

## Original article

# Mutations in the perforin gene can be linked to macrophage activation syndrome in patients with systemic onset juvenile idiopathic arthritis

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## Abstract

**Objective.** Macrophage activation syndrome (MAS) in systemic onset juvenile idiopathic arthritis (SoJIA) is considered to be an acquired form of familial haemophagocytic lymphohistiocytosis (fHLH). FHLH is an autosomal recessive disorder, characterized by diminished NK cell function and caused by mutations in the perforin gene (*PRF1*) in 20–50% of patients. Interestingly, SoJIA patients display decreased levels of perforin in NK cells and diminished NK cell function as well. Here, we analysed *PRF1* and its putative promoter in SoJIA patients with or without a history of MAS.

**Methods.** DNA of 56 SoJIA patients (41 Italian and 15 Dutch) was isolated. Of these, 15 (27%) had a confirmed history of MAS. We sequenced *PRF1* and 1.5 kb of the 5'-upstream region. DNA sequence variations in the promoter region were functionally tested in transfection experiments using a human NK cell line.

**Results.** We detected a previously undescribed sequence variation (–499 C > T) in the promoter of *PRF1* in 18% of the SoJIA patients. However, transfection experiments did not show functional implications of this variation. Secondly, we found that 11 of 56 (20%) SoJIA patients were heterozygous for missense mutations in *PRF1*. In particular, we found a high prevalence of the Ala91Val mutation, a variant known to result in defective function of perforin. Interestingly, the prevalence of Ala91Val in SoJIA patients with a history of MAS (20%) was increased compared with SoJIA patients without MAS (9.8%). One SoJIA patient, heterozygous for Ala91Val, showed profound decreased perforin levels at the time of MAS.

**Conclusions.** These findings suggest that *PRF1* mutations play a role in the development of MAS in SoJIA patients.

**Key words:** Systemic JIA, Macrophage activation syndrome, Perforin, Lymphocytes, NK cells.

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## Introduction

Systemic onset juvenile idiopathic arthritis (SoJIA) is characterized by systemic signs of inflammation besides arthritis and constitutes 4–17% of all JIA [1]. Its adult equivalent is adult-onset Still's disease.

Arthritis in SoJIA is often severe and debilitating and has a polyarticular course. Systemic signs of inflammation in SoJIA include a spiking fever, a skin rash, generalized lymphadenopathy, hepatosplenomegaly and sometimes serositis (more often pericarditis), making SoJIA a multisystem inflammatory disease.

Intriguingly, especially SoJIA patients are at risk for developing the serious inflammatory complication, macrophage activation syndrome (MAS), in the course of their disease [2, 3]. In general, MAS is not seen in patients with other types of JIA. MAS is thought to occur in ~10% of the SoJIA patients, although recent observations suggest that subclinical forms of MAS can be found in as many as 30% of the SoJIA patients [4].

The systemic symptoms and occurrence of MAS in SoJIA, along with the abrupt onset, the near equal ratio of affected male to female patients and the patterns of cytokines and laboratory abnormalities suggest that SoJIA is a distinct entity, strikingly different from other types of JIA [5]. Likewise, the pathogenesis of SoJIA seems to differ from other types of JIA in some important aspects. Unlike the other types of JIA, there are no known associations of SoJIA with HLA type [6] and autoantibodies or autoreactive T cells have not been identified so far.

These and other observations made in SoJIA patients suggest that, unlike the other types of JIA, in SoJIA, the role of adaptive immune responses may be limited. Instead, the role of the innate arm of the immune system, including monocytes/macrophages, NK cells and also neutrophils, seems to be more prominent [7, 8]. Many clinical features of SoJIA can be explained by immune responses involving IL-1, -6, -8, -18 and monocyte chemoattractant protein-1 [9–13].

Several groups have reported decreased levels of perforin in cytotoxic T cells and in particular NK cells, along with decreased NK cell function in SoJIA [14–16]. Perforin is a key molecule in the granule-mediated exocytosis pathway. This pathway is vital in immune responses to (intracellular) micro-organisms and in surveillance for malignancies [17]. Moreover, it has an important role in the maintenance of lymphoid and myeloid immune homeostasis, as shown in children with familial haemophagocytic lymphohistiocytosis (fHLH), a rare autosomal recessive disorder [17]. Mutations in several genes related to the granule-mediated exocytosis killing pathway cause fHLH [18–20]. Of these, the perforin gene (*PRF1*) coding for the pore-forming protein perforin (PRF) seems to be the most important.

