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EXTENDED REPORT

MEFV mutations affecting pyrin amino acid 577 cause autosomal dominant autoinflammatory disease

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ABSTRACT

Objectives Autoinflammatory disorders are disorders of the innate immune system. Standard genetic testing provided no correct diagnosis in a female patient from a non-consanguineous family of British descent with a colchicine-responsive autosomal dominant periodic fever syndrome. We aimed to unravel the genetic cause of the symptoms.

Methods Whole exome sequencing was used to screen for novel sequence variants, which were validated by direct Sanger sequencing. Ex vivo stimulation with peripheral blood mononuclear cells was performed to study the functional consequences of the mutation. mRNA and cytokine levels were measured by quantitative PCR and ELISA, respectively.

Results Whole exome sequencing revealed a novel missense sequence variant, not seen in around 6800 controls, mapping to exon 8 of the *MEFV* gene (c.1730C>A; p.T577N), co-segregating perfectly with disease in this family. Other mutations at the same amino acid (c.1730C>G; p.T577S and c.1729A>T; p.T577S) were found in a family of Turkish descent, with autosomal dominant inheritance of familial Mediterranean fever (FMF)-like phenotype, and a Dutch patient, respectively. Moreover, a mutation (c.1729A>G; p.T577A) was detected in two Dutch siblings, who had episodes of inflammation of varying severity not resembling FMF. Peripheral blood mononuclear cells from one patient of the index family showed increased basal interleukin 1β mRNA levels and cytokine responses after lipopolysaccharide stimulation. Responses normalised with colchicine treatment.

Conclusions Heterozygous mutations at amino acid position 577 of pyrin can induce an autosomal dominant autoinflammatory syndrome. This suggests that T577, located in front of the C-terminal B30.2/SPRY domain, is crucial for pyrin function.

INTRODUCTION

Autoinflammatory disorders are disorders of the innate immune system characterised by recurrent episodes of fever and systemic inflammation, with localised inflammation predominantly affecting serosal surfaces, skin, joints and eyes. Attacks are often self-resolving, and there are no signs of autoantibodies or infection.[1 2]

There are a number of inherited autoinflammatory syndromes, such as familial Mediterranean fever (FMF), tumour necrosis factor receptor-associated periodic syndrome, hyperimmunoglobulinaemia D and periodic fever syndrome (also known as mevalonate kinase deficiency), cryopyrin-associated periodic syndromes (CAPS), Blau syndrome, deficiency of the interleukin (IL)-1 receptor antagonist (DIRA), pyogenic arthritis, pyoderma gangrenosum and acne (PAPA). In other cases, the genetic background is uncertain or multifactorial, such as periodic fever with aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA), or thought to be unlikely, such as Schnitzler syndrome. Despite similarities in symptoms, the clinical picture is often different, as well as the mode of inheritance, duration, frequency of attacks, and response to treatment.[2 3] In addition, many patients suspected of having autoinflammatory disease do not fulfil diagnostic criteria for the known syndromes or test negative for the known mutations. Therefore, correctly diagnosing these patients can be challenging, often shown by the long period needed for diagnosis.[4]

In a family with an autosomal dominant autoinflammatory disorder, standard genetic testing did not provide a diagnosis. We aimed to reveal the genetic cause of the undiagnosed symptoms in this family by using a hypothesis-free, exome-wide sequencing approach.

