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

3
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33 **ABSTRACT**

34
35 **Background**

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

38 **Objective**

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

42 **Methods**

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

47 **Results**

48 We identified, in three patients, mosaic mutations affecting the same *NLRP3* aminoacid
49 (Glu569). This residue belongs to one of the two mosaic mutational hotspots that face each
50 other in the core of the *NLRP3* ATPase domain. The review of the 90 *NLRP3* mutations
51 identified in 277 patients revealed that those hotspots account for 68.5% (37/54) of patients
52 with mosaic mutations. Glu569 is affected in 22% (12/54) of the patients with mosaic
53 mutations and in 0.4% (1/223) of those with germline mutations. Only 8/90 mutations were
54 found in mosaic and germinal state. All of the germline mutations were associated with a
55 severe phenotype. These data suggest that mutations found only in mosaic state could be
56 incompatible with life if present in germinal state. None of the five most frequent germline
57 mutations was identified in mosaic state. Mutations found only in germinal state could,
58 therefore, be asymptomatic in mosaic state.

59 **Conclusion**

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

63 **KEY MESSAGES**

- 64
- 65 • This study, which reviews and reinterprets all previously reported *NLRP3* variations,
66 shows on the human *NLRP3* cryo-electron microscopy structure that the mosaic
67 mutations are mainly situated in the core of the *NLRP3*-inflammasome activating
68 domain, while germline mutations are scattered throughout this domain.
 - 69 • Only a minority of *NLRP3* mutations is found both in germinal and in somatic mosaic
70 state. Noteworthy, in the germinal state, those mutations were all found in sporadic
71 cases presenting with the most severe AID phenotype. The mutation localization in the
72 *NLRP3* protein but also the aminoacid change, could account for the occurrence of
73 mutations in mosaic or in germinal state.

74

75 **CAPSULE SUMMARY**

76

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

80

81 **KEY WORDS**

82

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

85

86 **ABBREVIATIONS**

87

88 ACMG: American College of Medical Genetics

89 AID: autoinflammatory disease

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

91 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 β : interleukin 1 β
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

119 INTRODUCTION

120
121 Autoinflammatory disorders (AIDs) associate recurrent episodes of fever, sterile
122 inflammation of joints and serosal membranes, skin rashes, lymphadenopathy,
123 musculoskeletal symptoms and biological inflammation. *NLRP3*-AIDs are a group of
124 monogenic AIDs related to germline or somatic *NLRP3* mutations leading to NLRP3-
125 inflammasome activation¹. The NLRP3-inflammasome is an intracellular multiprotein
126 signaling complex that assembles around NLRP3, the adaptor protein ASC (apoptosis-
127 associated speck-like protein containing a CARD) and the pro-caspase 1 leading to
128 autoproteolytic activation of caspase-1 and secretion of proinflammatory cytokines (mainly
129 IL1 β)².

130 The NLRP3 protein comprises a N-terminal pyrin domain (PYD), a central nucleotide-
131 binding and oligomerization domain (NACHT) and C-terminal leucine-rich repeats (LRRs).
132 NACHT is an adenosine triphosphatase (ATPase) domain including a nucleotide binding
133 domain (NBD) that contains a Walker A (ATP binding) and a Walker B (Mg²⁺ binding)
134 catalytic motifs, the helical domain 1 (HD1), a winged helix domain (WHD), and the helical
135 domain 2 (HD2). Through the NBD, HD2 and LRRs regions, NLRP3 interacts with the
136 Ser/Thr kinase NEK7 that mediates NLRP3 inflammasome activation³.

137 *NLRP3*-AIDs, previously known as cryopyrin-associated periodic syndromes
138 (CAPSs), include three overlapping entities with increasing severity: (i) the familial cold
139 autoinflammatory syndrome (FCAS, OMIM#120100) characterized by cold-induced
140 inflammatory episodes of fever, arthralgia and urticaria; (ii) the Muckle-Wells syndrome
141 (MWS, OMIM#191900) associating recurrent attacks of fever, urticaria and arthritis with
142 subsequent sensorineural hearing loss; and (iii) the chronic infantile neurological, cutaneous
143 and articular syndrome (CINCA, OMIM#607115) characterized by central nervous system
144 involvement, continuous urticarial rash and deformative joint arthritis.

