

ISSAID/EMQN Best Practice Guidelines for the Genetic Diagnosis of Monogenic Autoinflammatory Diseases in the Next-Generation Sequencing Era

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BACKGROUND: Monogenic autoinflammatory diseases are caused by pathogenic variants in genes that regulate innate immune responses, and are characterized by sterile systemic inflammatory episodes. Since symptoms can overlap within this rapidly expanding disease category, accurate genetic diagnosis is of the utmost importance to initiate early inflammation-targeted treatment and prevent clinically significant or life-threatening complications. Initial recommendations for the genetic diagnosis of autoinflammatory diseases were limited to a gene-by-gene diagnosis strategy based on the Sanger method, and restricted to the 4 prototypic recurrent fevers (*MEFV*, *MVK*, *TNFRSF1A*, and *NLRP3* genes). The development of best practices guidelines integrating critical recent discoveries has become essential.

METHODS: The preparatory steps included 2 online surveys and pathogenicity annotation of newly recommended genes. The current guidelines were drafted by European Molecular Genetics Quality Network members, then discussed by a panel of experts of the International Society for Systemic Autoinflammatory Diseases during a consensus meeting.

RESULTS: In these guidelines, we combine the diagnostic strength of next-generation sequencing and

recommendations to 4 more recently identified genes (*ADA2*, *NOD2*, *PSTPIP1*, and *TNFAIP3*), nonclassical pathogenic genetic alterations, and atypical phenotypes. We present a referral-based decision tree for test scope and method (Sanger versus next-generation sequencing) and recommend on complementary explorations for mosaicism, copy-number variants, and gene dose. A genotype table based on the 5-category variant pathogenicity classification provides the clinical significance of prototypic genotypes per gene and disease.

CONCLUSIONS: These guidelines will orient and assist geneticists and health practitioners in providing up-to-date and appropriate diagnosis to their patients.

Introduction

Patients suffering from systemic autoinflammatory diseases (SAIDs) present with seemingly unprovoked inflammatory manifestations such as fever, serositis, skin lesions, arthralgia/arthritis, acute abdominal pain, and occasionally with lesions of the central nervous system (1). Study of the hereditary recurrent fevers (HRFs), the first identified group among monogenic SAIDs, has

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provided an insight into SAIDs physiopathology. A common defect is dysregulation and/or overactivation of intracellular pathways of innate immune cells (2).

Reliable diagnosis of SAIDs is crucial for early access to treatment adapted to the underlying disease. As disease manifestations may overlap, SAID diagnosis is highly dependent on genetic testing. To this end, registries for patients with SAIDs (Eurofever) (3) and for SAID gene variants (Infervers) (4) have been established. In 2012, we developed specific recommendations for the genetic testing and reporting of the 4 prototypic HRFs: familial Mediterranean fever (FMF), *MEFV* (Mediterranean FeVer) gene, MIM 608107, mevalonate kinase deficiency (MKD), *MVK* (Mevalonate Kinase) gene, MIM 251170, tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), *TNFRSF1A* (TNF receptor superfamily 1) gene, MIM 191190 and cryopyrin-associated periodic syndromes (CAPS or NLRP3-AID), *NLRP3* (NOD-like receptor family, pyrin domain-containing 3) gene, MIM 606416 (5). The guidelines referred to the classical Mendelian inheritance of HRFs, being either autosomal recessive (FMF and MKD) or autosomal dominant (TRAPS and NLRP3-AID) and to a limited number of variants detected at that time, mainly by Sanger sequencing of variant hotspots. Since then, with the advent of next-generation sequencing (NGS), unexpected phenotypes, nonclassical modes of inheritance, and postzygotic variants were described in HRFs (6). This prompted a reappraisal of the routine scope and interpretation of HRFs genetic diagnosis.

We have incorporated several recent discoveries to the current recommendations:

FMF, the typical phenotype associated with *MEFV*, is caused by biallelic variants mainly located in exon 10 (encoding the pyrin B30.2 domain) (7, 8); however, a substantial number of patients with FMF with only one *MEFV* variant in exon 10 have been described (9). Monoallelic substitutions in other exons can result in a dominant and severe FMF-like phenotype; examples of predicted changes at the (p.) protein level are: p.(Pro373Leu) (exon 3), p.(His478Tyr) (exon 5) (10), and p.(Thr577/Ser/Asn/Ala) amino acid changes (exon 8) (11). The corresponding complementary (c.) DNA changes for these variants, and those cited in this special report can all be found in the Infervers registry (4). In addition, new *MEFV*-associated phenotypes were reported. Pyrin-associated autoinflammation with neutrophilic dermatosis is another severe and dominantly inherited disease caused by missense variants in exon 2 substituting the p.(Ser242) or p.(Glu244) residues (12). These variants disrupt the phosphorylation-dependent binding of pyrin with its endogenous inhibitor, the 14-3-3 protein. All *MEFV*-associated phenotypes are now collectively called pyrin-associated autoinflammatory diseases (13).

Several heterozygous, loss of function *MVK* variants are associated with a rare dominant skin disorder of adult onset called disseminated superficial actinic porokeratosis (DSAP), first described in the Chinese population. A recent study demonstrated a second, postnatal hit in the skin lesions of patients with DSAP rendering epidermal cells biallelic for *MVK* mutations (14). Patients with DSAP have no clinical features of MKD and the *MVK* variants linked to DSAP are distributed throughout the *MVK* gene. Interestingly, a search in the Infervers registry retrieved 5 variants causing DSAP, i.e., p.(Gly140Argfs*47), p.(Gly212del), p.(Ser272Phe), p.(Gly335Asp) and p.(Gly376Ser), which have also been found in combination with a second loss of function *MVK* variant in patients with MKD.

Gene mosaicism resulting from postzygotic variants in the *NLRP3* gene accounts for up to 30% of NLRP3-AID and NLRP3-AID-like patients in whom conventional sequencing did not detect pathogenic variants (15–17). Postzygotic pathogenic variants were initially found in children with severe forms of NLRP3-AID (15), subsequently in adult patients with typical, milder NLRP3-AID (16).

