RE-EVALUATION OF *MEFV* GENE VARIANTS: UTILITY OF A GENE SPECIFIC THRESHOLD IN REDUCING THE NUMBER OF VARIANTS OF UNKNOWN SIGNIFICANCE

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Abstract

Familial Mediterranean Fever (FMF) is an inherited autoinflammatory syndrome caused by mutations in the *MEFV* gene. *MEFV* variants are still in large part classified as variant of uncertain significance (VOUS), or with classification unresolved, posing significant challenges in the clinical diagnosis of Familial Mediterranean Fever (FMF). REVEL is a recently developed variant metapredictor tool. To reduce the number of *MEFV* variants with ambiguous classification we extracted the REVEL score for all missense variants reported at the locus specific database INFEVERS, and analyzed its correlation with expert-based classification and localization in the *MEFV*-encoded pyrin protein functional domains.

The data set of 216 missense variants was divided in four classification categories (BENIGN, VOUS, PATHOGENIC and UNRESOLVED). *MEFV* variants were plotted onto the pyrin protein, the distribution of REVEL scores in each category was computed and means, confidence intervals, and area under the receiver operating curve were calculated.

We observed a non-random distribution of pathogenic variants along the functional domains of the pyrin protein. The REVEL scores demonstrated a good correlation with the consensus classification of the International Study Group for Systemic Autoinflammatory Diseases (INSAID). Sensitivity, specificity, and accuracy were calculated for different cutoff values of REVEL scores and a gene-specific threshold was computed with confidence boundary limits. A REVEL score of 0.298 was the best performing cut-off to reclassify 96 *MEFV* gene variants previously of uncertain significance or unsolved thus reducing their proportion from 61.6% to 17.6%.

In conclusion, the combination of available expert information with highly sensitive predictor tools yields to more accurate interpretation of clinical consequences of *MEFV* gene variants. This approach should bring to a better genetic counseling and patient management.

50 **Author summary**

51 We aimed to refine *MEFV* gene variants classification using the metapredictor REVEL. We demonstrate that
52 a gene-specific threshold is effective for accurate variants' classification. Using this threshold, we reduced
53 significantly the proportion of *MEFV* variants with an ambiguous classification. The proposed classification
54 could represent a useful resource for variant interpretation in the context of FMF diagnosis.

Introduction

Familial Mediterranean Fever (FMF) is the most common of the Hereditary recurrent fevers (HRFs) with an estimated prevalence of 1 in 1-5/ 10 000. FMF has been first described more than sixty years ago [1,2], and since the identification of the causative gene (*MEFV*, NM_000243.2) it has been intensively studied [3,4]. The *MEFV* gene encodes for an 86 kDa protein (Pyrin or marenosttrin, TRIM20) prevalently expressed in immune cells (neutrophils, eosinophils, monocytes, dendritic cells and synovial fibroblasts), and is related to abnormal response of the innate immune system and IL-1 β during periodic attacks of fever and serositis [5,6]. In spite of several reports describing large FMF patients' datasets, a complete understanding of the pathogenic mechanisms responsible for this disease has not been reached yet. Recent studies suggested the possibility of dominant inheritance, at least for some mutations. This hypothesis is casting doubts on the traditional autosomal recessive mode of inheritance [7-10], a paradigm in FMF for long time. For FMF, a well-curated locus specific database is available at INFEVERS (<https://infevers.umai-montpellier.fr/web/>), containing genotype and clinical information for more than 500 variants. Recently, the International Study Group for Systemic Autoinflammatory Diseases (INSAID) has critically reviewed the clinical information for 858 variants in the four genes (*MEFV*, *TNFRSF1A*, *NLRP3* and *MVK*) responsible for hereditary recurrent fevers (HRFs) reaching a consensus classification for 94% of variants analyzed [11]. The notable exception was represented by the *MEFV* gene, where only 55.7% of the variants could be successfully classified with almost a third of them being variants of unknown significance (VOUS). As a result, clinicians face the harsh reality of frequently receiving an inconclusive genetic referral for FMF diagnosis. Internationally recognized guidelines have been reported by the American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) [12], providing a five-tier classification of genetic variants in: pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VOUS or VUS), likely benign (LB), and benign (B). However, since even the mode of inheritance in FMF families has been recently jeopardized by several reports of apparently dominant mutations, the ACMG/AMP guidelines might be of limited value in FMF. In this work, we used REVEL (a recently developed ensemble method which has been demonstrated to outperform most commonly used web-based predictors) [13], to improve the classification of many

unsolved, unclassified, or of uncertain significance *MEFV* gene variants. We also report a non-random distribution of variants along the coding sequence of the *MEFV* gene. This observation possibly identifies novel hotspots for variants with a putative pathogenic role.

