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Original article

Next generation sequencing panel in undifferentiated autoinflammatory diseases identifies patients with colchicine-responder recurrent fevers

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Abstract

Objectives. The number of innate immune system disorders classified as systemic autoinflammatory diseases (SAID) has increased in recent years. More than 70% of patients with clinical manifestations of SAID did not receive a molecular diagnosis, thus being classed as so-called undifferentiated or undefined SAID (uSAID). The aim of the present study was to evaluate a next-generation sequencing (NGS)-based clinically oriented protocol in patients with uSAID.

Methods. We designed a NGS panel that included 41 genes clustered in seven subpanels. Patients with uSAID were classified into different groups according to their clinical features and sequenced for the coding portions of the 41 genes.

Results. Fifty patients were enrolled in the study. Thirty-four patients (72%) displayed recurrent fevers not consistent with a PFAPA phenotype. Sixteen patients displayed a chronic inflammatory disease course. A total of 100 gene variants were found (mean 2 per patient; range 0–6), a quarter of which affected suspected genes. Mutations with a definitive diagnostic impact were detected in two patients. Patients with genetically negative recurrent fevers displayed a prevalent gastrointestinal, skin and articular involvement. Patients responded to steroids on demands (94%) and colchicine, with a response rate of 78%.

Conclusion. Even with a low molecular diagnostic rate, a NGS-based approach is able to provide a final diagnosis in a proportion of uSAID patients with evident cost-effectiveness. It also allows the identification of a subgroup of genetically negative patients with recurrent fever responding to steroid on demand and colchicine.

Key words: systemic autoinflammatory diseases, next generation sequencing, recurrent fevers, colchicine

CLINICAL SCIENCE

Rheumatology key messages

- More than 70% of patients with systemic autoinflammatory diseases did not obtain a molecular diagnosis.
- A novel 41-gene next generation sequencing panel showed a diagnostic rate of 4% in patients with undefined systemic autoinflammatory disease.
- A novel group of patients with systemic undifferentiated recurrent fevers responding to colchicine is described.

Introduction

Systemic autoinflammatory diseases (SAID) are a heterogeneous group of innate immune system disorders characterized by sterile inflammation without evidence of pathogenic autoantibodies or auto-reactive T lymphocytes [1, 2]. Originally, these disorders were limited to a handful of rare monogenic diseases (recurrent fevers), with the first autoinflammatory gene, *MEFV*, responsible for FMF, discovered in 1997 [3, 4]. Since then, substantial progress has been made, with the identification of at least 30 autoinflammatory genes accounting for conditions showing

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overlapping features (http://fmf.igh.cnrs.fr/ISSAID/Classification_AID/page1.html) [5–12]. Depending on the mode of inheritance and on either *de novo* or inherited occurrence of the mutation(s), the identification of one or more variants with a known pathogenic impact and high penetrance represents an essential final step for the diagnosis of monogenic SAID. However, in a considerable proportion of patients (70–80%), molecular analysis is unable to provide a diagnostic confirmation. In daily practice a relevant percentage of patients with clinical manifestations clearly consistent with SAID are classified as affected by undifferentiated or undefined SAID (uSAID) [13–16]. Little information is available on the clinical presentation, outcome and response to treatment in this heterogeneous group. On the other hand, next-generation sequencing (NGS) technologies are revolutionary diagnostic tools for genetic conditions, allowing the simultaneous analysis of different genes associated with a given group of inherited disorders [17]. Massive sequencing, therefore, represents a powerful approach to enable a definitive diagnosis in patients with uSAID, as preliminarily shown by our group with a 10-gene panel [18–23].

This study aimed to test a novel 41-gene NGS diagnostic panel tool and to explore its possible utility in improving the diagnostic yield in a population of uSAID followed in a tertiary centre for SAID.

Methods

NGS panel design and patients recruitment

An NGS diagnostic panel with 41 genes related to SAID was developed, including genes reported in the Infevers database in 2015, the 15 genes known at that time to be responsible for interferonopathies and the SERPING1 geneand the SERPING1 gene. Ion AmpliSe (Thermo Fisher Scientific, Waltham, MA, USA) designer software was used to design the panel, composed of the gene coding portions plus 20 bp flanking each exon, for a total of 139.35 kb, covered with 501 amplimers (two primer pools). Preparation of libraries was carried out according to the protocols of Thermo Fisher Scientific. According to the main clinical manifestations, genes were clustered in seven subpanels: A, recurrent fevers; B, urticarial rash; C, skin, bone and articular involvement; D, intestinal involvement; E, panniculitis, vasculopathy (type I interferonopathies); F, Aicardi–Goutières syndrome; G, others (Supplementary Table S1, available at *Rheumatology* online). The inclusion criteria for the study were patients with symptoms related to SAID with a pediatric onset; exclusion of other common aetiologies, such as neoplasms, infections, autoimmune diseases and immunodeficiency; negative or not conclusive molecular diagnosis based on Sanger sequencing of suspected genes; and for patients with recurrent fevers, exclusion of periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA) syndrome by means of the absence or low frequency (<30% of the fever episodes) of the classical clinical symptom triad of pharyngitis, aphthous stomatitis and cervical adenitis [24]. Ethical approval was obtained from the G. Gaslini Institute Ethical Board and consent was obtained from all the participants. All clinical, laboratory and radiological data were available in the private software of the Pediatric Rheumatology Clinic and the Medical Genetics Unit of our Institute.

Sequencing and variant validation

FastQ data were generated by Ion PGM™ (Thermo Fisher) semiconductor and analysed by Ion Reporter™5.0. Coverage analysis was performed by the Ion Coverage Analysis plug-in (Thermo Fisher) v5.2.1.2. Coding regions that were not included in the experimental design and amplimers covered <10× in the requested genes were analysed by Sanger sequencing. All missense, frameshift and splice-site variants were confirmed by the Sanger method if reported with a frequency lower than 3% in the general population, according to the ExAC or 1000 Genomes databases [25, 26].

Genotype–phenotype correlation

For patients without a confirmatory genotype, each variant was considered consistent with the clinical phenotype or not according to the data regarding associated symptoms in the literature (Supplementary Table S2, available at *Rheumatology* online). Furthermore, each variant was considered possibly pathogenic (PPV) if reported as damaging by three or more software tools between the five available at http://varsome.com, or if it showed a CLINVAR score higher than 3 [27–33]. PPVs were labelled as either likely pathogenic, variants of unknown significance or likely benign according to different criteria (Supplementary Material, section Methods, available at *Rheumatology* online). Family segregation analysis was performed when parental samples were available. Four groups of patients were identified: patients with a confirmatory genotype (group 1), patients with PPV consistent with clinical phenotype (group 2), patients with PPV not consistent with clinical phenotype (group 3), and patients with no new pathogenic, benign variants or no variants (group 4).

Statistical analysis

Categorical data were expressed as number and percentage, and continuous variables as median and range. Comparison of disease characteristics between patients groups were performed by χ^2 test or *t* test as appropriate and where significant interaction was determined a *post hoc* analysis was performed to determine whether significant differences existed between different populations and adjusted for multiple comparisons. A *P*-value <0.05 was considered statistically significant. R statistics version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses.

