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Evidence for genetic overlap between adult onset Still's disease and hereditary periodic fever syndromes

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Abstract

Objective Adult onset Still's disease (AOSD) is a severe, autoimmune disease that can be challenging to treat with conventional therapeutics and biologicals in a considerable number of cases. Therefore, there is a high need to understand its pathogenesis better. As major clinical symptoms overlap between AOSD and hereditary periodic fever syndromes (HPFS), we analysed four known HPFS genes in AOSD.

Methods We performed Sanger sequencing and quantitative analysis of all coding regions of *MEFV*, *TNFRSF1A*, *MVK* and *NLRP3* in 40 AOSD patients. All rare coding variants ($n=6$) were evaluated for several aspects to classify them as benign to pathogenic variants. Statistical analysis was performed to analyse whether variants classified as (likely) pathogenic were associated with AOSD.

Results We identified three rare variants in *MEFV*, one previously not described. Association to the three likely pathogenic *MEFV* variants was significant ($p_c=2.34E-03$), and two of the three carriers had a severe course of disease. We observed strong evidence for significant association to mutations in *TNFRSF1A* ($p_c=2.40E-04$), as 5% of patients (2/40) carried a (likely) pathogenic variant in this gene. Both of them received a biological for treatment.

Conclusion Our results indicate *TNFRSF1A* as a relevant gene in AOSD, especially in patients with a more challenging course of disease, while causal variants remain to be identified in the majority of patients.

Keywords Adult onset Still's disease · Biological therapy · Familial Mediterranean fever · Hereditary periodic fever syndromes · TNF receptor-associated periodic syndrome · Autoinflammatory syndromes

Abbreviations

ACMG	American College of Medical Genetics
ANA	Antinuclear antibodies
AOSD	Adult onset Still's disease
BDGP	Berkeley Drosophila genome project

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CAPS	Cryopyrin-associated periodic syndromes
CINCA	Chronic infantile neurological, cutaneous, articular syndrome
DMARD	Disease-modifying anti-rheumatic drugs
EVS	Exome variant server
ExAC	Exome aggregation consortium
FMF	Familial Mediterranean fever
GERP++	Genomic evolutionary rate profiling
GPT	Glutamate-pyruvate-transaminase
HPFS	Hereditary periodic fever syndromes
HSF	Human splicing finder
IL	Interleukin
LRT	Likelihood ratio test
MAF	Minor allele frequency
MEFV	Familial Mediterranean Fever gene
MKD	Mevalonate kinase deficiency
MLPA	Multiplex ligation-dependent probe amplification
MVK	Mevalonate kinase gene
MWS	Muckle–Wells syndrome
NFE	Non-Finnish Europeans
NLRP3	NLR Family Pyrin Domain Containing 3
RT-PCR	Reverse transcriptase polymerase chain reaction
SIFT	Scale-invariant feature transform
TNF	Tumour necrosis factor
TNFRSF1A	Tumour necrosis factor receptor superfamily member 1A
TRAPS	Tumour necrosis factor receptor-associated periodic syndrome
VUS	Variant of uncertain significance
γ-GT	γ-Glutamyltransferase

Introduction

Bywaters [1] was the first one to describe adult onset Still's disease (AOSD) in 1971, when reporting a group of 14 female patients suffering from symptoms very similar to the more common juvenile form of Morbus Still. The main symptoms described by Bywaters were high spiking fever, peripheral joint involvement and a fleeting rash. In contrast to the juvenile Morbus Still manifesting before the age of 16 years [2], age of onset in this group ranged between 17 and 35 years. For current classification of AOSD, at least 5 of the 8 criteria, introduced by Yamaguchi et al. [3] and including symptoms such as fever and Still's exanthema, have to be fulfilled.

The course of disease is not always benign and can be challenging to address by conventional therapeutics and biologics. Some patients show continuous disease activity and joint damage over years; furthermore, complications such as a diffuse intravascular coagulation or acute hepatic failure can occur [4]. NSAIDs (non-steroidal anti-inflammatory drugs), glucocorticosteroids, disease-modifying anti-rheumatic drugs (DMARD) (methotrexate, cyclophosphamide, hydroxychloroquine) are basic therapeutics for AOSD, while biological agents such as blockers of tumour necrosis factor (TNF), blockers of the interleukin-1 receptor (IL-1) and IL-6 receptor antibodies are given in patients not responding sufficiently [5–7].