Results

Non-random distribution of variants. The REVEL score was extracted for all the 216 missense variants present at the INFEVERS database. Each variant was mapped along the coding sequence of *MEFV* gene, and with respect to the functional domains of the pyrin protein (Fig 1). We observed that PATHOGENIC variants (as defined in the methods) clustered in the SPRY and zf-B_box domains. In contrast, BENIGN variants were markedly underrepresented in functional domains of the pyrin protein being localized, in large majority, in the exon 2 of the *MEFV* gene. Finally, VOUS occurred along the entire coding sequence of the *MEFV*, whereas distribution of UNRESOLVED variants appeared to mostly overlap with the PATHOGENIC one.

REVEL variants score. The means of REVEL scores for BENIGN (n=41, 0.18), VOUS (n=89, 0.25), PATHOGENIC (n=41, 0.39) and UNRESOLVED (n=44, 0.31) variants showed statistically significant differences among the four categories (F 21.04 p<0.0001, ordinary one-way ANOVA) demonstrating an appreciable level of correspondence between the INSAID consensus-based variant classification and an in silico computational scoring method such as REVEL (Fig 2). The results of unpaired t-tests among each category demonstrated statistically significant differences between BENIGN and UNRESOLVED, BENIGN and PATHOGENIC, and VOUS vs PATHOGENIC (Fig 2). Only one variant classified by INSAID as benign or likely benign had an unexpectedly high pathogenic score (E230Q, 0.737, identified as an outlier using ROUT method). In contrast, 28 of 41 INSAID pathogenic or likely pathogenic variants had a score below the REVEL threshold for pathogenicity (<0.4).[15] Worthy of note, the M694V variant, considered to be responsible for the most severe FMF phenotype, barely reached the threshold for pathogenicity (REVEL score 0.403). Finally, the area under the ROC curve (AUC) was 0.879 (p<0.0001, Fig 3)

Development of a new classifier and reclassification of *MEFV* variants. Since the REVEL precision in scoring variants was markedly different for BENIGN and PATHOGENIC categories we hypothesized that

106 *MEFV* gene variants with putative pathogenic effects may act more subtly than variants used to train
107 REVEL. Therefore, we decided to build a model trained on a dataset formed exclusively by the *MEFV*
108 variants classified by INSAID panel as BENIGN or PATHOGENIC.

109 Of the five algorithms tested LDA had the best performance, and was therefore further explored on the
110 validation set. Prediction results on this set demonstrated an accuracy of 75%, with a specificity of 87.5%,
111 and a sensitivity of 62.5%, and identified an approximate cut-off value of 0.29.

112 Next, we focused on the unclassified variants and aimed to assess their pathogenicity based on this
113 previously trained model. To further optimize the performance of this new cut-off, we calculated the
114 sensitivity and the specificity for different REVEL score thresholds (Fig 4).

115 A nearly identical REVEL score of 0.298 yielded the best performance as cutoff, showing the highest
116 accuracy (80.2%), positive predictive value (PPV, 83.8%) Matthews' correlation coefficient (60.8%) and
117 Youden index (58.2%) values, with a sensitivity of 76% and a specificity of 83%, estimated on the entire
118 dataset of BENIGN and PATHOGENIC variants (S1 file). Thus, when using 0.298 as the cutoff value, 34 of 41
119 BENIGN variants had a score under the threshold for pathogenicity, while 31 of 41 PATHOGENIC variants
120 had a score above the cutoff. The classifier we developed had an overall AUC of 0.878 ($p < 0.0001$).