As it shares important clinical, immunological and histopathological features with familial HLH, MAS in SoJIA is considered to be an acquired form of HLH [21]. Here, we studied a large cohort of Italian and Dutch SoJIA patients, both with and without a history of MAS. We analysed *PRF1* and its proximal 5'-regulatory region and performed luciferase-reporter assays to functionally test promoter variations addressing the question of whether mutations in *PRF1* and its putative promoter contribute to the pathogenesis of SoJIA in general or MAS in SoJIA specifically.

## Patients and methods

### Patients

We included 41 Italian and 15 Dutch patients with SoJIA, diagnosed according to the ILAR criteria [22]. Mean age at

disease onset was 4.7 (range 0.5–15) years. Of these, 15/56 (27%) had a confirmed history of MAS. MAS was diagnosed either on clinical grounds by the attending physician and/or confirmed on bone marrow biopsy (performed in 11 of 15 MAS patients).

Peripheral blood of all patients was obtained during outpatient visits or during admission to the hospital to isolate DNA. Moreover, fresh samples were used to determine perforin expression in NK cells and NK cell lytic function in all Dutch patients ( $n = 15$ ), including one SoJIA patient during an episode of MAS. Controls consisted of 15 polyarticular JIA patients and 15 healthy controls.

Informed consent was obtained either from parents/guardians or from the children directly when they were >12 years old of both JIA patients and healthy controls. The study was approved by the local ethics committees of the Wilhelmina Children's Hospital in Utrecht (METC number 03/211) and by the Institutional Review Boards of the IRCCSG Gaslini in Genova and the IRCCS Ospedale Pediatrico Bambino Gesù in Rome.

### Flow cytometry

Intracellular staining of perforin in NK cell subsets was performed in aliquots of 100  $\mu$ l of whole blood. Briefly, whole blood was incubated with appropriately diluted PE, PerCp- or APC-labelled mAbs against human CD3 (BD Biosciences, San Jose, CA, USA; clone SK7), CD16 (clone 3G8; BD Biosciences) or CD56 (clone C5.9; Dako, Carpinteria, CA, USA). For intracellular staining of perforin (clone  $\Delta$ G9; Kordia Life Sciences, Leiden, The Netherlands), the cells were first surface stained, then fixed and permeabilized in cytofix/cytoperm solution (BD Biosciences). Cells were incubated with 2% human AB serum to block aspecific binding and subsequently incubated with a FITC-labelled antibody to perforin. Finally, stained mononuclear cells were diluted in sheath fluid and run on a FACSCalibur (Becton Dickinson, San Jose, CA, USA). CellQuest software was used for analysis.

### NK cell cytotoxicity assays

NK cell activity was assessed by a standard 4-h  $^{51}\text{Cr}$ -release assay with K562 cells as target cells. Briefly, K562 cells were washed in RPMI-1640 medium supplemented with 5% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD, USA), 200 mg/ml glutamine and penicillin/streptomycin (Gibco BRL), counted and loaded with  $^{51}\text{Cr}$  at 37°C. After 1 h, cells were washed thrice and 40 000 K562 cells (100  $\mu$ l) were co-cultured with 100  $\mu$ l of whole blood of patients or healthy controls. After 4 h,  $^{51}\text{Cr}$  release was assessed in 100  $\mu$ l of the supernatant in a Packard Cobra gamma counter (GMI, Ramsey, MN, USA) as a measure of NK cell killing of K562 cells. Spontaneous lysis of cells was assessed by determining the  $^{51}\text{Cr}$  release of K562 cells incubated with medium alone, whereas maximum lysis was assessed by determining  $^{51}\text{Cr}$  release of K562 cells incubated with 3% Triton. Results are expressed as specific lysis per cent calculated as follows: (sample  $^{51}\text{Cr}$

release – spontaneous release)/(maximum release – spontaneous release) × 100%.

### DNA sequence analysis of *PRF1*

DNA of all patients was isolated (PureGene Genome DNA Isolation kit; Gentra Systems, Minneapolis, MN, USA) and used for DNA sequence analysis of *PRF1* (GenBank NC 000010.9 and NM005041.3) and its immediate upstream region. PCRs were carried out using Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). All reagents were obtained from Applied Biosystems (Roche Molecular Systems, Branchburg, NJ, USA). The coding region (exons 2 and 3) of perforin was amplified using primers as previously described [17]. In addition, ~1.5 kb upstream of the initiator ATG as well as the complete untranslated region (UTR), covering exon 1 and part of intron 1, was amplified using additional primers (supplementary methods are available at *Rheumatology* Online). PCR products were purified before DNA sequence analysis using the Qiaquick PCR purification Kit (Qiagen, Valencia, CA, USA) or EXOSap-IT kit (USB, Cleveland, OH, USA). Automated DNA sequence analysis was performed with the ABI Prism dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions. Sequencing reactions were carried out in forward and reverse direction, and samples were analysed on an Applied Biosystems ABI 310 Genetic Analyzer (Applied Biosystems).

