How to prescribe a genetic test for the diagnosis of autoinflammatory diseases?

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Introduction

The spectrum of systemic autoinflammatory disorders (SAIDs) continues to grow with over 30 different disorders identified to date. SAIDs are associated with dysregulation of the innate immune system, affecting proinflammatory cytokines and apoptosis pathways {McDermott, 2002 #6487; Pathak, 2017 #7067; Goldbach-Mansky, 2012 #7070}. The best-characterised recessively inherited SAIDs are: familial Mediterranean fever (FMF) and mevalonate kinase deficiency (also known as Hyperimmunoglobulin D syndrome (HIDS)) (Table 1). FMF (MIM #249100) is caused by defects in the *MEFV* gene (MIM #608107), which was identified by positional cloning in 1997 by the International FMF Consortia {Consortium, 1997 #1927; Consortium, 1997 #1928}. *MEFV* is located on chromosome 16, consists of 10 exons and encodes a 781-amino acid protein called pyrin. Most pathogenic variants are clustered in the C-terminal, in a relatively short section of exon 10, but genetic changes in other exons have also been identified in FMF {Touitou, 2004 #5577}. There are considerable phenotypic differences among the different *MEFV* mutations; but those located in the hot spot region of exon 10, between residues 680 and 694, are thought to cause more severe disease, in particular the p.M694V variant is known to be associated with more severe clinical features and an increased risk of developing AA amyloidosis {Yalçinkaya, 2000 #2378; Shohat, 1999 #2466; Livneh, 1999 #2162; Cazeneuve, 1999 #2164}. javascript:showcontent('active','references');A

simple heterozygous deletion of p.M694 residue, which is likely to produce marked structural disruption, has been reported to cause autosomal dominant FMF {Booth, 2000 #2309; Rowczenio, 2017 #7077}. Other MEFV substitutions altering amino acid at position 577 in exon 8 (p.T577N, p.T577S and p.T577A) also have been reported to cause colchicine-responsive autosomal dominant SAID resembling FMF {Stoffels, 2014 #6439}. Furthermore, MEFV variants in exon 2 were identified in patients with dominantly inherited pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) presenting with recurrent episodes of neutrophilic dermatosis, fever, elevated acute phase reactants, arthralgia and myalgia/myositis {Masters, 2016 #7065}{Moghaddas, 2017 #7066}.

Mevalonate kinase deficiency (MIM #260920, also know as HIDS) is caused by the deficiency of the mevalonate kinase enzyme {van der Meer, 1984 #3281; Houten, 1999 #6488} encoded by *MVK* gene (MIM #251170) mapped to chromosome 12q24 {Drenth, 1999 #3282}. The *MVK* consists of 11 exons {Houten, 2001 #5115} and four mutations; V377I, I268T, H20P/N and P167L account for more than 70% of all variants associated with MKD {van der Hilst, 2008 #4823}. Mutations in MVK that abolish enzyme activity result in mevalonic aciduria (MIM #610377), an autosomal recessive disease characterized by developmental delay, dysmorphology, hepatosplenomegaly and failure to thrive.

The two most common dominantly inherited SAIDs are: TNF receptor associated periodic syndrome (TRAPS), previously known as familial Hibernian fever and cryopyrin-associated periodic syndromes (CAPS) (Table 1). TRAPS (MIM #142680), is caused by mutations in the tumour necrosis factor receptor superfamily 1A (*TNFRSF1A*) gene (MIM #191190) located on chromosome 12p13, consisting of 10 exons{McDermott, 1999 #3123}. Substitutions affecting the highly conserved

cysteine residues causing disruption to the disulphide bonds are likely to introduce changes to the three-dimensional structure and folding of TNF receptor 1 (TNFR1) and previous studies suggest that the clinical phenotype attributed to these variants was more severe {Aksentijevich, 2001 #4259}. By contrast, p.R121Q (R92Q), the commonest *TNFRSF1A* variant, found at frequency of 2% in the North Americans and Irish populations, is associated with mild clinical features and incomplete penetrance.

CAPS comprise a spectrum of three distinctive disorders of increasing severity. The most severe is chronic infantile neurologic cutaneous articular syndrome (CINCA, MIM #607115) also known as neonatal-onset multisystem inflammatory disease (NOMID) {Ansell, 1975 #5110}, in the middle of the spectrum is Muckle-Wells syndrome (MWS, MIM #191900) described in 1962 by Muckle and Wells in a Derbyshire family with recurrent bouts of urticarial rash, progressive sensorineural deafness and AA amyloidosis {Muckle, 1962 #2487} and the mildest is familial cold autoinflammatory syndrome (FCAS, MIM #120100), first described by Kile and Rusk in 1940 {Kile, 1940 #2488}. CAPS is caused by gain-of-function mutations in the *NLRP3* gene (MIM #606416) {Hoffman, 2000 #2477; McDermott, 2000 #2533; Cuisset, 1999 #2476} also known as *CIAS1*, which is located on chromosome 1q44, consists of nine exons and encods the death domain protein called cryopyrin. This gene was identified by linkage analysis of large families with FCAS and MWS {Hoffman, 2000 #2477; McDermott, 2000 #2533; Cuisset, 1999 #2476}. The mode of inheritance is usually autosomal dominant although both incomplete penetrance and sporadic cases are well recognised. Dominant inheritance is evident in about 75% of patients with MWS and FCAS, whereas CINCA is usually due to *de novo*