121 Then, we re-classified all the 89 "variant of uncertain significance", 31 "unsolved" and 13 "not classified"
122 variants from the INFEVER database with more precision, using the information deriving from the
123 descriptive statistics of the BENIGN and PATHOGENIC REVEL scores variants to narrow the VOUS to a
124 smaller fraction. To move from binary to graded classifications, all variants with a REVEL score lower than
125 0.225 (upper 95% confidence interval of the BENIGN mean) were classified as BENIGN, while variants with a
126 score higher than 0.340 (lower 95% confidence interval of the PATHOGENIC mean) were classified as
127 PATHOGENIC. The remaining variants ($0.225 \leq \text{REVEL score} \leq 0.340$) were considered VOUS.

128 Applying these criteria to the 133 VOUS and UNRESOLVED variants, 61 and 35 could be reclassified as
129 BENIGN and PATHOGENIC, respectively (S1 Table and Fig 5). The same criteria changed the classification of

3 likely benign variants into PATHOGENIC (including the E230Q variant) and 5 likely pathogenic into BENIGN (S1 table). Notably, all but one of these 8 variants were only provisionally classified by INSAID experts.[11]

Finally, we mapped the newly classified variants along the MEFV protein aminoacid sequences which confirmed the preferential distribution of pathogenic variants in the SPRY and zf-B_box domains (Fig 5).

Discussion

The advent of massive parallel next generation sequencing technology has greatly increased the power and the sensitivity of genetic testing in the clinical setting. However, the vast amount of genomic data generated poses the significant challenge of accurately assessing the pathogenicity of identified variants. While many bioinformatic resources are currently available to aid sequence variant interpretation, several inconsistencies are present in variant classification across laboratories and reference databases [16,17]. The ultimate diagnosis of FMF is linked to life-time therapy in order to prevent the complications due to secondary amyloidosis, and to definitively improve patients' quality of life [18]. Thus, FMF presents all the above-mentioned challenges in addition to still developing effective clinical criteria for identifying truly affected patients [19,20].

In fact, of the 119 *MEFV* missense variants present in ClinVar only 24 have an ACMG classification different from VOUS or conflicting interpretations. Therefore, geneticists dealing with post-test counselling in FMF, find little help in the largest available database of clinical variation in human genome. Recently, an expert consensus panel classified the clinical significance of gene variants identified in four genes causing hereditary recurrent fevers (*MEFV*, *TNFRSF1A*, *NLRP3* and *MVK*) [11]. However, 30% of *MEFV* gene variants remained unclassified or with uncertain significance.

With this initial limitation in mind, we designed the present analysis. First, by mapping variants along the coding sequence of the *MEFV*, we confirmed that pathogenic changes cluster in the SPRY domain. Two further domains, the PYRIN domain (also denoted as PYD) and the zf-B_box domain, appear to host an excess of likely pathogenic variants, although differences did not reach statistically significant difference.

154 The N-terminal PYRIN domain is present in more than 20 proteins modulating apoptosis and inflammation
155 and is responsible for interaction, among others PYRIN domain-containing proteins, with the apoptosis-
156 associated speck-like protein with a CARD (ASC) [21,22]. The zf-B_box domain is necessary and sufficient for
157 binding the proline serine threonine phosphatase-interacting protein [PSTPIP1, or CD2-binding protein 1
158 (CD2BP1)]. This protein is involved in cytoskeletal organization, and its mutations cause pyogenic arthritis,
159 pyoderma gangrenosum, and acne (PAPA), a different autoinflammatory disorder [23]. In contrast, benign
160 variants occur largely in non-functional regions of the pyrin protein.

161 Next, we used REVEL, which has been demonstrated to outperform most of the available predictors, to
162 score both classified and unclassified variants of the *MEFV* gene. When using the originally reported best
163 threshold [13], REVEL scores were almost perfect in identifying the benign and likely benign variants
164 (97.2%) while only 13 pathogenic/likely pathogenic variants (31.7%) demonstrated a score above the
165 threshold for pathogenicity. We hypothesized that mechanisms conferring pathogenic effects to *MEFV*
166 variants are subtler than mechanisms of disease-causing variants used to train many prediction algorithms
167 including REVEL.

