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MEFV Variants in Patients with PFAPA Syndrome in Japan

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Abstract: *Background*: The pathogenesis of PFAPA (periodic fever, aphthous stomatitis, pharyngitis, adenitis) syndrome is unknown as yet. In order to understand whether genes implicated in other auto-inflammatory diseases might be involved in the pathogenesis of PFAPA, all variants in the genes causing familial Mediterranean fever (FMF), tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), and Hyper IgD syndrome were analyzed in children with PFAPA.

Patients and Methods: All variants in *MEFV*, *TNFRSF1A*, and *MVK* were analyzed in 20 patients with PFAPA. PFAPA were diagnosed by previous published criteria. The findings of all analyses in PFAPA patients were compared with those of unaffected normal subjects (n=62).

Results: In the 13 children of 20 with PFAPA, the heterozygous variants of *MEFV* (5 patients: *E148Q-L110P*, 2 patients: *E148Q*, 1 patient: *E148Q-L110P/E148Q*, 1 patient: *E148Q-P369S-R408Q-E84K*, 1 patient: *E148Q-L110P-P369S-A408G*, 1 patient: *R202Q*, 1 patient: *P115R*) were found. No variants belonging to *TNFRSF1A* or MVK were detected in children with PFAPA. The frequency of the *E148Q-L110P* variants in children with PFAPA was significantly higher than that observed in unaffected normal subjects (7/20 versus 8/62). The duration of the episodes of illness in PFAPA children with *MEFV* variants was shorter than that of patients without variants.

Conclusion: Genes involved in the development and progression of *MEFV* may affect the incidence and the phenotype of PFAPA in children.

Keywords: PFAPA, *MEFV*, FMF, Variant, Japanese.

INTRODUCTION

In 1987 [1], Marshall firstly described, the PFAPA (periodic fever, aphthous stomatitis, pharyngitis and adenitis) syndrome, which is characterized by recurrent episodes of fever associated with cervical adenitis, pharyngitis and aphthous stomatitis. The prognosis of this disease has been reported to be better than that of another autoinflammatory diseases [2]. Corticosteroids are effective in controlling episodes of illness in PFAPA, but they do not cure the ailments or prevent recurrence of the symptoms of this syndrome [3]. Interventions like tonsillectomy and administration of H_2 blockers have been reported to be partially effective for prophylaxis [3]. However the complete pathogenesis of PFAPA is unknown yet, and hence the therapeutic regimens have not yet been established for PFAPA [4].

Familial Mediterranean fever (FMF) is a recessively inherited disorder characterized by acute attacks of fever, and serositis usually lasting for 1–3 days. FMF is caused by mutations in the ME diterranean FeVer gene (*MEFV),* which encodes the protein pyrin (marenostrin)1[5,6]. Colchicine has been shown to be effective for prophylaxis in only 90% of the patients with FMF [7,8].

Recent studies have described that heterozygous variants of the *MEFV* gene were found in patients with PFAPA [9-11]. However, it still remains unclear whether these variants are the causative factors of PFAPA.

The purpose of this study was to understand whether heterozygous variants of *MEFV* may be associated with the onset of PFAPA. We have also tried to understand whether these mutations act as accessory factors and modify the phenotype of patients with PFAPA.

PATIENTS AND METHODS

Twenty children with frequent PFAPA episodes who visited our pediatric outpatient clinic were consecutively selected over a 5-year period (from January 2005 to January 2010). The diagnosis of PFAPA syndrome was established using previously established criteria [1,3,9]. These criteria include recurring fevers associated with exudative tonsillitis, negative throat culture, and possibly, aphthous stomatitis and cervical lymphadenopathy. The additional clinical criteria included completely asymptomatic intervals between the episodes, normal growth and development and exclusion of

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FMF, Behcet' s disease, and cyclic neutropenia. Oral low dose of prednisolone (0.3-0.5mg/kg/dose, 1 or 2 doses per day) was effective on all enrolled patients. *MEFV*, mevalonate kinase (*MVK*), and tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1A*) genes of all enrolled patients were sequenced. After obtaining a written informed consent approved by the Institutional Review Board of Kyoto University, peripheral blood was collected from all patients, and, if needed, their family members. Genomic DNA was extracted, and all the exons including exon-intron junctions of the *MVK, MEFV,* and *TNFRSF1A* genes were amplified by polymerase chain reaction and then sequenced using the ABI3130.

The results are shown as a mean ± SD or proportion, as appropriate. Differences between the groups in discrete variables were evaluated using Fisher's exact test at 5% significance.

Two-sided *P* values were adjusted for multiplicity using Hochberg's method.

Comparisons of continuous variables were done using unpaired Student's *t*-test. All *P* values given are 2-sided. *P* values less than 0.05 were considered significant. Statistical calculation was conducted by SAS version 9.1.3.

RESULTS

Twenty patients (9 boys, 11 girls) diagnosed with PFAPA were followed up in our clinic. Thirteen of these patients had a single *MEFV* (M^+ group). No variant of *TNFRSF1A* and *MVK* was detected in all patients. The genotypes of the *MEFV* gene in the 13 patients are seen in Table **1**. The most common *MEFV* variant patterns seen were *E148Q-L110P* (5 patients) and *E148Q* (2 patients). One patient was homozygous of *E148Q* and heterozygous of *L110P* of *MEFV*. One patient was heterozygous of *E148Q-P369S-R408Q*. One patient was heterozygous for *E148Q-P369S-R408Q-E84K*. One patient had *E148Q-L110P-P369S-R408Q*. The minor variants, *P115R* and *R202Q*, were detected in 2 patients. More than 2 *MEFV variants* were on 1 allele in all PFAPA patients. In 7 patients, no *MEFV* mutations were found. The allele frequencies of *E148Q*, *L110P*, *P369S, R408Q* and G304R in 20 PFAPA patients were 0.3, 0.175, 0.075, 0.075 and 0, respectively (Table **3**).

Table 1. The Genotypes of *MEFV* Genes of 13 Patients with PFAPA

MEFV Variant	No. of Patients
E148Q-L110P	5
E148Q	2
E148Q-L110P/E148	1
E148Q-P369S-R408Q	1
E148Q-P369S-R408Q-E84K	1
E148Q -L110P-P369S-R408Q	1
R202Q	1
P115R	1

Clinical and laboratory data were compared between *MEFV* positive group (n=13) and negative group (n=7) and are presented in Table **2**. Patients carrying an *MEFV* variant showed shorter duration of episodes of illness than patients without variants (3.6±0.86 days versus 5.3±1.89 days,

Table 2. Clinical Characteristics of PFAPA Patients with Variants in the *MEFV* Gene Compared with those of PFAPA Patients without *MEFV* Variants

	Patients with *MEFV* Variants (n=13)	Patients without *MEFV* Variants (n=7)	*P* Value
Age at onset (years)	2.8±1.9	3.2±1.9	NS
Age at Diagnosis (years)	4.3±2.2	4.9±1.9	NS
Male: female ratio	5/8	4/3	NS
Family history of PFAPA	4/9	3/4	NS
Attack duration (days)	3.6±0.86	5.3±1.89	*P*=0.0174
Interval between attacks (weeks)	4.9±1.59	5.5±0.96	NS
Cyclic periodic attacks	5/13	4/7	NS
Pharyngitis	13/13	7/7	NS
Aphthae	7/13	5/7	NS
Enlarged tonsillitis	13/13	7/7	NS
Abdominal pains	1/13	2/7	NS
Musculoskeletal Pains	1/13	2/7	NS
Headaches	4/13	5/7	NS
WBC/μL	143±41	142±41	NS
ESR mm/h	87±23	72±14	NS
CRP levels mg/dL	6.52±3.53	5.87±2.85	NS

NS: not significant.

p=0.0174). No significant differences in all other clinical and laboratory data were found between the 2 groups (Table **2**).

We also analyzed all sequences of *MEFV* genes in normal Japanese subjects (n=62). These individuals were healthy adult volunteers and had no recurrent episodes of fever. There was no difference in recruitment between the PFAPA patients and the control group. A comparison of these results between normal and PFAPA subjects is shown in Table **3**. In normal individuals, no significant allele frequencies were observed for the 4 variants found in PFAPA patients. In addition, the frequencies of *E148Q-L110P* and *P369S-R408Q* in the 2 groups were compared. A significant difference in the frequency of these variants was observed between the 2 groups (Table **4**, p = 0.043 and p = 0.026, respectively).

Table 3. Allele Frequencies of *MEFV* Variants in PFAPA Subjects and Normal Unaffected Subjects

Variant	PFAPA Subjects (n=40)	Unaffected Subjects (n=124)	*P* Value
E148Q	30.0%	18.5%	NS
L110P	17.5%	6.5%	NS
G304R	0.0%	3.2%	NS

NS: not significant.

Table 4. Frequencies of *MEFV* Variants in PFAPA Subjects and Normal Unaffected Subjects

Variant	PFAPA Subjects (n=20)	Unaffected Subjects (n=62)	*P* Value
E148Q-L110P	35%	13%	P=0.043

DISCUSSION

We studied 20 patients with PFAPA who were diagnosed by Marshall criteria [1]. Our aim was to access the roles of the predominant variants in genes that cause other febrile illnesses like FMF, TNF receptor-associated periodic syndrome (TRAPS) and the MVK deficiency. We did not find any incidence of variants of TRAPS and MVK deficiency in PFAPA patients. However several heterogeneous variants of *MEFV* were detected in 13 out of 20 patients with PFAPA. We analyzed the frequency of the *E148Q-L110P* and *P369S-R408Q* variants in PFAPA and control subjects. Our analyses indicate that the incidence of these 2 variants is significantly higher in patients with PFAPA than in normal individuals.

Amongst autoinflammatory disease, only PFAPA syndrome has been described as a non-inherited syndrome, since familial inheritance has not been reported in previous studies [3,12,13]. However some studies have reported familial cases that included siblings or a sibling and the sibling's mother [14-16]. Therefore, the hereditary nature of this syndrome is still a matter of debate. With respect to the genetic factors that may cause the PFAPA syndrome, one study has strongly argued against the involvement of the *MEFV* gene [10], but another article [11] has shown that mutations of the *MEFV* genes were found in 27% of cases diagnosed with PFAPA syndrome on the basis of Marshall's clinical criteria. Our observations of the high frequency (65%) of *MEFV* variants are in agreement with that reported by Dagan [11]. The differences in the findings may be attributed to the ethnic differences between the individuals studied, the small sized of the study, and the study population that was selected.