DNA of 98 Italian and 50 Dutch healthy controls was screened for the presence of the novel *PRF1* Arg28Cys variant and the novel C > T change at nucleotide –499 in the putative promoter by restriction enzyme digestion with *Nsp1* and *TaqI* $\alpha$ , respectively. Allele frequencies of formerly described mutations and variations in our study populations were compared with published data where appropriate.

### Cell culture, promoter constructs and transfections

The main body of the promoter construct used in this study was kindly provided by Dr S. K. Anderson (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD, USA) and contained 1136 bp upstream of the transcriptional start site (P2) [23]. This construct did not contain the 5'-UTR that comprises exon 1 and a small part (the first 4 bp) of exon 2. Since it has been described for other genes that the 5'-UTR may contain regulatory elements [24], we developed a construct including the complete UTR (P2 + UTR). This was done by using the Pfu DNA Polymerase Kit (Stratagene, La Jolla, CA, USA), sense primer 5'-GTTTC TAGAAGAGGGTGGGGAC-3' (nt –1237 to –1216 relative to the start codon) and antisense primer 5'-GGAGCTGGA ATCCCGTATAGAGAAGC-3' (from –26 to –5 bp with a GGAG-tag attached). The blunt-end PCR fragment was cloned into the PCR-Blunt vector using Top-10 competent cells according to instructions (Invitrogen, Paisley, UK). It was subsequently excised and inserted into the *MluI* and *XhoI* sites of the pGL3-Basic vector

(Promega, Madison, WI, USA). The sequence of the construct in the pGL3-basic vector was verified by DNA sequence analysis before mutagenesis.

Mutagenesis was performed using the QuickChange Site-Directed Mutagenesis System developed by Stratagene. The following primer pairs were used: sense 5'-CACTGC TGGTGATAATCGAGCTGCCCAAGCC-3' and antisense 5'-GGCTTGGGCAGCTCGATTATGCACCAGCAGTG-3' for mutation –499 C > T.

All constructs were verified by DNA sequence analysis before transfection. We cultured the human NK cell line YT Indy, known to constitutively express high levels of perforin, in RPMI medium, supplemented with 5% FCS, 1% penicillin/streptomycin and glutamine at 37°C. Cells were transiently transfected using Superfect (Qiagen) according to the manufacturer's instructions. Luciferase activities of wild-type promoter constructs were compared with those of the mutant promoter constructs (–499 C > T). The promoterless pGL3-Basic Luciferase Reporter Vector (Promega) was used as a negative control.

In order to investigate the influence of other, more or less SoJIA-specific conditions on the expression of perforin in NK cells in patients with different promoter variations, we compared normal transfection conditions with conditions where 10.0 or 100.0 ng/ml human recombinant IL-18 (MBL, Nagoya, Japan), 1.0 or 10.0 ng/ml human recombinant IL-6 (Strathmann Biotec AG, Hamburg, Germany) and 100 ng/ml human recombinant IL-6-soluble receptor (R&D Systems, Minneapolis, MN, USA) were added to the culture system or where the culture temperature was elevated (incubator temperature 40°C) mimicking fever.

### Statistical analysis

Comparisons of NK activity, perforin expression and luciferase activity were performed with the non-parametric Mann-Whitney U-test. Frequencies of mutations or sequence variations were compared using chi-square or Fisher's exact test. *P*-values were two tailed and the significance cut-off was *P* < 0.05.

## Results

For this study, we included 56 SoJIA patients from Italy (*n* = 41) and The Netherlands (*n* = 15). Of these, 15 (27%) had a confirmed history of MAS. As the diagnosis of MAS in active SoJIA patients is difficult, we used recently published clinical criteria for diagnosing MAS in SoJIA patients [25]. Table 1 shows patient characteristics of all SoJIA patients with a history of MAS with diagnostic parameters at the time of MAS.

