Current and future advances in genetic testing in systemic autoinflammatory diseases

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Abstract

Systemic autoinflammatory diseases (SAIDs) are a group of inflammatory disorders caused by dysregulation in the innate immune system that leads to enhanced immune responses. The clinical diagnosis of SAIDs can be difficult since individually these are rare diseases with considerable phenotypic overlap. Most SAIDs have a strong genetic background, but environmental and epigenetic influences can modulate the clinical phenotype. Molecular diagnosis has become essential for confirmation of clinical diagnosis. To date there are over 30 genes and a variety of modes of inheritance that have been associated with monogenic SAIDs. Mutations in the same gene can lead to very distinct phenotypes and can have different inheritance patterns. In addition, somatic mutations have been reported in several of these conditions. New genetic testing methods and databases are being developed to facilitate the molecular diagnosis of SAIDs, which is of major importance for treatment, prognosis and genetic counselling. The aim of this review is to summarize the latest advances in genetic testing for SAIDs and discuss potential obstacles that might arise during the molecular diagnosis of SAIDs.

Key words: autoinflammatory diseases, mosaicism, digenic inheritance, genetic testing, gene panel, NGS

Rheumatology key messages

- Diagnosis of systemic autoinflammatory diseases is hampered by paucity of clinical criteria and functional diagnostic tests.
- Genetic testing can greatly assist clinicians with diagnosis and selecting an appropriate treatment.
- About 60% of patients with suspected monogenic systemic autoinflammatory diseases are still molecularly undiagnosed.

Introduction

Autoinflammatory diseases are caused by defects in genes that regulate innate immunity leading to excessive production of proinflammatory cytokines such as IL-1, IL-6, TNF and type I interferons [1]. Typically, patients present with flares of inflammation manifesting with fever, elevated acute phase reactants, and with a range of tissue- and organ-specific manifestations. Although there are distinct clinical features that can help with the differential diagnosis of systemic autoinflammatory diseases (SAIDs), there is a considerable overlap in clinical presentations. The most predictive criteria include age of onset, duration of fever and disease flares, presence and type of skin rash, CNS involvement, positive family history, and patient's ancestry [2]. However, several problems exist regarding the applicability of clinical diagnoses for SAIDs. First, with the exception of prototypic autoinflammatory disorders, hereditary recurrent fevers (HRFs), clinical classification and/or clinical diagnostic criteria have not been developed for the majority of known disorders. Second, the proposed clinical criteria lacked accuracy, i.e. they were neither highly specific nor sensitive. This is comprehensible considering that mutations in different genes may lead to a similar disease (locus heterogeneity) as is the case in patients with familial cold autoinflammatory syndrome and Aicardi–Goutières syndrome, which have been linked to causal variants in three and eight different genes, respectively [3–6]. Mutations in the same gene can present with vast differences in disease severity and manifestation, as is the case with in flammasomopathies and pyrin-associated diseases (allelic heterogeneity). Differential diagnosis for SAIDs can also be challenging because patients with pathogenic
mutations in different genes may present with similar clinical features (focus heterogeneity), as is the case in patients with familial cold autoinflammatory syndrome. Furthermore, disease expressivity is likely influenced by the presence of modifying genetic alleles, epigenetic modifications and environmental factors including therapies. New evidence-based classification criteria for four prototypic HRFs has been recently published and they include a combination of clinical features and genotypes and as such they have much higher specificity and sensitivity [7]. However, none of the existing clinical classification criteria have been developed or validated in genetically diverse populations. Thus, molecular diagnostics have become an integral part of clinical management and can assist clinicians in the process of choosing an appropriate treatment [8]. Biological therapies that target specific cytokines driving inflammation in SAIDs have been highly effective in suppressing disease activity in these oftentimes life-long and devastating conditions.