METHODS
Patients
Family 1
A female patient (IV:5) from a non-consanguineous family of British descent with an apparent autosomal dominant inherited periodic fever syndrome (figure 1A) had recurrent episodes of synovitis, pleuritis or peritonitis, and skin rash (figure 2A) since the age of 6 (table 1). Her grandfather (II:2) had inflammatory episodes of fever, arthritis, pleuritis, peritonitis and skin rash from the age of 10. His father, two of his siblings and his daughter (mother of the proband; III:4) had similar attacks. They experienced a beneficial response to colchicine. However, initial DNA mutation screening for *MEFV* exons 2, 5, 10 as well as *TNFRSFR1A* exons 2, 3, 4, 5, 6, 7 and *NLRP3* in at least two family members did not show mutations.
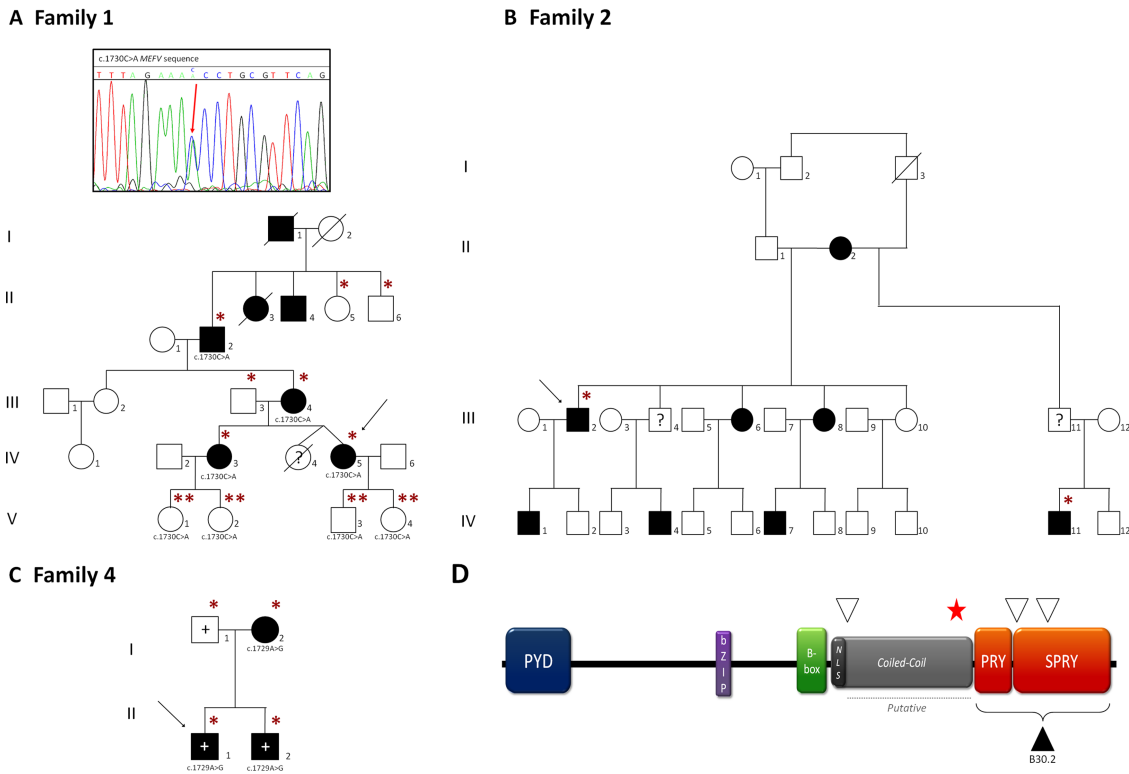
Figure 1 *MEFV* mutations. (A) The mutation found in patients in family 1 is a heterozygous autosomal dominant c.1730C>A mutation (shown in the chromatogram), leading to a p.T577N amino acid substitution. (B) Pedigree of family 2: c.1730C>G; p.T577S. (C) Pedigree of family 4: c.1729A>G; p.T577A. (A–C) Clinically affected family members are indicated in black. A diagonal line indicates that the individual is deceased. Males and females are indicated by squares and circles, respectively. Arrows indicate the proband. * represents genetic confirmation of clinical data, whereas ** represents genetic confirmation, but no symptoms are displayed yet because of very young age of the individuals. Question marks indicate that no information is available about these individuals. The + in family 4 indicates that these individuals carry a p.P369S/p.R408Q complex allele. (D) The p.T577 amino acid position in front of the B30.2 domain of the pyrin protein is indicated with a red star. Most familial Mediterranean fever mutations are in the B30.2 domain (solid arrow). The open arrows indicate previously described *MEFV* mutations with dominant phenotype.

This study was approved by the local ethics committee; patients gave informed consent.