Results

Demography and clinical features

Fifty patients were enrolled at the median age of 11 years (Table 1). All were Caucasian with a median disease duration of 7 years, with at least 2 years of follow-up in our centre. All the patients had previously been screened for

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Table 1 Overview of our cohort

Characteristics	Study cohort ($n = 50$)
Demography	
Male, *n* (%)	27 (54)
Adults, *n* (%)	7 (14)
Age at enrolment, median (range), years	11 (5–38)
Age at onset, median (range), years	4 (0–21)
Disease duration, median (range), years	7 (2–23)
Tested genes per patient, median (range)	3 (1–7)
Clinical manifestations, *n* (%)	
Periodic fever	36 (72)
Chronic urticaria	2 (4)
Prevalent skin/bone/joints involvement	6 (12)
Prevalent intestinal involvement	1 (2)
Suspected type 1 interferonopathies	5 (10)
Episodes of macrophage activation syndrome	3 (6)
NGS results	
Variants per patient (median, range)	2 (0–6)
Variants in suspected subsets, *n* (%)	29 (23)
New diagnosis, *n* (%)	2 (4)
Recurrent variants[a], *n* (%)	38 (30)

[a]See Supplementary Table S5, available at *Rheumatology* online. NGS: next-generation sequencing.

at least one SAID-related gene, with an average of three genes for each patient (Supplementary Table S3, available at *Rheumatology* online). The majority (36 patients; 72%) presented recurrent fevers but 14 displayed a prevalent chronic inflammatory disease course (Supplementary Table S4, available at *Rheumatology* online). Two of them (nos 5 and 22 in Table 2) had urticarial rashes as major clinical feature. Both were negative for mutations or mosaicisms of the *NLRP3* or *NLRP12* genes. Six patients (nos 1, 8, 9, 19, 25 and 48 in Table 2) showed a peculiar skin, bone or joints inflammatory involvement, presenting cutaneous abscesses, periostitis or osteitis. Except for two patients, all achieved a complete or partial response with steroids and one required hormonal therapy for steroid-caused puberty delay. Genes previously studied in these patients were *NOD2*, *PSTPIP1* and *ILRN1*. Only one patient (no. 43 in Table 2) presented a prominent intestinal involvement and five (nos 15, 18, 24, 31 and 50 in Table 2) displayed clinical features suggestive of a possible interferonopathy, as panniculitis, discoid lupus-like lesions, polyarthritis or myositis. All these patients were steroid-dependent, but colchicine, methotrexate and rapamycin were not effective in this subgroup. No patient was suspected for disease related to genes included in the F or G subpanels. Three patients (nos 27, 44, 50 in Table 2) displayed at least one episode of macrophage activation syndrome and were also screened for hereditary forms of haemophagocytic lymphohistiocytosis.

NGS results

A mean coverage of 318.7× was achieved in the 50 DNA samples, with only four amplicons represented at <10× (Fig. 1). A mean of 173.17 variants for each patient was detected, and 100 variants were finally considered (Fig. 2). All the validated variants were heterozygous (except for four homozygous variants of patient nos 1, 11, 19 and 38 in Table 2) and missense (except for the c.2807+1G>A substitution of the *IFIH1* gene of patient no. 5 in Table 2). Fifteen variants were detected in our dataset with an allele frequency higher than in public databases (Supplementary Table S5, available at *Rheumatology* online).

Genotype–phenotype correlation

Patients with a confirmatory genotype

Biallelic mutations of *MVK* gene with a clear pathogenic relevance were detected in two patients, who received a final diagnosis of mevalonate kinase deficiency (MKD). The first patient (no. 1 in Table 2) displayed an atypical presentation with a chronic and persistent inflammatory disease course dominated by a severe bone involvement (Supplementary Fig. S1, available at *Rheumatology* online). The urinary secretion of mevalonic acid confirmed the diagnosis. Anakinra was started with a prompt amelioration of the inflammatory manifestations. The other patient (no. 2 in Table 2) was previously analysed for *MVK* exons 2, 8, 9, 10 and 11 with standard Sanger sequencing, allowing the identification of a V377I variant only. At the time of examination the patient was adult and presented a sub-chronic disease course with persistent inflammation, arthralgia, sporadic abdominal pain and gastrointestinal complaints, beside displaying recurrent fevers during childhood. The NGS analysis detected the second *MVK* variant. Canakinumab was started with complete response.

Patients with PPV consistent with clinical phenotype

Two patients (nos 3 and 4 in Table 2) carried novel missense variants of the *PLCG2* gene. Patient no. 4 presented episodes of urticarial rash and systemic inflammation, while patient no. 5 had neither systemic inflammation nor immune abnormalities, as expected in PLAID [34]. Functional tests are ongoing and will help to determine the actual pathogenic relevance of the variants found in these patients.

Patients with PPV not consistent with clinical phenotype

Sixteen patients (30%) carried PPV specific for unrelated autosomal dominant diseases and nine displayed at least one PPV responsible for autosomal recessive disorders, being therefore asymptomatic carriers. Indeed, eight patients without skin involvement (nos 5–12 in Table 2) showed PPV associated with bone disorder, psoriasis or chronic urticarial rash, and two (nos 13 and 14 in Table 2) displayed *NOD2* variants but no inflammatory bowel manifestations. Six patients (nos 15–20 in Table 2) presented variants associated with type 1 interferonopathies. Four patients (nos 22–25 in Table 2) presented skin/joint inflammatory involvement and pathogenic variants. In particular, patients 22 and 23 presented *IL36RN* mutations and complete/partial response to anti-IL1 treatment, and

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Table 2 Genotype-phenotype correlation in 50 patients with uSAID

No.	Required subpanel	Clinical features and response to treatments	Gene	Mutation	CLINVAR	ExAC	Eur 1000 Genomes	Mutation Taster	Fathmm-MKL	Meta-SVM	PROVEAN	SIFT	CADD Phred	Final results
Group 1. Patients with a confirmatory genotype														
1	C	Poliarthritis, periostitis, generalized lymphadenopathy, hepatosplenomegaly, dysmorphisms (epicanthus, frontal bossing, saddle nose), small cerebral hemisphere with pachygyria. Complete response to anakinra	***MVK***	**Gly326Arg HOMO**	1	—	—	DC	D	D	D	T	4.40	MKD
2	A	Recurrent fever episodes since childhood with generalized lymphadenopathy, abdominal pain and erythematous rash. Complete response to steroids on-demand	***MVK***	**Val377Ile**	5	1.60E-03	—	DC a	D	D	N	T	20.3	MKD
			MVK	**Ala147Thr**	—	4.06E-6	—	DC	D	D	D	D	29.1	
Group 2. Patients with possibly pathogenic variant consistent with the clinical phenotype														
3	A	Recurrent fever episodes, every 8–10 days, with erythema nodosum and aphthosis. Lobar granulomatous panniculitis with interstitial and perivascular infiltrate of lymphocytes and histiocytes at skin biopsy. Complete response to steroids. No response to rapamycin, azathioprine, anakinra and thalidomide. Partial response to anti-TNF treatment	***PLCG2***	**Asn571Ser (LB)**	—	6.70E-03	1.39E-02	DC	N	T	N	D	22.9	Suspected APLAID[a]
			SH3BP2	Arg609Gly	—	1.966E-05	—	DC	N	T	N	T	14.22	
4	B	Recurrent episodes of urticarial rash, aphthosis, exudative pharyngitis, cervical lymphadenopathy, abdominal pain and arthromyalgia. Partial response to on-demand steroids. Infiltration of mast cells at skin biopsy. Partial response to anti-histaminic drugs and steroids. A sister with recurrent episodes of urticarial rash after cold exposure and recurrent upper airways infections with sinusitis	*PLCG2*	Ala1130Ser	—	8.12E-06	—	DC	D	T	N	D	24.5	Suspected PLAID
			PLCG2	**Pro1127 Arg (VUS)**	—	1.63E-05	—	DC	D	D	D	D	29.7	
Group 3. Patients with possibly pathogenic variant not consistent with the clinical phenotype in AD disease														
5	A	Recurrent febrile episodes of 7 days, every month, with sporadic exudative pharyngitis, arthritis, and headache. Complete response to steroids on-demand	*CARD14*	Ser200Asn	1	6.38E-03	2.00E-03	P	N	T	N	T	0.004	Undefined[a]
			TNFRSF11A	**Lys240Glu (VUS)**	0	1.07E-03	1.00E-03	DC	D	T	—	—	28.4	
			IFIH1	Glu627[a]	—	3.20E-03	8.90E-03	DC	D	—	—	—	26.8	