One of the major symptoms of AOSD is a recurrent quotidian fever of unknown origin. Recurrent episodes of fever, serositis, musculoskeletal symptoms and erysipeloid rashes or urticaria are typical symptoms of four different hereditary periodic fever syndromes (HPFS), comprehensively reviewed by Yao et Furst [8]. Namely, those HPFS are familial Mediterranean fever (FMF), tumour necrosis factor receptor-associated periodic syndrome (TRAPS), mevalonate kinase deficiency (MKD), and cryopyrin-associated periodic syndromes (CAPS). TRAPS and CAPS are autosomal dominantly inherited diseases and are associated with heterozygous mutations in *TNFRSF1A* and *NLRP3*, respectively, whereas FMF and MKD are autosomal recessive diseases and associated with homozygous or compound heterozygous mutations in *MEFV* and *MVK*, respectively.

Because of the considerably overlapping clinical features in HPFS and AOSD, we hypothesized that genes associated with HPFS can contribute to the pathogenesis of AOSD, as well. Therefore, we investigated whether point mutations or intragenic copy number variants affecting exons of any of these four candidate genes could be identified in a group of 40 AOSD patients.

Materials and methods

Patients

From 2013 to 2014, 40 AOSD patients were recruited by board certified rheumatologists either at the Department of Internal Medicine 3, Rheumatology and Immunology of the University Hospital Erlangen ($n=36$) or at the Department of Internal Medicine V, Division of Rheumatology at the University Hospital Heidelberg ($n=4$). 55% of the set of patients were female and 45% were male (Supplementary Table 1). The median age at onset [min; max] was 32 [11; 73] years and the median age at the time of recruitment was 41 [21; 77] years. Four patients did not receive any therapy at the time of recruitment, 29 received a therapy with biologicals and seven were treated with DMARDs only. An overview of clinical symptoms, laboratory findings and current treatment in each patient is given in Supplementary Table 1. In nine patients not fulfilling the Yamaguchi criteria infections and malignancies were excluded and other auto-inflammatory diseases less likely than AOSD. In all

but two of those nine patients, a high level of serum ferritin (defined as > 1000 ng/ml) and/ or a high level of IL-18 (defined as > 200 pg/ml) supported the diagnosis of AOSD further [9–11]. AOSD (and not HPFS) was also considered to be the most appropriate diagnosis, as the course of disease was sporadic, irregular and neither periodic nor recurrent or fever was not a major symptom. The nine patients had been seen for several years (median of 7 years [3; 22]) at the two clinical departments by experienced rheumatologists, and the diagnosis of AOSD was made clinically in a process of exclusion as indicated above and previously recommended by Yamaguchi et al. [3].

Parents and a sibling of one patient carrying the heterozygous variant c.184G>T/p.Gly62Trp in *MEFV* were available for targeted analysis of this variant. Written informed consent was obtained from each individual before enrolment in the study. The investigations were conducted according to the Declaration of Helsinki principles; the study was approved by the research ethics board of the FAU Erlangen Nurnberg.

Mutation analysis

DNA was extracted from whole blood ($n = 36$) or cell pellets ($n = 4$) with Chemagic DNA Blood kit special (PerkinElmer Chemagen, Baesweiler, Germany). All patients were sequenced for coding exons and flanking intronic sequences (± 10 base pairs) of the genes *TNFRSF1A, NLRP3, MVK* and *MEFV*. In general, primer design and the procedure of Sanger sequencing were performed as described before [12], while thermocyclers used were Mastercycler® Pro (Eppendorf AG, Hamburg, Germany). Sequences were analysed with the Software Sequencher 5.0 (Gene Codes Cooperation, Ann Arbor, USA). Genotyping rates of all amplicons were 100%. Variants were named according to the usual naming conventions with regard to reference sequences NM_001065.3 (coding for 455 amino acids) in case of *TNFRSF1A*, NM_001243133.1 (coding for 1036 amino acids) in case of *NLRP3*, NM_000243.2 (coding for 781 amino acids) in case of *MEFV* and NM_000431.3 (coding for 396 amino acids) as a reference sequence for *MVK*.

The frequency of all detected variants was determined in the largest European study group annotated in dbSNP as publicly available open resource [13]. We filtered for rare variants (minor allele frequency of less than 1%) and considered them as potential disease-causing/disease-contributing variants. In case of coding variants, they were evaluated for the following aspects: position in the gene, frequency in the Non-Finnish European population (NFE) given in the publicly available data of the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/) [14], conservation at nucleotide level with the tools GERP++ (Genomic Evolutionary Rate Profiling), PhyloP and SiPhy and predicted effects at protein level with the tools SIFT (Scale-invariant feature transform), Polyphen 2, LRT (likelihood ratio test), Mutation Taster and Mutation Assessor as implemented in Annovar2 [15].