mutations {Feldmann, 2002 #2828} and somatic variants {Nakagawa, 2015 #6492}{Saito, 2008 #5711}{Rowczenio, 2017 #6997}.

SAIDs are characterised by overlapping clinical features including recurrent bouts of fever and localised inflammation and occur periodically, irregularly or near constantly. Symptoms typically start during childhood, but the onset can also occur in adolescence or even in late adulthood. The fever episodes can occur sporadically and vary from less than one day to weeks and maybe accompanied by one or more of the following symptoms: serositis (peritonitis, pleuritis and pericarditis), myalgia, arthralgia, neurological manifestations, sensorineural hearing loss and rash. These attacks resolve spontaneously without anti-inflammatory, or immunosuppressive treatment and most patients feel well in between episodes. Although some fever attacks have an obvious trigger, for example exposure to cold in CAPS {Hoffman, 2001 #2835} or vaccination in MKD {Drenth, 2001 #5106} in most cases the cause remains elusive. These diseases are accompanied by acute phase response driven by cytokines. Prolonged, elevated levels of acute phase serum amyloid A (SAA) protein can result in the development of AA amyloidosis; a progressive disease leading to kidney failure. Rapid changes in the acute phase protein levels in response to stimuli allow SAA and CRP to be a good indicator for monitoring of treatment effects in the management of patients with SAIDs.

In the last decade, due to application of the next-generation sequencing (NGS) the genetic diagnosis in patients with SAIDs have greatly improved; novel diseases and disease-associated genes have been identified and remarkable progress has been made in the genetic characterization of the undiagnosed patients and the sporadic cases. The list of monogenic SAIDs is expanding and needs frequent updates. Below is an outline of some of the less frequently encountered monogenic SAIDs, also

listed in Table 1. Blau syndrome (MIM #186580) is caused by mutations in *NOD2/CARD15* gene {Arostegui, 2007 #5114} (MIM #607211) and is characterised by granulomatous inflammation of the skin, eye, and joints (the NOD2 variants are distinct from those in the same gene, seen in some cases of Crohn disease). The syndrome of pyogenic sterile arthritis, pyoderma gangrenosum and acne (PAPA) (MIM #604416) is associated with mutations in *PSTPIP1* gene {Wise, 2002 #4240} (MIM #606347) causing oligoarticular, destructive arthritis and severe cystic acne starting in early adolescence. A subset of patients may also develop pyoderma gangrenosum and pathergy-like sterile abscesses at injection sites. Deficiency of the interleukin-1 receptor antagonist (DIRA) (MIM #612852) resulting from mutations in *IL1RN* gene (MIM #147679) {Aksentijevich, 2009 #4978} characterized by recurrent episodes of generalized skin pustulation, fever, systemic inflammation, and leukocytosis resulting in a diffuse pustular skin rash, sterile osteomyelitis, and periostitis with articular pain but no fever. Deficiency of the interleukin-36 receptor antagonist (DITRA, MIM #614204) caused by mutations in the *IL36RN* gene (MIM #605507) manifests as recurrent episodes of generalized skin pustulation, fever, systemic inflammation, and leukocytosis {Marrakchi, 2011 #5719}. Chronic atypical neutrophilic dermatitis with lipodystrophy and elevated temperature syndrome (CANDLE, other name: autoinflammation, lipodystrophy, and dermatosis syndrome [ALDD], MIM #256040) results from mutations *PSMB8* gene (MIM #177046) and is characterised by recurrent skin eruptions, hepatomegaly, swelling, and systemic inflammation that appears to result from failure of the immunoproteasome to clear ubiquitinated proteins, resulting in excessive interferon signaling {Goldbach-Mansky, 2012 #7070; Xirotagaros, 2016 #7072}. CANDLE belongs to interferon-associated autoinflammatory disorders termed the interferonopathies {Rodero, 2016