168 Indeed, the mechanisms by which *MEFV* variants cause symptoms onset are poorly understood. In a mouse
169 model, human *MEFV* knock-in variants act as gain-of-function mutations although severity varied inversely
170 with respect to human phenotypes [24]. More recently, in FMF patients a small set of *MEFV* pathogenic
171 variants has been shown to act as hypermorphic mutations with a gene dosage effect [25]. Surprisingly, in-
172 vitro experiments have demonstrated that FMF mutations (including M694V) render pyrin inflammasome
173 insensitive to colchicine, while this drug is the mainstay treatment in FMF patients [26]. Although many
174 variants with a putatively pathogenic role lie in the C-terminus of the pyrin protein, no clear genotype-
175 phenotype correlations have emerged so far.

176 A further proof of the complex mechanisms leading to pathogenicity in FMF is demonstrated by the
177 markedly different REVEL scores variants interesting the same aminoacid. For instance, the well-known
178 polymorphism E148Q has a REVEL score of 0.274 while for the variant E148V the computed score of 0.617

179 has a clearly pathogenic value. Similar differences recur at five different positions along the *MEFV* coding
 180 sequence. Notably, the four most common *MEFV* variants on FMF chromosomes (M680I, M694V, M694I
 181 and V726A) would be all classified as neutral should the most stringent REVEL threshold score being used
 182 [27,28].

183 Therefore, using a classifier trained exclusively on *MEFV* variants unambiguously classified by INSAID as
 184 PATHOGENIC or BENIGN, we computed a novel REVEL threshold for pathogenicity which allowed us to
 185 propose a novel classification for all the *MEFV* variants previously considered as VOUS or UNSOLVED.
 186 Indeed, a larger dataset of variants with a definitive classification as pathogenic or benign would contribute
 187 to developing more accurate statistical models to predict the pathogenicity of variants of uncertain
 188 significance or newly identified.

189 The development of reliable functional assays able to assess precisely the effects *MEFV* gene variants on
 190 the pyrin inflammasome should help in expanding the set of variants with a robust classification.

191 The ACMG/AMP 2015 guidelines recommends that the usage of in silico predictors should have only a
 192 supporting role in establishing the pathogenic role of variants identified during genetic testing. In addition,
 193 these widely followed guidelines suggest that concordance among multiple algorithm-based predictions
 194 should be sought. However, while several metapredictors incorporating many of these older algorithms
 195 have been demonstrated to perform robustly in large heterogenous datasets, first generation algorithms
 196 such as SIFT, Polyphen, Mutation Taster, CADD and Provean are prevalently used even in recent work [29-
 197 31].

198 A further consideration should be mentioned about the thresholds used from the *in-silico* tools to predict
 199 functional consequences. Most of these predictors returns both a score and a prediction for the submitted
 200 variant. However, many others simply return a numeric value without any suggestion on the forecasted
 201 impact. Therefore, is up to the submitters to frame the returned score in a putative pathogenic
 202 classification and translate it in clinical recommendations for specialists and patients. REVEL, belongs to the
 203 second class of predictors, and two different threshold values for pathogenicity have been proposed by

204 authors using this metapredictor in their studies while a threshold was not given in the original paper
205 [13,15,28].

206 In this work, we identified a *MEFV* gene-specific threshold since causative mutations in FMF appears to act
207 through a mechanism that can't be considered neither loss-of-function (LOF) and gain-of-function (GOF).
208 Variants leading to either LOF or GOF represent the large majority in the training datasets used to develop
209 prediction algorithms. In contrast, *MEFV* mutations, according to a recent study seem to be hypermorphic
210 mutations conferring to the mutant pyrin protein an enhanced reactivity to yet unknown triggers of the
211 inflammasome [25]. Therefore, variant interpretation in FMF appears to be the prototypical case for a
212 gene-specific calibration of prediction algorithms, as suggested by recent work [28].

213 In addition, based on recent analyses of mutations present in locus-specific databases (LSDB), we found
214 that erroneous interpretations of the mechanisms responsible for the causative role of several variants can
215 lead to misuse of prediction tools [32]. This appears to be the case also for FMF, where nine different
216 frameshifting or nonsense variants are currently classified at INFEVERS as pathogenic or likely pathogenic in
217 apparent contrast to the described hypermorphic nature of *MEFV* mutations [25]. Thus, critical assessment
218 of genetic information is fundamental for translating precision medicine in everyday practice.