Molecular modelling of rare and interesting (see below) missense variants was performed based on experimental structures that were available for the respective protein domains: pyrin domain of *MEFV* [16], the B30.2 domain of *MEFV* [17], and the extracellular domain of *TNFRSF1A* [18]. Variants were modelled using Swiss-PdbViewer [19] and the lowest-energy rotamer was selected for each mutated side chain. RasMol [20] was used for structure analysis and visualization. We also performed a PubMed database search for all identified variants.

Classification

Six rare coding variants, considered as potentially damaging by our first evaluation, were classified according to guidelines of the American College of Medical Genetics (ACMG) [21]. For final classification, we used the freely available online tool (http://www.medschool.umaryland.edu/Genetic_Variant_Interpretation_Tool1.html/) as published recently [22] (Table 1).

Quantitative analysis

The 36 patients recruited in Erlangen were also analysed for intragenic deletions and duplications by the quantitative method Multiplex Ligation-dependent Probe Amplification (MLPA) [23]. We designed oligonucleotides for all coding exons of *TNFRSF1A* and *NLRP3* and for all but one exon of *MVK* according to the guidelines of the manufacturer (MRC Holland, Amsterdam, the Netherlands). In case of *MEFV*, we used SALSA MLPA P094-B01 *MEFV* probemix. MLPA was performed and analysed as described before [24]. The genotyping rate was 100% for each DNA.

Analysis of intronic variants

We analysed two intronic variants for potential splicing effects with the tools of BDGP (Berkeley Drosophila Genome Project, Berkeley, USA) [25] and the Human Splicing Finder 3.0 (HSF) [26].

Statistical analysis

As the group of patients comprised 40 patients, we determined median values of age at onset and of age at recruitment. We calculated the average value of the two middle elements, as the size of distribution was even.

The sum of mutations, classified as pathogenic, likely pathogenic or variant of uncertain significance (VUS)

Table 1 Classification of six coding variants according to ACMG guidelines. One variants was classified as "variant of uncertain significance", 4 as "likely pathogenic" and 1 as "pathogenic"

Gene	*TNFRSF1A*		*NLRP3*	*MEFV*		
Ref. seq.	NM_001065.3		NM_001243133.1	NM_000243		
Nucleotide	c.242G>A (Ex3)	c.596T>A (Ex6)	c.598G>A (Ex5)	c.184G>T (Ex1)	c.1958G>A (Ex10)	c.2230G>T (Ex10)
dbSNP	rs104895220	rs104895247	rs121908147	NR	rs104895085	rs61732874
pos. hg19	12:6442983	12:6440048	1:247587343	16:3306404	16:3293529	16:3293257
Variant	p.Cys81Tyr	p.Ile199Asn	p.Val200Met	p.Gly62Trp	p.Arg653His	p.Ala744Ser
Further names	p.Cys52Tyr [36]	p.Ile170Asn [38, 49]	p.Val198Met [50]	NR	NR	NR
PVS1	NF	NF	NF	NF	NF	NF
PS1	NF	NF	NF	NF	NF	NF
PS2	NF	NF	NF	NF	NF	NF
PS3	NF	[38]	NF	NF	NF	NF
PS4	NF	NF	NF	NF	NF	[31]
PM1	[35, 36]; 3D structure modelling[a]	[35, 36]; 3D structure modelling[a] [38]	[51]	3D structure modelling[a]	[30]; 3D structure modelling[a]	[30]; 3D structure modelling[a]
PM2	0/12,874 NFE controls (Cov. 25.3x) [14]	0/12,874 NFE controls (Cov. 51.9x) [14]	NF	0/33,341 NFE controls (Cov. 87.0x) [14]	2/66,624 NFE controls (Cov. 90.8x) [14]	NF
PM3	NF	NF	NF	NF	NF	NF
PM4	NF	NF	NF	NF	NF	NF
PM5	p.Cys81Phe in ClinVar [37]	NF	NF	NF	NF	NF
PM6	NF	NF	NF	NF	NF	NF
PP1	NF	[38]	[52]	NF	[53, 54]	NF
PP2	[36]	[36]	[55]	[32]	[32]	[32]
PP3	Annovar2 [15] (Supp.)	NF	NF	Annovar2 [15] (Supp.)	NF	NF
PP4	NF	NF	NF	NF	NF	NF
PP5	NF	NF	NF	NF	ClinVar (4) (45)	ClinVar (4) (45)
BP1	NF	NF	NF	NF	NF	NF
BP2	NF	NF	NF	NF	NF	NF
BP3	NF	NF	NF	NF	NF	NF
BP4	NF	Annovar2 [15] (Supp.)	Annovar2 [15] (Supp.)	NF	Annovar2 [15] (Supp.)	Annovar2 [15] (Supp.)
BP5	NF	NF	NF	NF	NF	NF
BP6	NF	NF	NF	NF	NF	NF
BP7	NF	NF	NF	NF	NF	NF
BS1	NF	NF	NF	NF	NF	NF
BS2	NF	NF	NF	NF	NF	NF
BS3	NF	NF	NF	NF	NF	NF
BS4	NF	NF	NF	NF	NF	NF
BA1	NF	NF	NF	NF	NF	NF
Classification	Likely pathogenic (IV)	Pathogenic (IIIb)	VUS	Likely pathogenic (V)	Likely pathogenic (V)	Likely pathogenic (II)