**Monogenic autoinflammatory diseases and mode of inheritance**

**Autosomal dominant and autosomal recessive inheritance**

Most monogenic SAIDs are inherited by one of the classical modes of inheritance, namely autosomal recessive or autosomal dominant (Table 1). An individual with an autosomal recessive genetic disorder must carry biallelic pathogenic variants in the same gene, either in a homozygous or in a compound heterozygous state. Each pathogenic mutation must be transmitted from each unaffected parent (in trans) unless one mutation arose as a de novo in the patient. Parental testing is highly recommended to confirm clinical diagnosis at the molecular level. The pathophysiology of recessively inherited diseases can usually be explained by a loss-of-function mechanism, which is when the loss of protein expression and/or function from both alleles causes the disease. These mutations are often found in genes that encode ubiquitously expressed enzymes and result in more global phenotypes that present early in life. The classic example of a recessive hereditary SAID is mevalonate kinase deficiency (MKD), caused by biallelic mutations in the MVK gene [9–11]. Other examples include deficiency of adenosine deaminase 2 (DADA2), caused by biallelic mutations in the ADA2 gene, and sideroblastic anaemia with B cell immunodeficiency, periodic fevers and developmental delay, which is caused by biallelic mutations in the TRNT1 gene [12–14].

Dominantly inherited disorders are caused by a single pathogenic variant that either arose de novo during gametogenesis or was inherited from an affected parent. However, there are examples of reduced penetrance in dominantly inherited traits whereby a causal mutation is inherited from an unaffected parent or is present in other unaffected family members. Typical examples of dominantly inherited SAIDs are inflammasomopathies, in which patients carry a heterozygous missense mutation that leads to gain in the protein function [15]. Another mechanism for dominantly inherited SAIDs is by a haploinsufficiency when the single functional copy of the gene is not enough to maintain the protein function. This example includes haploinsufficiency of A20 (HA20) that is caused by truncating mutations in the TNFAIP3 gene [16]. Parental testing is necessary to confirm de novo mutations. Most dominantly inherited pathogenic variants are novel, but some are reported at a very low frequency in large public databases of human gene alleles.

**Mosaicism**

Mosaicism has been described in SAIDs and it is one mechanism that can lead to atypical or unexpected modes of inheritance. Mosaicism is caused by de novo mutations that occur post-zygotically, so called somatic mutations, which result in two genetically distinct cell populations within a single individual. Pathogenic somatic mutations in NLRP3 were shown to cause neonatal onset multisystem inflammatory disease [also known as chronic infantile neurological cutaneous and articular syndrome (CINCA)], Muckle–Wells and Schnitzler syndrome and, depending on what cell types and tissues carry the altered genotype, disease manifestations and age of onset vary significantly [17–20]. Disease-causing somatic mutations are mainly seen in autosomal dominant inherited SAIDs and often only a small percentage of mutant cells, specifically myeloid lineage cells, is sufficient to initiate the inflammatory process [21].

If the somatic mutation is also found in other types of cells including gonadal tissue, it is called germline or gonadal mosaicism and the mutation has the potential to be passed on to the subsequent generation. Germline mosaicism has been reported in patients with Blau syndrome and with tumour necrosis factor receptor-1 (TNFR1)-associated periodic syndrome, caused by mutations in NOD2 and TNFRSF1A, respectively [22, 23]. More recently, somatic mutations have been identified in keratinocytes of patients with STING-associated vasculopathy with onset in infancy (SAVI) and in a patient with an NLR family CARD domain-containing protein 4 (NLRC4)-associated haemophagocytic lymphohistiocytosis (HLH) disease [24, 25].

**Digenic inheritance**

Digenism is another mechanism that causes non-Mendelian inheritance. While monogenic traits, which follow Mendelian inheritance, are caused by one (autosomal dominant) or two (autosomal recessive) mutations in a single gene, digenic disease manifests if mutations occur simultaneously in two distinct genes. Given that next generation sequencing (NGS) allows the sequencing of many genes in parallel, the discovery of disorders that exhibit a digenic or oligogenic inheritance is greatly facilitated.