The *L110P* variant, which is located in exon 2, was first reported in FMF patients in 2000 [17], and to date, several compound heterozygotes with other variants have been reported even in Japan [18,19]. In contrast, although the role of the *E148Q* variant, which is located in exon 2, in FMF patients was controversial, a recent study concluded that the variant is just a benign polymorphism [20]. In a Japanese study [18] of FMF patients, the most frequently observed *MEFV* variants observed were *E148Q/M694I* (25.0%), *M694I* (17.5%), and *L110P/E148Q/M694I* (17.5%). However, no patients had the *M694V* variant. These patterns are quite different from those in Mediterranean patients with FMF. The study also reported that the allele frequencies of *E148Q*, *M694I*, and *L110P* were 0.44, 0.35, and 0.31, respectively, and that these frequencies were significant difference from those seen in healthy controls. In our study, the allele frequencies of *E148Q*, *L110P*, and *G304* were 0.3, 0.175, and 0, respectively, and these frequencies did not differ significantly between patients and healthy controls. No mutation at the *M694I* was detected in our cohorts of patients and controls. The allele frequency of *L110P* is higher in patients with PFAPA than in healthy controls; however, the difference is not significant. The frequency of the *E148Q-L110P* variant combination is significantly higher in the PFAPA group than in the healthy control group. If the *E148Q* variant is non-functional, the L110P variant may be associated with the onset of PFAPA syndrome.

In several types of inflammatory such as Behcet's disease [21], Crohn's disease [22], ulcerative colitis [23], Henoch-Schönlein purpura [24,25] and the co-incidence of FMF variants has been investigated. These studies show increased incidence of genes involved in FMF in patients with these autoinflammatory diseases as well as the increased severity of the symptoms of each disease. On the other, in the patients with asthma, the incidence of FM mutation was decreased and the lower incidence correlated with reduced severity of symptoms [26]. Thus, FMF variants may affect the transition from Th2 to Th1 polarity in each disease. According to Berkun's study [27], PFAPA episodes in carriers of *MEFV* variants were shorter compared to those in patients without variants. In *MEFV* variant-positive patients, the regular cyclic pattern of attacks and the occurrence of oral aphthae was lower than those in patients without *MEFV* variants. In the present study, we found that the only affected variable was the duration of PFAPA episodes. Although no significant differences were observed in the regular cyclic pattern of attacks and the occurrence of oral aphthae between the 2 groups, the duration of PFAPA episodes was shorter in the variant-positive group than in the variant-negative group.

Taken together, these results show that the *MEFV* gene may not affect the onset of several autoinflammatory diseases, but is likely to modify the intensity and the displayed phenotype in terms of disease symptoms.

In conclusion, the *MEFV* variants, viz. *E148Q-L110P*, *P369-R408Q* may be associated with the onset of PFAPA, and some *MEFV* variants may affect the phenotype of PFAPA.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES

[1] Marshall GS, Edwards KM, Lawton AR. PFAPA syndrome. Pediatr Infect Dis J 1989; 8: 658-9.

[2] Wurster VM, Carlucci JG, Feder HM Jr, Edwards KM. Long-term follow-up of children with periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis syndrome. J Pediatr 2011; 159: 958-64.

[3] Thomas KT, Feder HM Jr, Lawton AR, Edwards KM. Periodic fever syndrome in children. J Pediatr 1999; 135: 15-21.

[4] Feder HM, Salazar JC. A clinical review of 105 patients with PFAPA (a periodic fever syndrome). Acta Paediatr 2010; 99: 178-84.

[5] The International FMF Consortium. Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. Cell 1997; 90: 797-807.

[6] French FMF Consortium. A candidate gene for familial Mediterranean fever. Nat Genet 1997; 17: 25-31.

[7] Medlej-Hashim M, Loiselet J, Lefranc G, Mégarbané A . [Familial Mediterranean Fever (FMF): from diagnosis to treatment. Sante 2004; 14: 261-6.

[8] Soylemezoglu O, Arga M, Fidan K, *et al.* Unresponsiveness to colchicine therapy in patients with familial Mediterranean fever homozygous for the M694V mutation. J Rheumatol 2010; 37: 182-9.

[9] Padeh S, Brezniak N, Zemer D, *et al.* Periodic fever, aphthous stomatitis, pharyngitis, and adenopathy syndrome: clinical characteristics and outcome. J Pediatr 1999; 135: 98-101.

[10] Cazeneuve C, Geneviève D, Amselem S, Hentgen V, Hau I, Reinert P. MEFV gene analysis in PFAPA. J Pediatr 2003; 143: 140-1.

[11] Dagan E, Gershoni-Baruch R, Khatib I, Mori A, Brik R. *MEFV, TNF1rA, CARD15* and *NLRP3* mutation analysis in PFAPA. Rheumatol Int 2010; 30: 633-6.

[12] Feder HM Jr, Bialecki CA. Periodic fever associated with aphthous stomatitis, pharyngitis and cervical adenitis. Pediatr Infect Dis J 1989; 8: 186-7.

[13] Tasher D, Somekh E, Dalal I. PFAPA syndrome: new clinical aspects disclosed. Arch Dis Child 2006; 91: 981-4.

[14] Sampaio IC, Rodrigo MJ, Monteiro Marques JG. Two siblings with periodic fever, aphthous stomatitis, pharyngitis, adenitis (PFAPA) syndrome. Pediatr Infect Dis J 2009; 28: 254-5.

[15] Valenzuela PM, Majerson D, Tapia JL, Talesnik E. Syndrome of periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) in siblings. Clin Rheumatol 2009 ; 28: 1235-7.

[16] Adachi M, Watanabe A, Nishiyama A, *et al.* Familial cases of periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis syndrome. J Pediatr 2011; 158: 155-9.

[17] Domingo C, Touitou I, Bayou A, *et al.* Familial Mediterranean fever in the 'Chuetas' of Mallorca: a question of Jewish origin or genetic heterogeneity. Eur J Hum Genet 2000; 8: 242-6.

[18] Tsuchiya-Suzuki A, Yazaki M, Nakamura A, *et al.* Clinical and genetic features of familial Mediterranean fever in Japan. J Rheumatol 2009; 36: 1671-6.

[19] Tomiyama N, Higashiuesato Y, Oda T, *et al. MEFV* mutation analysis of familial Mediterranean fever in Japan. Clin Exp Rheumatol 2008; 26: 13-7.

[20] Tchernitchko D, Legendre M, Cazeneuve C, Delahaye A, Niel F, Amselem S. The *E148Q MEFV* allele is not implicated in the development of familial Mediterranean fever. Hum Mutat 2003; 22: 339-40.

[21] Rabinovich E, Shinar Y, Leiba M, Ehrenfeld M, Langevitz P, Livneh A. Common FMF alleles may predispose to development of Behcet's disease with increased risk for venous thrombosis. Scand J Rheumatol 2007; 36: 48-52.

[22] Uslu N, Yüce A, Demir H, *et al.* The association of inflammatory bowel disease and Mediterranean fever gene (*MEFV*) mutations in Turkish children. Dig Dis Sci. 2010; 55: 3488-94.

[23] Giaglis S, Mimidis K, Papadopoulos V, *et al.* Increased frequency of mutations in the gene responsible for familial Mediterranean fever *(MEFV)* in a cohort of patients with ulcerative colitis: evidence for a potential disease-modifying effect? Dig Dis Sci 2006; 51: 687-92.

[24] Gershoni-Baruch R, Broza Y, Brik R. Prevalence and significance of mutations in the familial Mediterranean fever gene in Henoch-Schönlein purpura. J Pediatr 2003; 143: 658-61.

[25] Ozçakar ZB, Yalçinkaya F, Cakar N, *et al. MEFV* mutations modify the clinical presentation of Henoch-Schönlein purpura. J Rheumatol 2008; 35: 2427-9.

[26] Rabinovitch E, Harats D, Yaron P, *et al.* Familial Mediterranean fever gene and protection against asthma. Ann Allergy Asthma Immunol 2007; 99: 517-21

[27] Berkun Y, Levy R, Hurwitz A, *et al.* The familial Mediterranean fever gene as a modifier of periodic fever, aphthous stomatitis, pharyngitis, and adenopathy syndrome. Semin Arthritis Rheum 2011; 40: 467-72.

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Safety and efficacy of canakinumab in Japanese patients with phenotypes of cryopyrin-associated periodic syndrome as established in the first open-label, phase-3 pivotal study (24-week results)

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Abstract

Objectives

Cryopyrin-associated periodic syndrome (CAPS), a rare hereditary auto-inflammatory disease, is associated with mutations in the NLRP3 gene resulting in elevated interleukin-1β (IL-1 β) release. CAPS generally occurs in early childhood with most patients presenting with periodic fever, skin rash, osteoarthropathy, aseptic meningitis, sensorineural hearing loss and optic neuritis. Canakinumab, a fully human anti-IL-1β monoclonal antibody which binds selectively to IL-1β, has demonstrated good efficacy with CAPS. This is the first study to evaluate the safety and efficacy of canakinumab in Japanese patients with CAPS.

Methods

In this open-label study, 19 Japanese CAPS patients aged ≥2 years received canakinumab either 150 mg s.c. or 2 mg/kg for patients with a body weight ≤ 40 kg every 8 weeks for 24 weeks. The primary objective was to assess the proportion of patients who were free of relapse at week 24.

Results

A complete response was achieved in 18 (94.7%) patients with some requiring a dose and/or a frequency adjustment to attain full clinical response. The majority of patients (14/18; 77.8%) were in remission, i.e. free of relapse at week 24. Auto-inflammatory disease activity as assessed by physician's global assessment declined from baseline to end of the study (score of absent in 10.5% at baseline versus *31.6% at end of the study). Two patients had serious adverse events (SAEs), which resolved with standard treatment. One patient reported a mild injection-site reaction. No malignancies or deaths were reported during the study.*

Conclusion

Canakinumab 150 mg s.c. every 8 weeks was well-tolerated, highly efficacious and offered a convenient dosing regimen for treating Japanese patients with CAPS.

Key words

canakinumab, cryopyrin-associated periodic syndrome, interleukin-1β, auto inflammatory syndromes

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Competing interests: none declared.