145 Primarily related to germline heterozygous *NLRP3* mutations, *NLRP3*-AIDs were
146 described in familial forms of AIDs transmitted as an autosomal dominant trait⁴, as well as in
147 sporadic cases due to *de novo* *NLRP3* mutations^{5, 6}. The identification of somatic mosaic
148 *NLRP3* mutations has further broadened the clinical spectrum of *NLRP3*-AIDs^{7, 8} with the
149 reports of late onset diseases⁹⁻¹¹ and paucisymptomatic forms¹². The advances in next
150 generation sequencing (NGS) technologies have led to the identification of an increasing
151 number of *NLRP3* sequence variations in AID patients, raising the question of the
152 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.

162

163 PATIENTS AND METHODS

165 Patients

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

170 Study of the *NLRP3* gene

171 Genomic DNA was extracted from peripheral blood leukocytes of patients and parents
172 from Proband III using standard procedures. Peripheral blood mononuclear cells (PBMCs)
173 were isolated from peripheral blood using Pancoll density gradient (PAN-Biotech). PBMCs
174 were separated by positive selection using immunomagnetic microbeads (MACS, Miltenyi
175 Biotec), anti-CD14 (monocytes) followed by anti-CD15 (neutrophils), anti-CD19 (B cells)
176 and anti-CD3 (T cells). DNA was then extracted from monocytes, neutrophils, B cells, T
177 cells, urine and buccal cells using proteinase K digestion followed by a phenol-chloroform
178 extraction protocol.

179 Sanger sequencing: *NLRP3* exon 3 and its intronic junctions were amplified by
180 polymerase chain reaction (PCR) and sequenced using the Big Dye Terminator sequencing kit
181 (Applied Biosystems) on an ABI 3130XL automated capillary DNA sequencer (Applied
182 Biosystems). Sequences were analyzed against the reference sequence (NM_004895).

183 Next generation sequencing (NGS): NGS was performed using a custom sequence
184 capture (Nimblegen SeqCap EZ Choice system, Roche) of the exons and the flanking intronic
185 sequences of the main AID-causing genes: *ADA2*, *CARD14*, *IL1RN*, *IL36RN*, *LACC2*, *LPIN2*,
186 *MEFV*, *MVK*, *NLRC4*, *NLRP12*, *NLRP3*, *NOD2*, *PLCG2*, *PSMB8*, *PSTPIP1*, *RBCK1*,
187 *TMEM173*, *TNFAIP3*, *TNFRSF11A* and *TNFRSF1A*. Sequencing was performed on MiSeq or
188 Nextseq500 (Illumina) platforms according to the manufacturer's instructions. The
189 conventional bioinformatics pipeline, previously described¹³, was used to study germline
190 mutations. For the mosaic-dedicated bioinformatics pipeline sequence reads in fastq format
191 were aligned to the reference human genome (hg19) with Bowtie2. Variant calling was
192 performed with VarScan with a threshold for the minimum mutated allele percentage (MAP)
193 of 1%. Variant calls in VCF format were then annotated through ANNOVAR. All results
194 obtained by NGS were confirmed by Sanger sequencing.

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

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

201 HEK293T cells stably expressing green fluorescent protein (GFP)-tagged-ASC and
202 FLAG-tagged pro-caspase1 (designated ASC-GFP_C1-FLAG) were transfected with 375 ng
203 of mentioned plasmids using FUGENE HD (Promega). After 24h, the transfected cells were
204 observed using a Nikon Eclipse TS100 inverted fluorescent microscope. Manual counting of
205 cells containing ASC-GFP specks was then performed in 5 randomly selected fields at 20 X
206 magnification. The percentage of speck-positive cells was calculated as the number of specks
207 divided by the total number of counted cells.

208 THP1 cells were initially primed with 100 ng/ml PMA (Phorbol 12-Myristate 13-
209 Acetate) for 3h, then transfected with 500 ng of the plasmids using the FF-100 program in the
210 4D-Nucleofector, Amaxa (Lonza). Transfected THP1 cells were treated directly with 100
211 ng/ml LPS at 37C°. After 24h, cytokine measurements by ELISA assays were performed
212 following manufacturer's instructions (R&D).

213

214 **Methodology for literature review of *NLRP3* mutations**

215 Literature review of *NLRP3* mutations was performed in Pubmed using the terms
216 "NLRP3" and "mutation". Based on the classification of the American College of Medical
217 Genetics (ACMG)¹⁴, only pathogenic and likely pathogenic mutations were taken into
218 account.

219

220 **NLRP3 crystal structure analysis**

221 NLRP3 crystal structure analysis was performed using the human NLRP3 crystal
222 (Protein Data Bank accession number 6NPY) by Sharif et al³. The 3D structure was visualized
223 with the Pymol software.