Exome sequencing: library generation, reference alignment and variant calling

Genomic DNA samples were randomly fragmented using nebulisation. Bar-coded adapters were ligated to both ends of the resulting fragments, according the standard NEBNext DNA Library Prep Master Mix Set for Illumina protocol (New England Biolabs, UK).[5] Fragments with an average insert size of 220 bp were excised using the Caliper XT gel system, and extracted DNA was amplified by PCR. The quality of the product was verified on the Bio-Rad Experion instrument. When this met the criteria, the product was multiplexed in an equimolar pool of four similar products. This pool was hybridised to the Agilent SureSelect All exon V2 kit, according to the

provided protocol. After PCR amplification of enriched products, the quality was verified on the Bio-Rad Experion instrument followed by paired-end sequencing on the HiSeq2000 with 100 bp reads. Image files were processed using standard Illumina base-calling software, and generated reads were ready for downstream processing after demultiplexing.

The reads were aligned to the human reference genome build 37 using Burrows–Wheeler alignment.[6] To clean aligned data and perform variant calling, we applied Picard duplicate removal and The Genome Analysis Toolkit[7] quality score recalibration, indel realignment and unified genotyper. Using snpEff (http://snpeff.sourceforge.net), variants were annotated with information from dbSNP132, 1000 Genomes Project (phase 1, 2 and 3) and Ensembl build 37.64, which are all integrated into the pipeline MOLGENIS Compute developed by the Genomics Coordination Center of the Department of Genetics, University

Figure 2 Skin complications. An evanescent urticaria-like skin rash seen in individual IV:5 in family 1 (A) and individual II:1 (B) and II:2 in family 4 (C).
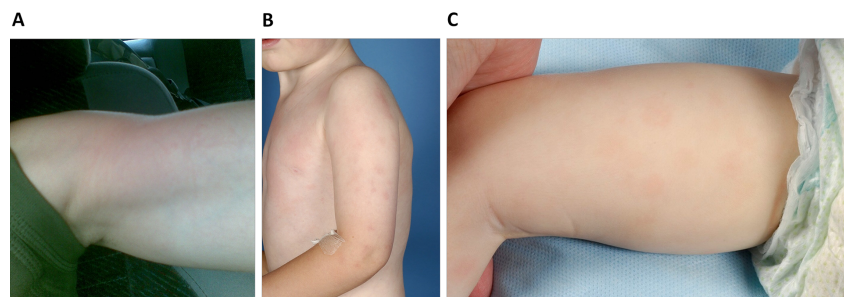
Table 1 Clinical characteristics

Characteristic	FMF	Current families			
		Family 1 c.1730C>A; p.T577N	Family 2 c.1730C>G; p.T577S	Family 3* c.1729A>T; p.T577S	Family 4* c.1729A>G; p.T577A
Inheritance	Autosomal recessive	Autosomal dominant	Autosomal dominant	?	?
Ethnic background	Mediterranean (Turkish, Arab, Armenian, Jewish, Italian)	British	Turkish	Dutch†	Dutch
Colchicine responsiveness	++	++	++	NA	−‡
Fever	++	++	++	NA	++
Serositis	++	++	++	NA	Arthritis
Skin lesions	++ Erysipelas-like erythema	+ Faint evanescent rash	−	NA	++
Hepatosplenomegaly	+/−	−	−	NA	++
Anaemia	+/−	−	−	NA	++
Psychomotor delay	−	−	−	NA	+ (II:1)
Duration of episodes	2–3 days	Days to weeks	1–1.5 weeks	NA	weeks

*In families 3 and 4, additional non-classic *MEFV* mutations were found: p.T267I and p.P369S/R408Q, respectively.
†Dutch patient of French origin.
‡Good response to anakinra. Symbols ranging from − to ++ indicate the absence or (degree of) presence of the mentioned symptoms.
FMF, familial Mediterranean fever; NA, not enough clinical data available from family 3.

of Groningen, Groningen, The Netherlands (http://wiki.gcc.rug.nl/wiki/GccStart).[8] Variant pathogeneity predictions were obtained with PolyPhen V.2.0, SIFT and Align GVD.[9–12]

Exome sequencing: step-wise filtering of sequence data

For our analysis we used a 'linkage-based strategy' analysis.[13] We chose all sequence variants (SVs) shared between two affected, related individuals (21 905 SVs). We excluded all variants that (1) had been reported in the dbSNP131 (including the 1000 Genomes Project) and in a private set of two unrelated samples (2225 SVs left), (2) mapped to the intronic regions of the genes, except to splice sites (565 SVs left), and (3) were present in the olfactory receptor genes[14] and high copy number genes.[15] We further reduced our dataset to 151 SVs by removing all variants in the homozygous state (dominant disease model), present on the X chromosome (autosomal model), and by using the updated SeattleSeq_Annotation tool[16] removing additional variants present in the 1000 Genomes Project but not yet present in the dbSNP131, at this point of the analysis. Finally, we removed all variants predicted as 'benign' by PolyPhen implemented in the SeattleSeq_Annotation tool,[16] resulting in 125 SVs for further consideration.