Introduction

Cryopyrin-associated periodic syndrome (CAPS) represents a group of rare inherited auto-inflammatory diseases and encompasses phenotypes of varying severity. An increase in severity is evident between phenotypes: familial cold auto-inflammatory syndrome (FCAS) is the mildest, while Muckle-Wells syndrome (MWS) is predominantly of intermediate severity, and neonatal-onset multisystem inflammatory disease (NOMID)/chronic infantile neurological cutaneous and articular syndrome (CINCA) is the most severe phenotype of CAPS. All phenotypes are characterised by urticaria-like rash, fever, variant degree of central nervous system and tissue inflammation, arthropathy, risk of development of amyloidosis (1) and other constitutional symptoms. CAPS is associated with mutations of the *NLRP3* gene encoding cryopyrin (2-6), an important component of inflammasome. Inflammasone activates caspase-1, leading to enhanced production of the cytokine interleukin-1beta (IL-1β) and subsequent inflammation (7, 8). The pathogenic role of IL-1β in CAPS has been demonstrated by the achievement of complete response after treatment with IL-1β inhibitors (9-13). Positive therapeutic effects of the IL-1 receptor antagonist and anakinra have been hampered by the need for frequent injections (14-17) associated with severe pain, which impairs the quality of life of patients, especially the paediatric population. Canakinumab (ACZ885, Ilaris®, Novartis Pharma), a fully human anti-IL-1β monoclonal antibody (18), has shown prolonged selective IL-1β inhibition (19, 20) and has demonstrated rapid (within hours), complete and sustained response in CAPS patients of mainly Caucasian origin without any consistent pattern of side effects (21). Canakinumab is approved by the US Food and Drug Administration (FDA) for FCAS and MWS (22) only and by EMA for treatment of all three phenotypes of CAPS (23).

At present, there are no approved therapies for CAPS in Japan. The present study was therefore conducted to evaluate safety and efficacy of canakinumab in Japanese paediatric and adult patients with CAPS. Herein we report the study data up to 24 weeks.

Materials and methods

Study design, patients and study definitions

This was an open-label, safety and efficacy study of canakinumab administered for 24 weeks (6 months) in Japanese patients diagnosed with FCAS, MWS or NOMID. Molecular diagnosis showed that 17 (89.5%) patients were positive for *NLRP3* mutations and two (10.5%) patients (one each with MWS and NOMID) were negative for the mutation. The study included an extension phase to provide canakinumab treatment to study patients until canakinumab is marketed in Japan. Two NOMID patients aged 2 and 3 years previously treated with anti-IL-1 agents (anakinra) were enrolled.

Patients received canakinumab 150 mg s.c. or 2 mg/kg for those patients with body weight ≤40 kg for every 8 weeks. In case of residual symptoms, stepwise increase of the dose up to 600 mg s.c. or 8 mg/kg s.c. (≤40 kg) and/or increased dosing frequency were allowed.

After a 6-hour washout period for those patients previously treated with anakinra, 19 patients were included. Ten had received anakinra prior to study initiation, of which five patients had reported a complete response, while the remaining had achieved partial response to anakinra. Patients requiring oral steroids, NSAIDs and/or disease-modifying anti-rheumatic drugs (DMARDs) were enrolled if they were on a stable dose (oral steroids: <20 mg/day or ≤0.4 mg/kg prednisone or prednisone equivalent, whichever applies) for at least 4 weeks prior to the screening visit. Steroid therapy was tapered after the first canakinumab treatment cycle (8 weeks between doses), at the discretion of the investigator. TNF-α inhibitors and IL-6 receptor blockers were not allowed during the study. Women of child bearing potential had to use an accepted form of contraception during the study and for at least 3 months after the last dose. Patients receiving live vaccine within 3 months before recruitment were excluded.

Complete response assessed at day 15 and day 29 was defined by (i) physician's global assessment of no or minimal auto-inflammatory disease (on a 5-point Likert scale ranging from absent, minimal, mild, moderate to severe) and assessment of no or minimal skin disease, and (ii) serological remission defined as serum CRP <1 mg/dL, and/or SAA <10 μg/mL. Patients who did not achieve (or maintain) complete response following canakinumab injection in any treatment period could receive a dose escalation (supporting Fig. 1). The possible step-wise up-titration regimens were: 300 mg s.c. (or 4 mg/kg for patients with a body weight ≤40 kg), 450 mg s.c. (or 6 mg/kg for patients with a body weight ≤40 kg), and 600 mg s.c. (or 8 mg/kg for patients with a body weight ≤40 kg).

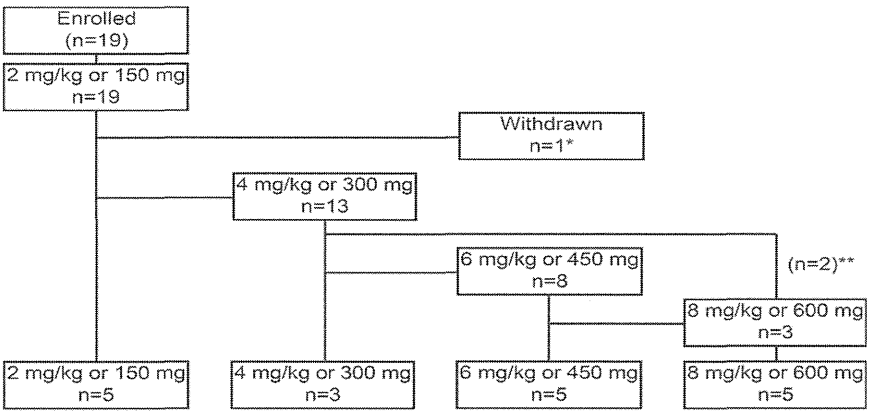
The primary efficacy endpoint was defined as the proportion of patients who did not experience a relapse at week 24. Relapse was defined as clinical relapse (physician's global assessment of both auto-inflammatory disease activity and assessment of skin disease, mild or greater) and serological relapse (serum CRP >3 mg/dL, and/or SAA >30 μg/mL).

Clinical improvement of the central nervous system (CNS) was assessed in NOMID patients only (defined as a mean weekly headache score [from the daily diary] <0.5 and a normal white cell count [≤15 cells/mm^3] in cerebrospinal fluid). Other key secondary endpoints included safety and tolerability of canakinumab, assessed by the occurrence of adverse events (AEs), serious AEs (SAEs) and immunogenicity.

This study was approved by the Independent Ethics Committee for each centre and performed in accordance to the ethical principles of the Declaration of Helsinki. All patients, parents or legal guardians (for patients aged <20 years) provided written informed consent.

Statistical analyses

Safety and full analysis set (efficacy analysis) included all patients who received at least one dose of study treatment. Only 19 patients were enrolled due to the low prevalence of CAPS, hence the estimation of statistical power was not applicable. Descriptive statistics were used to summarise demographics, baseline characteristics, efficacy and safety. Missing values were not imputed.



*One patient withdrew from this study by cancellation of the consent.

**Two patients needed two up titrations till Day 15 due to incomplete response to the first administration of canakinumab. Patients with incomplete response from the standard dosing regimen (2 mg/kg or 150 mg) received step-wise up-titration regimen. Patients who did not achieve complete response or had a relapse before the next planned administration received a dose up-titration.

Fig. 1. Patient disposition and dosing.

Results

Patients, demographic and baseline characteristics

A total of 19 CAPS patients (12 [63.2%] male/7 [36.8%] female) with a diagnosis of MWS (n=7; 36.8%) or NOMID (n=12; 63.2%) were enrolled in this study, of which 18 (94.7%) completed the 24-week study phase. One patient withdrew consent (Fig. 1). At study entry, there were 11 patients (57.9%) aged <16 years and eight patients (42.1%) aged 16 years or older. Median age was 14 years (range 2–48). Of 19 patients, five (26.3%) weighed >40 kg at baseline. Other key demographic and baseline characteristics are summarised in Table I.

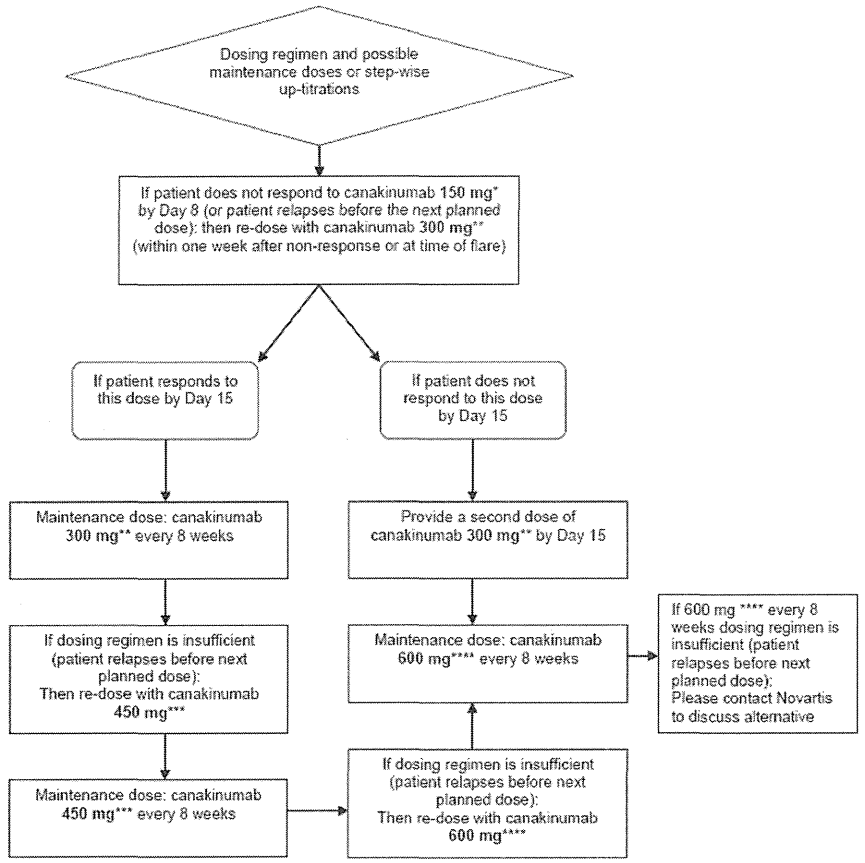
Treatment with canakinumab

At time of the 24-week analysis, the median treatment duration was 168 days (range 59–197 days) and patients received an average of 4.1 injections over 24 weeks of the study; 13 (68%) patients (MWS; n=4 and NOMID, n=9) received an up-titration of their dose, primarily due to absence of a complete response and in one patient the dose frequency was increased to every 6 weeks starting from day 49. In one NOMID patient aged 16 years, the

Table I. Baseline demographics and disease characteristics (safety population).