224

225 **Statistical analyses**

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

229

230 RESULTS

231 232 Clinical phenotype of *NLRP3*-AID patients

233 We studied three unrelated patients presenting with early onset of fever, urticaria,
234 arthralgia and inflammatory biological profile. Proband I and II, both 50 years old, also
235 developed sensorineural hearing loss around the age of 30 and were, therefore, suggestive of
236 MWS. Proband III, a 4-year-old boy, displayed a clinical phenotype evocative of CINCA
237 shortly after birth (**table 1** and **supplemental data 1**). None of the patients had a family
238 history of AID.

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

243 The c.1705G>A, p.(Glu569Lys) missense variation with a mutated allele percentage
244 (MAP) of 7% and 13% was identified in peripheral blood DNA of Proband I and II,
245 respectively (**table 2, supplemental figure 1**). This variation, which is absent from the
246 genome aggregation database (gnomAD), has already been reported in several *NLRP3*-AID
247 patients^{11, 12, 15-18}.

248 In Proband III, the NGS analysis revealed a novel heterozygous *NLRP3* variation, the
249 c.2305G>A, p.(Gly769Ser) which is not described in gnomAD and, according to the
250 MaxEntScan software, is not predicted to affect splicing. In the 3D structure of *NLRP3*,
251 Gly769 is located on an external part of a LRR motif (**supplemental figure 2**), outside the
252 region that interacts with NEK7. Secondly, the access to parents' DNA samples revealed
253 that p.(Gly769Ser) was inherited from the asymptomatic father, which argues against its
254 involvement in the AID phenotype. The NGS data were therefore subsequently reanalyzed
255 using a mosaic-specific pipeline and the c.1706A>G, p.(Glu569Gly) variation with a MAP of
256 9% in peripheral blood DNA was detected. This variation, absent from gnomAD, has never
257 been reported in *NLRP3*-AID patients.

258 The cellular distribution of the identified mosaic variations was then determined by
259 NGS and Sanger sequencing of DNA isolated from cells of different embryonic origins. In
260 Proband I, who carries the p.(Glu569Lys) variation, a wide cellular distribution of the mutated
261 allele was found with MAP ranging from 5% to 19%. It ranged from 11% to 15% in Proband
262 II who also carries the p.(Glu569Lys) variation. Noteworthy, Proband III, who had the most
263 severe phenotype (CINCA) and carries the p.(Glu569Gly) missense variation, displayed the

264 lowest rate of the mutated allele (ranging from 7% to 10%, depending on the analyzed cell
265 types) (**table 2, supplemental figure 1**).

266

267 **Functional consequences of identified *NLRP3* variations**

268 To assess the pathogenicity of the identified *NLRP3* variations, we studied ASC-speck
269 formation, a common readout of inflammasome activation¹⁹. In agreement with the known
270 role of *NLRP3* in inflammasome assembly and ASC-speck formation, cells transfected with
271 p*NLRP3*-WT displayed a significantly higher percentage of ASC-specks as compared to cells
272 transfected with the empty vector (EV) alone. However, no significant difference was
273 observed between the cells transfected with the constructs carrying either the germline variant
274 (p*NLRP3*-Gly769Ser) or the wild-type. In contrast, cells transfected with p*NLRP3*-
275 Glu569Gly or with p*NLRP3*-Glu569Lys (used as a positive control of inflammasome
276 activation) showed a significantly higher percentage of ASC-specks as compared to cells
277 transfected with p*NLRP3*-WT. Therefore, a gain-of-function effect on inflammasome
278 activation was observed for p.(Glu569Gly), leading us to classify this variation as pathogenic
279 (**figure 1**).

280 To further assess the functional consequences of the p.(Gly769Ser) variation, we
281 tested its impact on cytokine secretion. To this end, THP1 cells were transfected with the EV,
282 p*NLRP3*-WT, p*NLRP3*-Glu569Lys (used as positive control) or p*NLRP3*-Gly769Ser and
283 treated with lipopolysaccharide (LPS), a well-known activator of the *NLRP3* inflammasome.
284 IL1 β secretion was measured in the cell culture supernatants and significantly higher levels
285 were found in the supernatant of cells transfected with p*NLRP3*-Glu569Lys as compared to
286 cells transfected either with p*NLRP3*-WT or with the EV. When compared to WT-*NLRP3*,
287 similar amounts of secreted IL1 β were measured for the p.(Gly769Ser) variant (**supplemental**
288 **figure 3**). Taken together, these results led us to classify the p.(Gly769Ser) variant as
289 probably benign.