219 A limitation of our study is the small number of variants with a validated classification used to train our
220 classifier. However, this constraint could be only overcome with an expanding set of variants identified in
221 patients with a firm clinical evaluation that would certainly help in further narrowing the number of VOUS
222 variants in the *MEFV* gene.

223 Most in silico predictors have been trained on thousands of gene variants, possibly underscoring the
224 diverse molecular mechanisms responsible for pathogenicity in many genes. Thus, using a locus-specific
225 scoring system may result in improved specificity and sensitivity at least for some disease-causing genes.

226 In summary, combining validated clinical information with the development of a gene-specific variant
227 classifier we aimed to provide an improved framework for variant interpretation that should help in

reducing the uncertainty in FMF diagnostic and would benefit genetic counsellors for a better patient management.

Methods

Dataset of missense variants. The dataset is based on all the 216 missense variants reported in the INFEVERS database (<https://infevers.umai-montpellier.fr/web/>) last accessed on 30 December 2018. We excluded from the following statistical analyses all the synonymous changes, terminating and noncoding variants, insertions, duplications and indels. We merged the ‘pathogenic’ and ‘likely pathogenic’, ‘benign’ and ‘likely benign’, “unsolved” and “not classified” variants (as classified at the INFEVERS locus specific database) in the ‘PATHOGENIC’, ‘BENIGN’, and ‘UNRESOLVED’ categories, respectively. Notably, of the 216 missense variants listed at INFEVERS only one “benign” and six “pathogenic” variants are present. The VOUS category corresponded to variants classified as “VOUS” at INFEVERS. Therefore, our dataset was represented by 41 PATHOGENIC, 89 VOUS, 41 BENIGN, and 45 UNRESOLVED variants. All variants were mapped along the *MEFV* coding sequence and its functional domains using Lollipops [14].

Statistical comparison. We extracted the REVEL score of each variant and the mean, SD, and the 95% confidence intervals were determined for each category. The statistical significance of the differences between categories was evaluated by ordinary one-way ANOVA, and unpaired t-test between each category.

To evaluate the predictive potential of REVEL scores in the FMF context we built a classifier on the dataset represented by the BENIGN and PATHOGENIC variants (n=82). Five different algorithms were tested to identify the best model for predicting variants pathogenicity potential based on their REVEL score, namely, Linear Discriminant Analysis (LDA), Classification and Regression Trees (CART), k-Nearest Neighbors (kNN), Support Vector Machines (SVM) with a linear kernel, and Random Forest (RF). This set of algorithms was chosen as it represents a good combination of linear and non-linear models to test. The dataset was split into two parts, 80% was used to train the models through a 10-fold cross validation, and 20% was used as a

252 validation dataset. All models were weighed using the “accuracy” metric. Computations were performed in
253 R using the Caret package.

254 Finally, the specificity, sensitivity, accuracy, Matthews’ correlation coefficient and Youden index for each
255 REVEL score value in correctly identifying BENIGN and PATHOGENIC variants were calculated. Where not
256 otherwise stated GraphPad Prism V.8.0.1 (GraphPad Software, San Diego, California, USA) was used for all
257 statistical analyses.