Among other notable and recently documented SAIDs-associated genes are adenosine deaminase 2 (*ADA2*), nucleotide-binding oligomerization domain-containing protein (*NOD2*), proline-serine-threonine phosphatase-interacting protein 1 (*PSTPIP1*), and the tumor necrosis factor alpha-induced protein 3 (*TNFAIP3*) genes.

Deficiency of *ADA2* (*DADA2*, MIM 615688) typically presents with systemic vasculitis, early onset polyarteritis nodosa, and stroke (18, 19). The age at onset varies widely, and the spectrum of *DADA2* continues to expand to include hemorrhagic strokes, portal and systemic hypertension, hematologic abnormalities, immune deficiency, and bone marrow failure. Patients with *DADA2* have biallelic hypomorphic *ADA2* variants; more than 60 pathogenic variants described to date are located over the entire *ADA2* coding region. Small and large deletions have also been reported, and typically in combination with a missense variant on the opposite *ADA2* allele. The pathogenesis of *DADA2* remains uncertain. However, protein function can be assessed by a biochemical assay, which demonstrates low or absent plasma *ADA2* activity (20–22).

NOD2 is associated with 2 distinct diseases: the rare dominantly inherited Blau syndrome and multifactorial inflammatory bowel diseases (IBD) (23–25). Blau syndrome is typically characterized by a triad of noncaseating granulomatous inflammatory arthritis, uveitis, and dermatitis. Most pathogenic variants causing Blau syndrome localize in or near the central NOD domain of the *NOD2* protein; p.(Arg334Gln) and p.(Arg334Trp) are the most frequent ones (26, 27). In

addition, *NOD2* mosaicism has recently been reported in 4 unrelated patients with Blau syndrome (28). Early onset sarcoidosis is considered a sporadic form of Blau syndrome based on a similar phenotype and overlapping pathogenic variants. By contrast, several common variants including p.(Arg702Trp), p.(Gly908Arg), p.(Leu1007fs)) located in one of the C-terminal leucine-rich repeats domain of the *NOD2* protein have been repeatedly identified susceptibility alleles for Crohn disease. Up to 30% of patients with Crohn disease carry one or 2 copies of these variants in *NOD2* (25).

Pyogenic arthritis, pyoderma gangrenosum and acne (PAPA, MIM 604416) syndrome is caused by heterozygous missense *PSTPIP1* variants, predominantly p.(Ala230Thr) and p.(Glu250Gln), that reside in the protein's F-BAR domain (29). *PSTPIP1* interacts with pyrin. The mutant protein is hyperphosphorylated and binds more strongly to pyrin, provoking interleukin-1 β over-production. Another phenotype, the *PSTPIP1*-associated myeloid-related proteinemia inflammatory syndrome, characterized by neutropenia and markedly high myeloid-related protein 8/14 (calprotectin) concentrations with accumulation of zinc, has been linked to the p.(Glu250Lys) and p.(Glu257Lys) substitutions (30). Missense variants in the C-terminal domain of *PSTPIP1* may be risk factors for pyoderma gangrenosum, acne, and suppurative hidradenitis syndrome (31).

Heterozygous variants in the *TNFAIP3* gene cause haploinsufficiency of A20 (HA20, MIM 616744) (32). These patients present in childhood with fever, ulceration of mucosal surfaces, particularly in oral, genital, and gastrointestinal areas. Other disease features include skin rash, uveitis, polyarthritis, and a spectrum of autoimmune manifestations. Dominance in HA20 is attributed to insufficient concentrations of the A20 protein. The most frequent pathogenic genetic alterations found in HA20 are heterozygous frameshift, nonsense variants, and deletions, which disrupt the ubiquitin-editing activity of A20 on nuclear factor kappa B (NF- κ B) regulatory proteins and prolong NF- κ B induced inflammation. Substitutions such as p. Cys243Tyr resulting in increased production of human inflammatory cytokines by reduced suppression of NF- κ B activation have also been described (33) while several common *TNFAIP3* substitutions (allele frequency > 0.1) are risk factors for autoimmune conditions (rheumatoid arthritis, systemic lupus erythematosus, or Sjogren associated non-Hodgkin lymphoma).

The recent discoveries of new SAIDs, pathogenic copy-number variations (CNV) (34, 35) and low frequency gene mosaicism (19), along with the possibility of simultaneous screening of multiple genes using NGS-based methods provided genetic characterization of many previously undiagnosed patients. Conversely, one noticeable growing difficulty generated by large scale sequencing approaches is the expanding number of

patients with variants of unknown significance and or common variants in multiple genes, resulting in ambiguous or misleading conclusions. Among them are the terms "reduced penetrance," which concern monogenic diseases, and "risk factors," which concern multifactorial diseases. At-risk variants are associated with broader phenotypes spectrum and often with a higher overall population frequency than likely pathogenic variants. We therefore revised and updated the 2012 guidelines to comply with novel data generated by high throughput sequencing technologies and extended them with the 4 genes aforementioned as causing monogenic SAIDs (DADA2, Blau syndrome, PAPA, HA20).

Materials and Methods

Establishing these guidelines necessitated several preparatory steps, which are summarized in Fig. 1.

INITIAL MEETING

A first meeting was held during the European Society of Human Genetics 2018 conference (Milano, Italy) to identify elements to update, plan the new draft, and assemble critical data. Attendees were predominantly geneticists.