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Table 2 Continued

No.	Required subpanel	Clinical features and response to treatments	Gene	Mutation	CLINVAR	ExAC	Eur 1000 Genomes	Mutation Taster	Fathmm-MKL	Meta-SVM	PROVEAN	SIFT	CADD Phred	Final results
6	A	Recurrent fever episodes of 7–10 days, with complete response to colchicine. One episode of polyarthritis at the right foot	***AP1S3***	**Phe4Cys (LB)**	255	7.66E-03	9.90E-03	DC	D	D	D	D	27.2	Undefined[a]
7	A	Few self-limited fever episodes of 21 days, with severe malaise, erythematous rash, exudative pharyngitis, arthro-myalgia and abdominal pain. Recurrent aphthosis. Partial response to maintenance therapy with colchicine. No response to tonsillectomy. Twin sister with chronic entero-colitis and recurrent aphthosis, father with recurrent aphthosis and psoriasis	*IL10RA*	Val113Ile (LB)	0	6.75E-03	6.00E-03	P	N	T	N	T	0.006	Undefined[a]
			NLRP12	**Asp84Asn (VUS)**	—	8.16E-06	—	DC	D	T	D	D	28.1	
			RBCK1	Asn122His (LB)	—	—	—	P	N	T	N	T	10.66	
8	C	Non-suppurate nodular panniculitis with fever and arthralgia. Partial response to cyclosporine and anakinra. Complete response to steroids and anti-TNF	*CARD14*	Glu422Lys (LB)	—	2.30E-02	2.88E-02	P	N	T	N	T	8.682	Undefined
			CARD14	**Arg682Trp (VUS)**	1	1.10E-02	1.79E-02	DC	D	T	D	D	35	
			NLRC4	Gly786Arg (VUS)	—	—	—	—	N	T	N	T	23.2	
			NLRP3	Val200Met (LB)	5-3	8.49E-03	1.19E-02	DCa	N	T	N	T	0.002	
			NLRP7	Lys511Arg (LB)	2	1.31E-02	1.09E-02	P	N	T	N	T	0.001	
			NOD2	Arg684Trp (VUS)	—	4.04E-04	2.00E-04	P	D	T	N	T	23.2	
9	C	Chronic bilateral osteomyelitis at the lower limbs with cutaneous abscesses treated with long-term antibiotic treatment with complete response	***CARD14***	**Arg69Trp (VUS)**	—	1.49E-04	—	DC	D	T	D	D	24.3	Undefined
10	A	Recurrent fever episodes of 5–6 days, every 2–4 weeks, with exudative pharyngitis, aphthosis, hepatomegaly and arthritis. Complete response to steroids on demand	***CARD14***	**Arg610His (VUS)**	—	1.48E-04	—	DC	N	T	D	D	25.8	Undefined[a]
			MEFV	Glu148Gln (VUS)	2-3-5	7.08E-02	8.90E-03	Pa	N	T	N	D	23.3	
11	A	Recurrent fever episodes of 10 days, every 2–4 weeks, with abdominal pain, vomiting, diarrhoea, hepatosplenomegaly, periorbital oedema and urticarial rash. Complete response to steroids. Partial response to colchicine. Complete response to anakinra as maintenance therapy. Father with granulomatous hepatitis	*ADAR1*	Ser281Arg (LB)	—	—	—	P	N	T	N	T	0.015	Undefined[a]
			CARD14	Glu422LysHOMO (LB)	—	2.30E-02	2.88E-02	P	N	T	N	T	8.682	
			MEFV	Glu148Gln (VUS)	2-3-5	7.08E-02	8.90E-03	Pa	N	T	N	D	23.3	
			CARD14	**Arg682Trp (LB)**	1	1.10E-02	1.79E-02	DC	D	T	D	D	35	
12	A	Recurrent fever episodes of 5–6 days, every 10–15 days, with erythematous rash, cervical lymphadenopathy, exudative pharyngitis, arthralgia, abdominal pain and diarrhoea.	***PLCG2***	**Lys775Arg (VUS)**	—	1.37E-03	2.00E-03	DC	D	T	N	T	22.5	Undefined[a]

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Table 2 Continued

No.	Required subpanel	Clinical features and response to treatments	Gene	Mutation	CLINVAR	ExAC	Eur 1000 Genomes	Mutation Taster	Fathmm-MKL	Meta-SVM	PROVEAN	SIFT	CADD Phred	Final results
		Partial response to colchicine. Mild inflammation at gastroscopy and colonoscopy. Inflammatory bowel disease was ruled out during infancy												
13	A	Recurrent fever episodes with abdominal pain, headache and arthralgia. Sometimes arthritis. Partial response to colchicine	*ADAR1* *CARD14* ***NOD2***	Ser281Arg (LB) Pro506Leu (LB) **Gly908Arg (VUS)**	– – 255-0-3	– 1.38E-02 1.13E-02	– 1.39E-02 9.90E-03	P P DC	N N D	T T T	N N D	T T D	0.009 5.070 31	Undefined[a]
14	A	Few fever episodes with severe malaise, exudative pharyngitis, erythematous rash, arthromyalgia, generalized lymphadenopathy and hepatosplenomegaly. Laboratory exams showed hyperferritinemia and hypertransaminasemia. Complete response to high-dose steroids	***NOD2***	**Pro427Leu (VUS)**	–	1.10E-04	–	DC	D	D	D	D	25.7	Undefined[a]
15	E	Lipophagic panniculitis at the lower limbs with fever, arthritis and alopecia. Negative interferon signature. Complete response to steroids and methotrexate	*SH3BP2* *NLRP7* ***SAMHD1***	Arg534Trp (VUS) Cys399Tyr (VUS) **Met362Ile (VUS)**	3 0 –	4.62E-03 4.64E-04 –	4.20E-03 – –	P P DC	N N D	T T D	N D D	T D T	32 22.3 23.8	Undefined
16	A	Recurrent fever episodes of 4–5 days, every 2–3 weeks, with exudative pharyngitis, aphthosis, generalized lymphadenopathy, abdominal pain and urticarial rash. No response to colchicine and anakinra. Positive family history for rheumatoid arthritis	***TMEM173***	**Pro317Leu (VUS)**	–	4.07E-06	–	DC	D	T	N	D	22.4	Undefined[a]
17	A	Recurrent fever episodes of 5–6 days, every month, with malaise, abdominal pain, exudative pharyngitis and cervical lymphadenopathy. Complete response to steroids on demand. Brother with similar symptoms	***IFIH1***	**Lys349Arg (LB)**	–	3.22E-03	3.20E-03	DC	D	T	N	D	15.54	Undefined[a]
18	E	Recurrent fever episodes of 3–4 days, every 5–10 days, with maculo-papular rash, headache, abdominal pain and arthralgia. Sometimes aphthosis and diarrhoea. Normal colonoscopy. No response to colchicine as maintenance therapy. Positive family history for recurrent fever syndromes, multiple sclerosis and systemic lupus erythematosus	*IFIH1* ***SH3BP2***	Thr100Arg (LB) **Val438Met (VUS)**	– 0	2.03E-05 2.54E-04	– –	P DC	N D	T D	N N	D D	14.74 23.8	Undefined