#7071} and recently, it was demonstrated that it can also be caused by mutations in genes that encode other proteasome subunits, such as PSMB4, PSMB9, and PSMA3 {Brehm, 2015 #7075}. Majeed syndrome (MIM #609628) caused by mutations in the gene for lipin 2 (LPIN2) (MIM #605519) which result in perinatal onset of sterile osteolytic lesions, dyserythropoietic anemia, congenital anemia, and inflammatory dermatosis, resulting from the infiltration of neutrophils into the dermis {El-Shanti, 1993 #7076}. Stimulator of interferon genes (STING)-associated vasculopathy with onset in infancy (SAVI) (MIM #615934) is an autoinflammatory disease in the interferonopathy family caused by gain-of-function mutations in the gene TMEM173 (MIM #612374) {Liu, 2014 #6918}. Most patients have recurrent low-grade fevers, and all develop marked vascular inflammation limited to the capillaries and manifestations of vascular and tissue damage. Pulmonary manifestations include interstitial lung disease, paratracheal or hilar lymphadenopathy, and lung fibrosis {Xirotagaros, 2016 #7072}. Autoinflammation and PLCG2-associated antibody deficiency and immune dysregulation (APLAID) (MIM #614878) is a form of familial atypical cold urticaria caused by mutations of the PLCG2 gene (MIM #600220) and characterized by recurrent blistering skin lesions, ocular inflammation, enterocolitis, bronchiolitis, arthralgia and mild immunodeficiency {Ombrello, 2012 #7073; Zhou, 2012 #7074}. Autoinflammation with infantile enterocolitis (AIFEC) (MIM #616050) arise from mutations in the *NLRC4* gene (MIM #606831) which have been reported to cause neonatal-onset enterocolitis, periodic fever, and fatal or near-fatal episodes of autoinflammation and macrophage pyroptosis (inflammatory cell death) {Romberg, 2014 #6968}. In a different study *NLRC4* variant was reported in a seven-year-old girl who presented with recurrent episodes of fever, malaise, splenomegaly, vomiting, loose stools with mild duodenitis, intermittent rash, and

additional features consistent with macrophage activation syndrome (MAS) {Canna, 2014 #6967}. Deficiency of Adenosine Deaminase 2 (DADA2) (MIM #615688) is caused by mutations in the *CECR1* (cat eye syndrome chromosome region, candidate 1) gene (MIM #607575). Common clinical signs are early onset recurrent stroke, neurologic manifestations, and fever {Zhou, 2014 #6989}.

To date more than 800 nucleotide variants have been identified and recorded in Infevers database, which collects information on the autoinflammatory genes sequence variants (http:// fmf.igh.cnrs.fr/ISSAID/infevers/) {Touitou, 2004 #5577}. Some of these variants are clearly pathogenic, but majority are unconfirmed or seemingly non-pathogenic. Given the variable phenotype of SAIDs, genetic testing remains a crucial tool in making the correct clinical diagnosis.

Table 2.1 List of the systemic hereditary autoinflammatory diseases

Syndrome	Gene	Inheritance	Age at onset	Distinctive Features
FMF (MIM #249100)	*MEFV* Chr. 16	AR	Childhood	Fever episodes accompanied by abdominal pain, pleurisy, arthralgias or arthritis, serositis and synovitis
TRAPS (MIM #142680)	*TNFRSF1A* Chr. 12	AD	Childhood, but also later in life	Flares accompanied by conjunctivitis, periorbital oedema, myalgia, rash, abdominal pain, cervical lymphadenopathy
MKD/HIDS (MIM #260920)	*MVK* Chr .12	AR	Infancy	Immunisation induced attacks of fever, GI symptoms, cervical lymphadenopathy and abdominal pain, headache, arthralgias or arthritis, aphthous ulceration
CAPS: FCAS1 (MIM #120100) MWS (MIM #191900 NOMID/ CINCA (MIM #607115)	*NLRP3* Chr. 1	AD or Sporadic	Neonatal / Late onset in some sporadic cases	Cold induced attacks, urticarial rash, chronic meningitis, sensorineural deafness, cartilaginous/bony deformities and mental impairment in the most severe type
FCAS2 (MIM #611762)	*NALP12* Chr.19	AD	Infancy	Cold induced attacks, urticarial rash, sensorineural deafness
DIRA (MIM #612852)	*IL1RN* Chr. 2	AR	Infancy	Diffuse pustular skin rash, sterile osteomyelitis and periostitis with articular pain
PAPA (MIM #604416)	*PSTPIP1* Ch.15	AD	Childhood	Pyogenic destructive arthritis typically involving the elbow, knee and/or ankle, pyoderma gangrenosum, severe cystic acne
Blau Syndrome (MIM #186580)	*NOD2*(CARD15) Ch.6	AD	Childhood	Granulomatous inflammation of the skin, eye and joints
Majeed Syndrome (MIM #609628)	*LPIN2* Ch.18	AR	Infancy	Multifocal osteomyelitis, and dermatosis