290

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

292 In an attempt to identify possible germline-specific and mosaic-specific characteristics
293 of *NLRP3* mutations, we re-interpreted all previously reported *NLRP3* variations according to
294 both the associated disease severity and their localization in the protein domains. The three
295 unrelated *NLRP3*-AID patients with mutations involving the same residue but associated with
296 different phenotypic severity were incorporated in this study. Based on the ACMG
297 classification of sequence variations¹⁴, we classified as likely pathogenic or pathogenic 90

298 distinct *NLRP3* mutations identified in 277 patients: 33 somatic mosaic mutations in 54
299 unrelated patients and 65 germline mutations reported in 223 unrelated patients (**figure 2,**
300 **supplemental table 1**). Noteworthy, half (27/54 patients) of the reported cases with mosaic
301 mutations carry mutations located in the HD2 domain of *NLRP3*, including the
302 p.(Gly569Lys) mutation, whereas only 9% (20/223 patients) of the patients with germline
303 mutations carry mutations located in this region (**figure 2**). A second mosaic mutational
304 hotspot (10/54 - 18.5%) involves Phe304 to Gly309 aminoacids that overlaps the Walker B
305 motif of the NBD. Among all reported mutations, only 8 of them have been found in both a
306 mosaic and a germinal state (**figure 3**). These 8 mutations were identified in a germinal state
307 in 16 patients (16/223 – 7.2%) and, noteworthy, all these patients presented with the severe
308 CINCA phenotype. The same mutations were identified in mosaic state in 22 patients (22/54 –
309 40.7%) who displayed moderate (MWS) to severe (CINCA) phenotypes (**figure 3**). On the
310 other hand, the most frequent germline mutations, such as p.(Thr350Met), p.(Arg262Trp),
311 p.(Asp305Asn), p.(Leu355Pro) and p.(Ala441Val), have never been reported in a mosaic state
312 (**figure 4a**), whereas some of these mutations involve aminoacids that are changed in other
313 substitutions in mosaic state [p.(Arg262Pro), p.(Asp305His), p.(Asp305Ala) and
314 p.(Ala441Pro)] (**figure 2**).

315 With the aim to further assess the functional impact of the mutations, we located on
316 the crystal structure of *NLRP3*³ the five aminoacids involved in the most frequently reported
317 mutations in mosaic and germinal state. Noteworthy, we found that the mosaic mutations are
318 located in the core of the NACHT domain close to the ATPase domain, while the germline
319 mutations are scattered throughout the NACHT domain (**figure 4b**).

320

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 disease¹¹. 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 mutations⁷. The c.1705G>A p.(Glu569Lys) mutation has been reported (including the current study) in ten patients^{11, 12, 15-18}. Of note, this G>A transition is located on a CpG dimer that favors this type of mutational event²⁰. Two other mosaic mutations implicate the same aminoacid, c.1705G>C p.(Glu569Gln)¹¹ 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)^{11, 12, 15-18}. Noteworthy, the single patient with a germline mutation involving this residue had a very severe neonatal CINCA syndrome²¹, 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)

355 of the HD2 subdomain of the NACHT domain, whereas only 9% (20/223) of the patients with
356 a germline mutation carry a mutation in that region. In an attempt to explain this striking
357 observation, we highlighted the mutated aminoacids on the crystal structure of NLRP3. The
358 region spanning Tyr565 to Tyr572 is located in the core of the NACHT domain facing the
359 Walker B motif, a sequence involved in ATP hydrolysis and essential for NLRP3-
360 inflammasome activation²². Interestingly, the region of the Walker B motif includes the
361 second hotspot (Phe304 to Gly309) for mosaic mutations (18.5%, 10/54 patients). The two
362 mutational hotspots account for 68.5% (37/54) of the patients with mosaic mutations, but only
363 26.9% (60/223) of those with germline mutations. Secondly, only a minority of the reported
364 *NLRP3* mutations (8/90) were identified in both somatic mosaic and germinal state. In
365 germinal state, these 8 mutations concern 16 unrelated patients (16/223 – 7.1%) who are all
366 sporadic cases presenting with the severe CINCA phenotype^{5, 21-28}. Thirdly, the most frequent
367 germline mutations have never been reported in *NLRP3*-AID patients in a mosaic state despite
368 their location on CpG dimers for four of them (**figure 2** and **4a**). It is tempting to speculate
369 that those mutations reported in a germinal state only, may display a very mild phenotype (or
370 even be asymptomatic) if present in a mosaic state and could, therefore, escape diagnosis. In
371 keeping with this hypothesis, the single report of the p.(Thr350Met) mutation in a mosaic
372 state concerns an asymptomatic mother who transmitted the mutation to her child who
373 presented with a MWS phenotype²⁹. Conversely, somatic mosaic mutations could be
374 responsible for severe phenotypes or be incompatible with life if present in a germinal state¹⁸.
375 A negative selection of gametes carrying the most activating mutations is another attractive
376 hypothesis. Indeed, recent studies have shown a prominent NLRP3 staining of peritubular
377 cells of testis from patients with impaired spermatogenesis^{30, 31}. Moreover, two different
378 studies suggested hypofertility in *NLRP3*-AID patients^{32, 33}.