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Table 2 Continued

No.	Required subpanel	Clinical features and response to treatments	Gene	Mutation	CLINVAR	ExAC	Eur 1000 Genomes	Mutation Taster	Fathmm-MKL	Meta-SVM	PROVEAN	SIFT	CADD Phred	Final results
19	C	Discoid skin lesions and polyarthritis. C3 deposit at dermal–epidermal junction. Partial response to steroid	***SH3BP2***	**Ala212Val (VUS)**	2-3	5.62E-03	4.0E-03	P, DC	D	T	N	D	22.6	Undefined
			RNASEH2A	Asp205Glu	2-3	1.09E-02	8.40E-03	DC	D	T	N	T	14.56	
20	A	Recurrent fever episodes of 6 days, every month, with urticarial rash, myalgia and exudative pharyngitis. Low immunoglobulin A and autoimmune neutropenia. Complete response to steroids on demand	***ADAR1***	**Pro193AlaHOMO (LP)**	5-3	2.14E-03	4.00E-03	DC	D	D	D	D	—	Undefined[a]
			NLRP12	Asp979His (LP)	—	2.03E-05	—	P, DC	D	T	D	T	23.6	
			IL10RB	Glu25Lys (VUS)	0	7.07E-04	—	P	N	T	N	T	21.3	
Group 3. Patients with possibly pathogenic variant not consistent with the clinical phenotype in AR diseases														
21	A	Recurrent fever episodes with abdominal pain, periorbital oedema, exudative pharyngitis, cervical lymphadenopathy, headache and arthromyalgia. Sometimes aphthosis. No response to maintenance therapy with colchicine, anakinra, mesalazine or rapamicine	***SLC29A3***	**Ser203Pro (LP)**	5	1.25E-05	—	DC	D	D	D	D	25.6	Undefined[a]
			TNFRSF1A	Arg121Gln (VUS)	5	1.32E-02	6.00E-03	P	N	D	D	D	15.30	
			IFIH1	Hys460Arg (LB)	—	9.53E-02	1.19E-02	P a	N	T	N	T	9.6	
22	B	Recurrent/intermittent episodes of diffuse urticarial rash, bilateral conjunctivitis, erythema nodosum and arthralgia/arthritis. Laboratory exams during episodes showed low C4 and monoclonal light chain gammopathy. Complete response to steroids on demand and canakinumab. No response to colchicine	*DNASE1L3*	Met1Leu (LP)	5	1.58E-04	1.60E-03	DC	D	-—	—	—	22	Undefined
			IL36RN	**Ser113Leu (LP)**	—	2.90E-03	1.60E-03	DC	D	T	N	D	27.8	
23	A	Recurrent fever episodes of 7–10 days, every 2 weeks, with aphthosis, generalized lymphadenopathy and malaise. Sometimes arthritis. No response to colchicine. Complete response to steroids on demand and anakinra as maintenance therapy	*CARD14*	Glu422Lys (LB)	—	2.30E-02	2.88E-02	P	N	T	N	T	8.682	Undefined[a]
			DNASE1	Val185Ile (VUS)	—	3.58E-03	4.00E-03	DC	N	T	N	T	0.086	
			IL36RN	**Ser113Leu (LP)**	5	2.29E-03	1.60E-03	DC	D	—	—	D	27.8	
24	E	Recurrent fever episodes of 15–20 days, with abdominal pain, painful urticarial-like rash or panniculitis, polyarthritis or arthromyalgia at the lower limbs. Complete response to steroids, partial response to colchicine	*PSMB8*	Gly8Arg (VUS)	3	1.94E-02	2.88E-02	DC	N	T	N	D	—	Undefined
			NLRC4	Ala929Ser (LB)	—	7.07E-03	1.29E-02	N	N	T	N	T	0.381	
			RNASEH2B	**Ala177Thr (VUS)**	5	1.31E-03	—	DC a	D	D	N	T	19.86	
25	C		*TNFRSF1A*	Pro75Leu (VUS)	2	5.50E-03	—	P	N	T	D	D	22.2	Undefined

(continued)

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Table 2 Continued

No.	Required subpanel	Clinical features and response to treatments	Gene	Mutation	CLINVAR	ExAC	Eur 1000 Genomes	Mutation Taster	Fathmm-MKL	Meta-SVM	PROVEAN	SIFT	CADD Phred	Final results
		Recurrent erythema nodosum, sometimes with fever, arthritis, uveitis and generalized lymphadenopathy. Complete response to steroids. Lipoblastoma arborescens of the knee	***PSMB8***	**Thr74Ser (LP)**	—	4.49E-03	2.00E-03	DC	D	T	D	D	31	
26	A	Recurrent fever with skin rash, arthromyalgia, myositis and hepatosplenomegaly. No response to cyclosporine. Complete response to steroids and anakinra as maintenance therapy. Positive family history for autoimmune trombocitopenia	*CARD14*	Pro506Leu (LB)	—	1.38E-02	1.39E-02	P	N	T	N	T	5.070	Undefined[a]
			NLRP3	Val200Met (LB)	5-3	8.49E-03	1.19E-02	DC a	N	T	N	T	0.002	
			LPIN2	**Cys874Phe (VUS)**	0	1.37E-03	9.20E-03	DC	D	T	D	T	21.9	
27	A	Some episodes of MAS during neonatal period with complete response to steroids. Recurrent infections and self-limited fever episodes of urticarial rash, panniculitis, splenomegaly, generalized lymphadenopathy and malaise. High immunoglobulin A and low platelet count. Mild dismorphisms and growth delay. Consanguineous parents	*IL10RB*	Val148Met (VUS)	—	1.14E-03	—	P	N	T	N	D	15.82	Undefined[a]
			CECR1	Met309Ile (VUS)	—	1.74E-03	2.00E-03	P	D	T	N	T	2.643	
			LPIN2	**Glu601Lys (VUS)**	2	8.88E-03	9.90E-03	DC	D	T	N	T	22	
28	A	Episodes of polyarthritis and pericarditis, sometimes with fever and urticarial rash, treated with steroids with complete response. No response to methotrexate. Complete response to colchicine as maintenance therapy	*C1NH*	Thr48Ala (VUS)	—	4.87E-04	—	P	N	T	D	T	0.001	Undefined[a]
			LPIN2	**Glu601Lys (VUS)**	2	8.88E-03	9.90E-03	DC	D	T	N	T	22	
29	A	Recurrent fever episodes of 8–9 days, every 20–25 days, with headache, exudative pharyngitis and aphthosis. Complete response to steroids on demand. Now recurrent episodes of arthritis and livedo reticularis at the lower limbs. Partial response to colchicine as maintenance therapy	*NLRC4*	Gly786Arg (VUS)	—	—	—	—	N	T	N	T	23.2	Undefined[a]
			LPIN2	**Glu601Lys (VUS)**	2	8.88E-03	9.90E-03	DC	D	T	N	T	22	
			CARD14	Glu422Lys (LB)	—	2.30E-02	2.88E-02	P	N	T	N	T	8.682	
Group 4. Patients with not new pathogenic variants or benign variants or no variants														
30	A	Recurrent fever episodes of 3–4 day, every week. Complete response to colchicine as maintenance therapy	*CARD14*	Glu422Lys	—	2.30E-02	2.88E-02	P	N	T	N	T	8.682	Heterozygous FMF
			MEFV	**Val726Ala**[b]	5	2.17E-03	1.00E-03	DC a	N	T	N	T	0.001	
			PLCG2	His641Gly	—	—	—	—	—	—	—	—	28.5	
			RNASEH2A	Asp205Glu	3	1.09E-02	1.09E-02	DC	D	T	N	T	14.56	