All Dutch SoJIA patients (*n* = 15) were sampled, and freshly drawn peripheral blood mononuclear cells were FACS-stained to determine perforin expression in CD3-CD16+CD56<sup>dim</sup> NK cells. These were compared with the perforin expression in NK cells of polyarticular JIA patients and healthy controls. At the same time, lytic NK cell function was determined in <sup>51</sup>Cr-release assays. Interestingly, we were able to sample one SoJIA patient

TABLE 1 Patient characteristics of SoJIA patients at the time of MAS

	CRP, mg/l	WBC count, ( $<4.0 \times 10^9/l$ )	Ferritin, $\mu g/l$	Platelets ( $<262 \times 10^9/l$ )	Fibrinogen ( $<2500 mg/l$ )	ALAT ( $>59 U/l$ )	Persistent fever	CNS dysfunction	Haemorrhages	Hepatosplenomegaly	Haemophagocytosis on BMA
P1	UK	-	50 000	+	+	+	+	+	+	+	+
P2	10	+	ND	+	ND	+	+	-	-	+	+
P3	186	-	ND	-	-	-	-	-	-	+	ND
P4	287	-	7891	-	ND	+	-	-	-	+	+
P5 <sup>a</sup>	20	-	12 746	+	ND	+	+	+	+	+	+
P6	45	+	1792	+	-	+	-	-	-	+	ND
P7 <sup>a</sup>	140	+	ND	+	+	+	-	-	-	-	ND
P8	57	-	ND	+	+	-	+	-	-	+	+
P9	127	+	ND	+	+	+	+	-	-	+	+
P10	7	-	47 815	+	+	+	-	-	-	+	ND
P11	10	-	14 300	+	+	+	-	-	-	+	+
P12	40	+	10 043	-	+	+	-	-	-	+	+
P13	49	-	82 617	+	+	+	-	-	-	+	-
P14	300	-	58 265	+	+	-	+	-	-	+	+
P15 <sup>a</sup>	286	-	46 500	-	ND	+	+	-	-	+	+

<sup>a</sup>Patients suffered from two or more episodes of MAS. WBC: white blood cell count; BMA: bone marrow aspiration; ALAT: alanine aminotransferase; UK: data unknown; ND: data not determined.

during an episode of MAS and perform perforin staining of NK cell subsets as well as functional lytic NK cell assays.

As is shown in Fig. 1A, perforin expression in CD3-CD16+CD56<sup>dim</sup> NK cells of SoJIA patients was significantly decreased compared with healthy controls and poly-JIA patients. Furthermore, lytic NK cell function was deficient in SoJIA patients compared with healthy controls and poly-JIA patients, illustrated by decreased killing of K562 cells in a standard <sup>51</sup>Cr-release assay (Fig. 1B). Intriguingly, the SoJIA patient sampled at the time of MAS showed profoundly decreased perforin levels in CD3-CD16+CD56<sup>dim</sup> NK cells and severely decreased NK cell lytic function (indicated by arrows in Fig. 1A and B and by the overlay-histogram in Fig. 1C) compared with healthy controls and SoJIA patients not sampled at the time of MAS.

These results confirm laboratory findings of our lab and others showing deficiency of the granule-mediated cytotoxic pathway in NK cells in SoJIA patients [11, 12, 26]. Analysis of the SoJIA patient at the time of MAS suggests that during MAS, perforin expression and lytic NK cell function are even more depressed than during active SoJIA.