379 From a general viewpoint, it is therefore important to underline that the phenotypes
380 observed in *NLRP3*-AID patients (from FACS to CINCA) represent only a part of the
381 phenotypic spectrum associated with a mutation in *NLRP3*. Indeed, on the basis of the
382 epidemiological data presented in this study, it appears that the *NLRP3* mutations cover a
383 phenotypic spectrum larger than that revealed by the patients' phenotype: some mutations
384 could be lethal or asymptomatic (and therefore not reported) depending on their molecular
385 characteristics (i.e. germinal/mosaic status, localization in the protein and aminoacid change).
386 In conclusion, as illustrated here, the early detection of an *NLRP3* mosaicism is of key
387 importance to establish the diagnosis of *NLRP3*-AID and to initiate anti-IL1 treatment. Our
388 study also sheds light on one diagnostic pitfall due to the identification of several *NLRP3*

389 sequence variations in the same patient, thereby underlining the importance of functional and
390 segregation studies to assess the pathogenicity of the identified sequence variations. The two
391 hotspots for mosaic mutations are located around the Walker B motif, essential for NLRP3-
392 inflammasome activation. The phenotypic spectrum of *NLRP3*-AIDs appears to be related to
393 the germinal/mosaic status and localization of the underlying mutations.

394

395

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397

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399

400 **CONFLICT OF INTEREST**

401

402 The authors have no conflict of interest to declare.

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520 **FIGURE LEGENDS**

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522 **Figure 1. Impact of *NLRP3* mutations on ASC speck formation.** HEK (ASC-GFP_C1-
523 FLAG) cells were transfected with 375ng of the expression plasmids encoding the wild-type
524 *NLRP3* or the *NLRP3* protein carrying the missense variations p.(Glu569Gly), p.(Glu569Lys)
525 and p.(Gly769Ser) or the empty vector (EV). The percentage of ASC specks was calculated as
526 described in “Patients and Methods”. Results represent the mean + SD from 3 independent
527 experiments. P values were calculated using unpaired Student’s t test as compared to the wild-
528 type. * indicates significant P value <0.05; ** P value <0.01; ns indicates non-significant P
529 value.