BA benign stand alone, *BP* benign supporting, *BS* benign strong, *Cov* coverage, *Ex* exon, *NFE controls* non-Finnish European controls in ExAC, *NF* not fulfilled, *NR* not reported, *PM* pathogenic moderate, *PP* pathogenic supporting, *PS* pathogenic strong, *PVS* pathogenic very strong, *ref. seq*. reference sequence, *Supp*. supplementary table 2, *VUS* variant of unknown significance, *x* times coverage

[a] According to 3D structure modelling with Swiss-PdbViewer and RasMol (more details in "Materials and Methods" and "Results")

according to the ACMG guidelines [21] was determined in the group of AOSD patients separately for *MEFV, NLRP3* and *TNFRSF1A*. In addition, the sum of the corresponding rare alleles in non-Finnish European control individuals of publicly available database (ExAC) was extracted. Absolute numbers of novel alleles were set in relation to the lowest number of wild-type alleles and used in the statistical analysis. In case of c.242G>A/p.Cys81Tyr in *TNFRSF1A*, we did not observe a single allele in this large cohort. Therefore we used two neighbouring variants, 7 bp upstream or 14 bp downstream of p.Cys81Tyr (namely c.249G>A/p.Ser32Asn and c.228G>A/p.Gly25Asp), to check whether the exonic region was covered sufficiently. We used the lower number of wild-type alleles (in this case of c.249G>A/p.Ser32Asn) for statistical analyses. The same was applied for c. 184G>T/p.Gly62Trp. Here we used data of the synonymous variant c.186G>A/p.Gly62Gly located in the same codon. All publicly available data had a genotype quality of 100% in carriers of the rare variants and the site quality metrics was above average. Fisher's exact test, as implemented in R (version 2.15.3) [27] was used to test for significant differences in allele frequency between AOSD patients and the control group as described before [28] (Table 2). To take the number of separate statistic tests into account, p values were adjusted by Bonferroni ($n=3$ tests).

Results

In total, 61 single nucleotide variants were detected in the group of 40 AOSD patients, while we did not observe any evidence for an intragenic deletion or duplication. Based on the variants' position and effect in the gene, 12 missense variants and five variants located in flanking intronic regions were observed (Fig. 1). In a second step, variants with a minor allele frequency of more than 1% in NFE controls were excluded, leaving ten variants (eight exonic and two intronic ones) for further assessment. The eight exonic variants were analysed with Annovar2. Two variants (*MEFV* c.866C>T/p.Ala289Val, c.926C>T/p.Thr309Met) were excluded from further analysis because of uniformly benign results in Annovar2 (low conservation and benign predictions on protein level). Two heterozygous intronic variants, namely IVS8-7T>G in *MVK* and IVS4+7G>A in *NLRP3*, were analysed with two splice site prediction programs. As both programs did not indicate alternative splicing, the variants were excluded from further classification and statistical analysis.

As a next step, 3D-structure of five of the six remaining missense variants was analysed (Supplementary Table 2). Two of them, namely c.1958G>A/p.Arg653His and c.2230G>T/p.Ala744Ser, were located on the surface of the B30.2 domain of *MEFV* gene. Globular B30.2 domain and a helical coiled-coil domain form the C-terminal part of *MEFV* (Fig. 2a). The coiled-coil domain is required for homodimer formation, whereas the B30.2 domain interacts with inflammasome components [29]. Both variants in the B30.2 domain might therefore affect ligand binding properties.

