, *NLRP3*, *NOD2*, *PLCG2*, *PSMB8*, *PSTPIP1*, *RBCK1*, *TMEM173*, *TNFAIP3*, *TNFRSF11A* and *TNFRSF1A*. Sequencing was performed on MiSeq or Nextseq500 (Illumina) 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. Variant calling was performed with VarScan with a threshold for the minimum mutated allele percentage (MAP) of 1%. Variant calls in VCF format were then annotated through ANNOVAR. All results obtained by NGS were confirmed by Sanger sequencing.



## **Mutagenesis, cell culture and functional tests**

Site-directed mutagenesis of the *NLRP3* wild-type 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.

HEK293T cells stably expressing green fluorescent protein (GFP)-tagged-ASC and FLAG-tagged pro-caspase1 (designated ASC-GFP\_C1-FLAG) were transfected with 375 ng of mentioned plasmids using FUGENE HD (Promega). After 24h, the transfected cells were observed using a Nikon Eclipse TS100 inverted fluorescent microscope. Manual counting of cells containing ASC-GFP specks was then performed in 5 randomly selected fields at 20 X magnification. The percentage of speck-positive cells was calculated as the number of specks divided by the total number of counted cells.

THP1 cells were initially primed with 100 ng/ml PMA (Phorbol 12-Myristate 13-Acetate) for 3h, then transfected with 500 ng of the plasmids using the FF-100 program in the 4D-Nucleofector, Amaxa (Lonza). Transfected THP1 cells were treated directly with 100 ng/ml LPS at 37C°. After 24h, cytokine measurements by ELISA assays were performed following manufacturer's instructions (R&D).

## **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 (ACMG)<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 3D structure was visualized with the Pymol software.

## **Statistical analyses**

Differences were analyzed using the unpaired student t-test and were plotted with the Prism 5, GraphPad software, (San Diego, CA). A p value <0.05 was considered as statistically significant.

## RESULTS

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

We studied three 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 1** and **supplemental data 1**). None of the patients had a family history of AID.

To identify the molecular defect responsible for the patients' phenotype, we followed a 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 mutated allele percentage (MAP) of 7% and 13% was identified in peripheral blood DNA of Probands I and II, respectively (**table 2**, **supplemental figure 1**). This variation, which is absent from the genome aggregation database (gnomAD), 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, is not predicted to affect splicing. In the 3D structure of *NLRP3*, Gly769 is located on an external part of a LRR motif (**supplemental figure 2**), outside the region that interacts with NEK7. Secondly, the access to parents' DNA samples revealed that p.(Gly769Ser) was inherited from the asymptomatic father, 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. Noteworthy, 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 2, supplemental figure 1**).

### **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 wild-type. 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 (**figure 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 lipopolysaccharide (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 to WT-NLRP3, similar amounts of secreted IL1 $\beta$  were measured for the p.(Gly769Ser) variant (**supplemental figure 3**). Taken together, these results led us to classify the p.(Gly769Ser) variant as probably benign.

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

In an attempt to identify possible germline-specific and mosaic-specific characteristics of *NLRP3* mutations, we re-interpreted all previously reported *NLRP3* variations according to both the associated disease severity and their localization in the protein domains. The three unrelated *NLRP3*-AID patients with mutations involving the same residue but associated with different phenotypic severity were incorporated in this study. Based on the ACMG 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 (**figure 2, supplemental table 1**). Noteworthy, half (27/54 patients) of the reported cases with mosaic mutations carry mutations located in the HD2 domain of *NLRP3*, including the p.(Gly569Lys) mutation, whereas only 9% (20/223 patients) of the patients with germline mutations carry mutations located in this region (**figure 2**). A second mosaic mutational hotspot (10/54 - 18.5%) involves Phe304 to Gly309 aminoacids that overlaps 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 (**figure 3**). These 8 mutations were identified in a germinal state in 16 patients (16/223 – 7.2%) and, noteworthy, all these patients presented with the severe CINCA phenotype. The same mutations were identified in mosaic state in 22 patients (22/54 – 40.7%) who displayed moderate (MWS) to severe (CINCA) phenotypes (**figure 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 (**figure 4a**), whereas some of these mutations involve aminoacids that are changed in other substitutions in mosaic state [p.(Arg262Pro), p.(Asp305His), p.(Asp305Ala) and p.(Ala441Pro)] (**figure 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 five aminoacids involved in the most frequently reported mutations in mosaic and germinal state. Noteworthy, we found that 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 (**figure 4b**).