Syndrome	Gene	Inheritance	Age at onset	Distinctive Features
DITRA (MIM #614204)	*IL36RN* Chr. 2	AR	Childhood	Recurrent episodes of sterile pustular rash & fever
CANDLE (MIM #256040)	*PSMB8* Chr.6	AR	Neonatal	Recurrent skin eruptions, arthralgia/arthritis and progressive lipodystrophy.
SAVI (MIM #615934)	*TMEM173* Chr. 5	AD	Childhood	Systemic inflammation, cutaneous rash and pulmonary manifestations
APLAID (MIM #614878)	*PLCG2* Chr. 16	AD	Neonatal	Recurrent blistering skin lesions, ocular inflammation, enterocolitis, bronchiolitis, arthralgia
AIFEC (MIM #616050)	*NLRC4* Chr. 2	AD	Neonatal	Enterocolitis, recurrent fevers, GI symptoms, intermittent rash and macrophage activation syndrome
DADA2 (MIM #615688)	*CECR1* Chr. 22	AR	Childhood	Early onset recurrent stroke, neurologic manifestations and fever

Recommendations for genetic testing in SAIDs

Since effective treatment for many SAIDs diseases is now available, correct diagnosis is crucial for patients to access therapy tailored to their underlying disease. Such early and accurate diagnosis can dramatically improve quality of patient's life. Many of the SAIDs can display overlapping, partial or atypical symptoms, which makes the differential diagnosis extremely difficult and thus heavily dependent on genetic testing. Moreover, the current diagnostic approach in children may not be adequate for adults, who are often underdiagnosed. Thus there is the need for specific diagnostic recommendations for most SAIDs {Muscari, 2012 #7078; Cantarini, 2011 #7079}. Various attempts have been aimed at improving the efficiency of SAIDs diagnosis by proposing a set of clinical criteria to guide the genetic analysis of the SAIDs.

In 2006 Federici et al. proposed a decision tree helping to rationalize molecular diagnosis in patients with periodic fever in uncommonly affected areas {Federici, 2006 #7061}. After analyzing the *MEFV, MVK*, and *TNFRSF1A* genes in a large cohort of adult patients the authors concluded that meeting the FMF criteria and being of Mediterranean origin should be considered clear indications for undergoing molecular analysis of the *MEFV* gene. In the absence of these features or in the presence of a non-informative result from the *MEFV* genetic testing, the choice of analysis of the next gene should be made on the basis of expert advice.

In the same year Simon et al. examined whether more rigorous and non-restricted genetic screening would allow the classification of patients with undiagnosed SAIDs {Simon, 2006 #7060}. The authors looked at certain clinical features of the inflammatory episodes, such as the duration of attacks accompanying symptoms, the age of onset, prognosis, ethnic origin, family history, elevated acute

phase response during the attacks and assessed their contribution when making the clinical judgment. This study concluded that the clinical phenotypes and ethnic distribution of each of these syndromes was much more variable than anticipated and that genetic testing failed to make a definite diagnosis in a substantial proportion of patients who had clear periodic fever symptoms. It was also found that mutations in two or more autoinflammatory genes might be more common than expected, in particular the combination of disease-causing mutations with the low-penetrance variants, which probably reflects the high prevalence of the latter in the general population.

In 2008 Gattorno et al. published a diagnostic score for molecular analysis of SAIDs in children {Gattorno, 2008 #7059}. This study identified specific clinical variables, namely: presence of the family history, age at onset, abdominal and chest pain and diarrhoea, to be strongly associated with the probability of detecting relevant mutations in known SAIDs genes, thus also suggesting the order in which the genes should be screened. The diagnostic score suggested by the authors had high sensitivity (87%) and good specificity (72%) for the identification of genetically positive and negative patients.

Despite these attempts, the current guidelines for the classification of the four "classical" SAIDs (FMF, MKD, TRAPS, CAPS) lack accuracy and do not consider the results of genetic analyses. This urged the international experts (clinicians and geneticists) in the field of SAIDs to work on the new classification criteria, making the genetic analysis a compulsory requirement for the accurate diagnosis. These new guidelines aim at identifying patients who should undergo genetic testing and help to classify patients in countries were genetic analysis is not widely available (Gattorno *et al*, 2018, unpublished).

Classification of genetic variants in SAIDs

Diagnosis of SAIDs relies on a combined clinical information and genetic results. The interpretation of genetic testing might be straightforward if the pathogenicity of a given variant is well documented, but for many DNA changes, encountered during genetic screening, especially since the implementation of the NGS, the current knowledge of their consequences is insufficient. Furthermore, even for the best characterised SAIDs, such as FMF and TRAPS, there are no functional assays to corroborate the genotype-phenotype correlation, although in CAPS, the pathogenicity of the *NLRP3* variants can be validated by the inflammasome activation studies {Baroja-Mazo, 2014 #6909; Agostini, 2004 #2974} and in MKD enzymatic activity and elevated levels of mevalonic acid can support the diagnosis {van der Hilst, 2008 #4823; Steichen, 2009 #5775}. Another diagnostic dilemma concerning recessive SAIDs, in particular FMF, is that in a significant number of patients clinically diagnosed with FMF, a disease-associated mutation has been detected on only one *MEFV* allele {Dodé, 2000 #2375; Bayram, 2011 #6299} and certain variants, such as p.M694del, p.T577N, p.T577S and p.T577A, have been associated with an autosomal dominant mode of inheritance {Booth, 2000 #2309; Stoffels, 2014 #6439}. The former variant was identified in patients with the classical FMF, although in some cases variable penetrance was observed and tendency for symptoms to begin later in life. The substitutions affecting amino acid at position 577 had displayed a very broad range of clinical features from classical FMF to symptoms overlapping with other SAIDs. Interestingly, two other *MEFV* variants located in exon 2 caused dominantly inherited pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND), a disease that is distinct