Identification of two or more pathogenic variants in different genes in a single patient is not a confirmation of
| Disease acronym | Gene(s) | Disease name | Mode of inheritance | Disease mechanism | OMIM number* |
|-----------------|--------|--------------|---------------------|------------------|--------------|
| ADAM17 deficiency | ADAM17 | ADAM17 deficiency | AR | LoF | 614328 |
| AGS (Type 1-7) | ADAR, DNASE2, IFIH1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, TREX1 | Aicardi-Goutières syndrome | AR (type 2-6) or AD/AR (type 1) or AD (type 7) | LoF (type 1-6, Dnase II deficiency), GoF (type 7) | 615010, 615846, 610333, 610181, 610329, 612952, 225750 |
| AIADK | NLRP1 | Autoinflammation with arthritis and dyskeratosis | AD/AR | GoF | 617388 |
| AIFEC/NLRC4-MAS/FCAS4 | NLRC4 | Autoinflammation with infantile enterocolitis/NLRC4 macrophage activation syndrome/familial cold autoinflammatory syndrome 4 | AD | GoF | 616115, 616050 |
| AILJK | COPA | Autoimmune interstitial lung, joint, and kidney disease | AD | Dominant negative | 616414 |
| Blau syndrome | NOD2 | Blau syndrome/early-onset sarcoidosis | AD | GoF | 186580 |
| CAPS | NLRP3 | Cryopyrin-associated periodic syndromes (FCAS, MWS, NOMID/CINCA) | AD | GoF | 120100, 191900, 607115 |
| CAMPS/PSORS2 | CARD14 | CARD14-mediated psoriasis | AD | GoF | 173200, 602723 |
| Cherubism | SH3BP2 | Cherubism | AD | GoF/dominant negative | 118400 |
| DADA2 | ADA2 | Deficiency of adenosine deaminase 2 | AR | LoF | 615688 |
| DIRA | IL1RN | Deficiency of IL-1-receptor antagonist | AR | LoF | 612852 |
| DITRA | IL36RN | Deficiency of IL-36-receptor antagonist | AR | LoF | 614204 |
| EOIBD/IL-10 deficiency | IL10, IL10RA, IL10RB | Early-onset inflammatory bowel disease | AR | LoF | 613148 |
| FCAS2 | NLRP12 | Familial cold autoinflammatory syndrome 2 | AD | LoF | 611762 |
| FMF | MEFV | Familial Mediterranean fever | AD | GoF | 249100 |
| PAAD/PAAND | MEFV | Pyrin-associated dominant diseases/pyrin-associated autoinflammation with neutrophilic dermatosis | AD | GoF | 134610 |
| AIADK/FKLC/MSPC | NLRP1 | Familial keratosis lichenoides chronica/multiple self-healing palmoplantar carcinoma | AD | GoF | 617388, 615225 |
| H syndrome | SLC29A3 | Histiocytosis lymph adenopathy plus syndrome | AR | LoF | 602782 |
| HA20 | TNFAIP3 | Haploinsufficiency of A20 | AR | LoF | 616744, 260920, 610377 |
| HIDS/MKD | MKV | Mevalonate kinase deficiency/hyperimmunoglobulinaemia D syndrome | AR | LoF | 615895 |
| HOIL1 deficiency | RBCK1 (HOIL1) | HOIL1 deficiency | AR | LoF | 615895 |
| HOIP deficiency | RNF31 (HOIP) | HOIP deficiency | AR | LoF | NA |
| LACC1-associated diseases | LACC1 | LACC1-associated systemic JIA and early-onset IBD | AR | LoF | NA |
| LPIN2 deficiency/ Majeed | LPIN2 | Majeed syndrome | AR | LoF | 609628 |
| OTULIN deficiency/ORAS | OTULIN | OTULIN-associated autoinflammatory syndrome | AR | LoF | 617099 |
| PAPA | PSTPIP1 | Pyogenic arthritis, pyoderma gangrenosum and acne syndrome | AD | Not known | 604416 |
| PFIT | WDR1 | Periodic fever, immunodeficiency, and thrombocytopenia | AR | LoF | NA |

(continued)
The digenic (double) pathogenic variants are usually identified in proteins that are part of the same multiprotein complex. A classic example of digenic SAIDs is the group of proteasome-associated autoinflammatory syndromes also known as chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) syndrome. Most CANDLE syndrome patients have recessively inherited homozygous or compound heterozygous rare or novel mutations in the gene encoding the proteasome subunit \( \beta \) type 8 (PSMB8; i5) [26–30]. However, in some patients with proteasome-associated autoinflammatory syndromes (PRAAS) only one mutation in PSMB8 was found, which led to the hypothesis that these patients may carry a second pathogenic variant in any of the other genes encoding the subunits of the constitutive proteasome or the immune cell-specific immunoproteasome. Subsequently, a subset of these patients was found to carry heterozygous mutations in two different genes (PSMB8/PSMA3 or PSMB8/PSMB4 or PSMB9/PSMB4) [31]. In addition, a severe CANDLE phenotype with prominent neutrophilic dermatosis and immunodeficiency is linked to heterozygous mutations in proteasome maturation protein (POMP), which is essential for maturation of proteasomes [32].