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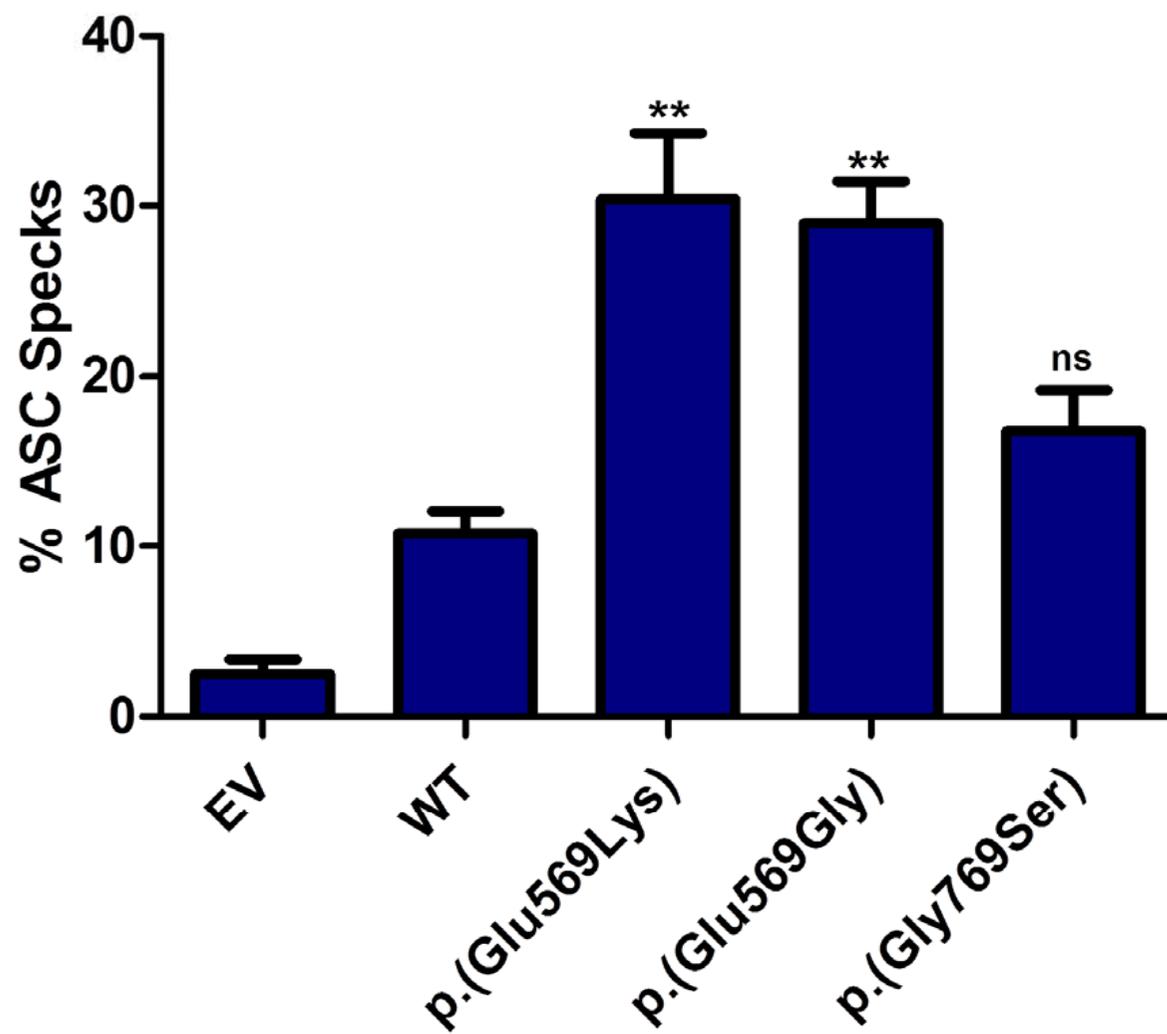
531 **Figure 2. Somatic mosaic and germline *NLRP3* mutations on a domain organization**
532 **model of the protein.** Top: all mutations reported in a mosaic state. Bottom: all mutations
533 reported in a germinal state. The five most frequently reported mutated aminoacids are in red.
534 The asterisks denote mutations identified in this study. PYD: Pyrin domain; NBD: Nucleotide
535 binding domain; HD1: Helical domain 1; WHD: Winged helix domain; HD2: Helical domain
536 2; LRR: Leucine-rich repeat domain. The Walker A and Walker B motifs are in yellow and
537 green respectively.

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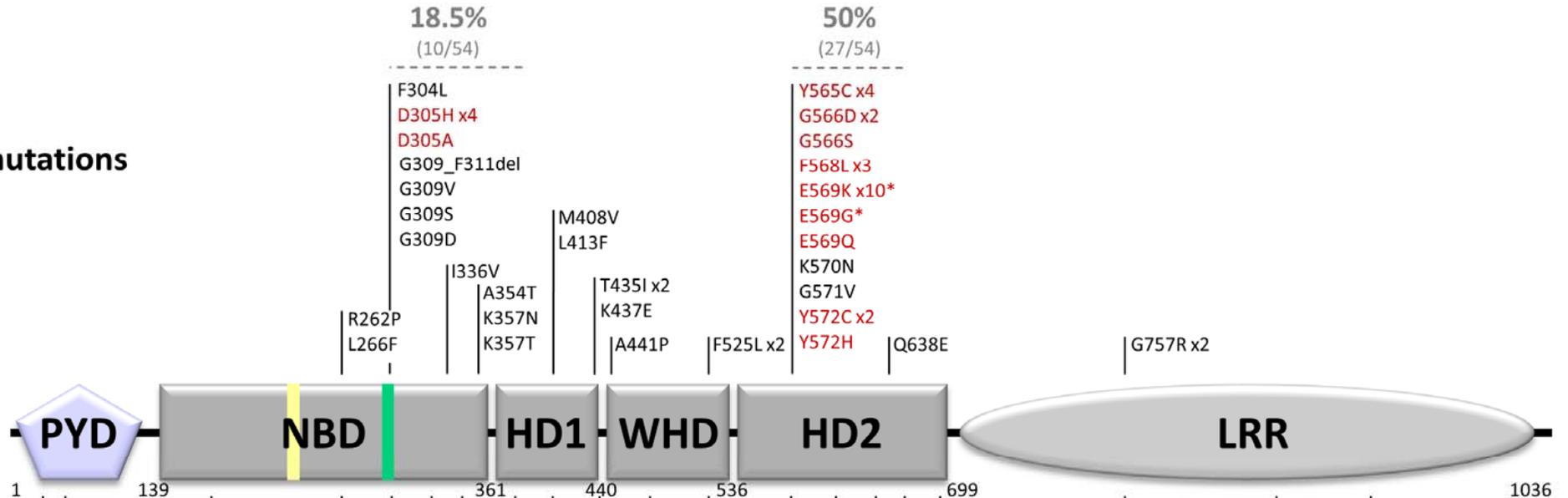
539 **Figure 3. Characteristics of the *NLRP3* mutations reported in both mosaic and germline**
540 **state.** Venn diagram of mosaic and somatic *NLRP3* mutations is represented on left part of the
541 figure. The 8 common mutations carried by 38 patients in either a mosaic or a germinal state
542 and the phenotypes of the corresponding patients are detailed on the right part of the figure.
543 For the p.(Glu569Lys) mutation (E569K) the levels of mosaicism are also given. MAP:
544 mutated allele percentage