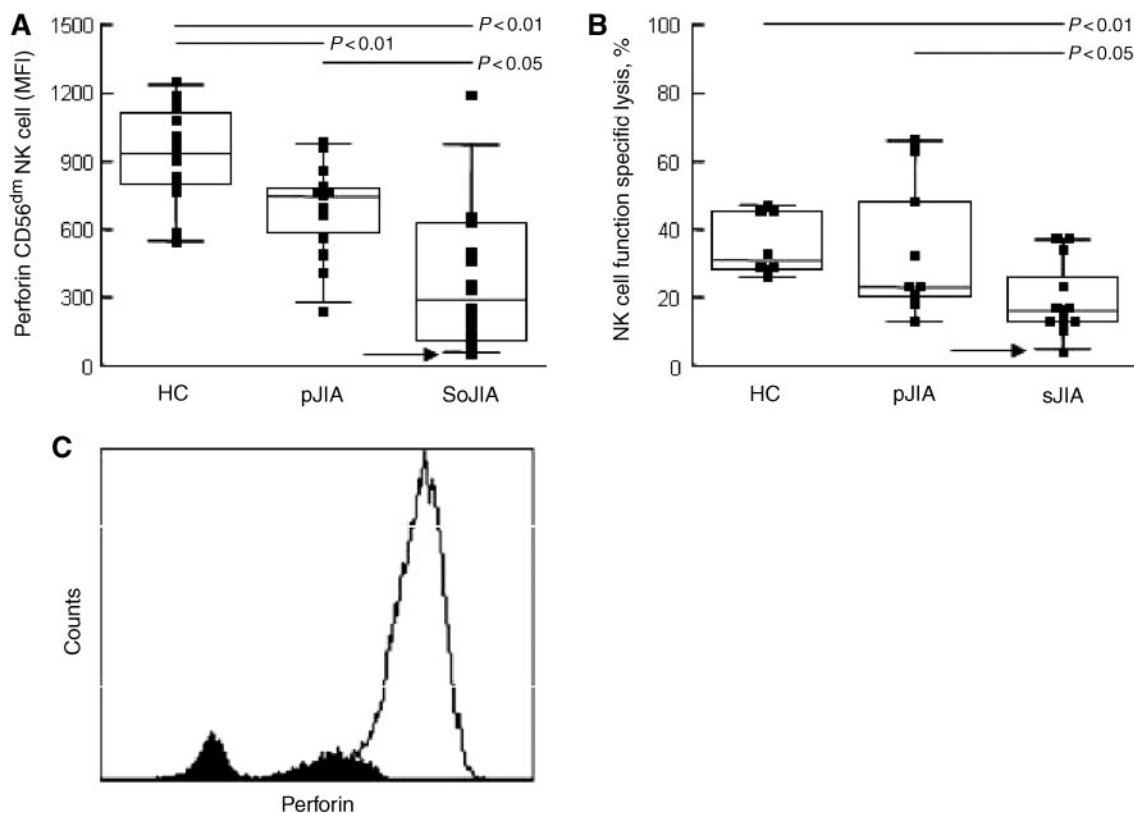
To test whether decreased perforin expression and MAS in SoJIA patients were associated with (heterozygous) mutations or promoter variations in *PRF1*, we characterized the coding region (exons 2 and 3), its 5'-UTR (exon 1 and the first 4 bp of exon 2) and 1.5 kb of its immediate 5'-upstream region in all 56 patients.

We found that 11 out of 56 SoJIA patients (~20%) were heterozygous for a missense mutation in *PRF1*. Four different mutations were identified: the formerly described Arg4His (one patient), Ala91Val (seven patients), Asn252Ser (three patients) and a novel Arg28Cys change (one Dutch patient). All mutations and sequence variations are listed in Table 2 with allele frequencies of the found mutations and variations in our study populations. For comparison, allele frequencies of formerly described variations and mutations in appropriate control populations are also listed. The yet undescribed mutation (Arg28Cys) found in one Dutch patient was not detected in 50 healthy Dutch controls (100 alleles).

Interestingly, we detected a rather high prevalence (7/56 patients, 12.5%) of heterozygosity for the Ala91Val variant in our cohort of SoJIA patients (Table 2). This variant is known to be of functional importance. Strikingly, heterozygosity for Ala91Val was twice as frequent in SoJIA patients with a history of MAS compared with SoJIA patients without a history of MAS (20 vs 9.8%, corresponding with allele frequencies of 10.0 and 4.9%, respectively). When compared with published control populations (in total more than 4000 healthy controls, of which more than 2500 Italian healthy controls as well as different northern-European control populations), the allele frequency of Ala91Val in our subgroup of sJIA with a history of MAS was increased at least 2-fold [27–32].

In addition to mutations or variations in the coding region, we also identified a C > T change at position

**Fig. 1** (A) Perforin expression in CD3-CD16+CD56<sup>dim</sup> NK cells of SoJIA patients, polyarticular JIA patients and healthy controls. The SoJIA patient sampled during MAS is indicated by an arrow. (B) Lytic NK cell function, measured by a standard <sup>51</sup>Cr-release assay using K562 target cells in different JIA groups and healthy controls. The SoJIA patient sampled during MAS is indicated by an arrow. (C) Perforin expression in CD3-CD16+CD56<sup>dim</sup> NK cells of a SoJIA patient at the time of MAS (filled graph) and healthy control (unfilled graph) stained in the same experiment. At the time of MAS, the fraction of perforin-negative cells within the CD3-CD16+CD56<sup>dim</sup> NK population is increased and the mean fluorescence intensity of perforin in the perforin-positive NK cells is decreased in a SoJIA patient compared with a healthy control.