## DISCUSSION

For an optimal medical care of patients with *NLRP3*-AID, an early molecular *NLRP3* diagnosis is essential. Indeed, only one of the three 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, two critical points are to be followed for an accurate molecular diagnosis of *NLRP3*-AIDs: i) high-depth sequencing and specific bioinformatics pipelines are necessary to detect low level mosaicisms; ii) intra-familial 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 three patients, nor in the previously reported ones (**figure 3**), thereby suggesting that additional genetic and/or environmental factors might modulate the expressivity of *NLRP3*-AIDs.

The aminoacid 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 ten patients<sup>11, 12, 15-18</sup>. Of note, this G>A transition is located on a CpG dimer that favors this type of mutational event<sup>20</sup>. Two other mosaic mutations implicate the same aminoacid, c.1705G>C p.(Glu569Gln)<sup>11</sup> and c.1706A>G p.(Glu569Gly) (Proband III) without involving a CpG dimer. Overall, Glu569 is affected in 22% (12/54) of patients carrying a mosaic mutation and in only 0.4% (1/223) of the patients 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>. Noteworthy, 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. Firstly, we noticed that in half of the patients with a mosaic mutation (27/54), the affected aminoacid lies on a short segment (Tyr565 to Tyr572)

of the HD2 subdomain of the NACHT domain, whereas only 9% (20/223) of the patients with a germline mutation carry a mutation in that region. In an attempt to explain this striking observation, we highlighted the mutated aminoacids 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 hotspot (Phe304 to Gly309) for mosaic mutations (18.5%, 10/54 patients). The two mutational hotspots account for 68.5% (37/54) of the patients with mosaic mutations, but only 26.9% (60/223) of those with germline mutations. Secondly, only a minority of the reported *NLRP3* mutations (8/90) were identified in both somatic mosaic and germinal state. In germinal state, these 8 mutations concern 16 unrelated patients (16/223 – 7.1%) who are all sporadic cases presenting with the severe CINCA phenotype<sup>5, 21-28</sup>. Thirdly, the most frequent germline mutations have never been reported in *NLRP3*-AID patients in a mosaic state despite their location on CpG dimers for four of them (**figure 2** and **4a**). 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 a 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, two different studies suggested hypofertility in *NLRP3*-AID patients<sup>32, 33</sup>.

From a general viewpoint, it is therefore important to underline that the phenotypes observed in *NLRP3*-AID patients (from FACS 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 (i.e. germinal/mosaic status, localization in the protein and aminoacid 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 one 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 two hotspots 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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## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.