from FMF and characterised by recurrent episodes of neutrophilic dermatosis, fever, elevated acute phase reactants, arthralgia and myalgia/myositis {Masters, 2016 #7065; Moghaddas, 2017 #7066}. Thus, the interpretation of genetic findings requires an in-depth knowledge and experience in the field of the SAIDs genetics.

To date more than 800 variants have been recorded on the Infevers database, an online repository for DNA changes in genes associated with SAIDs (http://fmf.igh.cnrs.fr/ISSAID/infevers/), but until recently, information regarding the clinical consequence of most variants was lacking. Preliminary recommendations on best practice guidelines for genetic testing in SAIDs, reporting of the genetic results and defining the clinical significance of certain DNA changes have been published in 2012 {Shinar, 2012 #6001}. These guidelines were prepared by an International Study Group for Systemic Autoinflammatory Diseases (INSAID) which consisted of an international panel of experts in genetic of the SAIDs and were based on the information deduced from previous SAIDs external quality assurance schemes and data obtained from the literature search. However, the limitation of this study was that the clinical significance of a small number of frequently identified variants was provided and the consequences of other, rare DNA changes was not available. Moreover, the clinical information of some variants was not always accurate, as it was derived from the experience of the laboratory or the clinician who originally reported the case. The need for consensus evidence-based criteria using a combination of clinical and genetic features lead to the same group of international experts recently publishing new guidelines for classification of genetic variants pathogenicity in the in four most recognised SAIDs genes: *MEFV, TNFRSF1A, NLRP3* and *MVK* {Van Gijn, 2018 #7063}. Applying a consensus-driven process on the pathogenicity assessment, this 'gold standard' classifications aimed to

dramatically speed up and synchronise diagnosis of SAIDs between geneticists and clinicians. The new pathogenicity classification of DNA changes are now uploaded onto the Infevers database. It complies with the recommendations made by the American College of Medical Geneticists {Richards, 2015 #7064} such that the DNA variants are assigned to one of the following categories: pathogenic, likely pathogenic, benign, likely benign and variant of unknown clinical significance. These classification criteria are subject to additional clarification on the basis of clinical findings, literature reports, population frequencies, mutation databases and possibly case-specific research data.

Reasons for Requesting Genetic Testing

Genetic tests can be carried out for a number of reasons, including: to diagnose a suspected genetic condition; to identify the specific underlying genetic cause of a disease; to determine the likelihood of developing a genetic condition in the future (in case of the late onset diseases); to identify carriers of a genetic condition; to work out the chance of passing a genetic condition onto children and to find out whether a developing embryo has a particular genetic condition.

Presymptomatic genetic testing is performed in a healthy person who may be at risk of developing a genetic condition with a delayed onset. This test is generally not recommended in SAIDs, although it may be advisable for asymptomatic family members whose relatives have been diagnosed with a serious SAIDs or if there is a family history of amyloidosis. Prenatal diagnosis and preimplantation genetic diagnosis are also not generally supported for the SAIDs, with the exception of families affected by a particularly severe disease such as CINCA/NOMID, which is characterised by debilitating complications including mental retardation, blindness,

deafness and bone deformation. CINCA/NOMID is usually caused by *de novo NLRP3* mutations, and because of the severity of the symptoms these patients rarely have children. Evaluation of carrier status is recommended to individuals from certain ethnic groups where there is an increased risk of specific genetic conditions; to healthy relatives of the affected patient diagnosed with recessive SAIDs or in cases in whom a novel genetic variant or variant of unknown consequences was identified. Carriers of a genetic condition do not generally have any associated health problems themselves but there may be implication for their children. In FMF testing for a carrier status in the *MEFV* gene is recommended to individuals living or originating from the Mediterranean region.

Diagnostic Strategy

The main indication for genetic referral is when a patient presents with clinical symptoms consistent with one or more of the SAIDs. Thus, in making a referral for DNA screening, clinical information that supports the choice for screening of one or more SAIDs genes is required. Genetic diagnosis for SAIDs is now widely available, in particular there are many laboratories providing FMF testing in Europe (the list is provided on the Orphanet at www.orpha.net). Such laboratories should work within a comprehensive quality management system (for example be part of an accreditation), use appropriate validated methods, participate annually in the external quality assessment schemes (for example, Hereditary Recurrent Fever (HRF) EMQN scheme) and the relevant techniques quality schemes (for example, EMQN scheme for DNA sequencing) and define their typical turn-around time.