Interestingly, a recent crystal structure [17] revealed that c.1958G>A/p.Arg653His was also capable of forming intramolecular contacts with p.Glu552/Gln555 that stabilized a

Table 2 Association analysis

Locus	Allele	AOSD patients ($n=40$)	Eur-controls[a]	p_c value[b]
NLRP3	c.598G>A/p.Val200Met	1 (0.01)	662 (0.01)	1.65
	Σ mutant alleles	1 (0.01)	662 (0.01)	
	Σ wild-type alleles	79 (0.99)	65,894 (0.99)	
TNFRSF1A	c.242G>A/p.Cys81Tyr	1 (0.01)	0 (0.00)	2.40E−04
	c.596T>A/p.Ile199Asn	1 (0.01)	2 (0.00)	
	Σ mutant alleles	2 (0.02)	2 (0.00)	
	Σ wild-type alleles	78 (0.98)	21,640 (1.00)	
MEFV	c.1958G>A/p.Arg653His	1 (0.01)	2 (0.00)	2.34E−03
	c.2230G>T/p.Ala744Ser	1 (0.01)	143 (0.00)	
	c.184G>T/p.Gly62Trp	1 (0.01)	0 (0.00)	
	Σ mutant alleles	3 (0.04)	145 (0.00)	
	Σ wild-type alleles	77 (0.96)	66,393 (1.00)	

Absolute numbers (frequencies) of one variant in *NLRP3*, two rare variants in *TNFRSF1A* and three in *MEFV*, and results of a gene wise association analysis comparing the frequency of rare alleles in the group of 40 AOSD patients to 66,556/10,821/33,269 NFE controls of ExAC

[a] Non-Finnish Europeans of ExAC, $n=66,556$ in *NLRP3,* $n=10,821$ in *TNFRSF1A* and $n=33,269$ in *MEFV*

[b] Corrected p values according to Bonferroni

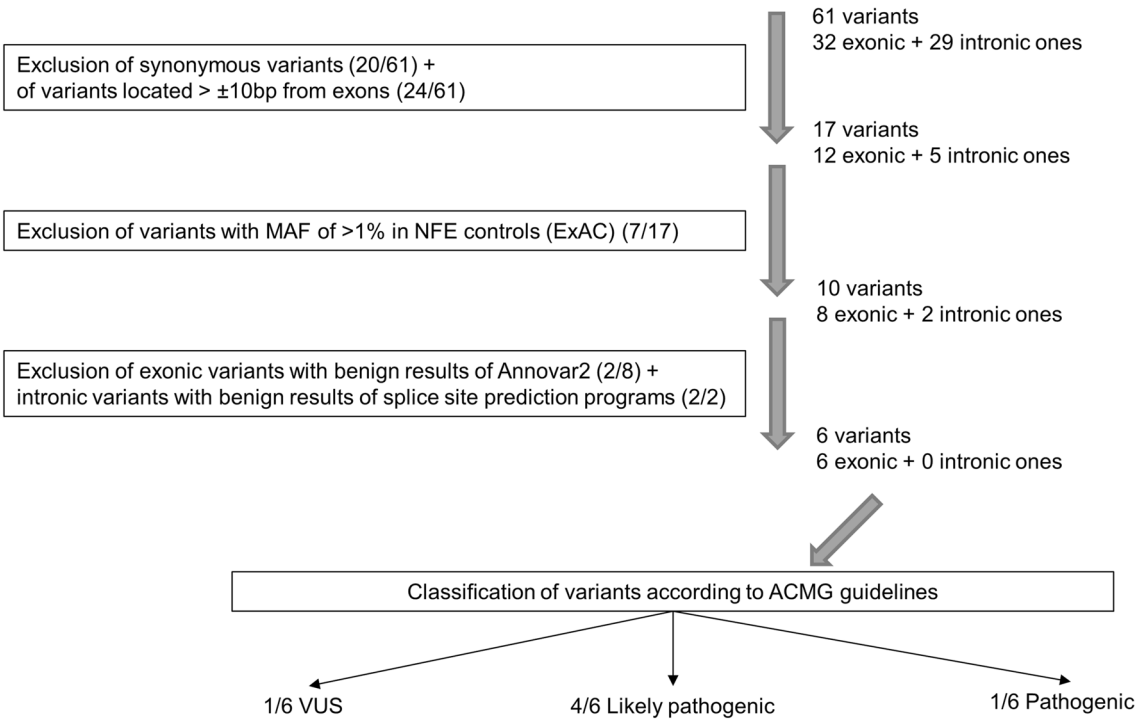

Fig. 1 Procedure of different analyses of variants in HPFS genes to identify disease-causing/ disease-contributing variants. All variants identified in this study were evaluated using the same aspects for all exonic and intronic variants, respectively. Those aspects are indicated on the boxes at the left side, while the number of variants remaining after each step is shown at the right side. *MAF* minor allele frequency, *NFE controls* non-Finnish Europeans controls

closed tetrameric conformation (Fig. 2a, b). It was speculated that ligand binding to the B30.2 domain competed with the respective interaction, thereby leading to an open *MEFV* conformation [17]. Our inspection of variant c.1958G>A/p.Arg563His showed that the interactions, which stabilized the closed conformation, could not be formed due to the shorter histidine side chain (Fig. 2c). Consequently, the c.1958G>A/p.Arg653His variant was expected to shift the equilibrium between the open and closed states of *MEFV*, and thereby also the binding affinity of B30.2 ligands.