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Table 2 Continued

No.	Required subpanel	Clinical features and response to treatments	Gene	Mutation	CLINVAR	ExAC	Eur 1000 Genomes	Mutation Taster	Fathmm-MKL	Meta-SVM	PROVEAN	SIFT	CADD Phred	Final results
31	E	Episodes of recurrent fever with polyarthritis and erythematous rash. Complete response to steroids	***MEFV***	**Val726Ala**[b]	5	2.17E-03	1.00E-03	DC a	N	T	N	T	0.001	Undefined
			NOD2	Ile836Thr	—	4.06E-05	—	P	N	T	N	T	1230	
32	A	Recurrent autoimmune haemolytic anaemia during early childhood treated with steroids. Recurrent febrile episodes, lasting 15–20 days, with abdominal pain, scrotal pain, diarrhoea, aphthosis, exudative pharyngitis and arthralgia. Pericarditis during one episode. Complete response to steroids on demand and colchicine as maintenance therapy	*CARD14*	Glu422Lys	—	2.30E-02	2.88E-02	P	N	T	N	T	8.682	Undefined[a]
33	A	Recurrent fever episodes of 1–2 days, every month, with abdominal pain, nausea, vomiting, headache, aphthosis and arthralgia at the lower limbs. Complete response to colchicine as maintenance therapy	*CECR1*	Met267Ile	—	1.74E-03	2.00E-03	P	D	T	N	T	2.643	Undefined[a]
34	A	Recurrent fever episodes, lasting 3–4 days, every 2 weeks, with abdominal pain and malaise. Complete response to on-demand steroids. Partial response to colchicine as maintenance therapy	*LPIN2*	Ser579Pro	0	2.49E-03	8.00E-04	P	N	T	N	T	16.44	Undefined[a]
			MEFV	Pro369Ser	255	1.42E-02	4.00E-03	P	N	T	D	D	15.64	
			MEFV	Arg408Gly	255	—	4.00E-03	P	N	T	N	T	7871	
			SH3BP2	Arg534Trp	3	4.62E-03	4.20E-03	P	N	T	N	T	32	
35	A	Low fever episodes with headache and arthromyalgia lasting 24–48 h every 1–2 months. Gilbert syndrome. Partial response to on-demand NSAIDs	*PSMB8*	Gly8Arg	3	1.94E-02	2.88E-02	DC	N	T	N	D	—	Undefined[a]
			PSTPIP1	Arg405Cys	0-3	5.44E-04	1.00E-03	DC	N	T	N	T	23.8	
			CARD14	Glu422Lys	—	2.30E-02	2.88E-02	P	N	T	N	T	8.682	
			NLRP7	Met192Leu	—	8.24E-06	8.00E-04	P	N	T	D	D	4.578	
36	A	Recurrent fever episodes of 3 days, every 3 weeks, with aphthosis, maculo-papular rash and arthralgia. Partial response to colchicine	*CARD14*	Glu422Lys	—	2.30E-02	2.88E-02	P	N	T	N	T	8.682	Undefined[a]
			NOD2	Ala725Gly	2	3.13E-03	—	P	D	T	N	T	3.629	
37	A	Recurrent fever episodes, every 15–20 days, with exudative pharyngitis, cervical lymphadenopathy, abdominal pain and arthralgia. Partial response to colchicine	*TREX1*	Glu321Gly	0-3	1.69E-03	—	P	N	T	N	T	0.046	Undefined[a]
			IFIH1	His460Arg	—	9.53E-02	1.19E-02	P a	N	T	N	T	9.6	
			PSTPIP1	Gly258Arg	2-3	—	4.00E-03	P	D	T	N	T	3.64	
38	A	Recurrent fever episodes of 7–10 days with oral aphthosis. No response to colchicine or anakinra as maintenance therapy. Partial response to mofetil mycophenolate	*CARD14*	Pro506Leu	—	1.38E-02	1.39E-02	P	N	T	N	T	5.070	Undefined[a]
			MEFV	Ala744Ser	4-5	1.84E-03	5.00E-03	P, DC	N	T	N	T	0.003	
			NLRP7	Lys511Arg HOMO	2	1.31E-02	1.09E-02	P	N	T	N	T	0.001	
			PSTPIP1	Ser323Leu	—	1.94E-05	—	P	N	T	N	T	17.62	

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Table 2 Continued

No.	Required subpanel	Clinical features and response to treatments	Gene	Mutation	CLINVAR	ExAC	Eur 1000 Genomes	Mutation Taster	Fathmm-MKL	Meta-SVM	PROVEAN	SIFT	CADD Phred	Final results
39	A	Recurrent fever episodes of 3–4 days, every 1–2 months, with severe malaise, abdominal pain, cervical lymphadenopathy and arthromyalgia. Sometimes aphthosis and genital ulcers. No response to steroids on demand. Partial response to colchicine	*DNASE1*	Glu35Asp	—	4.06E-06	6.00E-03	P, DC	N	T	N	T	8.484	Undefined[a]
40	A	Recurrent fever with abdominal pain, nausea and vomiting. Complete response to steroids and anakinra. Partial response to indometacine	*IL10RA* *IL10RB*	Val233Met Val148Met	— —	1.78E-03 1.14E-03	2.00E-03 —	P a P a	N N	T T	N N	D D	19.48 15.82	Undefined[a]
41	A	Recurrent fever episodes of 1–2 days, every 2–4 weeks, with urticarial rash and arthritis. Partial response to on-demand steroids. Positive family history for Hashimoto's thyroiditis	*SH3BP2*	Arg534Trp	—	4.62E-03	4.20E-03	P	N	T	N	T	32	Undefined[a]
42	A	Recurrent fever episodes of 3–5 days, with abdominal pain, aphthosis and arthralgia. Partial response to ibuprofen. Hyperintense bone lesions at total-body magnetic resonance					None							Undefined[a]
43	D	Chronic liver disease with neutrophilic infiltration and Budd–Chiari syndrome, ulcerative cutaneous lesions and pyodermagangrenosum. History of recurrent pneumonia and myeloid dysplasia					None							Undefined
44	A	Recurrent fever episodes with diffuse erythematous rash, generalized lymphadenopathy and arthritis. Episodes of MAS. Pulmonary alveolar proteinosis. Partial response to steroids and canakinumab as maintenance therapy					None							Undefined[a]
45	A	Recurrent fever episodes with cervical lymphadenopathy, abdominal pain and headache. Eosinophilia. Personal history of dental abscesses. Partial response to maintenance therapy with colchicine. Positive family history for recurrent fevers (father and cousin)					None							Undefined[a]