Characteristics	Canakinumab (n=19)
Sex, n (%)	
Male	12 (63.2)
Female	7 (36.8)
Age (years)	
Mean (SD)	14.8 (11.4)
Median (range)	14.0 (2–48)
≥2– <12 years, n (%)	8.0 (42.1)
≥12– <16 years, n (%)	3 (15.8)
≥16 years, n (%)	8 (42.1)
Weight (kg), n (%)	
≤40	14 (73.7)
>40	5 (26.3)
BMI (kg/m^2)	
Mean (SD)	17.6 (2.2)
Median (range)	17.2 (13.5–21.5)
Diagnosis, n (%)	
FCAS	0
MWS	7 (36.8)
NOMID	12 (63.2)
Molecular diagnosis of	
NLRP3 mutation, n (%)	19 (100.0)
Positive	17 (89.5%)
Negative	2 (10.5%)
Previous use of anakinra, n (%)	10 (52.6)
C-reactive protein (mg/dL) (normal value: <1mg/dL)	
Mean (SD)	4.52 (4.3)
Median (range)	3.3 (0.1–13.2)
Serum Amyloid A (μg/mL) (normal value: <10μg/mL)	
Mean (SD)	324.2 (364)
Median (range)	236 (2.6–1380)

BMI: body mass index; FCAS: familial cold autoinflammatory syndrome; MWS: Muckle-Wells syndrome; NOMID: neonatal-onset multisystem inflammatory disease; NLRP3: NOD-like receptor family, pyrin domain containing3; SD: standard deviation.



* canakinumab 150 mg s.c. for patients whose body weight is > 40 kg (or 2 mg/kg for patients with a body weight ≤ 40 kg)
** canakinumab 300 mg s.c. (or 4 mg/kg for patients with a body weight ≤ 40 kg)
*** canakinumab 450 mg s.c. (or 6 mg/kg for patients with a body weight ≤ 40 kg)
**** canakinumab 600 mg s.c. (or 8 mg/kg for patients with a body weight ≤ 40 kg)
There is currently no long-term safety information for doses greater than 600 mg s.c. available.
The above outlined decision tree may be applied to those patients who either did not achieve a complete response by Day 8 or Day 15 or to those patients who relapse prior to their next scheduled dose.

Supporting Fig. 1. Alternative dosing regimen for CAPS patients who do not experience sufficient symptomatic relief.

canakinumab dose was escalated to the highest dose of 600 mg. Four patients (8–25 years) with baseline body weight ≤40kg received a dose escalation to 8 mg/kg.

Proportionally higher mean last doses of canakinumab were required in patients ≤40 kg (n=12) *versus* >40 kg (n=6) at 6 mg/kg and 250 mg, respectively; in patients weighing >40 kg, the canakinumab dose administered was 350 and 150 mg for NOMID and MWS, respectively.

Efficacy

Relapse assessment. Overall, protocol-defined complete response was achieved in 18 (94.7%) patients. One patient achieved a complete response by day 148. This patient achieved clinical remission by day 29, but the inflammatory markers remained elevated until day 148. One non-responder patient achieved clinical remission, but the patient's CRP and SAA levels remained above normal during the study; however there was a significant decrease by week 24 compared to baseline. Some patients required either a dose escalation and/or a frequency adjustment to attain full clinical response (supporting Fig. 1); 15 (78.9%) patients achieved a complete response within 15 days, 2 patients were up-titrated within 29 days, and 1 patient by day 148. At week 24, the majority of patients (n=14/18 [77.8%]) were in remission, *i.e.* free of relapse (Table II).

Table II. Relapse at week 24 in MWS and NOMID patients (full analysis set).

Characteristics	Canakinumab n=19 n (%)
Number of complete responders by week 24	
Total	18 (94.7)
Day 15*	15 (78.9)
Day 29*	2 (10.5)
Day 148*	1 (5.3)
Relapse at week 24	4 (22.2)
No relapse at week 24	14 (77.8)
MWS patients	6 (85.7)
NOMID patients	8 (72.7)
No clinical/serological relapse at week 24	12 (66.7)
Discontinue prematurely prior to week 24	1 (5.6)

*Patients requiring either a dose and/or a frequency adjustment to attain full clinical response.
MWS: Muckle-Wells syndrome; NOMID: neonatal-onset multisystem inflammatory disease.

Of 12 NOMID patients, 11 achieved complete response by week 24 and nine achieved a complete response by day 15; one achieved complete response with dose adjustment by day 29 and one by day 148. Three (27.3%) out of the 11 complete responders (all NOMID patients) had a relapse at week 24. All patients with MWS (n=7) achieved complete response by week 24, though one patient had a relapse at week 24. All except one patient achieved complete response with canakinumab. All prior responders to anakinra also achieved a complete response with canakinumab.

Auto-inflammatory disease activity

The severity of auto-inflammatory disease activity as assessed by physician's global assessment declined from baseline to the end of the treatment period. This decrease in disease activity was apparent in all the individual symptom components including assessments of skin disease, headache/migraine, conjunctivitis and fatigue/malaise (Fig. 2).

Inflammatory markers

Canakinumab treatment induced a rapid decline in CRP levels within 15 days (Fig. 3a). Overall, mean CRP levels decreased by 2.94±2.99 mg/dL (38% decrease) from baseline to end of the study, day 169 (4.52 mg/dL *vs.* 1.19

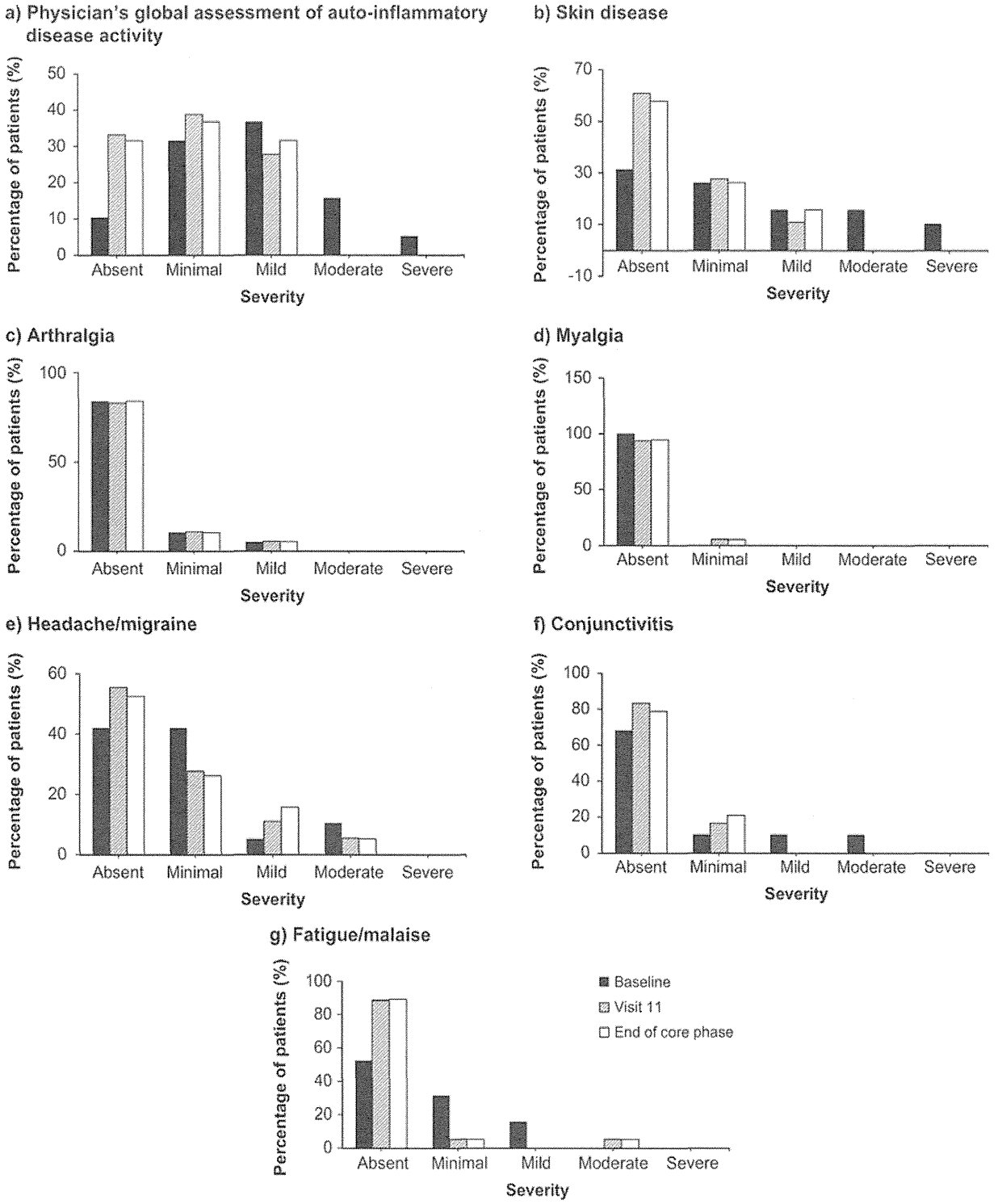


Fig. 2. Summary of assessment of auto-inflammatory disease activity (full analysis set).

mg/dL). A similar trend was observed for mean serum SAA level, which decreased from baseline to end of the study (324.19 μg/mL *vs.* 54.71 μg/mL) (Fig. 3b). On day 57, there was an increase in CRP and SAA levels, however this was driven by measurements from three patients whose mean values were near normal at other time points.

Immunogencity

Of the 19 patients, three were detected with anti-canakinumab binding antibodies during one of the post-dose assessments. However, no anti-canakinumab antibodies were detected afterwards.