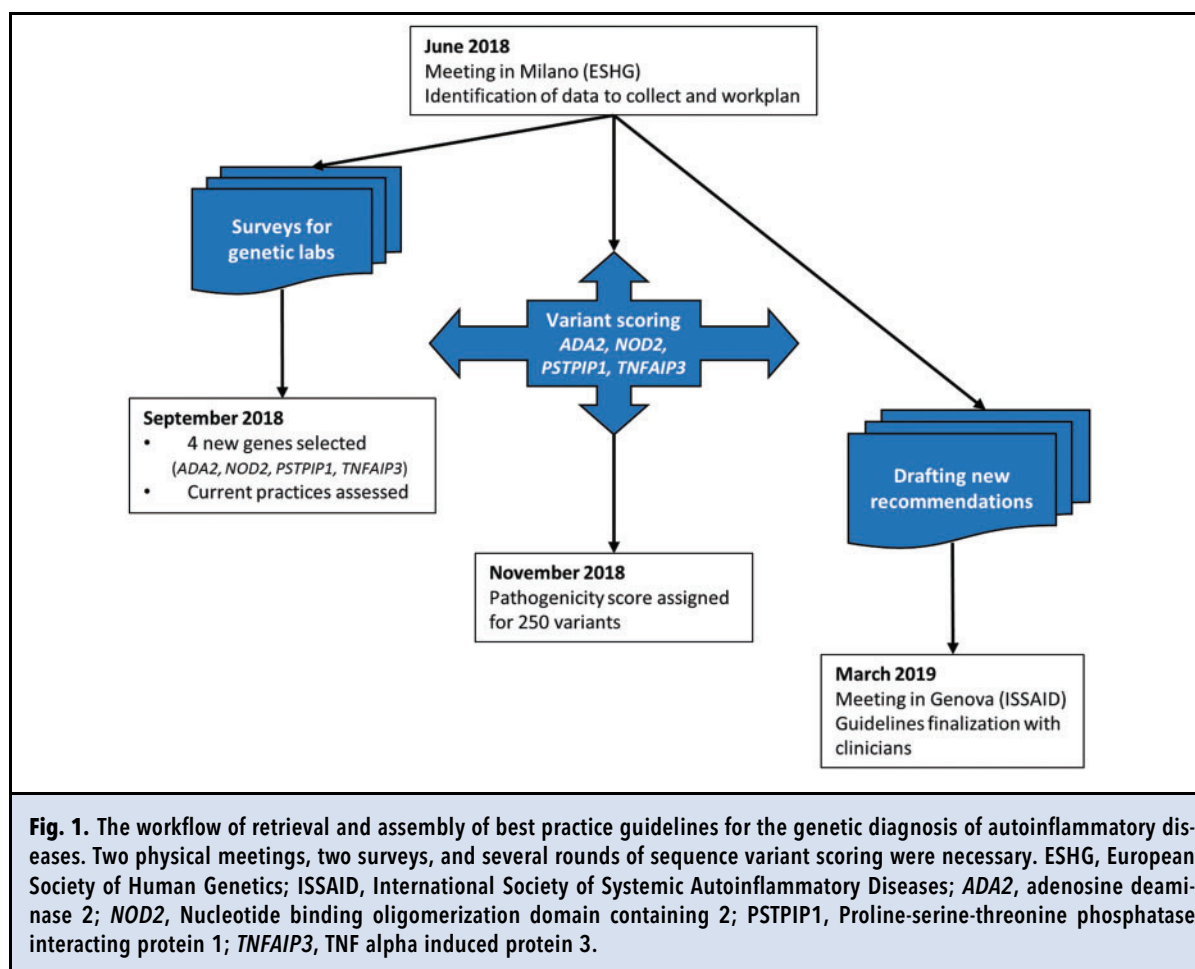
SURVEYS

Two parallel surveys were employed. The first survey aimed at selecting new SAIDs-associated genes to include in these guidelines. An exhaustive list of SAID genes available in Infervers was circulated by E-mail to relevant laboratories retrieved from Orphanet, Infervers contributors and participants of the European Molecular Genetics Quality Network (EMQN) HRF scheme. Laboratories were asked to select genes routinely included in their sequencing panels. This survey revealed a core list of 19 genes from which the writing group selected *ADA2*, *NOD2*, *PSTPIP1*, and *TNFAIP3* based on the following criteria: number of papers and functional assays available, large number of sequence variants reported, and individual requests received by members of the writing group. Four additional genes were considered a reasonable number to manage.

A second survey was developed to highlight current genetic practices in genetic testing of SAIDs as a basis to elaborate guidelines suitable for all laboratories. Six queries were defined and their answers summarized by Rowczenio et al. (36).

SCORING OF NEW GENETIC VARIANTS

We assigned pathogenicity scores (5 = Pathogenic, 4 = Likely pathogenic, 3 = Variant of uncertain significance (VUS), 2. Likely Benign, and 1. Benign) for variants of the *ADA2*, *NOD2*, *PSTPIP1*, and *TNFAIP3* genes using an original workflow we developed for the



HRF genes (37). In brief, data available in Infevers were downloaded, and the variants were scored by IC, DR, YS, IT, and MvG. The classification fulfilled the American College of Medical Genetics and Genomics recommendations and integrated data from both reports and the experts' own unpublished studies. A classification was considered validated if $\geq 75\%$ of the experts reached consistent votes. A provisional classification was assigned if between $>50\%$ and 75% of experts reached consistent votes. Consensus was reached after sequential rounds of voting. The experts could reach a similar classification while relying on different combinations of American College of Medical Genetics and Genomics classification items. Converging the experts' opinion at the classification level rapidly enhanced the consensus classification. Pathogenicity scores with their respective status were then made available in Infevers.

ISSUING OF THE NEW GUIDELINES

A writing group including organizers and assessors of the European Molecular Genetics Quality Network

(EMQN) HRFs scheme (YS, IC, DR, MvG and IT) drafted the first version of the guidelines. The draft was disseminated to molecular geneticists and clinicians working in the field of SAIDs, then discussed in a workshop held during the ISSAID 2019 conference (Genoa, Italy). A second amended version was disseminated by e-mail, after which the final document was ratified.

Results and Recommendations

WHERE TO REQUEST A GENETIC TEST FOR SAIDs?