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Table 2 Continued

No.	Required subpanel	Clinical features and response to treatments	Gene	Mutation	CLINVAR	ExAC	Eur 1000 Genomes	Mutation Taster	Fathmm-MKL	Meta-SVM	PROVEAN	SIFT	CADD Phred	Final results
46	A	Recurrent fever episodes of 3 days, every 3 weeks, with abdominal pain and erythematous rash. Complete response to colchicine					None							Undefined[a]
47	A	Recurrent fever episodes of 3–4 days, every 2 weeks, with abdominal pain, exudative pharyngitis, generalized lymphadenopathy and myalgia. Complete response to colchicine					None							Undefined[a]
48	C	Chronic non-bacterial osteomyelitis-like bone lesions at clavicle, humerus and femur with severe acne at trunk and face					None							Undefined
49	A	Recurrent fever episodes of 4 days, every 20–30 days, with abdominal pain, headache and arthromyalgia. Complete response to steroids on demand					None							Undefined[a]
50	E	Recurrent fever episodes of 10 days. An episode of acute demyelinating encephalopathy and MAS with complete response to steroids. Partial response to rapamycin as maintenance therapy					None							Undefined

For each patient, the most relevant variant is marked in bold. CLINVAR code: 0, uncertain significance; 1, not provided; 2, benign; 3, likely benign; 4, likely pathogenic; 5, pathogenic; 255, other. [a]Patients with undifferentiated recurrent fever episodes. [b]Variants already known. a, automatic; AD: autosomal dominant; D, damaging; DC, disease causing; LB: likely benign; LP: likely pathogenic; MAS: macrophage activation syndrome; MKD: mevalonate kinase deficiency; N, neutral; P, polymorphism; T, tolerated; uSAID: undifferentiated or undefined systemic autoinflammatory diseases; VUS: variant of unknown significance.

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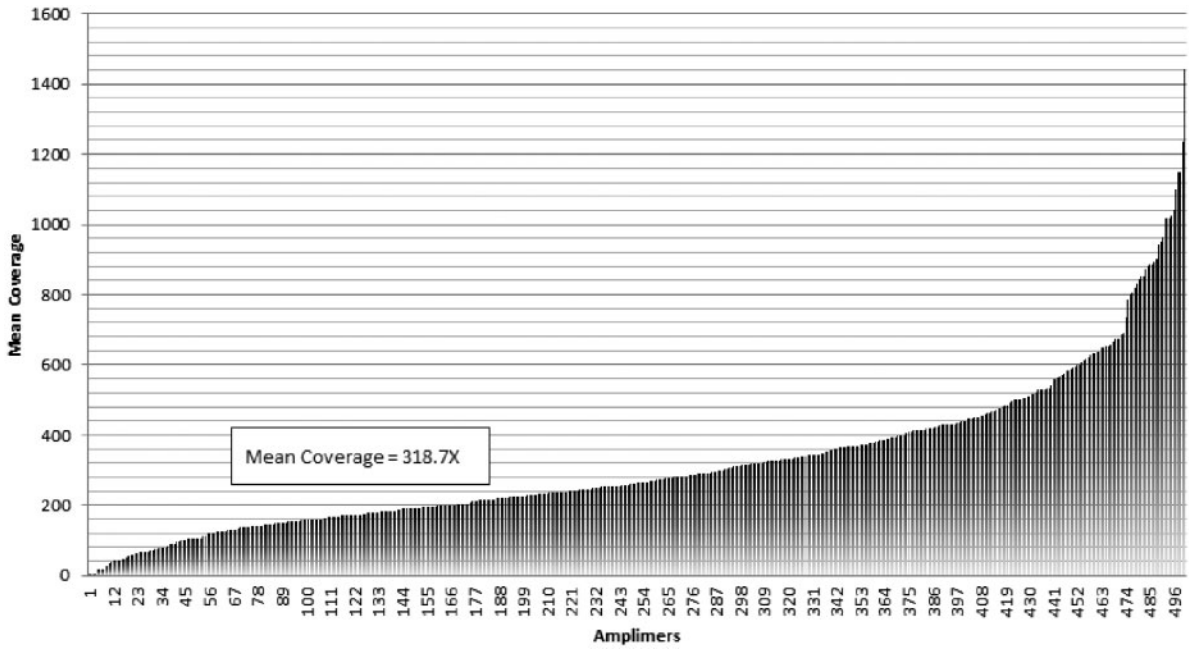
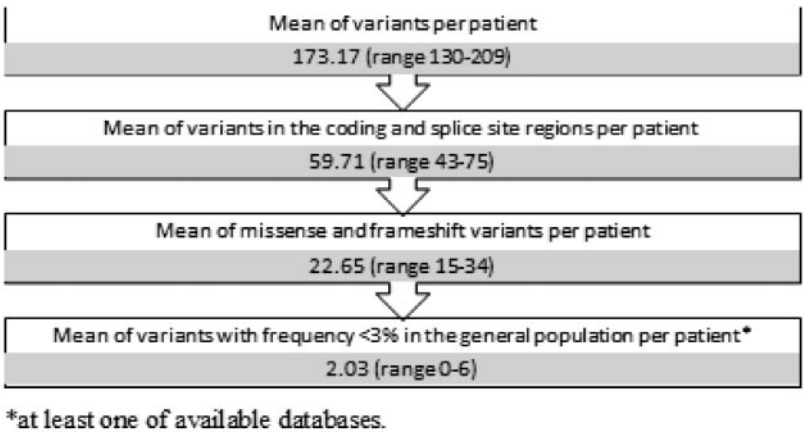
Fig. 1 Coverage analysis of all the amplimer reads obtained from all 50 samples



The 501 amplimers used for the enrichment of the gene target ordered according to the mean coverage, from the least to the most represented in the reads pool, as calculated among the 50 samples tested. The mean coverage is reported in the box.

Fig. 2 NGS variants validation workflow



The bioinformatics pipeline adopted to filter out variants unlikely to be involved in the uSAID disorders taken into consideration in this study. In particular, criteria such as position of the variants within either the coding portion or splice sites, missense, nonsense and missense nature of the coding change, and the frequency of the variants in the general population have been applied to reduce the number of candidate variants from an average of 173 to 2 per patient. NGS: next-generation sequencing.

it is known that individuals with *IL36RN* gene mutations up-regulate IL-1 production in response to IL-36 stimulation [35]. Furthermore, the p.Ser113Leu variant of the *IL36RN* gene has been found to be significantly more frequent in patients with inflammatory skin manifestations [36]. Patient no. 24 displayed the p.Ala177Thr variant of the *RNASE2B* gene, whose carriers have been reported to be asymptomatic, while patient no. 25 had a positive interferon signature [37, 38]. Four patients (nos 26, 27, 28 and 29 in Table 2) presented recurrent fevers that characterize

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the Majeed syndrome, but their *LPIN2* gene variants are considered of unknown significance.

Patients with no new pathogenic variants, benign variants or no variants

The NGS panel was not able to identify any PPV in 21 patients (42%). The heterozygous p.Val726Ala variant of the *MEFV* gene has previously been identified by Sanger analysis in patients 30 and 31 in Table 2. This variant is considered pathogenic: the lack of other variants in genes of the NGS panel together with the clinical manifestations associated with fever episodes prompted us to classify patient 30 as heterozygous FMF [39]. The patients had a complete response to colchicine. Conversely, patient 31 is still considered affected by uSAID because the clinical picture was inconsistent with FMF. The remaining 19 patients displayed only likely benign variants or did not show any noteworthy variant.