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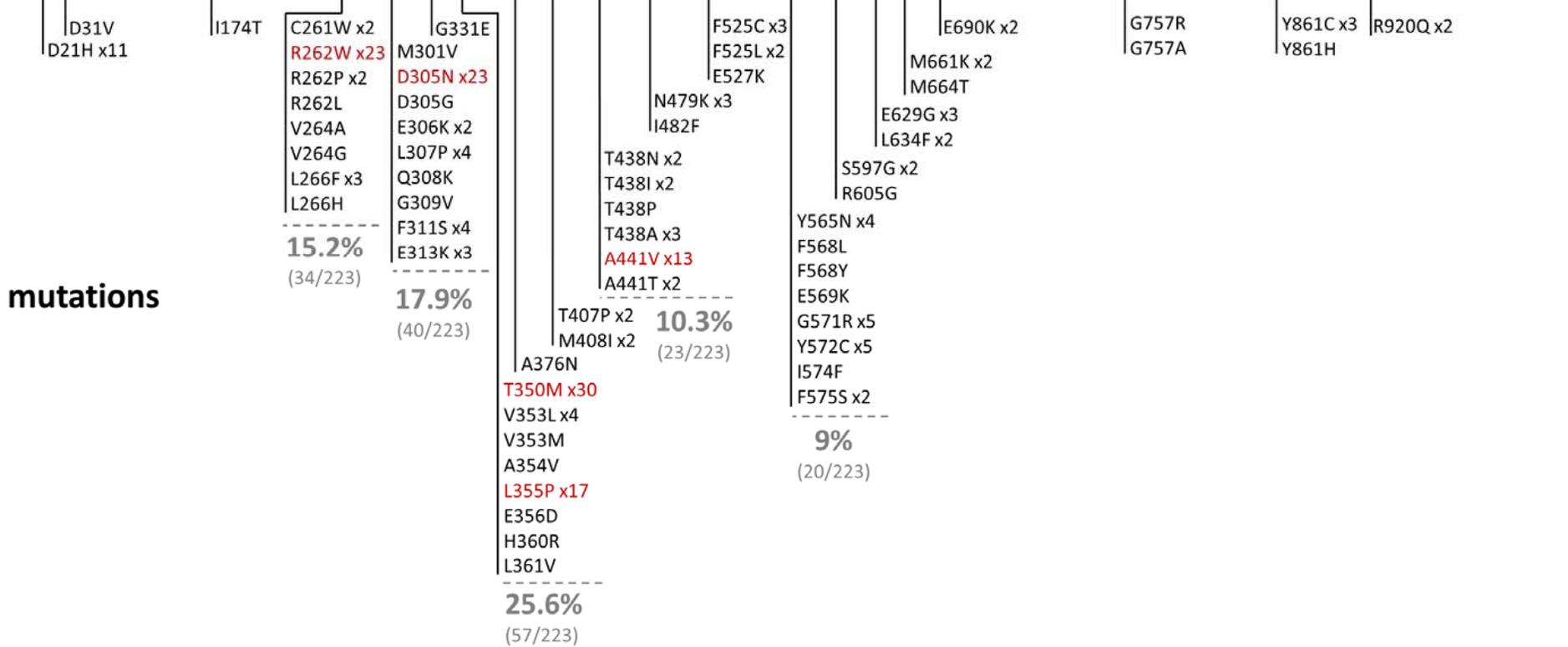
546 **Figure 4. Patients’ phenotypes and localization in the 3D structure of *NLRP3* of the**
547 **most frequently reported mutations. a.** Characteristics associated with the five most
548 frequent mosaic and germline mutations **b.** Highlight of the five most frequently mutated
549 aminoacids in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