An Infevers, which is an online registry of sequence variants in monogenic autoinfammatory syndromes (http://fmf.igh.cnrs.fr/ISSAID/infevers) was developed to assist molecular geneticists when evaluating the relevance of DNA results in SAIDs. It provides a comprehensive and updated list of gene variants and for the four most recognised SAIDs diseases: FMF, MKD/HIDS, TRAPS and CAPS the information on the phenotype–genotype correlation is also included. Other disease variant databases, such as the Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/) and the ClinVar Database (http://www.ncbi.nlm.nih.gov/clinvar/) are also helpful. It is recommended to use in silico prediction tools when assessing the pathogenicity of novel or rare variants such as: AGVGD (http://agvgd.iarc.fr/), Sorts Intolerant From Tolerant (SIFT) (http://sift.bii.a-star.edu.sg/), Polyphen-2 (http://coot.embl.de/PolyPhen)/ and Combined Annotation Dependent Depletion (CADD) score (http://cadd.gs.washington.edu/). Identification of one pathogenic variant in dominantly inherited SAIDs, homozygosity or compound heterozygosity (confirmed by studying the parental alleles) in recessively inherited SAIDs, should be enough to confirm the diagnosis. However, interpretation of DNA result should always take into account the sensitivity of the molecular screening strategy. Failure to identify a causal mutation in a given gene can almost never exclude the diagnosis. Genetic testing should be referred to a specialised laboratory to ensure that relevant tests are performed and proper information is reported to clinicians, particularly those who are inexperienced with SAIDs.

Molecular techniques used in the analysis of SAIDs

In order to maximize the benefits of genetic testing, it is essential to target the test to the patient. This requires an interaction between the clinician, who must use all of the clinical information to make a differential diagnosis, and the laboratory performing the DNA analysis.

In SAIDs a variety of techniques have been used for DNA analysis including restriction enzyme digest, allele-specific PCR, but direct mutation screening by DNA sequencing is the method employed by most laboratories. In some instances, a two-step strategy involving an initial search for the most common pathogenic variants followed, if necessary, by an extended search of the complete coding sequence of the various genes has been employed by many laboratories. For example, patients who meet the clinical diagnosis for FMF, MKD and CAPS would, in the first instance, undergo analysis of exons 2 and 10 of the *MEFV* gene, 9 and 11 of the *MVK* gene and 3 of the *NLRP3* gene respectively.

Patients with SAIDs can display overlapping, partial or atypical symptoms, which makes extremely difficult to make a differential diagnosis if only clinical symptoms are being considered. The molecular analysis based solely on the candidate gene screening has low efficiency (close to 20%) and is time consuming and expensive. Moreover, since most of the causative mutations are located in specific regions of the genes called 'hot spots' only some exons or part of exons tend to be analysed {D'Osualdo, 2006 #5767}. For more than a decade Sanger sequencing has been considered the 'gold standard' method for detection of point mutations and small indel variants. This test has high sensitivity and specificity, but its limitations permit the detection of large structural changes and longer reads can deteriorate quality for base calling. Furthermore, since the discoveries of new SAIDs and their causative genes, genetic analysis needs to be performed in an efficient manner permitting

screening of multiple genes in a relatively short time. As the analytical validity of the NGS technologies has improved enormously in the last years and the plummeting cost of NGS sequencing reagent is now equivelent to or below that of Sanger sequencing, NGS has become the preferred method for molecular testing in SAIDs {Omoyinmi, 2017 #7081; Rusmini, 2016 #7080}. This is especially true for NGS targeted gene panels. Targeted sequencing uses massively parallel sequencing for screening of several genes of interest from multiple patients in a single run and thus far has proven to be fast and accurate in the discovery of disease causing mutations in many Mendelian disorders. Such targeted approaches have great analytical sensitivity for detection of heterozygous changes, however reliable interpretation of the multiple and *de novo* variants found through NGS requires experienced personnel and disease dedicated software. Since the implementation of targeted NGS panels for testing of SAIDs, remarkable progress has been made in the detection of disease-associated mutations and in improving genetic characterization of many previously undiagnosed patients, some of whom have been diagnosed with new diseases {Omoyinmi, 2017 #7081; Rusmini, 2016 #7080}. Furthermore, it has been shown by several authors that NGS methods have superior sensitivity over Sanger sequencing allowing for detection of somatic variants in SAIDs {Tanaka, 2011 #5750; Rowczenio, 2017 #6997}.