The third *MEFV* variant c.184G>T/p.Gly62Trp was located in the N-terminal pyrin domain, which constitutes a protein interaction domain. Gly62 was located in a sterically demanding turn and adopted backbone dihedral angles that were only feasible for glycine ($\Phi = +137°; \psi = +169°$) (Fig. 2d). Replacement of Gly62 by tryptophan leads to steric clashes between the Cβ-carbon of Trp62 and the carboxyl oxygen of Tyr61 (Fig. 2e; magenta arrow). This clash will at least result in local structural changes and might additionally cause the unfolding of the entire domain.

The c.242G>A/p.Cys81Tyr variant in *TNFRSF1A* was located in the extracellular domain of this protein, involved in TNF binding. Cys81 formed a disulfide bond with Cys62 (Fig. 3a), thereby stabilizing a β-sheet structure of the domain. The c.242G>A/p.Cys81Tyr exchange disrupted this disulfide bond (Fig. 3b), which was expected to destabilize the domain fold significantly and to affect TNF binding properties.

At this stage, we classified the six missense variants according to the ACMG guidelines (Table 1) [21], as this classification allowed a more standardised evaluation of the pathogenic potential of genetic variants. All three variants in *MEFV* gene were classified as likely pathogenic (c.184G>T/p.Gly62Trp, c.1958G>A/p.Arg653His, c.2230G>T/p.Ala744Ser). p.Arg653His and Ala744Ser are both located in exon 10, a mutational hotspot in *MEFV* according to Dodé et al. [30]. This location and the extreme low frequency of 0.003% in NFE controls of ExAC [14] were regarded as two moderate pathogenic criteria according to ACMG. Coker et al. [31] identified a prevalence of 0.85% of p.Ala744Ser among FMF patients, which is markedly higher than the prevalence of 0.2% in NFE controls in ExAC [14], corresponding to a strong pathogenic criterion of ACMG classification.

c.184G>T/p.Gly62Trp was absent in ExAC, EVS (Exome Variant Server) and 1000 genomes, corresponding to a moderate pathogenic criteria. Furthermore, missense mutations in *MEFV* have been identified as the main type of mutations in FMF [32]. Analysis of c.184G>T/p.Gly62Trp with Annovar2 indicated a high conservation and evidence for

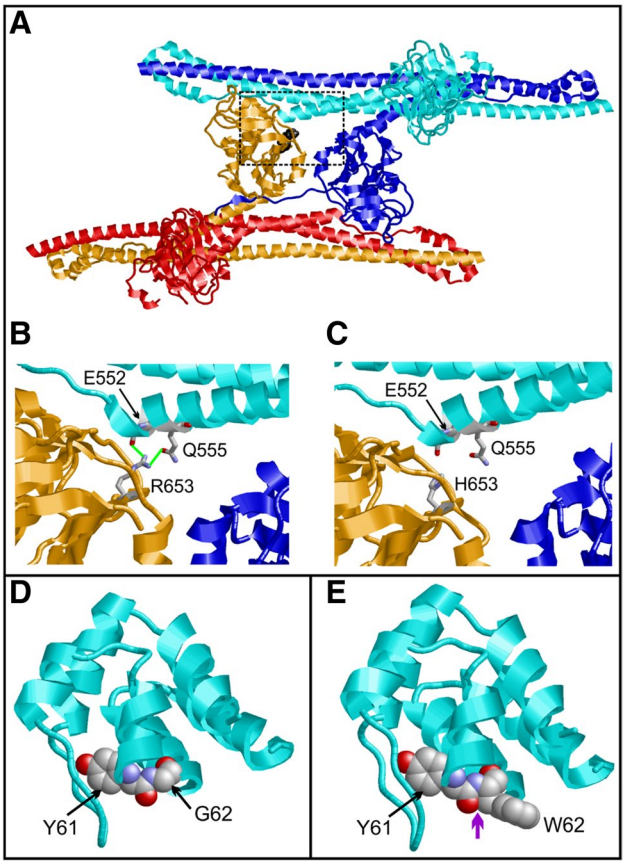

Fig. 2 Molecular modelling of two *MEFV* variants. **a** Structure of the coiled-coil and B30.2 domain structure of *MEFV*. The coiled-coil domains form dimers (red/orange, cyan/blue). In the closed conformation, the B30.2 domains mediate interactions between two dimers (e.g. orange/cyan). R653 of one subunit is shown in black its vicinity as an enlargement in panels (**b, c**). **b** Intermolecular interactions between R653 and E552/Q555 of two *MEFV* subunits. **c** In H653, interactions cannot be formed due to shorter histidine side chain. **d** G62 is located in a tight turn in the pyrin domain of *MEFV*. **e** A G62W replacement results in steric clashes (magenta arrow)