Digenic and oligogenic inheritance has also been reported in other types of initially uncharacterized SAIDs [33–35]. The oligogenic type of inheritance is in the continuum between monogenic and polygenic inheritance, whereby a phenotype results from cointerhertance of several low frequency variants (allele frequency 1–5%). The allele frequency is not low enough to explain association with rare diseases and these variants are often identified in unaffected parents. As the impact of these alleles on protein function is largely unknown, they are typically reported as variants of unknown clinical significance. These variants are often found in patients with non-specific and milder inflammatory phenotypes compared with patients who carry novel/rare variant(s) of the same gene [36]. They are thought to act by a synergistic effect to induce inflammation. Best known examples include p.R121Q (R92Q) and p.P75L (P46L) in TNFRSF1A, p.E148Q and p.P369S in PSMB9, p.V198M (V200M), p.R488K (R490K) and p.Q703K (Q705K) in NLRP3. Some of these variants have been identified at a higher frequency (also known as burden of variants) in patients with periodic fever, aphthous stomatitis, pharyngitis and adenitis syndrome [37–41].
Multifactorial inheritance and the contribution of environmental and epigenetic factors

Most known SAIDs are monogenic diseases, but there are also a number of multifactorial and complex SAIDs such as Behçet’s disease, systemic juvenile idiopathic arthritis (sJIA), and Crohn’s disease. The underlying hypothesis is that these common and genetically complex autoimmune/autoinflammatory disorders can be attributed to synergistic effects of common reduced-penetrance variants (population allele frequencies >5%) in the presence of appropriate environmental factors (e.g. pathogens). These susceptibility variants are identified by genome-wide association studies in large cohorts of unrelated patients and healthy controls. Known examples include risk alleles in \(\text{IL1A-IL1B, IL-10, IL23-IL12RB2, ERAP1}\) and HLA class I loci in patients with BD and in \(\text{AJAP1, COL11A1, HDAC9, ENC1}\) and HLA class II loci in patients with (sJIA) [42–44].

These complex genetic diseases are not suitable for genetic diagnosis and genetic counselling, as these risk variants or haplotypes are also found in asymptomatic people. However, without proper genetic testing of patients with presumably multifactorial diseases, a possible Mendelian genetic cause cannot be ruled out.

Over the past few years it has become apparent that the pathogenicity of SAIDs is not only determined by genetic factors, but that environmental and epigenetic factors additionally play a crucial role in disease expressivity. Epigenetic factors are defined as heritable changes in gene expression that do not affect the genomic DNA sequence. Because epigenetic changes are less stable and easier to modulate than DNA mutations, they serve to integrate environmental signals and to mediate interactions between environment and the resulting genomic output. The best understood epigenetic mechanisms comprise DNA methylation, histone modifications, remodelling of chromatin, and non-coding RNAs, and some of them have been associated with the pathogenesis of SAIDs [45–50].

Genetic testing methods for autoinflammatory diseases

Genetic testing for SAIDs is not absolutely required for classical and well-characterized diseases in certain populations (e.g. FMF and MKD in the Mediterranean and Dutch populations, respectively), but it might be important for predicting a disease course and for genetic counselling in a family. At the present time, universal recommendations for genetic testing in SAIDs do not exist except for prototypic HRFs: FMF, MKD, TNFR1-associated periodic syndrome and cryopyrin-associated periodic syndromes [51]. An appropriate genetic testing approach should be chosen depending on the phenotype of the patient and the suspected disease-causing gene [52]. Due to significant clinical overlap between different SAIDs, a differential diagnosis might require the sequential or simultaneous analysis of several genes. Prenatal diagnosis is not generally recommended, although this may change as new recessively inherited diseases with severe and potentially lethal phenotype are being identified, e.g. linear ubiquitin chain assembly complex deficiencies and Aicardi–Goutières syndrome [6, 53–55].