Somatic mosaicism is defined as the 'occurrence of two genetically distinct populations of cells' within an individual. It is caused by a mutation that occurs postzygotically and can either affect a subtype of cells {Frank, 2010 #6491; Forsberg, 2017 #6908}. The proportion and distribution of affected cells might vary depending upon when the mutational event had occured; if it arises at a very early stagees, the mutated cells will be widely distributed, potentially even including the

germ cells and thus will be transmissible to future offspring (this is known as gonosomal mosaicism). The mutation which is acquired later, is usually restricted to specific cell populations. Low frequency alleles have been found in a number of SAIDs, CAPS {Tanaka, 2011 #5750; Saito, 2008 #5711; Omoyinmi, 2014 #6496; Arostegui, 2010 #5777; Nakagawa, 2015 #6492; Zhou, 2015 #6897; Mensa-Vilaro, 2016 #6885}, SAVI syndrome {Liu, 2014 #6918}, Blau syndrome {de Inocencio, 2015 #6533} and TRAPS {Rowczenio, 2016 #6907}.To date several studies have identified somatic variants in the *NLRP3* gene in children with CINCA/NOMID and MWS {Tanaka, 2011 #5750; Saito, 2008 #5711; Omoyinmi, 2014 #6496; Arostegui, 2010 #5777}. Patients with CAPS typically present in infancy, thus it was particularly interesting to find *NLRP3* mosaicism in sporadic adult patients, who had typical, but late onset CAPS phenotype. Finding that acquired CAPS was caused by the mutational event, which most likely occurred late in life, changed the spectrum of patients referred for the *NLRP3* mutation screening. Indeed, in the past such cases may have been diagnosed as atypical acquired CAPS-like disorder known as Schnitzler syndrome. This rare, adult-onset disease was first described in 1972 by French dermatologist Liliane Schnitzler {Schnitzler, 1974 #5562} and just over 300 cases have been reported to date. The clinical phenotype of Schnitzler syndrome varies to some extent between patients, but the presence of a recurrent or chronic urticarial-looking rash and a monoclonal IgM or IgG paraprotein are the obligate Strasbourg criteria to make a diagnosis. Elevated levels of proinflammatory cytokines, particularly interleukin-1β, and the extracellular apoptosis-associated speck-like protein with caspase recruitment domain (ASC) aggregates during an active disease state suggests the disease is associated with upregulated inflammasome activation {de Koning, 2014 #6502}. Indeed, treatment with the IL-1

blocking therapies resuled in complete resolution of inflammatory symptoms in more than 80% of patients {de Koning, 2011 #5785; Rowczenio, 2017 #7023} and may prevent the development of the systemic amyloid A (AA) amyloidosis, which occurs as a consequence of chronic inflammation. Thus far, despite intensive investigations also including searching for germline and somatic variants using NGS panel consisting of 32 autoinflammatory genes, no genetic influence has been identified in Schnitzler syndrome {Rowczenio, 2017 #7023}. Several cases with transient or no detectable paraprotein have been reported as 'atypical Schnitzler syndrome' but the discovery of adult patients with the late-onset CAPS caused by somatic *NLRP3* mosaicism suggest that such diagnosis could be made in these patients {Rowczenio, 2017 #7023}.

Limitations and future applications of genetic testing in SAIDs

Most laboratories offering molecular testing for SAIDs are moving towards NGS targeted sequencing. This method of screening produces a much larger number of genetic variants when compared with Sanger sequencing and the genetic diagnosis in some cases has become more complex. Deciphering the information from NGS analysis is not trivial and many laboratories face analytical and interpretative challenges ranging from the validation of large numbers of genomic changes to managing the terabytes of data. Beside the identification of clearly pathogenic variants, some of the DNA results may be equivocal, for example finding low penetrance variants, variants of unknown significance, monoallelic variants in autosomal recessive diseases and DNA changes identified in more than one SAIDs gene. In such cases the classification of SAIDs might be challenging. Specific pipelines filtering out the pathogenic DNA changes from common

polymorphisms and variants of unknown significance need to be employed to help with the data analysis. Special precautions needs to be employed when analysing the DNA results as somatic mutations have been identified in several SAIDs, thus high sensitivity and sufficient read depth are necessary in order to detect these low frequency variants. Ideally, the interpretation of the genetic results in SAIDs should be performed by persons (clinical scientists or clinicians) who have an in-depth experience with SAIDs. The online disease databases including Infevers, Human Gene Mutation Database and ClinVar are very good resources for obtaining the genotype-phenotype correlation.

Whole Exome sequencing (WES) and the whole genome sequencing (WGS) should be recommended in patients in whom targeted gene panels failed to identify genetic cause. The former covers coding regions of approximately 20,000 genes which comprise about 2% of the human genome and is cheaper and less complicated in the bioinformatical/statistical analysis than the WGS, which examines all genes including their non-coding regions. The relevance of genetic changes found in the non-coding DNA has been reported in some the SAIDs (http://fmf.igh.cnrs.fr/ISSAID/infevers), but these are much harder to interpret than changes in the coding regions, which means that clinicians can be left with uncertainty about whether or not a genetic change found in the intron is the cause of the patient's symptoms.