SAIDs genetic testing is available in generalist and expert laboratories, dedicated medical reference centers, and networks, formally nominated or recognized in several countries (France, Italy, Spain, UK, Germany, Turkey, Israel, USA, Armenia, Japan, and the Netherlands). In 2019, for example, 147 laboratories offered FMF genetic testing worldwide, and 62 of them participated in an HRF specific external genetic quality assessment and proficiency scheme provided by EMQN (online [Supplemental Information](#) file). Laboratory

certification data is available at Orphanet and the NCBI genetic testing registry.

WHEN TO REQUEST A GENETIC TEST FOR SAIDs?

Most laboratories currently do not have specific administrative prerequisites for genetic testing apart from legal ones, i.e., medical prescription and informed consent (36). Laboratory geneticists appreciate inclusion of medical information at referral but do not necessarily consider it mandatory to perform genetic analysis. Interaction between clinicians and laboratory geneticists is mandatory (Supplemental document); we suggest the following:

Patients with overt disease. Clinical data documenting the SAID phenotype and, where available, defining specific manifestations or the fulfillment of clinical criteria should be provided for better diagnostic orientation, choice of the sequencing strategy and interpretation of the genetic results. To limit the workload for clinicians, we suggest that laboratories request a minimal core of items (online Supplemental Table 1).

Predictive diagnosis. In the context of a familial situation, even equivocal symptoms should suggest genetic testing, since both the genetic and environmental backgrounds may reduce the expressivity of the familial genotype. For diseases with risk of irreversible damage such as severe NLRP3-AID (risk of central nervous system lesions or AA-type amyloidosis) or DADA2 (risk of stroke), predictive testing of asymptomatic individuals may be justified and should be discussed in a multidisciplinary team of experts. Follow-up of at-risk individuals may avoid occurrence of life-threatening complications (for example, renal amyloidosis by monitoring serum amyloid A or urinalysis). However, whether such cases should be given prophylactic treatment remains controversial.

Prenatal diagnosis. We do not recommend prenatal or preimplantation genetic diagnosis in families without a history of deleterious SAIDs, as inconclusive genetic results in case of reduced penetrance genotypes or novel VUS may create unnecessary anxiety. In addition, most SAID conditions considered here are successfully treatable, and symptoms may subside over time. In the case of deleterious diseases, genetic counseling should always precede prenatal diagnosis or preimplantation genetic diagnosis.

HOW TO CHOOSE THE BEST DIAGNOSTIC STRATEGY?

Elements collected through the prerequisite form (online Supplemental Table 1) may orient the testing strategy. However, for those patients presenting with general or atypical SAID features, selecting a genetic screen may be very challenging and is dictated in part by the methodologies available in the laboratory.

Test methods and scope. Both Sanger and NGS sequencing are in use in most laboratories providing genetic diagnosis of SAIDs (36). Sequencing of a specific gene (Sanger or NGS) is advised when clinical criteria apply or a biochemical test is positive (e.g., decreased MVK or ADA2 activity); A limited initial *MEFV* exon 10 sequencing is highly recommended for FMF diagnosis, however using an NGS gene panel is preferable in other cases. We suggest sequencing the 8 genes at a minimum, and if possible additional SAID genes from the list referenced in Infevers (which is constantly updated). Information on assessing genetic performance is available in the online Supplemental Information file. We do not yet recommend both exome sequencing and genome sequencing in routine settings, but these can be selectively applied to patients whose gene panels are not informative.

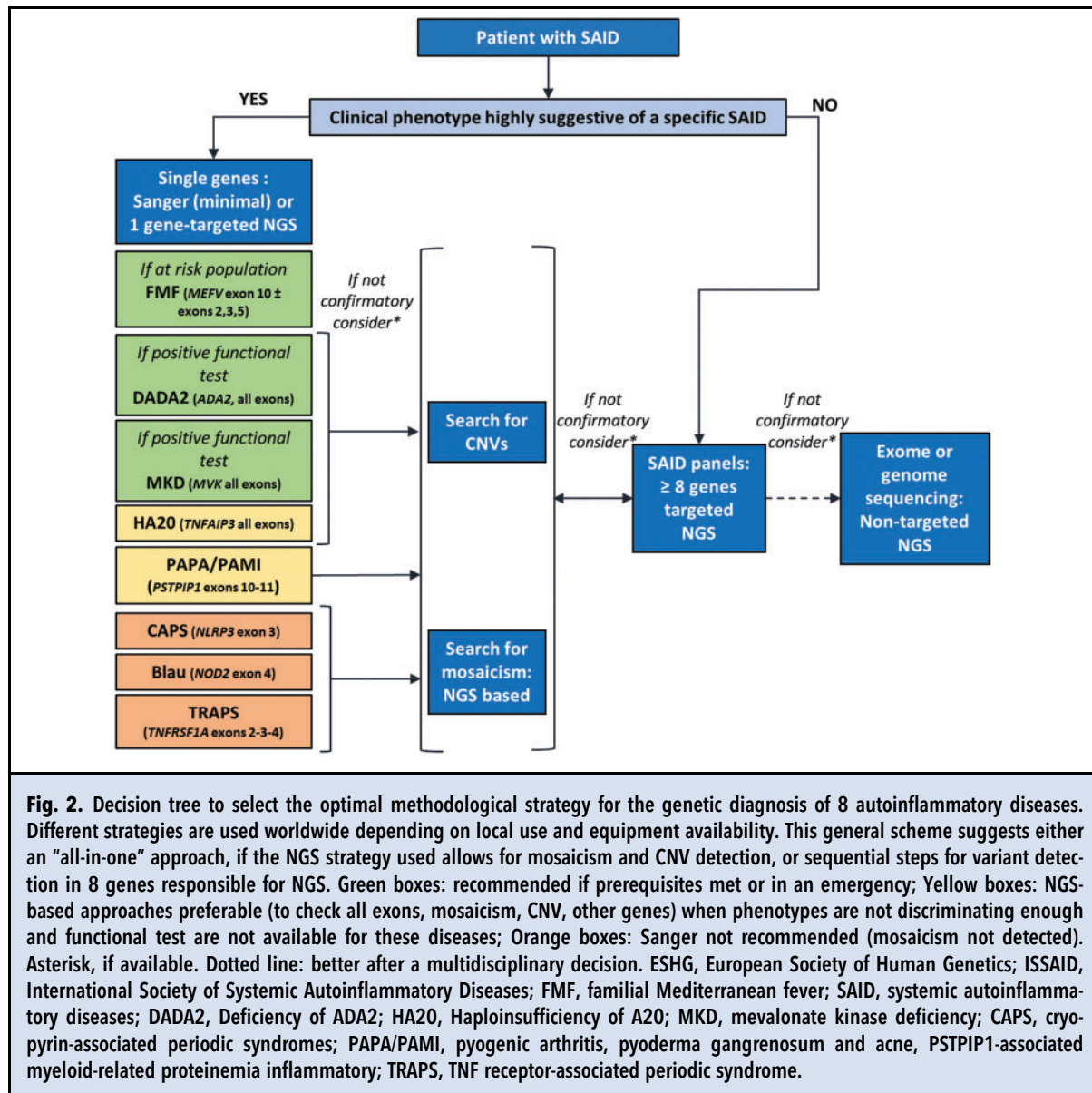
Complementary approaches. Given the growing number of pathogenic CNVs (34, 35, 38) or postzygotic variants found in SAID-associated genes (15–17), we recommend mosaicism detection and that implementation of CNV detection approaches be added to the routine diagnostics in those patients with no confirmatory genotype obtained with strategies that do not allow for CNV or mosaicism detection. Chromosomal imbalances involving either loss or gain of large genomic regions may be detectable with molecular cytogenetic techniques such as comparative genomic hybridization or single nucleotide polymorphism arrays. Validation of small rearrangements by a second method, such as quantitative PCR (qPCR or real-time PCR) is advisable. Multiplex ligation-dependent probe amplification analysis, long-range PCR and long read sequencing can also prove helpful to detect smaller CNVs. Quantitative RT-PCR can be used to search for alterations in gene expression, in case other DNA-based confirmatory methods are not available.