Sanger sequencing

Sanger sequencing still constitutes the mainstay of molecular testing for most SAIDs. This method is well-suited for low-throughput laboratories for which an NGS approach would not be profitable. It is recommended for patients with a clear clinical diagnosis (e.g. FMF, neonatal onset multisystem inflammatory disease/CINCA) or for testing mutational hotspots (e.g. exon 10 of the \(\text{MEFV}\) gene; exons 2–5 of the \(\text{TNFRSF1A}\) gene, exon 3 in the \(\text{NLRP3}\) gene) or to test for mutations in founder populations (e.g. FMF, MKD). Sanger sequencing can also assist in the confirmation of a biochemical diagnosis in patients with MKD or DADA2 and thereby contributes important information to genetic counselling. Furthermore, Sanger sequencing is also still indispensable in confirming variants that were identified via NGS methods. Nevertheless, performance and efficiency of Sanger sequencing in SAIDs is low, except for patients from founder populations with FMF and MKD.

NGS approaches

NGS panels are the current method of choice for patients who fulfil criteria for several possible diseases and/or present with ambiguous phenotypes. Initially, NGS was relatively expensive, but rapid advances in methodology and technology have turned it into a time- and cost-efficient sequencing technique. NGS is based on a new technology that generates and analyses millions of sequence-reads per run. Even though the different commercially available NGS platforms vary in their specific methodologies, the general sequencing process encompasses the same steps. First, the DNA samples are fragmented by either enzymatic digestion or sonication. The short fragments of DNA are then ligated to sample-specific adapters \(\text{in vitro}\), followed by a so-called bridge-PCR step on a solid surface that is coated with complementary primers. Subsequently, the amplification products are sequenced by pyropsequencing, sequencing by ligation, or sequencing by synthesis. The resulting sequence-reads are then aligned to a reference genome, and finally variant-calling algorithms are applied to compare mapped reads to the reference genome and to identify potential variation [56].

NGS gene panels

NGS is now increasingly used for the diagnosis of autoinflammatory disorders in the clinical setting, mainly in the context of targeted gene panels. The complete coding sequence of a selected set of genes is specifically enriched and sequenced in these gene panels (Table 2). The SAID gene panels used by commercial and research diagnostic laboratories vary in their exact composition but usually coincide in the inclusion of the classical autoinflammatory genes (\(\text{ELANE, LPIN2, MEFV, MVK, NLRP12, NLRP3, NOD2, PSTPIP1 and TNFRSF1A}\)) [57–60]. An overview of the available SAID gene panels can be found in the Genetic Testing Registry database.
with digenic or oligogenic inheritance. The syndrome also increases the probability of diagnosing SAIDs using a gene panel as a genetic testing approach for an individual. Given the multiple mutations that are already known to cause disease, and even novel genes not yet associated to human disease may be uncovered.

Many genetic testing providers also offer a so-called exome-slice technology. This approach still captures and sequences the whole exome, but the analysis is informatically limited to a specific gene list. This method is best suited for individuals with a clearly defined phenotype or where a comprehensive gene panel is not available. Since the analytic pipeline will only report data on genes included in the predefined list the burden of false-positive variants will be reduced.

### Whole genome sequencing

Compared with WES, whole genome sequencing (WGS) is intended to capture the complete human genome in an unbiased manner (Table 2). In addition to the detection of all disease-causing variants found by conventional approaches, WGS also has the potential to identify deep intronic variants and other cryptic mutations in non-coding regulatory regions and therefore increases the diagnostic yield [65–67]. Moreover, the genome-wide uniformly distributed coverage allows a much more reliable identification of CNVs as compared with WES. An additional important advantage of WGS is the opportunity to periodically reanalyse the genome-wide data for recently discovered or re-classified variants [68]. Despite the emerging evidence on the effectiveness and benefit of WGS in clinical diagnosis for a variety of genetic disorders, it is still mainly used for the discovery of novel disease genes in a research setting [69–71].