–499 in the putative promoter region of *PRF1* in 10/56 patients (18%). The –499C>T promoter variant was again found in an increased frequency in SoJIA patients with a history of MAS (26.6%) compared with the cohort SoJIA patients without a history of MAS (14.6%, Table 2). However, we found comparable allele frequencies in our Italian ( $n=98$ ) and Dutch ( $n=50$ ) healthy control populations.

Intriguingly, the Dutch SoJIA patient sampled during MAS with profoundly decreased perforin levels and NK cell lytic function, as shown in Fig. 1, was heterozygous for both the –499 C > T variation and the Ala91Val mutation. To test whether –499 C > T imposed functional effects upon perforin expression in NK cells, we performed transient transfection assays of wild-type and mutated promoter constructs (P2 + UTR) in a human NK cell line. Promoter activities are shown in Fig. 2A. Under standard culture conditions, we did not find differences in luciferase activity between the wild-type putative promoter and mutant promoter constructs (Fig. 2A). To investigate the influence of other, more or less SoJIA-specific conditions on the expression of perforin

in NK cells, we also performed transfection experiments with *in vitro* culture conditions mimicking some features of active SoJIA. Therefore, human rIL-18 or rIL-6 + human rIL-6-soluble receptor was added in concentrations comparable to levels present in plasma of patients with active SoJIA. In addition, fever was mimicked by elevating culture temperature during transfection. The results of these *in vitro* simplified ‘multiple-hit’ experiments are demonstrated in Fig. 2B–D. No significant differences between wild-type and mutated constructs could be demonstrated.

## Discussion

In the past few years, our group and others [7, 14, 26] have shown that NK cell dysfunction is a distinguishing feature of SoJIA compared with other types of JIA, and suggested this to be of importance in the pathophysiology of this disease [16]. In the present study, we found an even more marked decrease in perforin levels of NK cells along with a further decrease of lytic NK cell function, in a SoJIA patient sampled at the time of MAS (Fig. 1A–C).

TABLE 2 *PRF1* genotypes in SoJIA patients

Patient	MAS	Genotype				
		–499 C > T	Arg4His	Arg28Cys	Ala91Val	Asn252Ser
P16	No					Ht
P20	No	Ho			Ht	
P22	No	Ht				
P23	No	Ht			Ht	
P29	No					Ht
P37	No	Ht			Ht	
P45	No	Ht			Ht	
P46	No	Ht				
P51	No		Ht			
P54	No			Ht		Ht
Allele frequency in SoJIA without MAS ( <i>n</i> = 41), %		8.5	1.2	1.2	4.9	3.7
P7	Yes	Ht				
P13	Yes	Ht			Ht	
P14	Yes	Ht			Ht	
P15	Yes	Ht			Ht	
Allele frequency in SoJIA with MAS ( <i>n</i> = 15), %		13.3	0	0	10.0	0
Allele frequency in control populations, %		5.0–10.2	1.0 [30]	0	1.0–5.8 [26–31]	0.4–0.7 [28, 31]

Almost 20% of the SoJIA patients harbour heterozygous mutations in *PRF1*. Displayed are all SoJIA patients with mutations in *PRF1* subdivided into SoJIA patients without (upper part) or with (lower part) a history of MAS. For comparison, allele frequencies in control populations are displayed in the lower row. Ht: heterozygous change, Ho: homozygous change.

This suggests that impaired NK cell cytotoxicity contributes to the susceptibility of SoJIA patients to develop MAS.