Gene mosaicism can be detected (or validated) with other NGS technologies such as single molecule molecular inversion probes or amplicon-based deep sequencing.

Optimal strategy at a glance. Online Supplemental Table 2 summarizes an optimal strategy for identifying and validating a pathogenic variant in each gene. Figure 2 shows a decision tree for genetic diagnosis of the 8 genes described in this special report, based on the patient's medical referral.

HOW TO INTERPRET THE GENETIC TEST?

Classification and validation of sequence variants identified in SAID-associated genes. We recommend to use an approach which considers a range of evidence for the classification of variants, e.g., the American College



of Medical Genetics and Genomics guidelines (39). An expert consensus interpretation of about 1300 sequence variants for the 8 selected genes relying on both public and unpublished, lab generated data (Supplemental Table 3 and Supplemental document) is available at the Infervers website. For the final consensus score, we used the rules previously described (37), and in the Materials and Methods section. Online Supplemental Table 3 demonstrates the progression made by the current work for variants listed in Clinvar, compared to the data from computational tools for semi-automated variant interpretation and to the classification made by geneticists experienced in SAIDs and EMQN. Of note,

classifications, especially VUS, may change when more information on function and regulation of aberrant proteins becomes available.

De novo and postzygotic variants. De novo variants, occasionally generated by a postzygotic mutational event during embryonic development or the lifespan, may cause dominantly inherited SAIDs in sporadic cases (17). Pathogenic postzygotic variants are predominantly present in the myeloid cells, which are the main effector cells in SAIDs. Pathogenicity of a new variant should be assigned based on a well-defined phenotype and functional assays are suggested, although typically performed

Table 1. Guidelines for interpreting and reporting genetic results in 8 selected autoinflammatory diseases.

	Transmission	Genetic results	Interpretation	Additional remarks to be reported
Confirmatory genotypes	Classically dominant	1 (likely) pathogenic variant ^a in (NLRP3, NOD2, PSTPIP1, TNFAIP3, TNFRSF1A) or 1 pathogenic ^a dominant variant in (MEFV, MVK)	This genotype confirms clinical diagnosis of (CAPS, Blau, PAPA, HA20, TRAPS) or (PAAANDs, Porokeratosis etc.).	Genetic counseling recommended – if available add: screening of asymptomatic parents is recommended to identify de novo variants or low-grade parental mosaicism
		Biallelic (likely) pathogenic variants ^a in (ADA2, MEFV, MVK). The patient is (homozygote or compound heterozygote)	This genotype confirms clinical diagnosis of (DADA2, FMF, MKD).	– if relevant add: known phenotype-genotype association ^b Genetic counseling recommended
Consistent genotypes	Classically dominant	1 novel ^c likely pathogenic variant in (NLRP3, NOD2, PSTPIP1, TNFAIP3, TNFRSF1A)	This genotype is consistent with clinical diagnosis of (CAPS, Blau, PAPA, HA20, TRAPS).	– if relevant include known phenotype-genotype association ^b Genetic counseling recommended
		2 (likely) pathogenic ^a not phased or 1 (likely) pathogenic ^a + 1 rare/novel VUS biallelic variants in (ADA2, MEFV, MVK)	This genotype is consistent with clinical diagnosis of (DADA2, FMF, MKD). Diagnosis relies on clinical judgment or criteria	Diagnosis relies on clinical judgment or criteria If available add: – Screening of other affected relatives is recommended to help interpret this variant – Screening of asymptomatic parents is recommended to identify de novo variants or low-grade parental mosaicism

Continued

Table 1. (continued)