Specific assessments in NOMID patients

A protocol-defined CNS remission was achieved in 33.3% (n=4/12) of the NO-

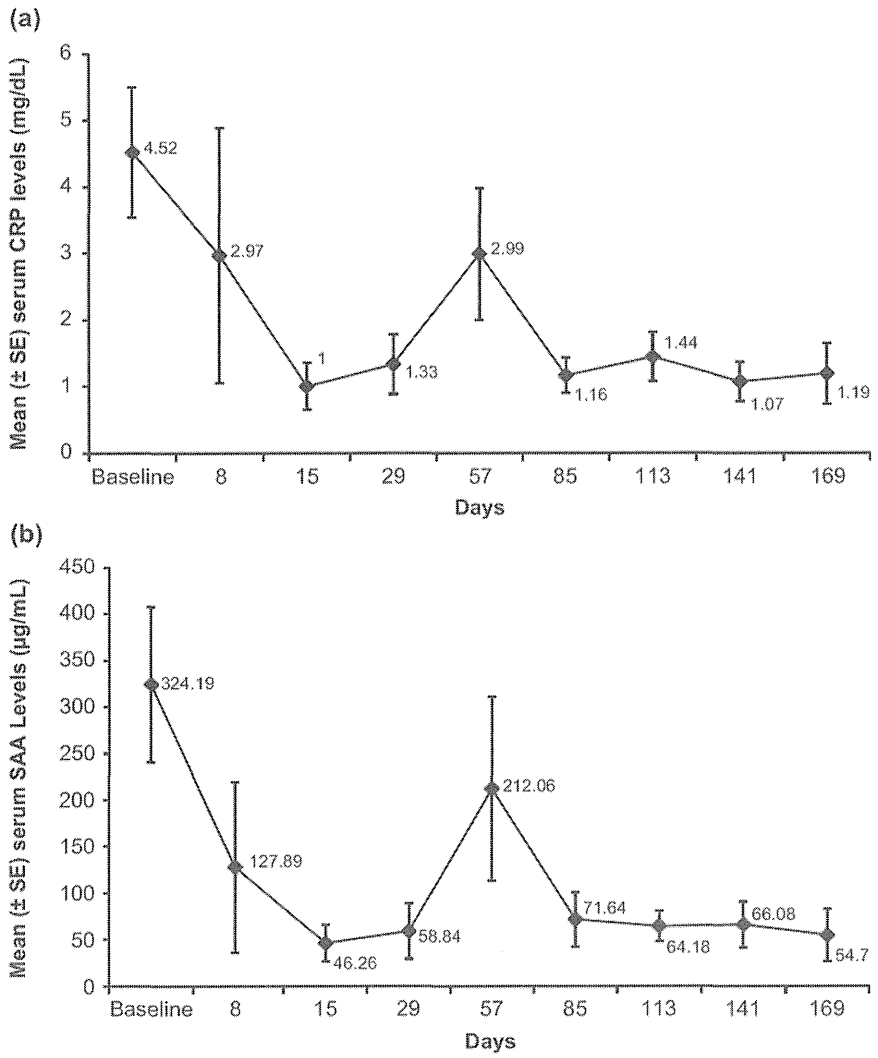


Fig. 3. (a) Serum CRP level across time points (full analysis set); (b) Serum SAA levels across time points.

MID patients by day 8 and in 41.7% (n=5/12) at the end of study; 9/12 patients had CNS remission at week 24 (with just the headache score). Lumbar puncture was only performed in 7/12 patients, of which five were in CNS remission based on the headache score and normal white cell count. A CNS relapse was reported in two (16.7%) patients on day 57 and in one patient (9.1%) on day 113. Of the three patients with a protocol-defined CNS relapse, one was uptitrated from 4mg/kg to 6mg/kg due to a concomitant clinical and serological relapse. In the other two patients, no uptitration was performed for CNS relapse. In these three patients, there was no association between the CNS relapse and clinical flare. The results of key cerebrospinal fluid assessments in NOMID patients were available in only 6 patients, who had both baseline and week 24 values. In these patients (n=6), mononuclear cells (lymphocytes, macrophages, monocytes) remained unchanged or elevated slightly from baseline to week 24 (normal values: adult ≤5 WBC/mm^3, newborns ≤20 WBC/mm^3). Absolute neutrophils which markedly reduced in two NOMID patients remained largely unchanged in the other three patients, even though it was elevated in one patient at week 24 compared with baseline. None of these patients reported headache, but they were noted to have elevated CRP and/or SAA levels. In addition to elevated SAA levels, one patient had physician's global assessment of autoinflammatory disease activity above minimal and had a relapse at week 24.

Table III. Most frequently occurring (>10%) adverse events regardless of study drug relationship (safety population).

Primary system organ class/ preferred term	Canakinumab n=19 n (%)
Total patients with AEs	18 (94.7)
Gastrointestinal disorders	7 (36.8)
Abdominal pain upper	2 (10.5)
Diarrhoea	2 (10.5)
Stomatitis	2 (10.5)
General disorders and administration site conditions	3 (15.8)
Infections and infestations	16 (84.2)
Nasopharyngitis	7 (36.8)
Gastroenteritis	6 (31.6)
Upper respiratory tract infection	3 (15.8)
Nervous system disorders	2 (10.5)
Respiratory, thoracic and mediastinal disorders	5 (26.3)
Rhinorrhoea	3 (15.8)
Cough	2 (10.5)
Skin and subcutaneous tissue disorders	6 (31.6)
Acne	2 (10.5)
Dry skin	2 (10.5)
Urticaria	2 (10.5)
Vascular disorders	2 (10.5)
Hypertension	2 (10.5)

A patient with multiple occurrences of an AE is counted only once in the AE category. A patient with multiple adverse events within a primary system organ class is counted only once in the total row. AE: adverse event.

Safety

Overall, 18 (94.7%) patients experienced at least one AE. The most commonly reported AEs (≥15% of patients) were nasopharyngitis (n=7, 36.8%), gastroenteritis (n=6, 31.6%), upper respiratory tract infection (n=3, 15.8%), and rhinorrhea (n=3, 15.8%). Twelve (63.2%) patients reported AEs, which were suspected to be study drug-related (Table III). The majority of AEs were mild (n=13, 68.4%) or moderate (n=3, 15.8%) in severity. Severe AEs of diffuse vasculitis and pneumonia were each reported in one (5.3%) patient. All but one MWS patient experienced at least one AE. Nasopharyngitis was reported in a higher proportion of NOMID patients (n=6, 50%) compared to MWS patients (n=1, 14.3%). All other AEs in NOMID and MWS patients occurred at similar frequencies or in less than three patients in each group. Two patients had serious AEs, which were suspected to be treat-

ment-related (Parvovirus infection and Epstein-Barr virus infection [n=1] and pneumonia [n=1]), but resolved with standard treatment. Of the 19 patients, only one reported a mild injection-site reaction. No deaths were reported during the study. Higher canakinumab s.c. doses (>150mg or 2mg/kg q8wks) did not appear to be associated with a differential safety profile.

Discussion

The present study confirms the clinical and serological efficacy of canakinumab in a Japanese population of paediatric and adult CAPS patients presenting with the most severe NOMID and MWS-phenotypes. Eighteen (94.7%) out of 19 patients enrolled in this study have achieved a complete response with some patients requiring either a dose and/or a frequency adjustment to attain full clinical response. For most patients (78.9%), irrespective of CAPS phenotype, a complete response was achieved with the standard subcutaneous canakinumab dose (13), *i.e.* 150 mg (>40 kg body weight) or 2 mg/kg (≤40 kg body weight) every 8 weeks.

All clinical symptoms frequently observed in CAPS patients such as inflammation of skin, eyes, bones, joints and meninges accompanied by recurrent fever, severe fatigue, myalgia and headache, showed an improvement during canakinumab treatment. Improvement in clinical outcomes with canakinumab therapy such as autoinflammatory disease activity, and reduction in the levels of acute phase proteins such as CRP and SAA confirms the pivotal role of IL-1β and its inhibition in CAPS.

The sustained effects of canakinumab on patient's clinical symptoms have been associated with its mean terminal half-life of 26 days and a possibly disease-modifying effect through autocrine down-regulation of IL-1β production (19). The canakinumab administration schedule of one injection every 8 weeks and the low incidence of injection-site reactions, as previously observed in other phase II and III canakinumab CAPS studies (21, 25, 26), may be beneficial, especially to paediatric patients.

In the present study, individualised up-titration in patients with an incomplete response proved to be a safe and an efficacious approach for the majority of patients achieving a complete response within one month. Patients with incomplete response, as shown by changes in clinical symptoms (headache, fever or rash according to CAPS) and raised inflammatory marker levels (elevated CRP >3 mg/dL, and/or SAA >30 μg/mL), had initially received canakunimab titrated up to 8 mg/kg. The dosage interval was shortened by up to four weeks if patients failed to achieve a complete response. There was no clear correlation between the genotype, phenotype, and treatment response. The mean dose requirement for patients ≤40 kg was found to be proportionally higher (6 mg/kg) than for those with a body weight >40 kg (250 mg). In the group of patients with a body weight >40kg, the NOMID patient subgroup required a higher mean dose compared to the MWS patient subgroup, in line with the level of severity of the disease.

At baseline, 12 NOMID patients presented with CNS symptoms that included headache and pleocytosis and 9 showed improvement in these symptoms by week 24. Patients showed no significant changes, either worsening or improvement, based on audiogram and neurological or ophthalmic assessments. Two patients showed normalisation in auditory acuity and one patient showed normalisation in visual acuity. There were no organic changes observed on magnetic resonance imaging (MRI). This may be attributed to the fact that the observation period was relatively short and approximately 53% of patients were pre-treated with anakinra at the time of the study entry.

In the present study, no patients discontinued due to unsatisfactory therapeutic effect, suggesting that an effective individual canakinumab dosing regimen was determined. The safety profile was comparable to that observed in previous canakinumab studies (21, 24), with no new or unexpected safety findings. Consistently with previous studies and other biologics, infections were the most frequent AEs and the patients responded well to standard therapy. There were no deaths, discontinuations nor dose adjustments/or interruptions due to AEs. In 3 out of 19 patients, anti-canakinumab binding antibodies were detected in one of the post-dose visits, however these patients showed no evidence of immunogenicity related AEs or impaired efficacy. The overall safety profile observed in previous canakinumab studies in CAPS was confirmed in this Japanese population including the paediatric and NOMID sub-populations.

The present study has limitations, including the small size of the patient population, the non-controlled design and the relatively short-term observation period, each of which were addressed in previous studies. Additionally, the small sample size and short follow-up period did not allow detailed assessment of side effects related to anti-IL-1 therapy such as malignant disease and autoimmunity. Long-term observation with a large population is needed to address these issues (27).

Conclusion

Canakinumab 150 mg s.c. dosed every 8 weeks proved to be efficacious and provided a convenient dosing regimen for treating Japanese patients with CAPS. Higher canakinumab doses in younger patients and in adult patients with more severe CAPS disease were efficacious in achieving a complete response and were well tolerated without any evidence of increased AEs. While these results for the treatment of CAPS with canakinumab for up to 197 days are encouraging, the long-term safety of canakinumab in CAPS patients will be further evaluated in this ongoing study.