The subset of patients with undifferentiated recurrent fever episodes

This study has provided the opportunity to describe a large subgroup of patients presenting with recurrent fever episodes inconsistent with a classical PFAPA phenotype and negative for genes associated with hereditary recurrent fevers [40–44]. The main clinical features of this subgroup (marked with footnote symbol a in Table 2) are described in Fig. 3. Fever episodes lasted on average 6 days ($P < 0.0001$), with a median symptoms-free period of 3 (range 1–6) weeks, similar to PFAPA syndrome. Abdominal pain (usually not associated with nausea, vomiting or diarrhoea) and limb pain were the most common symptoms during the fever episodes (Supplementary Table S6, available at *Rheumatology* online). The classic PFAPA triad (pharingotonsillitis, apthousis and cervical lymphadenopathy) was less frequently reported ($P < 0.0001$). On the other hand, skin rash and arthritis were significantly more frequent ($P < 0.0001$). Atypical for PFAPA syndrome, hepatosplenomegaly was reported in 15% of our patients. Rare symptoms in our cohort were periorbital oedema (two patients) and pericarditis (one patient). Eighteen patients were exclusively treated with steroid on demand with a high response rate (Supplementary Table S6, available at *Rheumatology* online). In 18 patients colchicine treatment was used with an overall complete or partial response in 14 of them (78%). Anakinra was ineffective in three out of four colchicine-resistant patients, suggesting an IL1β-independent pattern of inflammation in these patients. Only a partial response was achieved with mycophenolate, mofetil or etanercept in some cases (Supplementary Table S5). Other disease modifying anti-rheumatic drugs (azathioprine or methotrexate) were also used without response. Tonsillectomy was ineffective in one patient.

We also analysed six patients with disease onset after 10 years of age (nos 8, 22, 23, 31, 44 and 48, in Table 2) and not discovered relevant differences of clinical manifestations and treatment response from the entire study cohort. Furthermore, variants are randomly distributed among the different phenotypes according to their main pathogenic pathways (Supplementary Table S7, available at *Rheumatology* online).

Discussion

In this study, we tested a 41-gene NGS diagnostic panel in a group of 50 consecutive patients referred to our tertiary-care centre and affected by uSAID to explore the pros and cons of such a NGS approach in their diagnostic work-up. The 41-gene panel allowed us to achieve a definitive diagnosis in two patients. Interestingly, they presented an atypical form of MKD. This might have been one of the reasons why, despite initial suspicion, the solution of these cases came only after NGS-based testing.

Indeed, after long-standing experience and with broad literature data, the Sanger sequencing approach has turned out ineffective unless the clinical picture is totally consistent with one of the known monogenic simple SAID and a clinical diagnosis can already be made with a high degree of certainty. Therefore, NGS may represent a valuable and effective technique when either the first determination by Sanger sequencing results are inconclusive or the clinical picture is not typical for a specific condition.

However, the final diagnostic yield achieved by the present NGS panel in uSAID was rather low, with 2 out of 50 patients receiving a genetic confirmatory diagnosis and only two other patients displaying genetic variants possibly causative of their condition. On the other hand, many patients were carriers of possible pathogenic variants of genes that were not consistent with the clinical phenotype, and another relevant group displayed either no pathogenic variants or monoallelic variants for autosomal recessive diseases. These cases raise the question of the careful interpretation of data coming from the NGS analysis. Indeed, variants should be assessed in the context of a multidisciplinary discussion among geneticists and expert clinicians. To facilitate the NGS data interpretation, and to homogenize diagnosis among different centres, editing of proper guidelines for genotype interpretation and variant reporting would be of great value. A first attempt has recently been carried out by the INSAID project for the four inherited periodic fevers (cryopyrin-associated periodic syndromes, TNF receptor-associated periodic syndrome, MKD and FMF) [45] and the classifications are available in the Infevers database (http://fmf.igh.cnrs.fr/ISSAID/infevers/).

One possible concern about the systematic use of the NGS in the diagnostic work-up is related to the costs of this technique. Patient 1 had a very intense history of admissions in different local hospitals during his first 6 years of disease trying to reach the correct diagnosis. A rough calculation of the direct costs imputable to these admissions reveals the presumptive amount of 30 000 euros. Conversely, the costs of the present study (including materials and manpower for the personnel involved) are calculated to be <500 euros/sample, including the final Sanger Seq validations, with a total of almost 25 000 euros for all the 50 samples. Thus, at least in the present experience, the diagnosis of patient 1 alone provides the justification of the costs of our whole study. Indeed the

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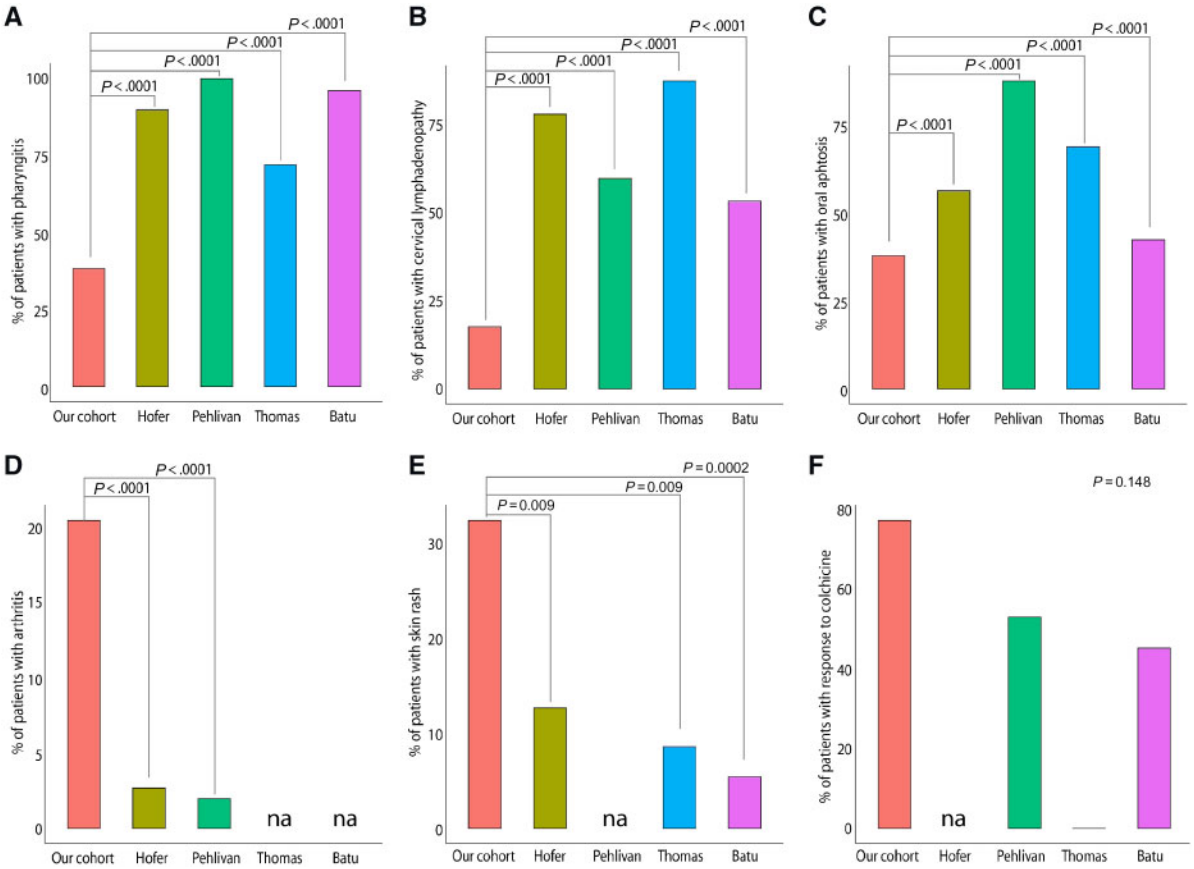
FIG. 3 Clinical features of patients with SURF compared with different cohorts of PFAPA syndrome available in the literature



Values are number of patient (%). *P*-values were assessed using χ^2 test or *t*-test as appropriate and where significant interactions were determined *post hoc* test for multiple comparison were performed. All the post-hoc analyses highlighted significant differences ($P < 0.05$) between our cohort and the other PFAPA populations reported by Hofer *et al.* [41], Pehlivan *et al.* [44], Thomas *et al.* [40] and Batu *et al.* [42]. na: not available; PFAPA: periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis; SURF: systemic undifferentiated recurrent fevers.

actual economic impact of the NGS approach was far behind the aims of the present study. In fact, such an evaluation would require a specialized methodological approach using specific pharma economic tools.