Transmission	Genetic results	Interpretation	Additional remarks to be reported
			<ul style="list-style-type: none"> – If relevant add: biomarker test for ADA2 or MVK is suggested – if available add: Parental testing is recommended to resolve the issue of complex allele (not mandatory for 2 variants in MEFV exon 10 since they have never been reported in complex alleles)
Inconclusive genotypes	1 rare VUS in (NLRP3, NOD2, PSTPIP1, TNFAIP3, TNFRSF1A)	This genotype is inconclusive.	Refer to an expert clinician to consider other SAIDs
Classically dominant		If screening of a limited number of exons, add: Rare undetected variants may exist.	If available: Screening of the other affected relatives is recommended to help interpret this variant
Classically recessive	1 (likely) pathogenic ^a or	This genotype is inconclusive.	Refer to an expert clinician to consider [name of the suspected disease]-like or other SAIDs
	2 rare VUS in (ADA2, MEFV, MVK)	If screening of a limited number of exons, add: Rare undetected variants may exist.	Diagnosis relies on clinical judgment or criteria
			<ul style="list-style-type: none"> – If relevant add: biomarker test for ADA2 or MVK is suggested
			If relevant:
			<ul style="list-style-type: none"> – parental testing is recommended to resolve the issue of complex allele – screening of other affected relatives is recommended to help interpret this/these variant(s)
No variant	No pathogenic variant was identified in the [name] gene, within the sequence investigated and using our routine analysis (see technique used)	This result does not support the involvement of [name gene] in the phenotype of your patient.	Refer to an expert clinician to consider [name of the suspected disease]-like or other SAIDs
Recessive and dominant		If screening of a limited number of exons, add: Rare undetected variants may exist.	

Continued

Table 1. (continued)

Specific situations	Transmission	Genetic results	Interpretation	Additional remarks to be reported
	Dose effect (dosage)	1 (likely) pathogenic variant ^a in (<i>MEFV</i>) and consistent segregation analysis or very consistent phenotype	Cases of sporadic heterozygotes associated with this genotype were published.	Refer to an expert clinician to discuss the diagnosis
	Mosaicism	1 (likely) pathogenic variant ^a in (<i>NLRP3</i> , <i>NOD2</i> , <i>TNFRSF1A</i>)	This genotype could be consistent with clinical diagnosis of (FMF).	Genetic counseling recommended
	Phenotype not related to mutated gene (NGS panel, exome or genome)	1 (likely) pathogenic variant ^a in classically recessive, or transmitted by one asymptomatic parent in classically dominant genes	Re-evaluate the phenotype with the prescribing clinician before reporting. [%] detected mosaicism.	Refer to an expert clinician to discuss the diagnosis
			Better not to report.	
			If reported ^d , mention that the genotype is not known to be associated with the phenotype.	
	Frequent VUS	See Supplemental Table 2 for well-known examples	Better not to report. If reported mention that these VUS may be defined as risk alleles, but do not confirm the diagnosis genetically.	Refer to an expert clinician to consider other SAIDs

^aRecognized validated or provisional pathogenicity scores (infivers, clinvar...) are available.

^bE.g., in FMF: homozygosity for M694V is known to be associated with elevated risk of amyloidosis.

^cIn all cases: refer any new variant or new supporting data to expert databases.

^dCarrier status may be relevant to report in at-risk populations. How to deal with secondary findings is outside the scope of the guidelines.

VUS: Variant of uncertain significance; Frequent and rare VUS: as defined by the American College of Medical Genetics and Genomics provided that the relevant ethnicity is well represented in the data set; NGS: Next-generation sequencing; SAID: Systemic autoinflammatory diseases.

MEFV: Mediterranean fever; MKK: Mevalonate kinase; TNFRSF1A: TNF receptor superfamily 1; NLRP3: NOD-like receptor family, pyrin domain-containing 3; NOD2 Nucleotide-binding oligomerization domain-containing 2; PSTPIP1: Proline-serine-threonine phosphatase-interacting protein 1; TNFAIP3: TNF alpha-induced protein 3; ADA2: Adenosine deaminase.

CAPS: Cryopyrin-associated periodic syndrome; PAPA: Pyogenic arthritis, pyoderma gangrenosum and acne; HA20: Haploinsufficiency of A20; TRAPS: TNF receptor-associated periodic syndrome; PAAND: Pyrine associated autoinflammation with neutrophilic dermatosis; DADA: deficiency of ADA2; FMF: familial Mediterranean fever; MKD: Mevalonate kinase deficiency.

in research laboratories. Follow-up on its possible germline transmission is advised (online [Supplemental Information](#) file).

Geographic and ethnic considerations. Studies of disease expressivity in ethnically matched FMF patients have indicated that Western world geography and/or environmental factors may reduce FMF disease severity, possibly by epigenetic mechanisms (40). Therefore, genotype–phenotype correlations should be reassessed regionally.

Confirmation of clinical diagnosis. Confirmation of a dominant disease requires identification of a pathogenic or likely pathogenic variant. Confirmation of a recessive disease requires 2 pathogenic or likely pathogenic variants in trans phase. We strongly recommend parental testing for this purpose. A combination in trans of a pathogenic or likely pathogenic variant with a rare or novel VUS should be reported as consistent with the clinical disease diagnosis. Identification of the de novo nature of a variant may further support its pathogenicity. A genotype including a common VUS (often found in patients with SAID that do not exhibit a typical phenotype for the disease) should not be regarded as confirmatory. A broader screen of genes may identify the responsible pathogenic variant in another autoinflammatory gene. [Table 1](#) provides detailed recommendations for genotype interpretation.

HOW TO REPORT GENETIC RESULTS?

We suggest only reporting rare class 3, class 4, and class 5 variants. [Table 1](#) displays examples of reporting these variants in the context of phenotype. When the diagnosis is not confirmed at the genetic level, the report should state that the diagnosis relies on clinical judgment or criteria.

Descriptions of autoinflammatory manifestations in patients heterozygous for classically recessive diseases such as FMF are becoming more frequent (6). However, the genetic laboratory report should not state automatically that the diagnosis is consistent with pyrin-associated autoinflammatory diseases. This statement should be restricted to certain pathogenic variants located outside exon 10, such as variants in codons 242, 244, 373, 478, or 577, along with a comment on the consistency with the patient's phenotype and familial segregation if available. In some populations where high carrier rates due to a founder effect are encountered (e.g., M694V in the *MEFV* gene in individuals of Mediterranean descent), NGS panels can detect the concomitant presence of heterozygous pathogenic variants in SAID genes other than those identified as causal (based on the phenotype and genotype). These variants

should be interpreted cautiously. We suggest considering them at best as possible modifier alleles when they converge into the same inflammatory pathway as the confirmed genetic disease.