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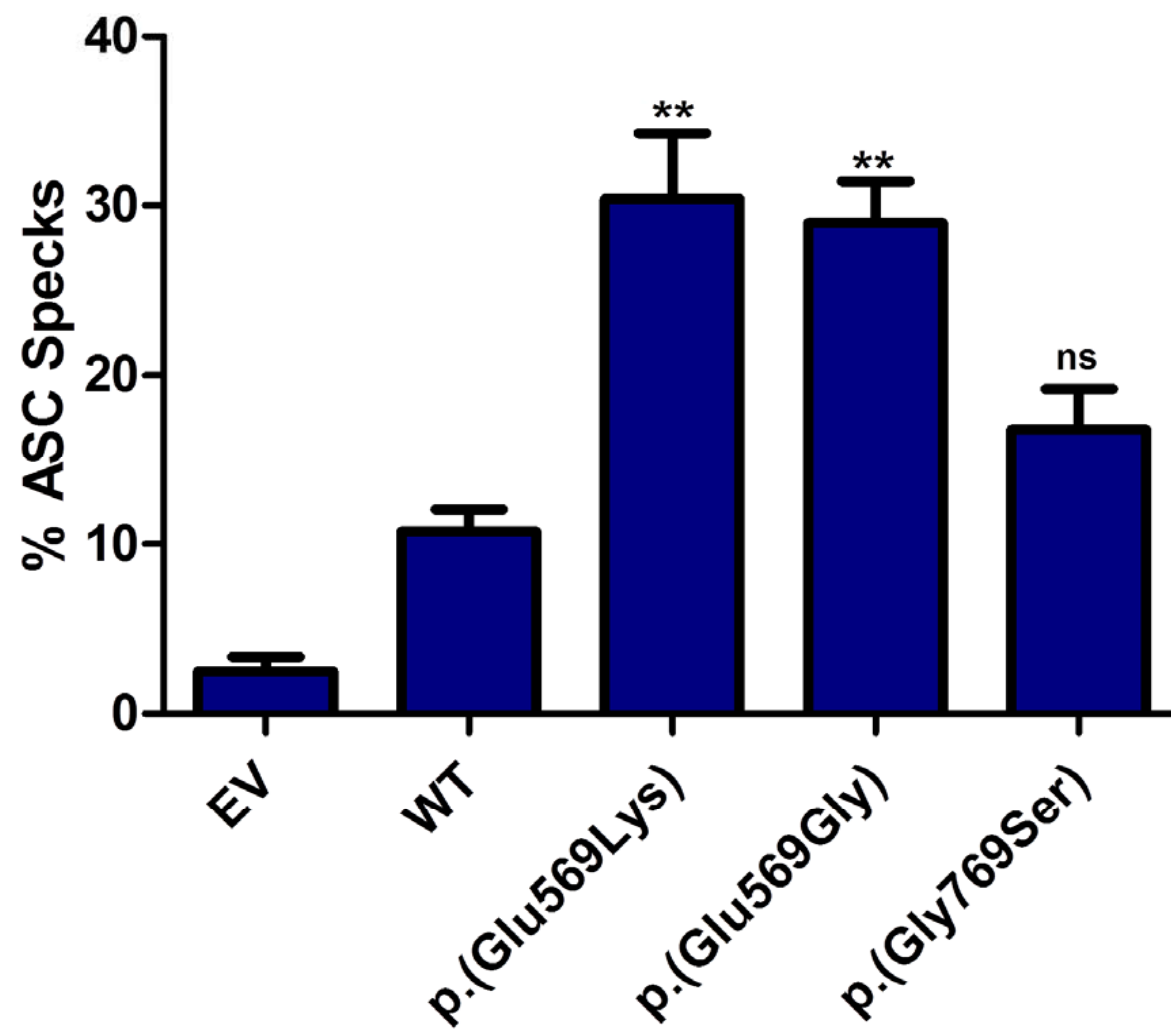
## FIGURE LEGENDS

**Figure 1. Impact of *NLRP3* mutations on ASC speck formation.** HEK (ASC-GFP\_C1-FLAG) cells were transfected with 375ng of the expression plasmids encoding the wild-type *NLRP3* or the *NLRP3* protein carrying the missense variations p.(Glu569Gly), p.(Glu569Lys) and p.(Gly769Ser) or the empty vector (EV). The percentage of ASC specks was calculated as described in “Patients and Methods”. Results represent the mean + SD from 3 independent experiments. P values were calculated using unpaired Student’s t test as compared to the wild-type. \* indicates significant P value <0.05; \*\* P value <0.01; ns indicates non-significant P value.