### Classification criteria for genetic variation

As the number of variants detected by NGS approaches is much higher compared with Sanger sequencing, the interpretation and comprehension of sequence variation and causality have become much more complex. The
correct estimation of the clinical significance of genetic variation therefore necessitates clear and thorough guidelines in order to standardize the interpretation process. This need led to the development of a more structured workflow for variant interpretation by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology in 2015 [72]. Their guidelines recommend an assessment of the different pieces of evidence for or against pathogenicity for each variant. The different criteria are then combined according to a scoring scheme in order to apply a final variant classification from a five-tier classification system (benign, likely benign, variants of unknown clinical significance, likely pathogenic, or pathogenic). Specifically, for HRFs the International Study Group for Systemic Autoinflammatory Diseases recently applied a new workflow for the classification of genetic variants in four genes (MEFV, TNFRSF1A, NLRP3 and MVK) [73].

The most common pathogenic variants seen in the genes associated with prototypic HRF syndromes are non-synonymous nucleotide changes, and except for MVK, structural mutations (deletions, duplications, rearrangement) are infrequent. More recently, protein truncating mutations were reported in IL1RN, IL36RN and TNFAIP3 genes associated with deficiency of IL-1 receptor antagonist, deficiency of IL-36 receptor antagonist (DITRA) and HA20. Rare large genomic deletions were identified in the IL1RN, IL36RN and TNFAIP3 loci [76]. A comprehensive list of classified variants associated with autoinflammatory diseases can be found in the online database Infevers (https://infevers.umaine-montpellier.fr/web/index.php) [77].

Evidence based on computational predictions

Most of the American College of Medical Genetics and Genomics /Association for Molecular Pathology variant interpretation criteria rely on in silico prediction of the effect of a specific variant on protein function. In general, these criteria use current knowledge on functional and clinical impact of similar variants to predict the pathogenicity of the variant in question. Clearly, the predictive value of this category increases with more knowledge on the molecular disease mechanism as well as the functional domains of a specific gene or protein. For instance, the very strong criteria for pathogenicity can be applied for protein-truncating variants (i.e. nonsense, frameshift, canonical splice site, single-exon or multiexon deletion) only if the underlying mechanism of pathogenesis is known to be loss of function. Other criteria within this category rely on the existing knowledge of variants within important functional regions of a protein.

Evidence based on population data

Population data are an important line of evidence to support the pathogenicity of identified variants. The allele frequency of severe disease-causing alleles is expected to be rare in the general population and a frequency of >5% in any population is considered a ‘stand-alone’ benign classification (exceptions exist for well-known founder alleles including several variants in the MEFV gene).

Population-wide variation resources include the Exome Aggregation Consortium (http://exac.broadinstitute.org), the Genome Aggregation Database (http://gnomad.broadinstitute.org), and the 1000 Genomes Project (http://www.internationalgenome.org) [78, 79]. However, when applying these criteria, it is essential to recognize factors that can affect allelic population frequency such as disease prevalence, expressivity, penetrance and genetic heterogeneity [80].

Evidence based on functional studies

Molecular and cellular biology data on the effect of a specific variant on protein function is taken into account in this category of evidence. Not all functional experiments are useful in predicting the pathogenicity of a specific variant and it is extremely important to question the biological relevance of a specific assay to disease mechanism and manifestation. For example, although many autoinflammatory diseases are mediated by the cytokine IL-1β, pathogenicity of novel NACHT, LRR and PYD domains-containing protein 3 (NLRP3) variants should not be assessed by measuring the cytokine production in patients’ serum samples as the serum levels of IL-1β are functionally irrelevant. A more reliable functional assay is to measure the IL-1β production in supernatants of stimulated peripheral blood cells. The appropriate functional IL-1β test should be consistently used in research and clinical testing. In addition, the subjective nature and variability of functional experiments can cause significant interlaboratory differences in the interpretation of clinical relevance of candidate variants and substantial efforts are made to increase concordance in variant prediction [81]. It is therefore essential that the functional tests have been validated in a clinical diagnostic laboratory setting to achieve high clinical validity (accuracy) and utility. Specific functional tests for SAID causal genes are still scarce with the exception of diseases caused by enzyme deficiencies: MKD, DADA2 and phospholipase Cγ2 (PLCγ2)-associated diseases [82, 83].