258

259

References

1. Reimann HA, Moadie J, Semerdjian S, Sahyoun PF. Periodic peritonitis; heredity and pathology: report of seventy-two cases. JAMA 1954;154: 1254-1259.
2. Heller H, Sohar E, Sherf L. Familial Mediterranean fever. AMA Arch Intern Med 1958;102: 50-71.
3. French FMF Consortium. A candidate gene for familial Mediterranean fever. Nat Genet 1997;17: 25-31.
4. The International FMF Consortium. Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. Cell 1997;90: 797-807.
5. Centola M, Wood G, Frucht DM, Galon J, Aringer M, Farrell C, et al. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. Blood 2000;95: 3223–3231.
6. Seshadri S, Duncan MD, Hart JM, Gavrilin MA, Wewers MD. Pyrin Levels in Human Monocytes and Monocyte-Derived Macrophages Regulate IL-1 Processing and Release. J Immunol 2007;179: 1274-1281.
7. Stoffels M, Szperl A, Simon A, Netea MG, Plantinga TS, van Deuren M, et al. MEFV mutations affecting pyrin amino acid 577 cause autosomal dominant autoinflammatory disease. Ann Rheum Dis 2014;73: 455-461.
8. Sarabay G, Touitou I. Dominant familial Mediterranean fever. *Rheumatology* 2017;56: 173-175.
9. Rowczenio DM, Iancu DS, Trojer H, Gilbertson JA, Gillmore JD, Wechalekar AD, et al. Autosomal dominant familial Mediterranean fever in Northern European Caucasians associated with deletion of p.M694 residue-a case series and genetic exploration. *Rheumatology* 2017;56: 209-213.
10. Stella A, Cortellessa F, Scaccianoce G, Pivetta B, Settimo E, Portincasa P. Familial Mediterranean fever: breaking all the (genetic) rules. *Rheumatology* 2018 doi: 10.1093/rheumatology/key328. [Epub ahead of print].
11. Van Gijn ME, Ceccherini I, Shinar Y, Carbo EC, Slofstra M, Arostegui JI, et al. New workflow for classification of genetic variants' pathogenicity applied to hereditary recurrent fevers by the

- International Study Group for Systemic Autoinflammatory Diseases (INSAID). J Med Genet 2018; 55: 530-537.
12. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17: 405-424.
13. Ioannidis NM, Rothstein JH, Pejaver V, Middha S, McDonnell SK, Baheti S, et al. REVEL: An Ensemble Method for Predicting the Pathogenicity of Rare Missense Variants. Am J Hum Genet 2016;99: 877-885.
14. Jay JJ, Brouwer C. Lollipops in the Clinic: Information Dense Mutation Plots for Precision Medicine. PLoS One 2016;11: e0160519.
15. Li J, Zhao T, Zhang Y, Zhang K, Shi L, Chen Y, et al. Performance evaluation of pathogenicity-computation methods for missense variants. Nucleic Acids Res 2018;46: 7793–7804
16. Amendola LM, Jarvik GP, Leo MC, McLaughlin HM, Akkari Y, Amaral MD, et al. Performance of ACMG-AMP Variant-Interpretation Guidelines among Nine Laboratories in the Clinical Sequencing Exploratory Research Consortium. Am J Hum Genet 2016; 99:247. doi: 10.1016/j.ajhg.2016.06.001
17. Pepin MG, Murray ML, Bailey S, Leistriz-Kessler D, Schwarze U, Byers PH. The challenge of comprehensive and consistent sequence variant interpretation between clinical laboratories. Genet Med 2016;18: 20-24.
18. Bonfrate L, Scaccianoce G, Palasciano G, Ben-Chetrit E, Portincasa P. A novel cluster of patients with Familial Mediterranean Fever (FMF) in southern Italy. Eur J Clin Invest 2017;47: 622-629.
19. Giancane G, Ter Haar NM, Wulffraat N, Vastert SJ, Barron K, Hentgen V, et al. Evidence-based recommendations for genetic diagnosis of familial Mediterranean fever. Ann Rheum Dis 2015;74: 635-641.
20. Federici S, Sormani MP, Ozen S, Lachmann HJ, Amaryan G, Woo P, et al. Evidence-based provisional clinical classification criteria for autoinflammatory periodic fevers. Ann Rheum Dis 2015;74: 799-805.