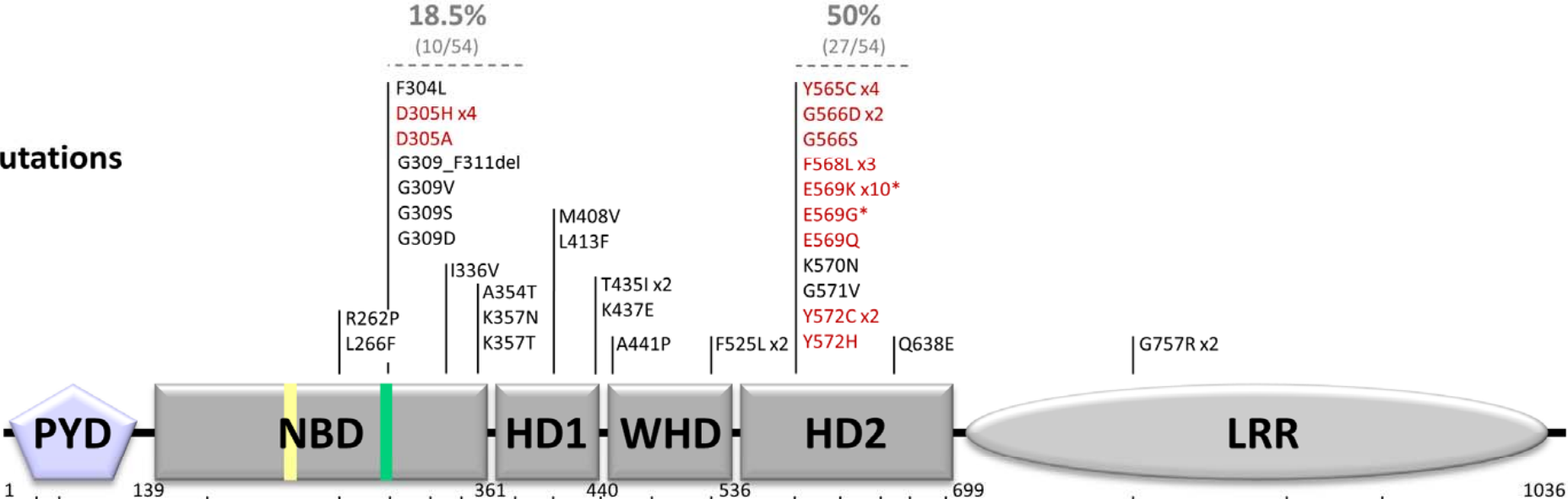
**Figure 2. Somatic mosaic and germline *NLRP3* mutations on a domain organization model of the protein.** Top: all mutations reported in a mosaic state. Bottom: all mutations reported in a germinal state. The five most frequently reported mutated aminoacids are in red. The asterisks denote mutations identified in this study. PYD: Pyrin domain; NBD: Nucleotide binding domain; HD1: Helical domain 1; WHD: Winged helix domain; HD2: Helical domain 2; LRR: Leucine-rich repeat domain. The Walker A and Walker B motifs are in yellow and green respectively.

**Figure 3. Characteristics of the *NLRP3* mutations reported in both mosaic and germline state.** Venn diagram of mosaic and somatic *NLRP3* mutations is represented on left part of the figure. 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 on the right part of the figure. For the p.(Glu569Lys) mutation (E569K) the levels of mosaicism are also given. MAP: mutated allele percentage

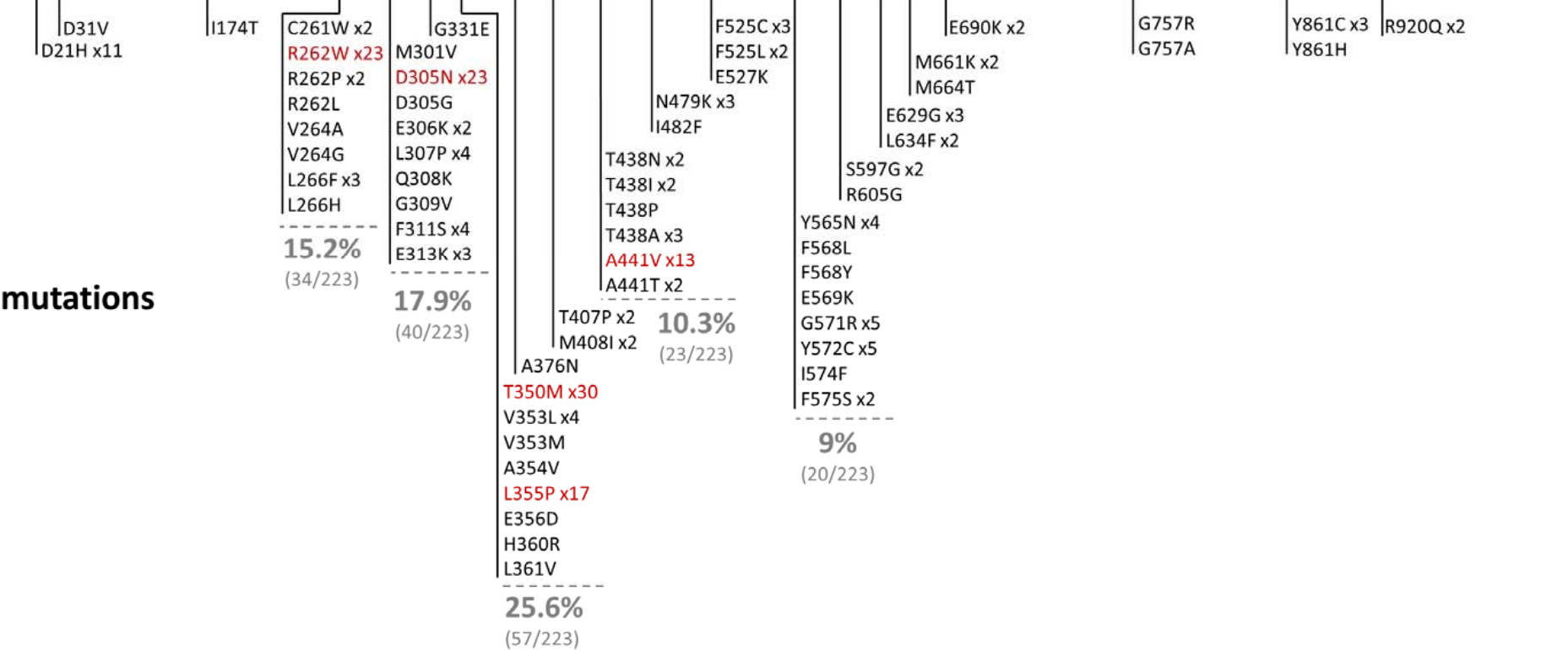
**Figure 4. Patients’ phenotypes and localization in the 3D structure of *NLRP3* of the most frequently reported mutations.** **a.** Characteristics associated with the five most frequent mosaic and germline mutations **b.** Highlight of the five most frequently mutated aminoacids in mosaic state (in red), in germline state (in blue) and in both mosaic and germinal state (in green). Cyan: adenosine di-Phosphate (ADP); yellow: Walker A motif; green: Walker B motif.