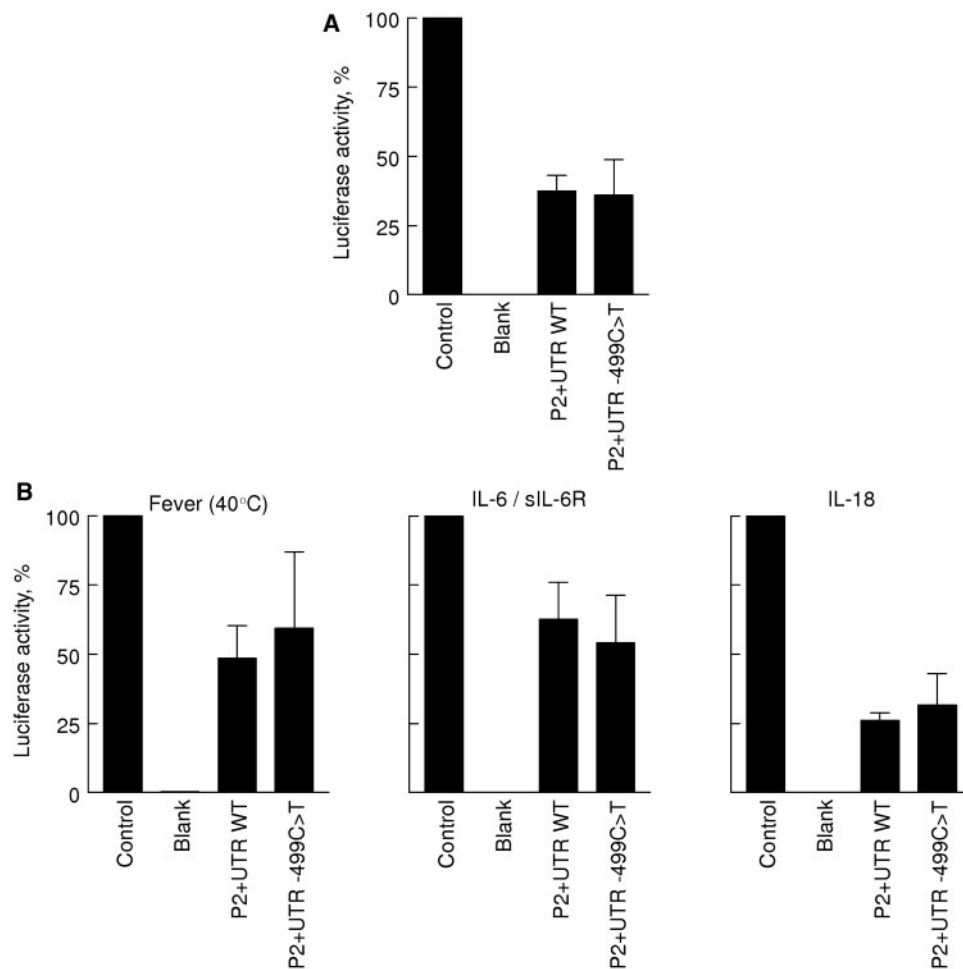
As SoJIA is not a monogenically inherited disease, it is likely that decreased levels of perforin in cytotoxic lymphocytes of SoJIA patients are the result of multiple, possibly interacting factors. In the current study, we performed a comprehensive analysis with regard to the role of genetic variations in *PRF1* in the pathogenesis of SoJIA in general, or MAS in SoJIA specifically. Our study included 56 SoJIA patients from four European paediatric rheumatology centres, representing one of the largest cohorts described so far in the literature. Importantly, our cohort includes a significant subgroup with a confirmed history of MAS. Besides the coding region of *PRF1*, we analysed a large segment of the putative promoter to include putative regulatory elements of *PRF1* gene expression.

We found that 11 out of 56 SoJIA patients (~20%) were heterozygous for a missense mutation in *PRF1*. Interestingly, we found one heterozygous mutation (Ala91Val) with a particularly high incidence (7/56 patients, 12.5%) in our cohort of SoJIA patients. Moreover, the Ala91Val prevalence was particularly high in SoJIA patients with a history of MAS (3/15; 20.0%) when compared with SoJIA patients without MAS (4/41; 9.8%;  $P > 0.05$  likely due to low numbers of patients).

The Ala91Val substitution in perforin is a polymorphic variant with an allele frequency of 1.0–5.5% in large population-based control studies (more than 4000 healthy controls, including more than 2500 Italians and different northern-European control populations) [27–29, 31]. The alanine at residue 91 is conserved [33] and several studies have suggested a causal link between Ala91Val and atypical (late) presentation of fHLH [34, 35]. The Ala91Val variant has been studied extensively in multiple datasets in the past few years. These studies have shown that substitution of alanine into valine results in both pre- (expression in lymphocytes) and post- (lytic capacity) synaptic defective function of perforin [33, 36, 37]. We therefore propose that in patients with systemic onset JIA, a complex disorder caused by multiple interacting genetic and environmental factors, heterozygosity for Ala91Val is a risk factor for the development of MAS. This is supported in our cohort by the observation of extremely low perforin levels in NK cells along with severely depressed lytic NK cell function in Patient 15, sampled at the time of confirmed MAS.