Validation of mutations

Variations were validated by direct Sanger sequencing and analysed using DNA Variant Analysis software (Mutation Surveyor). To validate the presence of mutations in *MEFV* in affected patients of family 1, DNA was isolated from whole blood, and conventional PCR and Sanger sequencing of exon 8 were performed. Primer sequences are available upon request.

Additional patients

Retrospectively, three other patients/families had been linked to mutations at this location of the *MEFV* gene in our centres shortly before or simultaneously with family 1; details are included in the Results section (figure 1 and table 1). In families 2 and 3, it was discovered by sequencing the entire *MEFV* gene, and in family 4 it had been discovered via a sequencing array of

120 inflammasome-related genes. This had been achieved by generating a bar-coded whole genome fragment library for the index patient, which was enriched for the coding regions of these 120 genes using a custom Agilent 1M microarray, and the enriched library was sequenced on the SOLiD4 sequencer as described previously.[17] This array did not yield mutations in any of the other genes.

In vitro cytokine production

We characterised the immune responses of peripheral blood mononuclear cells (PBMCs) from one affected family member (family 1, III:4) and one healthy control subject. PBMCs were isolated using Ficoll-Paque PLUS (Bio-Sciences AB). A total of 5×10^5 cells in RPMI 1640 medium (Dutch modification; Invitrogen, Paisley), supplemented with 1 mM sodium pyruvate (Sigma-Aldrich), 2 mM L-glutamine (Merck) and 50 µg/ml gentamicin (Centrafarm), were incubated in round-bottomed 96-well plates (Greiner) at 37°C. After 24 h of incubation with various stimuli, supernatants were collected and stored at −80°C until further analysis. In another experimental setup, cells were stimulated for 3 h with 1 µg/ml lipopolysaccharide (LPS) (Sigma; *Escherichia coli* serotype 055:B5, and purified as described elsewhere[18]), followed by 15 min with 1 mM ATP to induce IL-1β release.[19]

Cytokine assays

Cytokine concentrations in supernatants were measured using commercial ELISA kits from R&D Systems (IL-1α, IL-1β) or PeliPair Reagent sets from Sanquin (IL-6), according to the manufacturer's instructions.

Quantitative reverse transcriptase-PCR

Freshly isolated PBMCs (1×10^6) were incubated with various stimuli at 37°C. Total RNA was extracted using TRIzol reagent (Invitrogen), subjected to DNAse treatment (Ambion DNA-free Kit; Invitrogen) and reverse-transcribed into cDNA (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, California, USA). Quantitative reverse transcriptase-PCR for human IL-1β and

β₂-microglobulin was performed using an Applied Biosystems 7300 real-time PCR system. Primer sequences are available upon request.

RESULTS

Genetic cause of the autoinflammatory disease in family 1

We used whole exome sequencing in two patients from family 1 (IV:5 and II:2). We aligned the raw sequencing to the human reference build 37, obtaining 27 Gb of high-quality reads. The mean on-target coverage was 76-fold and 135-fold for patients II:2 and IV:5, respectively, of which 92% and 93%, respectively, was covered at least 10 times, indicating high-quality data. The concordance of sequenced data with genotyping data (HumanCytoSNP-12 beadchip; Illumina) was 99.9%. We identified approximately 31 600 SVs of high quality per individual which, after filtering, resulted in a list of 125 candidate SVs present in both patients. One of these was a mutation in exon 8, c.1730C>A, of the *MEFV* gene (NM_000243.2), resulting in a missense mutation, p.T577N, in the pyrin protein (figure 1D).