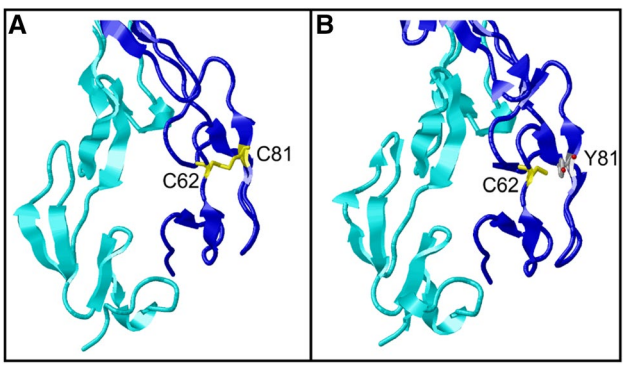

Fig. 3 Molecular modelling of one *TNFRSF1A* variant. **a** C81 of *TNFRSF1A* forms a disulfide bond with C62 in the extracellular domain of the protein. **b** The disulfide bond cannot be formed in the C81Y variant resulting in a drastic destabilization of the domain

pathogenicity by three of five prediction programs (Supplementary Table 2), supporting the pathogenic effect of p.Gly62Trp. 3D-structure modelling suggests that the variant leads to local structural changes and might additionally cause the unfolding of the entire domain. Overall, this variant was also classified as a likely pathogenic variant. The genetic testing of the family members revealed that the unaffected mother and sister carried the variant as well.

The variant c.598G>A/p.Val200Met in *NLRP3* was classified as VUS. On the one hand, the location in exon 5 as the mutational hotspot of *NLPR3* argued for a disease-relevant variant, but on the other hand, the frequency of 0.99% among NFE controls in ExAC [14] rendered pathogenicity unlikely. The variant is controversial discussed in the literature, e.g. [33, 34] consistent with a classification as VUS in our study.

TNFRSF1A variant c.242G>A/p.Cys81Tyr was classified as likely pathogenic, as it fulfilled three moderate pathogenic criteria: its location in the extracellular domain, a mutational hotspot, [35, 36], its absence in NFE controls [14] and the pathogenic classification of a different variant (c.242G>T/p.Cys81Phe) by ClinVar that affected the same amino acid [37].

The second variant in *TNFRSF1A*-c.596T>A/p.Ile199Asn—was graded as pathogenic. Kriegel et al. [38] analysed structural effects of the variant, leading to a disturbed cleavage of the TNF1 receptor. Additionally, complete segregation of the clinical phenotype with the variant within the published family was observed. Like p.Cys81Tyr, the variant is located in the extracellular domain and was absent in NFE controls of ExAC [14]. Overall, one strong pathogenic criterion, two moderate and three supporting pathogenic criteria were fulfilled.

Interestingly, two of the three carriers of interesting *MEFV* variants obtained a combination of therapies including biologicals and the two carriers of *TNFRSF1A* received biological therapy (Supplementary Table 1), indicating a more severe course of disease in carriers of those variants.

Last but not least, we performed a statistical analysis, comparing the frequency of the six variants categorized as VUS, likely pathogenic or pathogenic in AOSD patients to the frequency of those variants in NFE controls of ExAC [14] in *NLRP3, MEFV* and *TNFRSF1A* separately (Table 2). Thereby, we observed no significant association with the variant in *NLRP3* ($p_c = 1.65$), but with the two variants in *TNFRSF1A* ($p_c = 2.40\text{E}-04$) and the three *MEFV* variants ($p_c = 2.34\text{E}-03$).