Discussion

In 2012, we proposed a consensus set of best practice guidelines for genetic testing of a selection of 4 HRFs aimed at improving the quality of their molecular diagnostics, and for promoting harmonization and standardization of laboratory test reports (5). As the field of SAIDs has been steadily evolving with the discovery of novel genes and sequence variants, novel modes of inheritance, and increased genetic and allelic heterogeneity, we have now extended the routine diagnosis to include new SAID-associated genes, and have revised the guidelines to include newer NGS approaches. Online [Supplemental Table 4](#) summarizes the evidences for recommendation grading, and online [Supplemental Table 5](#) summarizes what has changed and improved since 2012.

Finally, we anticipate several future challenges for genetic diagnosis of SAIDs that will require continuous re-assessment of genetic guidelines and classification criteria. A number of recently identified SAID-associated genes still lack substantial databases and guidelines due to the rarity of disease-associated variants in these genes. Because NGS-based sequencing platforms are now widely used in many countries, the extensive sequencing will ultimately identify a higher number of new sequence variants in any given patient, challenging their expert interpretation. Epigenetic risk factors and modifiers will also be identified and need to be incorporated in SAID diagnosis. These challenges will only be met by collaborative efforts and international cohort studies. We believe the guidelines proposed here will be of immediate help to the SAID medical community and the practice of precision medicine.

Supplemental Material

[Supplemental material](#) is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: EMQN, European Molecular Genetics Quality Network; ISSAID, International Society for Systemic Autoinflammatory Diseases; SAID, systemic autoinflammatory diseases; HRF, hereditary recurrent fevers; NGS, Next-Generation Sequencing; MIM, Mendelian inheritance in men; PAAND, pyrin-associated autoinflammation with neutrophilic dermatosis; DSAP, disseminated superficial actinic porokeratosis; DADA2, deficiency of ADA2; CNV, copy-number variations; HA20, haploinsufficiency of A20; VUS, variant of uncertain significance.

List of Genes: *ADA2*, adenosine deaminase 2; *MEFV*, Mediterranean fever; *TNFRSF1A*, TNF receptor super family 1A; *MVK*, mevalonate kinase; *NLRP3*, NLR family pyrin domain-containing 3; *NOD2*, nucleotide-binding oligomerization domain-containing 2; *PSTPIP1*, proline-serine-threonine phosphatase-interacting protein 1; *TNFAIP3*, TNF alpha-induced protein 3.