The heterozygous Arg4His and Asn252Ser changes are both formerly described polymorphisms and present, respectively, in 1/56 (1.8%) and 3/56 (5.4%) of patients in our cohort. These variants have been described to have no functional implications for the expression and lytic capacities of perforin [36], although Asn252Ser has been

**Fig. 2** Transfection assays with wild-type and mutant promoter constructs of *PRF1* in a human NK cell line (YT Indy). The promoter constructs include the UTR. There is no difference in luciferase activity between the mutant  $-499\text{ C} > \text{T}$  construct and the wild-type construct (**A**). This remains the case when certain SoJIA-specific features (i.e. fever, increased levels of IL-6 and soluble IL-6 receptor or IL-18, **B**) are included in the experimental settings, reflecting a simplified multiple hit model.



implied to be a susceptibility factor for the development of type I diabetes [29].

The novel Arg28Cys mutation, present in one Dutch patient in our cohort but absent in our healthy Dutch control population, may influence the function of the protein as it results in the loss of positive charge and may affect disulfide bridge formation. Online tools [38, 39] that predict effects of amino acid changes gave contradictory results concerning the change of arginine into cysteine at position 28 of the perforin protein. The patient concerned, P54 is 15 years old at this moment and has not developed MAS up till now. Repeated flow cytometry for perforin in cytotoxic lymphocytes and assessment of lytic NK cell function in Patient P54 showed intermediately decreased levels of perforin and lytic NK cell function (~20%).

Finally, we identified a variant ( $-499\text{ C} > \text{T}$ ) in the putative promoter region of *PRF1* in 10/56 patients

(nine heterozygous patients and one homozygote). This  $499\text{ C} > \text{T}$  variant was found in comparable allele frequencies in our healthy control populations (10.2% in Italian healthy controls compared with 5.0% in Dutch healthy controls), but was again found in higher frequencies in the subgroup of SoJIA patients with a history of MAS compared with SoJIA patients without MAS (13.3 vs 8.5%). Using predictive databases [40, 41], we found that the  $-499\text{ C} > \text{T}$  variation does not seem to affect binding of presently known transcription factors. Moreover, in transfection experiments assessing the functional consequences of promoter variation (luciferase reporter assays) in NK cells, we could not find statistically significant changes in luciferase activity of wild-type constructs compared with the mutant  $-499\text{ C} > \text{T}$  construct either in standard culture conditions or in 'SoJIA-specific' conditions (Fig. 2B). Therefore, we have no indications so far that this variant in the 5'-upstream region of *PRF1*

contributes to decreased levels of perforin in NK cells of SoJIA patients. However, the transcriptional regulation of *PRF1* is complex [42], and recently it was shown that the induction of perforin in activated cytotoxic lymphocytes is under control of distal *cis*-acting locus control regions (LCRs) upstream of *PRF1* [43]. Future studies may be aimed at the identification of mutations in these distal LCRs as putative candidates affecting perforin expression in SoJIA patients.

It is tempting to speculate that heterozygous mutations or promoter variations in other genes related to fHLH (UNC13D, STX11) could be risk factors for MAS in SoJIA as well. This is supported by a recent study [44], suggesting a role of UNC13D mutations in the pathogenesis of MAS in SoJIA. Moreover, a recent case report describes a SoJIA patient with a compound heterozygous mutation in UNC13D [45]. In contrast, a paper by Donn *et al.* [46] does not support a contributing role for genetic loci involved in fHLH to the pathophysiology of SoJIA. However, Donn *et al.* studied only a restricted number of specific SNPs in *PRF1* and other genes possibly related to fHLH (UNC13D, Rab27A and GZMB) and did not analyse the full sequence of these genes. Most importantly, the study of Donn *et al.* displays no information about MAS in the SoJIA patients, whereas our data point specifically to a contributing role of heterozygous *PRF1* mutations for the development of MAS in SoJIA patients and not to SoJIA itself.

Since it is hypothesized that there is a complex interplay of different factors and genes contributing to the increased risk of developing MAS in SoJIA, an even larger cohort (i.e. further international collaboration) is needed, including a sufficient number of patients with a history of MAS, to definitively sort this out.

In conclusion, in this study, we found indications that heterozygous missense mutations in *PRF1* can be a risk factor for the development of MAS in SoJIA patients. It is, therefore, tempting to assume that (heterozygous) mutations in other genes involved in the pathogenesis of HLH (such as genes encoding Syntaxin11 and MUNC13-4) could also be involved in the development of MAS in SoJIA patients.

## Supplementary data

Supplementary data are available at *Rheumatology* Online.

### Rheumatology key messages

- Low perforin expression and lytic function of NK cells in SoJIA patients are further depressed during MAS.
- Heterozygous mutations in *PRF1* can be risk factors for MAS in SoJIA patients.

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