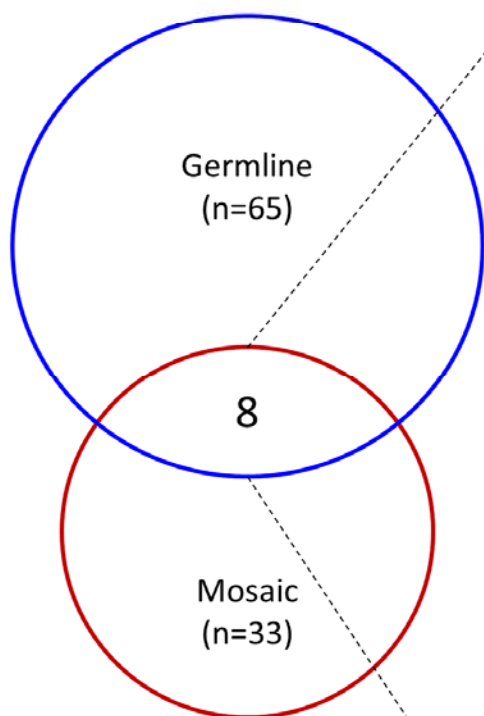
Mosaic mutations



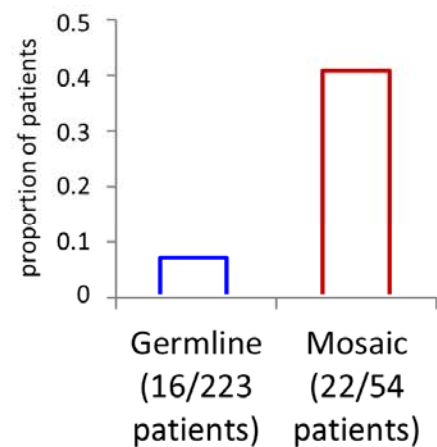
Germline mutations



## Mutations



## Molecular epidemiology

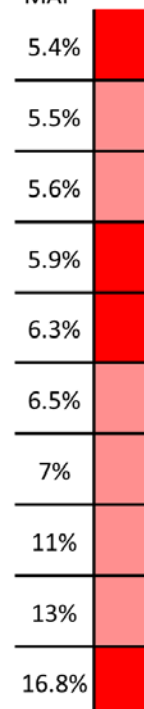


Germline Mosaic

	Germline	Mosaic
R262P	2	1
L266F	3	1
G309V	1	1
F525L	2	1
F568L	1	3
E569K	1	6
Y572C	5	1
G757R	1	2

## E569K

Whole blood  
MAP



Phenotypes ■ CINCA ■ MWS ■ Other

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 x2	No	MWS (N=2)	No
G566S	No	CINCA (N=1)	No
Y572C x2	No	MWS – CINCA (N=2)	CINCA (N=5)
Y572H	No	CINCA (N=1)	No

b