Considering the colchicine responsiveness and the autoinflammatory phenotype, this was the most likely candidate. As shown in figure 1A, Sanger sequencing confirmed the co-segregation of the mutation within the affected family members only. More importantly, this mutation was not found in 396 healthy Dutch controls, in addition to 500 Dutch controls from the GoNL Project, the CEU and YRI population from the 1000 Genomes Project,[20] 200 Danish and 120 UK controls, and the ESP5400 dataset. Neither could we detect this mutation in over 1000 patients with unexplained autoinflammatory symptoms.

Pyrin mutations in the same amino acid position in additional families with autoinflammatory diseases

Shortly before or simultaneously, three different mutations affecting the same amino acid location of pyrin were detected by our group in three other patients/families (figure 1 and table 1). After sequencing the whole gene, we found a c.1730C>G (p.T577S) mutation in the *MEFV* gene (without other *MEFV* gene mutations) in a family of Turkish descent (family 2, figure 1B). This family presented with an FMF-like phenotype, good therapeutic effect of colchicine, and also an autosomal dominant inheritance pattern.

In two other patients/families, the mutation was found in combination with an additional non-classic *MEFV* gene variant. By sequencing the entire *MEFV* gene, we detected a c.1729A>T (p.T577S) mutation in association with c.800C>T (p.T267I) in a Dutch patient with an autoinflammatory phenotype (family 3: Infevers database: http://fmf.igh.cnrs.fr/ISSAID/infevers/search. php?n=1; no additional data available).

The fourth family is also of Dutch ethnic background, with a severe autoinflammatory phenotype in an autosomal dominant inheritance pattern (family 4, figure 1C). DNA from the index patient was sequenced by an array for the coding region of 120 inflammasome-related genes. This revealed a c.1729A>G (p.T577A) mutation, in association with a p.P369S/R408Q complex allele. No other predicted pathogenic variants were detected. Since the age of 26 months, the index case (II:1) had recurrent skin rash (figure 2B,C) and episodes of arthritis and fever, accompanied by hepatomegaly, massive splenomegaly and severe anaemia (haemoglobin 3.5 mmol/l), resulting in delay of growth and psychomotor development. Other abnormalities included lymphadenopathy and conjunctivitis. Biopsy of the skin rash showed urticaria. Standard genetic testing for CAPS and DIRA was negative. Under anakinra treatment, this patient rapidly achieved sustained remission with near normalisation of

haemoglobin (6.5 mmol/l) within 1 month, improved growth and improvement in psychomotor development. His mother (I:2) had experienced repeated inflammatory attacks since the age of 6, albeit less severe than her son's. These episodes included fever, arthritis, anaemia, skin rash and splenomegaly. She had had erythema nodosum as a child, and also oral and vaginal aphthosis (HLA-B51 negative). She also had colitis, which, on biopsy, showed chronic inflammation with granulomas. Her disease responded well to anakinra. A brother of the proband (II:2) showed symptoms similar to the index patient from 4 days after birth, with failure to thrive, delayed psychomotor development, fever, urticarial skin rash, anaemia and hepatosplenomegaly. A brief trial of colchicine was not successful, but anakinra relieved the symptoms.

Functional studies

Since the T577 mutations are located in exon 8, just before the PRY-SPRY domain of pyrin, we hypothesised that it might alter the structure of the protein. However, a western blot of PBMC lysates from patient III:4 from family 1 did not show any differences in banding pattern compared with the control (not shown), indicating that the mutation did not affect the electrophoresis characteristics.

To study the functional consequences of the T577N mutation, we isolated PBMCs from this patient and a healthy volunteer. First, we examined IL-1β mRNA levels. As shown in figure 3A, PBMCs obtained from the T577N patient without colchicine treatment showed an almost six times higher expression of basal IL-1β mRNA. With colchicine treatment, expression levels fell and resembled control levels, indicating that colchicine acts on the expression of IL-1β mRNA and that this mutation causes higher IL-1β expression levels, either directly or indirectly.

Next, to evaluate functional consequences of T577N at the protein level, we performed LPS stimulation studies. As shown in figure 3B, cells from the patient secreted more of the proinflammatory cytokines, IL-1β and IL-6, after 3 h of LPS and LPS+ATP stimulation than cells from the control, even under colchicine treatment. After 24 h incubation in medium and with LPS (figure 3C), T577N PBMCs obtained from the patient without treatment showed higher production (not shown) and secretion of cytokines (IL-1β, IL-1α, IL-6), compared with the control. Treatment with colchicine normalised IL-1β secretion to control levels. In response to low-dose LPS, only the PBMCs from the patient not taking colchicine secreted excessive amounts of cytokines, illustrating that the cells bearing the mutation are more easily triggered to produce cytokines. This effect is most pronounced for IL-1β.