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References

1. AKSENTIJEVICH I, D PUTNAM C, REMMERS EF *et al.*: The clinical continuum of cryopyrinopathies: novel CIAS1 mutations in North American patients and a new cryopyrin model. *Arthritis Rheum* 2007; 56: 1273-85.
2. AGANNA E, MARTINON F, HAWKINS PN *et al.*: Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis. *Arthritis Rheum* 2002; 46: 2445-52.
3. DODÉ C, LE DÛ N, CUISSET L *et al.*: New mutations of CIAS1 that are responsible for Muckle-Wells syndrome and familial cold urticaria: a novel mutation underlies both syndromes. *Am J Hum Genet* 2002; 70: 1498-506.
4. HOFFMAN HM, MUELLER JL, BROIDE DH, WANDERER AA, KOLODNER RD: Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet* 2001; 29: 301-5.
5. FELDMANN J, PRIEUR AM, QUARTIER P *et al.*: Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. *Am J Hum Genet* 2002; 71: 198-203.
6. AKSENTIJEVICH I, NOWAK M, MALLAH M *et al.*: De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new member of the expanding family of pyrin-associated autoinflammatory diseases. *Arthritis Rheum* 2002; 46: 3340-8.
7. DINARELLO CA: Mutations in cryopyrin: bypassing roadblocks in the caspase 1 inflammasome for interleukin-1beta secretion and disease activity. *Arthritis Rheum* 2007; 56: 2817-22.
8. GATTORNO M, TASSI S, CARTA S *et al.*: Pattern of interleukin-1beta secretion in response to lipopolysaccharide and ATP before and after interleukin-1 blockade in patients with CIAS1 mutations. *Arthritis Rheum* 2007; 56: 3138-48.
9. YAMAZAKI T, MASUMOTO J, AGEMATSU K *et al.*: Anakinra improves sensory deafness in a Japanese patient with Muckle-Wells syndrome, possibly by inhibiting the cryopyrin inflammasome. *Arthritis Rheum* 2008; 58: 864-8.
10. LESLIE KS, LACHMANN HJ, BRUNING E *et al.*: Phenotype, genotype, and sustained response to anakinra in 22 patients with autoinflammatory disease associated with CIAS-1/NALP3 mutations. *Arch Dermatol* 2006; 142: 1591-7.
11. ROSS JB, FINLAYSON LA, KLOTZ PJ *et al.*: Use of anakinra (Kineret) in the treatment of familial cold autoinflammatory syndrome with a 16-month follow-up. *J Cutan Med Surg* 2008; 12: 8-16.
12. HOFFMAN HM, THRONE ML, AMAR NJ *et al.*: Efficacy and safety of rilonacept (interleukin-1 Trap) in patients with cryopyrin-associated periodic syndromes: results from two sequential placebo-controlled studies. *Arthritis Rheum* 2008; 58: 2443-52.
13. WITTKOWSKI H, KUEMMERLE-DESCHNER B, AUSTERMANN J *et al.*: MRP8 and MRP14, phagocyte-specific danger signals, are sensitive biomarkers of disease activity in cryopyrin-associated periodic syndromes. *Arthritis Rheum Dis* 2011; 70: 2075-81.
14. MAKSIMOVIC L, STIRNEMANN J, CAUX F *et al.*: New CIAS1 mutation and anakinra efficacy in overlapping of Muckle-Wells and familial cold autoinflammatory syndromes. *Rheumatology* (Oxford) 2008; 47: 309-10.
15. O'CONNELL SM, O'REGAN GM, BOLGER T *et al.*: Response to IL-1-receptor antagonist in a child with familial cold autoinflammatory syndrome. *Pediatr Dermatol* 2007: 24: 85.
16. HOFFMAN HM: Rilonacept for the treatment of cryopyrin-associated periodic syndromes (CAPS). *Expert Opin Biol Ther* 2009; 9: 519-31.
17. GOLDBACH-MANSKY R, SHROFF SD, WILSON M *et al.*: A pilot study to evaluate the safety and efficacy of the long-acting interleukin-1 inhibitor rilonacept (interleukin-1 Trap) in patients with familial cold autoinflammatory syndrome. *Arthritis Rheum* 2008; 58: 2432-42.
18. LACHMANN HJ, LOWE P, FELIX SD *et al.*: In vivo regulation of interleukin 1beta in patients with cryopyrin-associated periodic syndromes. *J Exp Med* 2009; 206: 1029-36.
19. CHURCH LD, MCDERMOTT MF: Canakinumab, a fully-human mAb against IL-1beta for the potential treatment of inflammatory disorders. *Curr Opin Mol Ther* 2009; 11: 81-9.
20. Novartis. Ilaris prescribing information. http://wwwpharmausnovartiscom/product/pi/pdf/ilarispdf2010, Accessed Feb 17. http://wwwpharmausnovartiscom/product/pi/pdf/ilarispdf
21. LACHMANN HJ, KONE-PAUT I, KUEMMERLE-DESCHNER JB *et al.*: Use of canakinumab in the cryopyrin-associated periodic syndrome. *N Engl J Med* 2009; 360: 2416-25.
22. http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/DrugandBiologicApprovalReports/PriorityNDAandBLAApprovals/UCM090995.pdfhttp://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/DrugandBiologicApprovalReports/PriorityNDAandBLAApprovals/UCM090995.pdf accessed July 2010.
23. http://www.ema.europa.eu/pdfs/human/opinion/illaris_44782909en.pdfhttp://www.ema.europa.eu/pdfs/human/opinion/illaris_44782909en.pdf.accessed 2010.
24. NEVEN B, PRIEUR AM, QUARTIER DIT MAIRE P.: Cryopyrinopathies: update on pathogenesis and treatment. *Nat Clin Pract Rheumatol* 2008; 4: 481-9.
25. KUEMMERLE-DESCHNER JB, RAMOS E, BLANK N *et al.*: Canakinumab (ACZ885, a fully human IgG1 anti-IL-1β mAb) induces sustained remission in pediatric patients with cryopyrin-associated periodic syndrome (CAPS). *Arthritis Res Ther* 2011; 13: R34 [Epub ahead of print].
26. KUEMMERLE-DESCHNER JB, HACHULLA E, CARTWRIGHT R *et al.*: Two-year results from an open-label, multicentre, phase III study evaluating the safety and efficacy of canakinumab in patients with cryopyrin-associated periodic syndrome across different severity phenotypes. *Ann Rheum Dis* 2011; 70: 2095-102.
27. FDA DRUG SAFETY COMMUNICATION: Early communication about an ongoing safety review of Tumor Necrosis Factor (TNF) blockers (marked as Remicade, Embrel, Humira, and Cimzia).

PLOS ONE

The Contribution of *SAA1* Polymorphisms to Familial Mediterranean Fever Susceptibility in the Japanese Population

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Abstract

Background/Aims: Familial Mediterranean Fever (FMF) has traditionally been considered to be an autosomal-recessive disease, however, it has been observed that substantial numbers of patients with FMF possess only 1 demonstrable *MEFV* mutation. The clinical profile of familial Mediterranean fever (FMF) may be influenced by *MEFV* allelic heterogeneity and other genetic and/or environmental factors.

Methodology/Principal Findings: In view of the inflammatory nature of FMF, we investigated whether serum amyloid A (SAA) and interleukin-1 beta (IL-1β) gene polymorphisms may affect the susceptibility of Japanese patients with FMF. The genotypes of the -13C/T SNP in the 5′-flanking region of the *SAA1* gene and the two SNPs within exon 3 of *SAA1* (2995C/T and 3010C/T polymorphisms) were determined in 83 Japanese patients with FMF and 200 healthy controls. The same samples were genotyped for IL-1β-511 (C/T) and IL-1 receptor antagonist (IL-1Ra) variable number of tandem repeat (VNTR) polymorphisms. There were no significant differences between FMF patients and healthy subjects in the genotypic distribution of *IL-1β -511* (C/T), *IL-1Ra* VNTR and *SAA2* polymorphisms. The frequencies of *SAA1.1* allele were significantly lower (21.7% versus 34.0%), and inversely the frequencies of *SAA1.3* allele were higher (48.8% versus 37.5%) in FMF patients compared with healthy subjects. The frequency of -13T alleles, associated with the *SAA1.3* allele in the Japanese population, was significantly higher (56.0% versus 41.0%, $p = 0.001$) in FMF patients compared with healthy subjects.

Conclusions/Significance: Our data indicate that *SAA1* gene polymorphisms, consisting of -13T/C SNP in the 5′-flanking region and SNPs within exon 3 (2995C/T and 3010C/T polymorphisms) of *SAA1* gene, are associated with susceptibility to FMF in the Japanese population.

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Introduction

FMF is an inherited autoinflammatory disease characterized by recurrent self-limited fever, and serositis [1]. These episodes of inflammation are mainly mediated by a massive influx of neutrophils into serous cavities and are accompanied by an elevation of acute phase reactants [2]. The disease is associated with mutations in the *MEFV* gene that encodes pyrin, and is transmitted in an autosomal-recessive manner [3]. Therefore, heterozygotes are expected to be carriers or lack the clinical phenotype of FMF. However, mutations in the second *MEFV* allele have not been observed in 20–30% of patients with typical FMF [4]. Recent studies suggest that subjects with a single *MEFV* mutation may cross a threshold for the development of an FMF phenotype if they also express a combination of gene polymorphisms that favor increased inflammation [5]. These polymor-

phisms are thought to belong to genes of the interleukin-1β/innate immune system pathways [5]. The IL-1 family of cytokines is critical to the host's response to infection, and induction of innate immunity and acute phase inflammation [6]. The overproduction of IL-1β is responsible for a variety of autoinflammatory syndromes including FMF [7]. IL-1β requires cleavage via caspase-1 for proper secretion, which is facilitated by inflammasome activation [8]. The NOD-like receptor family, pryin domain containing 3 (NLRP3) inflammasome has emerged as a critical cytosolic sensor for a number of endogenous mediators, including amyloid protein [9].

Recent studies have shown that serum amyloid A (SAA) induced the expression of pro-IL-1β and activated the NLRP3 inflammasome in a cathepsin B and $P2X_7$-dependent manner resulting in secretion of mature IL-1β [10]. SAA is an acute-phase protein, which increases in the serum during inflammation and is susceptible to proteolytic cleavage to amyloid A (AA) protein, the major fibrillar protein in secondary amyloidosis [11]. An allelic variant of *SAA1.3*, was found to be associated with AA amyloidosis in Japanese rheumatoid arthritis (RA) patients [12]. In view of the recent genetic studies in FMF, other modifying genetic factors may contribute to the susceptibility or clinical expression of FMF in addition to *MEFV* mutations. Therefore, we attempted to determine the effect of gene polymorphisms on the susceptibility to FMF in the Japanese population.

Materials and Methods

Patients

In early 2007, a laboratory network collecting the genetic diagnosis of periodic fever was established at the Japan Autoinflammation Association (JAA), and *MEFV* gene analysis was carried out at the Clinical Research Center of National Hospital Organization (NHO) Nagasaki Medical Center. Up to October 2012, 481 consecutive unrelated patients with periodic fever were referred and underwent molecular diagnosis at the NHO Nagasaki Medical Center. All patient, who were originating from Japan, (East Japan n = 36, West Japan n = 47) were asked to complete a questionnaire that included demographics (sex, age of onset), family history (consanguinity of parents, family history of recurrent fever), and the presence of recurrent febrile attacks typical of FMF, including peritonitis, pleuritis, arthritis, and transient inflammatory responses. The genetic analysis of *MEFV* gene was approved by the Ethics Committee of Nagasaki Medical Center, and written informed consent was obtained from each individual. On the basis of Tel-Hashomer criteria [13], we divided the FMF patients in two groups: Group 1, typical FMF exhibiting the presence of 1 or more major criteria independent to the presence of minor criteria; Group 2, incomplete FMF exhibiting the absence of major criteria and 2 or more minor criteria. It is important to stress that response to colchicine was confirmed in almost all patients. As controls, 200 healthy Japanese individuals without pre-existing medical diseases (90 men and 110 women 14 to 64 years, with a mean age of 38.6±13.9 years) from East Japan (n = 86) and West Japan (n = 114) were enrolled in the study after obtaining informed consent.