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References

- Georgin-Lavialle S, Fayand A, Rodrigues F, Bachmeyer C, Savye L, Gateau G. Autoinflammatory diseases: state of the art. *Presse Med* 2019;48:e25-48.
- Manthiram K, Zhou Q, Aksentjevich I, Kastner DL. The monogenic autoinflammatory diseases define new pathways in human innate immunity and inflammation. *Nat Immunol* 2017;18:832-42.
- Toplak N, Frenkel J, Ozen S, Lachmann HJ, Woo P, Koné-Paut I, et al. An international registry on autoinflammatory diseases: the Eurofever experience. *Ann Rheum Dis* 2012;71:1177-82.
- Touitou I, Lesage S, McDermott M, Cuisset L, Hoffman H, Dode C, et al. Infevers: an evolving mutation database for auto-inflammatory syndromes. *Hum Mutat* 2004;24:194-8.
- Shinar Y, Obici L, Aksentjevich I, Bennetts B, Austrup F, Ceccherini I, et al. Guidelines for the genetic diagnosis of hereditary recurrent fevers. *Ann Rheum Dis* 2012;71:1599-605.
- Touitou I. Inheritance of autoinflammatory diseases: shifting paradigms and nomenclature. *J Med Genet* 2013;50:349-59.
- French FMF Consortium. A candidate gene for familial Mediterranean fever. *Nat Genet* 1997;17:25-31.
- Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. The International FMF Consortium. *Cell* 1997;90:797-807.
- 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 (Oxford)* 2017;56:209-13.
- Aldea A, Campistol JM, Arostegui JI, Rius J, Maso M, Vives J, et al. A severe autosomal-dominant periodic inflammatory disorder with renal AA amyloidosis and colchicine resistance associated to the MEFV H478Y variant in a Spanish kindred: an unusual familial Mediterranean fever phenotype or another MEFV-associated periodic inflammatory disorder? *Am J Med Genet A* 2004;124A: 67-73.
- 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;73:455-61.
- Masters SL, Lagou V, Jéru I, Baker PJ, Van Eyck L, Pary DA, et al. Familial autoinflammation with neutrophilic dermatosis reveals a regulatory mechanism of pyrin activation. *Sci Transl Med* 2016;8:332ra45.
- Ben-Chétrit E, Gattorno M, Gul A, Kastner DL, Lachmann HJ, Touitou I, et al. Consensus proposal for taxonomy and definition of the autoinflammatory diseases (AIDs): a Delphi study. *Ann Rheum Dis* 2018;77:1558-65.
- Atzmony L, Choate KA. Second-hit somatic mutations in mevalonate pathway genes underlie prokeratosis. *J Invest Dermatol* 2019;139:2409-11.
- Saito M, Fujisawa A, Nishikomori R, Kambe N, Nakata-Hizume M, Yoshimoto M, et al. Somatic mosaicism of CIAS1 in a patient with chronic infantile neurologic, cutaneous, articular syndrome. *Arthritis Rheum* 2005;52:3579-85.
- de Koning HD, van Gijn ME, Stoffels M, Jongekrijg J, Zeeuwen P, Elferink MG, et al. Myeloid lineage-restricted somatic mosaicism of NLRP3 mutations in patients with variant Schnitzler syndrome. *J Allergy Clin Immunol* 2015;135:561-4.
- Labrousse M, Kevorkian-Verguet C, Boursier G, Rowczenio D, Maurier F, Lazaro E, et al. Mosaicism in autoinflammatory diseases: cryopyrin-associated periodic syndromes (CAPS) and beyond. A systematic review. *Crit Rev Clin Lab Sci* 2018;55:432-42.
- Zhou Q, Yang D, Umbrello AK, Zavalov AV, Toro C, Zavalov AV, et al. Early-onset stroke and vasculopathy associated with mutations in ADA2. *N Engl J Med* 2014;370:911-20.
- Navon Elkhan P, Pierce SB, Segel R, Walsh T, Barash J, Padeh S, et al. Mutant adenosine deaminase 2 in a polyarteritis nodosa vasculopathy. *N Engl J Med* 2014;370:921-31.
- Hashem H, Kelly SJ, Ganson NJ, Hershfield MS. Deficiency of adenosine deaminase 2 (DADA2), an inherited cause of polyarteritis nodosa and a mimic of other systemic rheumatologic disorders. *Curr Rheumatol Rep* 2017;19:70.
- Caorsi R, Penco F, Grossi A, Insalaco A, Omenetti A, Alessio M, et al. ADA2 deficiency (DADA2) as an unrecognized cause of early onset polyarteritis nodosa and stroke: a multicentre national study. *Ann Rheum Dis* 2017;76:1648-56.
- Rama M, Duflos C, Melki I, Bessis D, Bonhomme A, Martin H, et al. A decision tree for the genetic diagnosis of deficiency of adenosine deaminase 2 (DADA2): a French reference centres experience. *Eur J Hum Genet* 2018;26:960-71.
- Miceli-Richard C, Lesage S, Rybojad M, Prieur AM, Manouvrier-Hanu S, Häfner R, et al. CARD15 mutations in Blau syndrome. *Nat Genet* 2001;29:19-20.
- Kanazawa N, Okafuji I, Kambe N, Nishikomori R, Nakata-Hizume M, Nagai S, et al. Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-kappaB activation: common genetic etiology with Blau syndrome. *Blood* 2004;105:1195-7.
- Bonen DK, Cho JH. The genetics of inflammatory bowel disease. *Gastroenterology* 2003;124:521-36.
- Caso F, Galozzi P, Costa L, Sfriso P, Cantarini L, Punzi L. Autoinflammatory granulomatous diseases: from Blau syndrome and early-onset sarcoidosis to NOD2-mediated disease and Crohn's disease. *RMD Open* 2015;1:e000097.
- Wouters CH, Maes A, Foley KP, Bertin J, Rose CD. Blau syndrome, the prototypic auto-inflammatory granulomatous disease. *Pediatr Rheumatol* 2014;12:33.
- de Inocencio J, Mensa-Vilaro A, Tejada-Palacios P, Enriquez-Merayo E, González-Roca E, Magri G, et al. Somatic NOD2 mosaicism in Blau syndrome. *J Allergy Clin Immunol* 2015;136:484-487.e2.
- Wise CA, Gillum JD, Seidman CE, Lindor NM, Veile R, Bashiardes S, et al. Mutations in CD2BP1 disrupt binding to PTP PEST and are responsible for PAPA syndrome, an autoinflammatory disorder. *Hum Mol Genet* 2002;11:961-9.
- Hashmi SK, Bergstrom K, Bertuch AA, Despotovic JM, Muscal E, Xia F, et al. PSTPIP1-associated myeloid-related proteinemia inflammatory syndrome: a rare cause of childhood neutropenia associated with systemic inflammation and hyperzincemia. *Pediatr Blood Cancer* 2019;66:e27439.
- Calderón-Castrat X, Bancalari-Díaz D, Román-Curto C, Romo-Melgar A, Amorós-Cerdán D, Alcaraz-Mas LA, et al. PSTPIP1 gene mutation in a pyoderma gangrenosum, acne and suppurative hidradenitis (PASH) syndrome. *Br J Dermatol* 2016;175:194-8.
- Zhou Q, Wang H, Schwartz DM, Stoffels M, Park YH, Zhang Y, et al. Loss-of-function mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease. *Nat Genet* 2016;48:67-73.
- Shigemura T, Kaneko N, Kobayashi N, Kobayashi K, Takeuchi Y, Nakano N, et al. Novel heterozygous C243Y

- A20/TNFAIP3 gene mutation is responsible for chronic inflammation in autosomal-dominant Behçet's disease. *RMD Open* 2016;2:e000223.
- 34.** Franco-Jarava C, Wang H, Martin-Nalda A, Alvarez de la SD, García-Prat M, Bodet D, et al. TNFAIP3 haploinsufficiency is the cause of autoinflammatory manifestations in a patient with a deletion of 13Mb on chromosome 6. *Clin Immunol* 2018;191:44-51.
- 35.** Uettwiller F, Sarabay G, Rodero MP, Rice GI, Lagrue E, Marot Y, et al. ADA2 deficiency: case report of a new phenotype and novel mutation in two sisters. *RMD Open* 2016;2:e000236.
- 36.** Rowczenio D, Shinar Y, Ceccherini I, Sheils K, Van Gijn M, Patton SJ, et al. Current practices for the genetic diagnosis of autoinflammatory diseases: results of a European Molecular Genetics Quality Network Survey. *Eur J Hum Genet* 2019;27:1502-1508.
- 37.** 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;55:530-7.
- 38.** Lee PY, Huang Y, Zhou Q, Schnappauf O, Hershfield MS, Li Y, et al. Disrupted N-linked glycosylation as a disease mechanism in deficiency of ADA2. *J Allergy Clin Immunol* 2018;142:1363-1365.e8.
- 39.** Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. 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;17:405-24.
- 40.** Touitou I, Sarkisian T, Medlej-Hashim M, Tunca M, Livneh A, Cattan D, et al. Country as the primary risk factor for renal amyloidosis in familial Mediterranean fever. *Arthritis Rheum* 2007;56:1706-12.