DISCUSSION

In this study, we show that alteration of the amino acid threonine at position 577 of pyrin, the protein associated with FMF, causes an autosomal dominant autoinflammatory phenotype including fever and systemic inflammation. In our patients, this disorder has some similarities to FMF but also clear differences (table 1 shows the comparison between the classic phenotype of FMF and our patients). FMF is a well-characterised autoinflammatory disease with autosomal recessive inheritance, in which (homozygous or compound heterozygous) mutations are most often located in the B30.2 domain. Our data suggest that T577 is crucial for pyrin function, as four different novel DNA variants detected in patients with autoinflammatory disease from different populations affect the same amino acid and are able to cause disease in the heterozygous form.

Figure 3 Ex vivo inflammasome stimulation. Peripheral blood mononuclear cells (PBMCs) were incubated for 3 h with 1 µg/ml lipopolysaccharide (LPS) or RPMI, after which the medium was removed. Cells were incubated for an additional 15 min with either RPMI or 1 mM ATP (A,B). In another experiment (C), cells were stimulated for 24 h with 1 or 10 ng/ml LPS. After incubation, the plates were spun, supernatants collected and mRNA was isolated. (A) IL-1β mRNA expression in cells from one patient from family 1, under colchicine treatment (red bar) or not (black bar), and cells from a control (white bar). Basal mRNA levels for IL-1β in the patient without treatment are 5.7 times higher than control basal IL-1β mRNA levels. Under colchicine treatment, these levels appear to normalise to control values. (B) Increased cytokine production by PBMCs after 3 h LPS stimulation and 15 min of ATP. (C) The PBMCs of the untreated patient are more sensitive to 24 h LPS stimulation, especially at low-dose LPS. IL-1β secretion normalises to control levels, when the patient is under colchicine treatment.
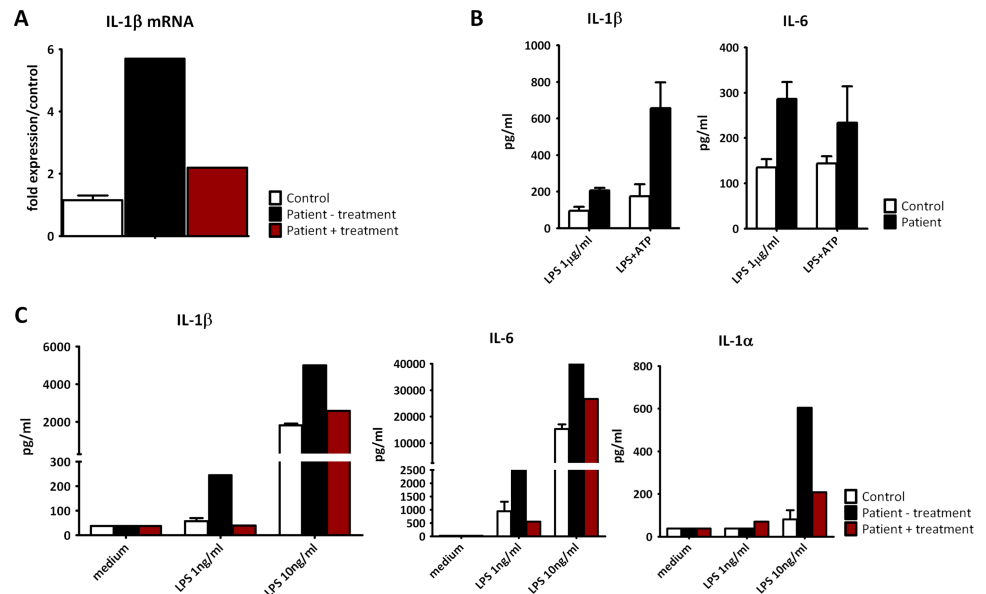
To date, the precise structure and function of pyrin have not been fully resolved. It consists of an N-terminal pyrin domain, a B-box, bZIP basic, coiled-coil domains and a B30.2/SPRY domain (figure 1D).[21–23] The crystal structure of this B30.2 domain was elucidated by Weinert et al,[24] who showed that many FMF-associated pyrin mutations are in close vicinity of a predicted peptide-binding site, suggesting that these are probably involved in altering binding properties of the B30.2 domain. However, the crystal structure of the remaining part of pyrin is unknown, which makes it impossible to perform predictive modelling of the T577 mutation effects. On the basis of the hypothetical presence of coiled coils,[25] we suggest it will lead to a structural change significantly affecting protein function/oligomerisation.