MEFV gene Mutation analysis

All patients were undergone genetic analysis of *MEFV* gene exons 1, 2, 3 and 10 by direct sequencing. 2 milliliters of blood samples were collected from all subjects. Genomic DNA was extracted from whole blood by means of the Promega Wizard® Genomic DNA Purification Kit (Promega, USA). Mutation analysis was performed by genomic sequencing as described previously [14].

Genotyping

***SAA1* gene.** The genotype of the SAA1 -13C/T in the 5-region of exon 1 (rs11024595) was determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method [15]. The primers used for the PCR reaction were 5′-ACATCT TGTTCCCTC AGGTTG-3′ (sense) and 5′-GCTGTAGCTGAGCTGCGG-3′ (antisense).

The 229-bp PCR products were digested with restriction enzyme AciI (BioLabs, Beverly, MA, USA) and electrophoresed on a 12.5% polyacrylamide gel [15].

The *SAA1.1*, *1.3*, and *1.5* alleles, corresponding to the T-C, C-T, and C-C haplotypes of the C2995T (rs1136743) and C3010T (rs1136747) polymorphisms were also determined by the PCR-RFLP [15]. The primers used for the PCR reaction were 5′-GCC AATTACATCGGCCTCAG-3′ (sense) and 5′-TGGCCA AAGAATCTCTGG AT-3′ (antisense).

The 518-bp PCR products were digested with restriction enzyme BclI (Promega, San Luis Obispo, CA, USA) and BanI (Promega) and electrophoresed on a 2.5% agarose gel [15].

The genotype of the *SAA2* (rs2468844) was determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. The primers used for the PCR reaction were 5′-AGAGAATATCCAGAGACTCACAGGC-3′ (sense) and 5′-CAGGCCAGCAGGTCGGAAGT-3′(antisense). The 115 bp PCR products were digested with the restriction enzyme Nco I. The digested products were separated by 3% agarose gels by ethidium bromide staining [15].

IL-1Ra. For the IL-1RA VNTR polymorphism, the region including variable numbers of identical 86-bp tandem repeats was amplified by PCR using the following primers: 5′-CTCAGC-CAACACTCCTAT-3′ (sense) and 5′-TCCTGGTCTGCAGG-TAA-3′ (antisense). PCR products of 240(allele 2, two repeats), 325 (allele 3, three repeats), 410 (allele 4, four repeats), and 500 bp (allele 5, five repeats) were distinguished by agarose gel electrophoresis [16].

IL-1B-511. A fragment containing the *AvaI* polymorphic site at promoter region -511 of the IL-1B gene was amplified by PCR. PCR was carried out with primers, forward primer 5′-GCCTGAACCCTGCATACCGT-3′ (sense). 5′-GCCAA-TAGCCCTTGTCT-3′ (antisense). Fragments were separated by electrophoresis on 3% agarose with ethidium bromide staining using appropriate commercially available size markers for comparison. The C allele was designated if two bands of 92 and 63 bp were obtained, and the T allele was designated if a signal band of the undigested 155 bp was obtained. Genotypes were designated as follows: C/C, two bands of 92 and 63 bp: C/T, three bands of 155, 92, and 63 bp; and T/T, a single band of 155 bp [16].

Statistical Analysis

Results are expressed as mean±SD. Statistical analysis was performed with SPSS18 for windows (SPSS Statistics, Illinois). The statistical significance of differences between groups was calculated by either the chi-square test for categorical data and Mann-Whitney's U-test for quantitative data. Deviation from Hardy-Weinberg equilibrium was assessed using the SNPAlyze software ver. 7.0 (Dynacom, Yokohama, Japan). A p value of <0.05 was considered significant.

Table 1. *MEFV* genotypes, gender, and the presence of amyloidosis in 83 Japanese patients with FMF.

MEFV genotypes	n(%)	Typical	(Male/Female)	Incomplete	(Male/Female)	Amyloidosis	*p* value
M694I/M694I	4(4.8)	4	(1/3)			1	
M694I/normal	4(4.8)	4	(4/0)				
M694I/E148Q	13(15.7)	13	(10/3)				
M694I/P751L	1(1.2)	1	(0/1)				
M694I/E148Q/E148Q	1(1.2)	1	(0/1)				
M694I/E148Q/L110P	5(6.0)	5	(3/2)			2	
P369S/R408Q	4(4.8)			4	(1/3)		
E148Q/P369S/R408Q	3(3.6)			3	(0/3)		
E148Q/E148Q/P369S/R408Q	4(4.8)	2	(2/0)	2	(0/2)		
E148Q/R202Q/P369S/R408Q	1(1.2)			1	(1/0)	1	
E148Q/G304R/P369S/R408Q	1(1.2)			1	(0/1)		
E148Q/E148Q/P369S/P369S/R408Q/R408Q	1(1.2)			1	(0/1)		
E148Q/normal	12(14.5)	6	(3/3)	6	(1/5)		
R202Q/normal	2(2.4)	1	(1/0)	1	(0/1)		
G304R/normal	1(1.2)			1	(1/0)		
E148Q/E148Q	1(1.2)	1	(0/1)				
E148Q/L110P	6(7.2)	1	(0/1)	5	(1/4)		
E148Q/R202Q	1(1.2)	1	(0/1)				
E148Q/E148Q/L110P	3(3.6)	1	(1/0)	2	(2/0)		
E148Q/L110P/R202Q	2(2.4)			2	(0/2)		
E84K/normal	8(9.6)	5	(3/2)	3	(1/2)		
E84K/E148Q	1(1.2)			1	(0/1)		
E84K/G304R	1(1.2)			1	(0/1)		
Normal	3(3.6)			3	(1/2)		
Gender (Male/Fmale)			(28/18)		(9/28)		$P<0.0001$
Age (years)			36.2±18.2		39.9±19.6		$p=0.419$
Total			46		37		

Data are expressed as number (percentage). ± ; standard deviation. *p* values were calculated with chis-square test for qualitative data and Mann-Whitney test for quantitative data.

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Results

Demographic data and MEFV genotypes

We diagnosed 83 subjects, all of Japanese origins, as FMF. Among these patients, 44 were diagnosed as typical FMF and 37 were diagnosed as incomplete FMF. The demographic data of the newly-diagnosed FMF patients are summarized in Table 1. The overall male: female ration in patients with FMF was 0.8 (37:46). In incomplete FMF patients, the more affected sex is female in contrast to typical FMF (Table 1). The mean age ± SD at diagnosis was 37.9±18.8 years. Age at diagnosis of patients with typical FMF was similar to those with incomplete FMF (36.2±18.2 and 39.9±19.6 years, respectively; $p=0.419$; Table 1). By mutation analysis, the *MEFV* gene mutation could not be identified in 3 of 83 patients (3.6%). The distribution of the *MEFV* genotype was heterogenous. The most frequent genotype was M694I/E148Q, followed by E148Q/normal and E84K/normal. AA amyloidosis was histologically confirmed in 4 patients with FMF, whose genotypes were M694I/M694I *SAA1.5/15*, M694I/E148Q/L110P *SAA1.1/1.1*, M694I/E148Q/L110P *SAA1.3/1.5* and E148Q/R202Q/P369S/R408Q *SAA1.3/1.5*.

IL-1β and *IL-1Ra* gene polymorphism

The genotype frequencies of IL-1β-511 (C/T), and IL-1Ra VNTR polymorphisms in FMF patients and healthy subjects are summarized in Table 2. There were no significant difference in the frequencies of these polymorphisms between FMF patients and healthy subjects.

Association between *SAA2* gene polymorphism and FMF

There was no significant difference in the frequencies of the *SAA2* genotype between FMF patients and healthy subjects (Table 2).

Association between *SAA1* gene polymorphisms and FMF

A segment of the genomic *SAA1* gene with polymorphic sites was subjected to PCR/restriction fragment length polymorphism (PCR-RFLP) analysis. Table 3 shows the frequencies of individuals with various genotypes and alleles at the *SAA1* locus in either FMF patients (n = 83) or Japanese healthy subjects (n = 200). The allele frequency of *SAA1.1* was significantly lower in FMF patients compared with healthy subjects (21.7% versus 34.0%). Conversely,

Table 2. Frequencies of the genotypes at the *IL-1β* -511, *IL-1Ra* and *SAA2* loci in patients with FMF and healthy subjects.

	FMF patients	Healthy subjects	*p* value
	n = 83(%)	n = 200(%)	
Genotype at *IL-1β* -511 locus			
C/C	27(32.5)	59(29.5)	$\chi^2 = 0.934$ $p = 0.627$
C/T	43(51.8)	100(50.0)	
T/T	13(15.7)	41(20.5)	
Genotype at *IL-1Ra* locus			
1/1	73(88.0)	167(83.5)	$\chi^2 = 2.451$ $p = 0.857$
1/2	5(6.0)	20(10.0)	
1/3	0	1(0.5)	
1/4	4(4.8)	7(3.5)	
2/2	0	2(1.0)	
2/4	1(1.2)	3(1.5)	
Genotype at *SAA2* locus			
A/A	62(74.7)	163(81.5)	$\chi^2 = 2.338$ $p = 0.276$
A/G	19(22.9)	35(17.5)	
G/G	2(2.4)	2(1.0)	

IL-1β; Interleukin-*1β* .*IL-1Ra*; Interleukin-1 receptor antagonist .*SAA2*; Serum amyloid A2. Chi-square test was used to examine differences of genotype and allele frequencies between FMF patients and healthy subjects.
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the allele frequency of *SAA1.3* was higher in FMF patients compared with healthy subjects (48.8% versus 37.5%).