In time, specialized, phenotype-driven targeted gene sequencing will give way to WES and WGS, which will transform clinical genetic testing, but reliable interpretation of the multiple and de novo variants found through NGS would still remain challenging and require experienced personnel who are confident in

validation of the DNA results before reporting them back to the clinicians or genetic councillors.

Challenges in genetic diagnosis of SAIDs

Despite the implementation of NGS into routine diagnostic practice of the SAIDs, a germline pathogenic mutation has not been identified in an estimated 50% to 60% of patients with suspected SAIDs {Savic, 2012 #7068}. This is particularly relevant to adult sporadic cases in whom the SAIDs are not typically suspected {Pathak, 2017 #7067}. Some of these cases may have somatic variants in the SAIDs genes. Such diagnosis remains a challenge as patients may present with variable clinical features. Furthermore, if the mutation was acquired late in life, it would be restricted to a specific cell populations, thus it can be missed if the mutated cell population is only a small fraction of the cells being tested. Detection of low frequency alleles continues to be technically highly demanding and expensive process.

Clinical information of certain variants can be conflicting of weather these are true disease-causing mutations or benign polymorphism, this include p.R121Q (common name R92Q) in the *TNFRSF1A* gene, p.V198M and p.Q703K in the *NLRP3* gene and p.E148Q in the *MEFV* gene {Ravet, 2006 #4228; Rowczenio, 2013 #6473; Verma, 2012 #5969}. The latter has been reported in classical FMF when coupled with various exon 10 mutations, but homozygosity for this variant alone is thought not to be associated with FMF in the vast majority of cases {Touitou, 2001 #5108}. Moreover p.E148Q prevalence in some populations is extremely high, for example 20% carrier rate has been estimated among Indians {Booth, 2001 #2669}.

Problems encountered during molecular diagnosis of the FMF, the most common SAID, are well characterised. In 20–25% of the patients with the clinical diagnosis of FMF and a positive response to colchicine therapy, the second variant in the *MEFV* gene has not been identified{Soriano, 2012 #6735}. Moreover, some *MEFV* variants have been reported to cause dominant FMF, namely p.M694del, p.T577N, p.T577S and p.T577A {Stoffels, 2014 #6439}. The exon 10 variant, p.M694del, was found to be associated with variable penetrance and tendency for symptoms to begin later in life than the classical FMF {Rowczenio, 2017 #7077; Booth, 2000 #2309} The phenotype associated with the exon 8 substitutions at position 577 was very broad, ranging from classical FMF to symptoms overlapping with other SAIDs including urticarial rash, arthritis, hepatosplenomegaly, conjunctivitis, severe anaemia with growth retardation and delayed psychomotor development. Interestingly, two other *MEFV* variants: p.S242R and p.E244K were discovered to cause dominantly inherited autoinflammatory disease Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis (PAAND) characterised by childhood-onset recurrent episodes of neutrophilic dermatosis, fever, elevated acute-phase reactants, arthralgia, and myalgia/myositis {Masters, 2016 #7065; Moghaddas, 2017 #7066}. This disease is distinct from FMF and resembles pyogenic arthritis, pyoderma gangrenosum and acne syndrome, which is caused by mutation in the PSTPIP1 gene {Wise, 2002 #4240}. The above mentioned mutations disrupt a binding motif of the regulatory 14-3-3 proteins within pyrin and cause spontaneous production of IL-1β and IL-18, associated with inflammatory cell death. The altered serum cytokine profile was found to be significantly greater in PAAND than in the wild-type *MEFV* and the p.M694V associated FMF, which may explain the different

clinical features exhibited by PAAND patients compared with those with FMF {Moghaddas, 2017 #7066}.

Conclusion

The spectrum of systemic autoinflammatory disorders continues to grow. During the last decade a remarkable progress has been made in the identification of disease-associated genes by using new molecular technologies, namely the NGS targeted gene panels and whole exome sequencing. This allowed for the genetic characterization in undiagnosed patients and in sporadic cases. The widespread application of NGS techniques resulted in novel gene discovery and in recognition of somatic mutations as a causes of several dominantly inherited SAIDs. With greater knowledge of the genotype-phenotype correlation of the most common SAIDs it became apparent that the clinical phenotypes and ethnic distribution is much more variable than previously anticipated. Despite the increasing use of genetic testing in SAIDs, the molecular diagnosis is still missing in a substantial proportion of patients, who have a clear periodic fever phenotype.

Clinical criteria in SAIDs guiding the genetic testing needs to be well defined as this will improve the efficiency of genetic diagnosis. Moreover, a larger number of DNA variants being discovered through NGS testing can make the genetic diagnosis more complex, thus detailed clinical information should be obtained during clinical assessment to help with the interpretation of genetic findings.