The exact function of pyrin in inflammation is still under discussion. The protein, or at least part of it, is found in the nucleus of granulocytes and dendritic cells,[26 27] where it might activate NF-kB. Pyrin is also found in the cytoplasm of monocytes, interacting with tubulin and colocalising with microtubules.[28] Through the pyrin domain, it can interact with ASC (apoptosis-associated speck-like) protein, which is involved in inflammasome complex formation,[29] thereby activating IL-1β. Experimental findings fit with two possible mechanisms: either pyrin inhibits IL-1β activation by competing with caspase-1 for ASC[30–33] or it forms an inflammasome complex by itself,[34–36] reviewed more extensively by Masters et al.[2] Although we cannot pinpoint the exact mechanism, our data show that the dominant T577N mutation results in increased cytokine secretion after LPS stimulation, which normalises when the patient is receiving colchicine treatment. This suggests that T577N imparts a gain of inflammatory function, contributing to the growing body of evidence that MEFV mutations are gain-of-function rather than loss-of-function mutations, as previously hypothesised on the basis of recessive inheritance of FMF.

Although FMF is known to be an autosomal recessively inherited disease,[37 38] it is known that in some patients only one (classical) mutation in the MEFV gene has been found or that the inheritance pattern in the family is more compatible with autosomal dominant inheritance.[39] To explain these observations, several reasons have been suggested: missing of the second mutation because of, for example, incomplete screening of the gene, presence of a large deletion, copy number variations (shown not to play a role[40]) or location in intron or promoter sequences; concomitant presence of a mutation in another autoinflammatory gene,[41–44] or modifications at the epigenetic[45] or post-translational level; pseudo-dominance (which might arise in offspring of a heterozygous carrier and a homozygous patient)[46–49]; mosaicism[50] (where individuals have cells of different genotypes). In the present cases, one may argue that these patients had a digenic disease with an as yet unidentified autoinflammatory gene mutation. However, the T577 mutation appears to be able to cause a true autosomal dominant phenotype; neither the whole exome sequencing in family 1 nor the array of 120 inflammasome-related genes in family 4 revealed a mutation in another innate immunity-related gene. Although in families 3 and 4, the patients do carry the T577 mutation in combination with another mutation in the MEFV gene, patient I:2 in family 4 shows that the T577 mutation is sufficient to cause disease. There have been a few other reports of MEFV mutations that also appear to cause an FMF phenotype in the heterozygous state—for example, a heterozygous H478Y mutation in a Spanish family,[51] and a number of mutations in exon 10, such as M694V and R653H or a deletion of M694.[44 46 47]

It is well known from several hereditary autoinflammatory diseases that the character and severity of the phenotype can vary considerably, despite mutations in the same gene.[2] For example, a wide spectrum of disease associated with mutations in the NLRP3 gene is now recognised as CAPS, but previously

described as three separate clinical diseases.[2] Another example is mevalonate kinase deficiency, in which severely affected patients have multisystemic disease with risk of death early in childhood, whereas more common mutations only cause the episodic fever syndrome, HIDS.[2] Recently discovered *MVK* mutations in disseminated superficial actinic porokeratosis seem to expand the spectrum even further.[52] The present study suggests that *MEFV* gene mutations are also linked to a spectrum of manifestations, in addition to the classical phenotype of FMF. At this time, we hesitate as to whether to use a name such as 'pyrin-associated periodic syndrome' to include this broader continuum of disease, or to designate this as an atypical form of FMF.

In summary, we describe four missense mutations that alter pyrin T577 in four families. It causes a characteristic autoinflammatory disease, which can be controlled with colchicine or IL-1 blockade. This suggests that the phenotype associated with *MEFV* gene mutations is more diverse than just FMF.

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