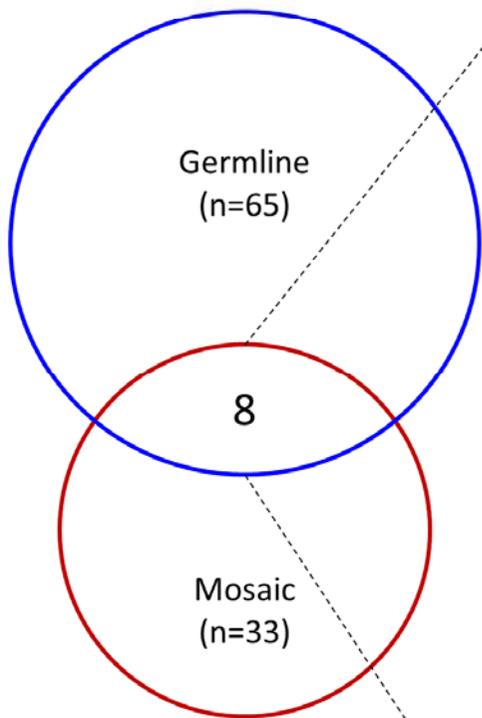
Mosaic mutations



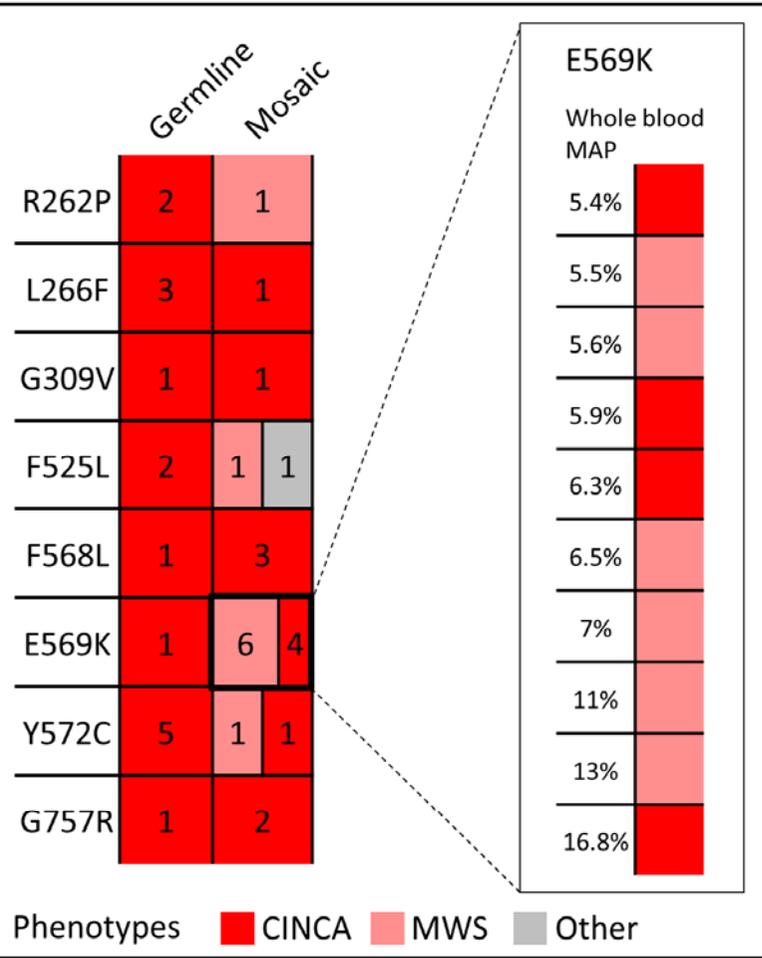
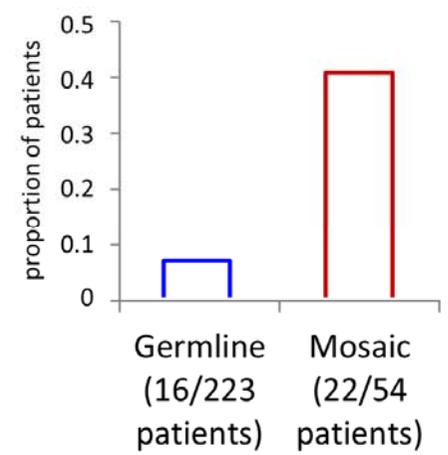
Germline mutations



Mutations



Molecular epidemiology



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