Evidence based on mode of inheritance and segregation

Because monogenic disorders are expected to be inherited in a Mendelian fashion, the evidence of segregation of a specific variant in a family can also be used for the interpretation of its clinical significance. For instance, the occurrence of a variant de novo is considered strong evidence of pathogenicity if several criteria are met: confirmation of maternity and paternity; presence in a gene associated to a disease that is consistent with the phenotype; parents must definitely be unaffected. For a dominantly inherited disorder, the co-segregation of disease in affected family members can be used as supporting evidence of pathogenicity. In contrast, inheritance of a variant from an unaffected parent is considered strong evidence that the variant is benign. The application of the described criteria relies heavily on an accurate and
correct phenotypic evaluation of all included family members and can therefore be influenced by factors such as penetrance, expressivity and age of disease onset.

Confirmation of the diagnosis
As for any Mendelian conditions, the definitive genetic diagnosis of SAIDs is based on the finding of unambiguous mutations in the causative genes. Theoretically, finding two biallelic clearly pathogenic mutations (assessed by studying the parental alleles) in recessive diseases or one mutation in dominant diseases would confirm the diagnosis. Heterozygous mutations must be either already known to cause the disease or, if they are novel and/or de novo variants, laboratories should carefully evaluate the clinical relevance of these variants. In all other cases, patient care should be based on clinical grounds and should not prevent initiation of therapies.

Limitations of molecular diagnosis

Limited sensitivity of molecular test
Limitations in sensitivity can occur if only a subset of the known disease-causing mutations are tested by a given method. For example, a sequencing approach that focuses on the coding regions of a specific gene might miss deep intronic variants or variants in cis regulatory elements that can affect protein translation and expression. However, to date, there are no deep intronic variants or variants in regulatory elements described for SAIDs.

In some cases, the second cryptic (non-coding) mutation may not be easily identified and require additional analyses. For DADA2, for instance, several pathogenic deletions and duplications comprising one or several exons of the ADA2 gene have been described [12, 84–86]. Depending on the size of those CNVs, Sanger sequencing alone would not be sufficient for their detection and more elaborate laboratory techniques such as multiplex ligation-dependent probe amplification, digital droplet PCR (ddPCR) or NGS approaches are needed.

Another explanation for limited testing sensitivity is somatic mutations that are below the detection threshold of a specific genetic testing method. Even though Sanger sequencing is a reliable and robust experimental verifying method, it is not sensitive enough to detect somatic mutations below a frequency of ~20% [87, 88].

Other factors contributing to an inconclusive genetic test
Other factors that may contribute to an apparently negative genetic test can come from misinterpretation of the results. The best example to illustrate is the MEFV gene that causes FMF. Based on segregation analyses, FMF has long been considered a recessive disorder. As the frequency of pathogenic variants in MEFV varies significantly between different populations, the estimated sensitivity for finding biallelic mutations ranges from 70% to 95% depending on the ethnic background of the patient [89, 90]. However, in some FMF cases, only a single known pathogenic mutation in MEFV is identified [91–94]. Furthermore, heterozygous mutations in other domains of pyrin have been found in families with clearly dominantly inherited pyrin-associated diseases [95, 96]. One of them is pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND), a dominantly inherited disease caused by amino acid substitution at position 242 (S242R) or 244 (E244K), which is critical for pyrin inhibition [97, 98]. The phenotype of PAAND patients is very similar to pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) syndrome that is caused by heterozygous mutations in the PSTPIP1 gene. Interestingly, proline–serine–threonine phosphatase-interacting protein 1 (PSTPIP1) and pyrin are proteins known to interact, and they associate with the cytoskeleton [99–101]. Similar to MEFV, a complex pattern of inheritance has been described for the NLRP1 gene-associated diseases [102, 103].

Finally, a negative genetic test result in a patient with an apparent SAID can simply be due to the fact that the disease-causing gene has not yet been identified.

Conclusion
The number of molecularly characterized SAIDs has grown rapidly over the past 20 years and has been fuelled by the development of new sequencing technologies. Despite the major progress in the identification of novel genes and pathways associated with SAIDs, many more disease genes are still unknown and over 50% of patients are mutation-negative [58, 104]. A periodic re-evaluation and re-analysis of the genetic testing opportunities is therefore recommended for undiagnosed patients. New NGS technologies and algorithms are being developed to help with detection and classification of all genetic variants. Novel concepts regarding modes of inheritance and interpretation of variant pathogenicity will require a systematic review and update of genetic diagnostic strategies and new guidance on standardization and reporting of genetic tests [51].

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