Discussion

To the best of our knowledge, our analysis in a cohort of AOSD patients covered qualitative and quantitative variants in the coding regions of HPFS genes for the first time and

included more genes than previously described [39–41]. In previous studies of HPFS genes in AOSD, single coding variants and/ or only hotspot regions of *MEFV* were sequenced for small coding variants. Association was either only marginal significant or not observed. For two individual carriers of relevant *MEFV* mutations, segregation analysis within the families was not possible, while in case of the third interesting one, classified as likely pathogenic (c.184G>T/p.Gly62Trp), two further healthy family members carried the variant. Based on the understanding of FMF as an autosomal recessively inherited disease, we assume that a combination of these rare *MEFV* variants with additional risk factors might cause AOSD. Most probably, the additional risk factors will be genetic ones in genes yet to be identified. The patient subgroup that obtained a combination of therapies including biologicals at the time of recruitment and that had therefore a more severe clinical course of disease comprised two of three carriers of *MEFV* variants. The numbers are fairly small to draw any conclusions, but might indicate the potential of genetic risk factors in predicting clinical outcome. A therapeutic regimen including colchicine will be considered in all three carriers of *MEFV* variants.

Moreover, we observed strong evidence for association with rare coding variants in *TNFRSF1A* for the first time. This result is in contrast to a previously published, smaller group of 20 AOSD patients: Cosan et al. [39] did not detect a single coding variant in the gene associated to TRAPS. In contrast to the carriers of single *MEFV* mutations, we consider the two variants in *TNFRSF1A* as the disease-causing ones and therefore as mutations, as TRAPS is known as an autosomal dominantly inherited disease. Unfortunately, we were unable to perform segregation analysis in these patients' families as well. Overall, our study provides some evidence that TRAPS should be considered as a differential diagnosis in AOSD, as 5% of our patients (2/40) carried a mutation in *TNFRSF1A*. Similarly, as in case of carriers of *MEFV* variants, the two carriers of *TNFRSF1A* variants obtained a therapy with a biological indicating a more severe clinical course. Moreover, our results might suggest screening AOSD patients with a challenging course of disease for variants in *TNFRSF1A*.

In contrast, we did not detect any noteworthy variant in *MVK*, while we identified variant c.598G>A/p.Val200Met in *NLRP3* once. In case of this latter variant, the ACMG classification lead to an assignment to a VUS, supporting the controversy over this missense variant. Our approach based on Sanger sequencing though did not cover any somatic mutations. Interestingly, somatic mutations in *NLRP3* have been described in a high percentage of patients (69%) with a clinical form of CAPS, named chronic infantile neurologic, cutaneous, articular syndrome (CINCA) [42].

Considering our findings of disease-causing variants in HPFS genes in only 5% of our AOSD patients, other risk factors will be probably more important as disease-causing or disease-contributing factors. Two publications on individual patients with AOSD described viral infections as a potential trigger for disease manifestation [43, 44]. These cases seem to be rare, but they might be underdiagnosed due to the often more complicated, prolonged diagnosis of AOSD; still, coincidental findings cannot be excluded. To show a relevant association, systematic studies that include more patients should be performed. In this context, results of a large study of 96 AOSD patients and 64 healthy controls are worth mentioning: similar rates of past infections for parvovirus B19 were observed (50 versus 47%) [45].

Even though larger epidemiological studies have been performed, there is no evidence for familial AOSD cases to the best of our knowledge. A single twin pair followed up for 8 years remained discordant for AOSD [46], while a small study of 11 twin pairs with juvenile Still's disease indicated partial genetic contribution [47]. Therefore, AOSD has previously been considered a sporadic disease [48], rendering autosomal dominant, autosomal recessive and X-linked inheritance less likely and suggesting somatic mutations or mutations that arose de novo as more probable disease models. Therefore, using *next generation sequencing* strategies to identify disease-causing genes will be promising.

Conclusion

In conclusion, we observed evidence for significant association of AOSD with variants in *MEFV* and *TNFRSF1A*, however, not in *NLRP3*. One rare variant in *MEFV* that has not been described before was classified as likely pathogenic. Two of the three carriers of *MEFV* variants had a severe course of disease, and the two carriers of *TNFRSF1A* variants received biological therapy, therefore, a more challenging course of disease might motivate to perform molecular genetic diagnostic of *TNFRSF1A*. Moreover, our study indicated that genetic risk factors other than mutations in HPFS genes remain to be identified in the majority of AOSD patients.

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Author contributions J.R., A.H. and U.H. designed the study. J.R., A.H. and N.B. recruited patients and collected clinical data. Genetic, statistical and bioinformatics analyses were performed by R.S., S.L. and H.S. All authors were involved in the interpretation of data. R.S. and U.H. wrote the manuscript that was read and approved by all authors.

Compliance with ethical standards

Ethical approval The study was conducted according to the Helsinki agreement and approved by the research ethics board of the FAU Erlangen Nürnberg under the protocol number 52_14 B in 2014 and changes were approved in 2015.

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