21. Martinon F, Hofmann K, Tschopp J. The pyrin domain: a possible member of the death domain-fold family implicated in apoptosis and inflammation. *Curr Biol* 2001;11: R118–R120.
22. Richards N, Schaner P, Diaz A, Stuckey J, Shelden E, Wadhwa A, et al. Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis. *J Biol Chem* 2001;276: 39320–39329.
23. Shoham NG, Centola M, Mansfield E, Hull KM, Wood G, Wise CA, et al. Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway. *Proc Natl Acad Sci U S A* 2003;100: 13501–13506.
24. Chae JJ, Cho YH, Lee GS, Cheng J, Liu PP, Feigenbaum L, et al. Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1 β activation and severe autoinflammation in mice. *Immunity* 2011;34: 755-768.
25. Jamilloux Y, Lefevre L, Magnotti F, Martin A, Benezech S, Allatif O, et al. Familial Mediterranean fever mutations are hypermorphic mutations that specifically decrease the activation threshold of the Pyrin inflammasome. *Rheumatology* 2018;57: 100-111.
26. Van Gorp H, Saavedra PH, de Vasconcelos NM, Van Opdenbosch N, Vande Walle L, Matusiak M, et al. Familial Mediterranean fever mutations lift the obligatory requirement for microtubules in Pyrin inflammasome activation. *Proc Natl Acad Sci U S A* 2016;113: 14384-14389.
27. Touitou I. Standardized testing for mutations in familial Mediterranean fever. *Clin Chem* 2003;49: 1781–1782.
28. Ghosh R, Oak N, Plon SE. Evaluation of in silico algorithms for use with ACMG/AMP clinical variant interpretation guidelines. *Genome Biol* 2017;18: 225.
29. Seifi M, Footz T, Taylor SA, Walter MA. Comparison of Bioinformatics Prediction, Molecular Modeling, and Functional Analyses of FOXC1 Mutations in Patients with Axenfeld-Rieger Syndrome. *Hum Mutat* 2017;38: 169-179.
30. Riera C, Padilla N, de la Cruz X. The complementarity Between Protein-Specific and General Pathogenicity Predictors for Amino Acid Substitutions. *Hum Mutat* 2016;37: 1013-1024.

31. Ernst C, Hahnen E, Engel C, Nothnagel M, Weber J, Schmutzler RK, et al. Performance of in silico prediction tools for the classification of rare BRCA1/2 missense variants in clinical diagnostics. BMC Med Genomics 2018;11: 35.
32. Stella A, Lastella P, Loconte DC, Bukvic N, Varvara D, Patruno M, et al. Accurate Classification of NF1 Gene Variants in 84 Italian Patients with Neurofibromatosis Type 1. Genes 2018);9: 4.

FIGURE CAPTIONS

Fig 1. Distribution of INFEVERS variants along Pyrin Protein. Distribution of 216 MEFV INFEVERS missense variants onto the Pyrin protein. Pops size is proportional to the number of variants at a single aminoacid. In light blue are shown the exons of the *MEFV* gene.

Fig 2. REVEL scores of MEFV variants. The mean and standard deviations are reported for each category. Unpaired *t*-tests were performed among all categories, two-tailed P values after Tukey's post hoc analysis for multiple comparison are shown. An asterisk denotes the only variant identified as outlier (E230Q).

Fig 3. Area under the ROC curve for REVEL scores. The area under the ROC curve (AUC) is shown based on REVEL scores for the 41 BENIGN and 41 PATHOGENIC MEFV gene variants corresponding to the INFEVERS classes benign and likely benign, pathogenic and likely pathogenic, respectively.

Fig 4. Sensitivity and specificity of different REVEL scores thresholds.

Different REVEL score values were used as thresholds to assess 41 BENIGN and 41 PATHOGENIC variants of the Pyrin protein. The dotted line indicates the best performing REVEL threshold.

Fig 5. Distribution of MEFV gene variants after reclassification. Distribution of 216 MEFV missense variants onto the Pyrin protein after reclassifying VOUS and UNSOLVED variants. Pops size is proportional to number of variants at single aminoacid positions.

368 **Supporting information**

369 **S1 Table. INFEVERS classification, REVEL scores, and proposed novel classification for 216 *MEFV* missense**
 370 **variants.** List of the 216 missense variants analyzed with previous and proposed classification.

371 **S1 File. Parameters used to assess the performances of different values of REVEL score.** The parameters
 372 used to evaluate the performance of different value of REVEL scores as threshold for pathogenicity are
 373 indicated with their results for the best performing threshold value of 0.298

Figure 1

REVEL SCORES OF VARIANTS

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Figure 2

AUC based on REVEL scores

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Figure 3

Sensitivity/Specificity

Figure 4

BENIGN

VOUS

PATHOGENIC

Figure 5