It is conceivable that the use of NGS panels with a larger number of genes would significantly increase the rate of diagnosis, as recently shown by a similar experience in an equivalent number of vasculitic and SAID patients screened with two panels of 113 and 166 genes, respectively [19]. In these panels, genes related to other immune-mediated diseases were included. The clinical overlap of SAID with the enlarging spectrum of monogenic conditions characterized by an immune dysregulation suggests adopting either larger panels or, ultimately, whole exome sequencing with an intermediate step of *in silico* analysis of all the genes possibly involved, including those of recent identification. This would overcome the present main limitation of a NGS panel that does not allow a fast update of the list of genes possibly involved. The progressive reduction of the costs for whole exome sequencing and the improvements in bioinformatics analysis will likely allow the adoption of whole exome sequencing for routinely diagnostic procedures in the near future. This approach would also provide the advantage of identifying new candidate genes, involved or not in pathways already known to be associated with SAID, with relevant implications also on the research side.

The low detection rate with the 41-gene panel observed in the present study is also likely due to the high percentage of patients with recurrent fever. It is conceivable that a small percentage of these patients could have a condition secondary to mutations of genes not identified yet. However, it is also possible that most of them might present a multifactorial autoinflammatory condition different from the well-characterized PFAPA syndrome. Indeed, the ambiguity of the available PFAPA criteria may allow classification of these latter patients as affected by PFAPA syndrome, independently of the actual frequency of the

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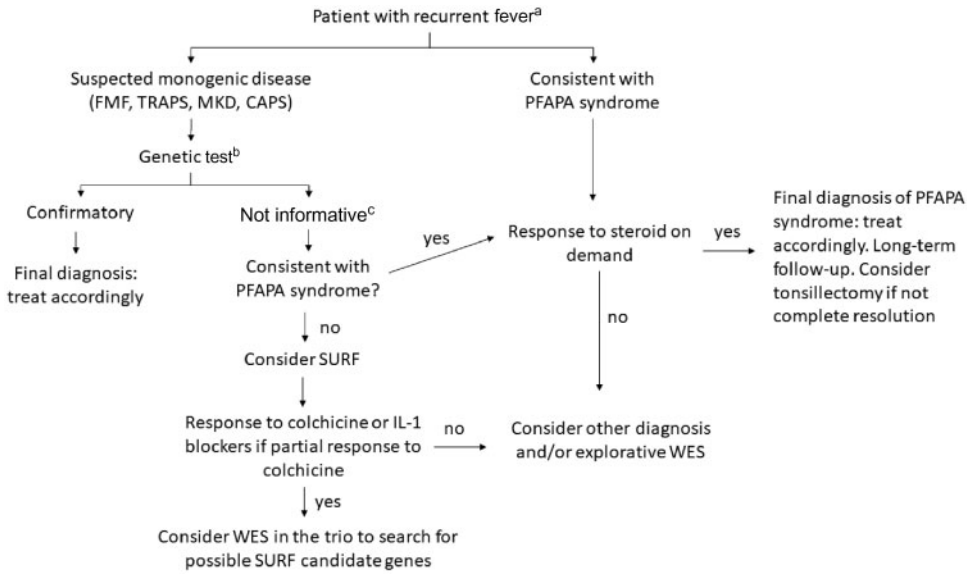
Fig. 4 Diagnostic and therapeutic flow-chart for patients with suspected autoinflammatory recurrent fever



[a]After careful exclusion of other possible causes (infections, neoplasms, etc.). [b]With target Sanger sequencing or NGS diagnostic panels. [c]Consider carefully variants of unknown origin and low-penetrance variants, consider the possibility of somatic mosaicisms. CAPS: cryopyrin associated periodic syndrome; MKD: mevalonate kinase deficiency; NGS: next-generation sequencing; PFAPA: periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis; TRAPS: TNF receptor associated periodic syndrome.

cardinal manifestations [24, 41]. In this subgroup, the presence of low penetrance variants or polymorphisms does not seem to influence the clinical phenotype. Of note the same variants could be also observed in a number of other multifactorial conditions, such as multiple sclerosis and recurrent pericarditis [46, 47]; however their actual impact in conferring susceptibility to mounting a pro-inflammatory response in these disorders is still largely debated.

The present study offers an original description of this particular subset of patients. Recurrent abdominal and limb pain and skin rash were the most frequent clinical manifestations. Whenever present, pharyngitis was reported to be sporadic and only seldom associated with a clear exudative tissue reaction. Beside steroid on-demand, which is usually effective, a relevant percentage of these patients display a complete or at least partial (i.e. evident reduction of the frequency and intensity of fever episodes) response to colchicine. This percentage is significantly higher than for classical PFAPA patients. A recent paper has described a good response to colchicine in a PFAPA cohort, but patients with one *MEFV* mutation were not excluded in that study and a lower frequency of fever episodes was considered sufficient to evaluate colchicine as effective [43]. Furthermore, at variance with PFAPA syndrome, in the present study tonsillectomy was not used as a possible strategy to reduce fever attacks, showing a failure in the single case in which it was performed.

This heterogeneous group of undefined periodic fever does represent an interesting subgroup for future genetic and functional investigations. According to the historical Tel Hashomer diagnostic criteria, the response to colchicine would allow, independently of the presence of *MEFV* mutations, the classification of these patients as clinical FMF [48]. However, this subgroup presented a longer duration of fever episodes (median 6 days) and a high prevalence of skin rash that is not usually observed in FMF. It is in fact possible that a proportion of these patients could be carriers of mutations typical of novel genetic defects, but it is also conceivable that most of them present a multifactorial inflammatory condition, possibly affecting the RhoA-dependent activation of pyrin inflammasome, independently of the presence of mutations of the *MEFV* gene. With the aim to characterize this distinct clinical subgroup of patients from the more common PFAPA syndrome, we suggest using the term systemic undefined recurrent fevers, as previously proposed [49], and a flow chart for the identification and treatment of these patients is proposed in Fig. 4.

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R.P. participated in the design of the study, collected and analysed the data, and drafted the manuscript; M.R. and A.G. participated in the design of the study, performed genetic analysis and helped to draft the manuscript; F.C. collected the samples and performed genetic analysis; S.V., R.C., P.P., V.M., L.O. and C.C. collected the data and helped to draft the manuscript; F.B. performed statistical analysis and helped to draft the manuscript; A.R., M.E.V.G., I.C. and M.G. conceived the study, participated

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in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript as presented.

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Consent to participate and publish: Consent to participate and publish was obtained in our Institute in accordance with local requirements.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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