The -13C/T polymorphism, in the 5′-flanking region of the *SAA1* gene is associated with the *SAA1.3* allele and susceptibility to amyloidosis in Japanese RA patients [17]. We analyzed the frequency of -13C/T polymorphisms in FMF patients and Japanese healthy subjects. Allele frequencies of -13C/T were different among these two groups (Table 4), and -13T allele was significantly increased in FMF patients compared with healthy subjects (56.0% versus 41.0%, $p = 0.001$). These data suggest that the -13T allele is associated with susceptibility to FMF in the Japanese population. Allele frequencies of -13 C/T polymorphisms were also analyzed in typical or incomplete FMF patients. There was no significant difference in the frequencies -13T allele between typical and incomplete FMF patients (Table 5). Among 83 patients with FMF, 30 patients had 0 to 1 *MEFV* mutation (no mutation 3; heterozygous 27) and 53 patients at least 2 mutations (homozygous or compound heterozygous). There was no significant difference in *SAA1* gene polymorphisms between FMF patients with different numbers of *MEFV* mutations (Table 6).

Hardy-Weinberg equilibrium test

Finally, Hardy-Weinberg equilibrium was estimated by chi-square test with Yates' correction. There was no significant difference between observed and experienced frequencies of each genotype (*SAA1* -13C/T, *SAA2*, *IL-1β-511*) in the both FMF patients (Table 7) and healthy subjects (Table 7). These results indicated that these populations had a relatively stable genetic background and were stable for genetic statistical analysis.

Table 3. Frequencies of the genotypes and alleles at the *SAA1* locus of Japanese patients with FMF and healthy subjects.

	FMF patients	Healthy subjects	*p* value
	n = 83(%)	n = 200(%)	
Genotype at *SAA1* locus			
1.1/1.1	4(4.8)	24(12.0)	$\chi2 = 12.553$ $p = 0.028$
1.1/1.3	22(25.6)	49(24.5)	
1.1/1.5	6(7.2)	39(19.5)	
1.3/1.3	15(18.1)	27(13.5)	
1.3/1.5	29(34.9)	47(23.5)	
1.5/1.5	7(8.4)	14(7.0)	
Allele at *SAA1* locus			
1.1	36(21.7)	136(34.0)	$\chi2 = 9.563$ $p = 0.008$
1.3	81(48.8)	150(37.5)	
1.5	49(29.5)	114(28.5)	

SAA1; Serum amyloid A1. Chi-square test was used to examine differences of genotype and allele frequencies between FMF patients and healthy subjects.
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Discussion

FMF is considered to be an autosomal recessive disease [18]. The gene causing FMF is *MEFV*, which encodes pyrin, expressed in the cytoplasm of myeloid cells [2]. Pyrin is postulated to act as a negative regulator of IL-1-mediated inflammation [19]. However, approximately 30% of FMF patients exhibit a single *MEFV* mutation, despite sequencing of the entire *MEFV* genomic region and other autoinflammatory genes [20]. More recently it was demonstrated that pyrin truncation in mice did not show an overt phenotype of FMF, however, pyrin-deficient and FMF-associated B30.2 mutations "knock in" mice showed severe spontaneous inflammatory phenotype, suggesting that FMF may be caused by a gain of function by disease-associated missense changes in pyrin

Table 4. Frequencies of the genotypes and alleles at -13C/T *SAA1* locus of Japanese patients with FMF and healthy subjects.

	FMF patients	Healthy subjects	*p* value
	n = 83(%)	n = 200(%)	
Genotypes at -13C/T *SAA1*			
C/C	13(15.7)	67(33.5)	$\chi^2 = 11.538$ $p = 0.003$
C/T	47(56.6)	102(51.0)	
T/T	23(27.7)	31(15.5)	
Alleles at -13C/T *SAA1*			
T	93(56.0)	164(41.0)	$\chi^2 = 10.682$ $p = 0.001$
C	73(44.0)	236(59.0)	

SAA1; Serum amyloid A1. Chi-square test was used to examine differences of genotype and allele frequencies between FMF patients and healthy subjects.
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Table 5. Allele frequencies of *SAA1* gene polymorphisms in typical and incomplete FMF patients.

	FMF criteria		*p* value
	Typical	Incomplete	
	2n = 92(%)	2n = 74(%)	
Allele at *SAA1* locus			
1.1	16(17.4)	20(27.0)	$\chi^2 = 3.733$ $p = 0.155$
1.3	44(47.8)	37(50.0)	
1.5	32(34.8)	17(23.0)	
Alleles at -13C/T *SAA1*			
T	51(55.4)	42(56.8)	$\chi^2 = 0.029$ $p = 0.865$
C	41(44.6)	32(43.2)	

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Table 6. Number of *MEFV* gene mutations and *SAA1* gene polymorphisms in FMF patients.

	Number of mutations		*p* value
	0~1 mutations	≧2 mutations	
	2n = 60(%)	2n = 106(%)	
Allele at *SAA1* locus			
1.1	11(18.3)	25(23.6)	$\chi^2 = 0.955$ $p = 0.620$
1.3	29(48.3)	52(49.1)	
1.5	20(33.3)	29(27.4)	
Alleles at -13C/T *SAA1*			
T	33(55.0)	60(56.6)	$\chi^2 = 0.040$ $p = 0.841$
C	27(45.0)	46(43.4)	

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and that FMF may not be a pure autosomal recessive disease due to the loss of protein function [21]. One explanation is that subjects having a single *MEFV* mutation may develop an FMF phenotype in the presence of other inflammasome-related genes or in the presence of other environmental factors [22]. Therefore, the role of potential modifier genes and polymorphisms within these gene families should be assessed in conjunction with genotype-phenotype association studies. Polymorphisms in genes associated with the inflammasome pathway can affect the development of FMF [5]. For example, TLR2 polymorphisms may be an important factor in the susceptibility of FMF [23,24].

In this study, we investigated the *SAA1* and *IL-1β* gene polymorphisms in Japanese patients with FMF. There was no significant difference in *IL-1β-511* (C/T) or *IL-1Ra* VNTR polymorphisms between FMF patients and healthy subjects in accord to the previous report [25]. However, we demonstrated that *SAA1* gene polymorphisms, which are attributed to AA amyloidosis, might be also responsible for susceptibility to FMF. It is clear that genotypes at the *SAA1* locus are associated with an increased susceptibility to AA amyloidosis [26]. However, the contribution of these genotypes to the occurrence of non-amyloid, inflammatory disease has not been elucidated. In this study, we investigated the allele frequencies of *SAA1.1* and -13 (C/T) polymorphisms of the *SAA1* promoter region in Japanese patients with FMF. Our data demonstrated that the -13T allele polymorphism was a major risk factor and that the *SAA1.1* allele was protective for the occurrence of FMF in Japanese case-control studies.

The presence of 2 single-nucleotide polymorphisms (SNPs) within exon 3 of the *SAA1* gene, 2995 C/T and 3010 C/T, defined 3 haplotypes that corresponded to the *SAA1.1*, *SAA1.3*, and *SAA1.5* isoforms [26]. In Japanese patients with RA, homozygote expression of the *SAA1.3* allele was a proven risk factor, whereas *SAA1.1* appeared to be protective for AA amyloidosis [27]. In contrast, a strong positive association with *SAA1.1* has been established in Caucasian patients with amyloidosis secondary to juvenile idiopathic arthritis and FMF [28–30]. Moriguch *et al.* identified another *SAA1* SNP, the -13T/C SNP in the 5′-flanking region of the *SAA1* gene [17]. They observed the -13T allele was associated with AA amyloidosis, and associated with the *SAA1.3* allele in Japanese RA patients [17]. Interestingly, a polymorphism in the *SAA1* promoter -13T allele was found to be significantly associated with increased AA amyloidosis risk in both populations and to be in linkage disequilibrium with *SAA1.1* and *SAA1.3* in Caucasian and Japanese patients, thus apparently explaining the previous discrepancy [31–35]. Functional studies have demonstrated that the -13T allele is responsible for a higher transcriptional rate [36]. However, this did not result in higher serum levels of SAA, possibly due to increased proteolytic processing rates of *SAA1.1* and *SAA1.3* compared to *SAA1.5* [37]. The mechanisms by which the -13T allele predisposes to FMF remains to be unraveled and many possibilities have been suggested.

The overproduction of IL-1β, induced by NLRP3 inflammasome activation, is responsible for a variety of autoinflammatory syndrome including FMF. The NLRP3 inflammasome has emerged as a critical cytosolic sensor for a number of endogenous mediators, including amyloid proteins [6]. Recent studies indicated that SAA activates the NLRP3 inflammasome in a cathepsin B and $P2X_7$-dependent manner, resulting in the secretion of mature IL-1β [10]. The accumulation of newly formed AA amyloid fibrils and aberrant processing of SAA is relevant to AA amyloidogenesis [38]. Therefore, in subjects with AA amyloidogenic genetic factors, such as -13T allele, the presence of SAA-derived AA amyloid fibrils may implicate the NLRP3 inflammasome activation pathway, which is thought to be relevant to the pathogenesis of FMF. Jeru *et al.* demonstrated that the *SAA1* genotype influenced the severity of FMF and disease susceptibility through a negative selection process, providing new insights into the role of *SAA1* in the pathophysiology of FMF [39]. Assuming that *SAA1* gene polymorphisms induce the formation of AA amyloid fibrils, this suggests that the polymorphisms may be associated with the NLRP3 inflammasome activation process and susceptibility to FMF. These findings may provide insights into modifier factors, other than *MEFV*, in the development of FMF.

The gender discrepancy (female dominant in incomplete FMF) seen in the present study may result from hormonal or associated environmental factors, which generate a disease of atypical or milder severity in female. For example, the risk for developing amyloidosis had been shown to be higher in male patients with FMF [40,41]. These findings suggest that clinical variability observed in FMF may be party attributed to the influence of environmental factors including gender. The main limitations of the study are its localization to a certain country, and a limited number of patients.

Table 7. Frequencies of *SAA1* -13C/T, *SAA2*, *IL1β* -511 genotypes in Japanese patients with FMF and frequencies of *SAA1* -13C/T, *SAA2*, *IL1β* -511 genotypes in healthy subjects.

Frequencies of *SAA1* -13C/T, *SAA2*, *IL1β*-511 genotypes in Japanese patients with FMF				
Locus	**Genotype**	**Observed number(%)**	**Expected number[a]**	***p* value**
SAA1 -13C/T	C/C	13(15.7)	16.1	$\chi^2=1.292$ $p=0.256$
	C/T	47(56.6)	40.9	
	T/T	23(27.7)	26.1	
SAA2	A/A	62(74.7)	61.6	$\chi^2=0.007$ $p=0.932$
	A/G	19(22.9)	19.8	
	G/G	2(2.4)	1.6	
IL1β -511	C/C	27(32.5)	28.3	$\chi^2=0.144$ $p=0.704$
	C/T	43(51.8)	40.3	
	T/T	13(15.7)	14.3	
Frequencies of *SAA1* -13C/T, *SAA2*, *IL1β*-511 genotypes in healthy subjects				
Locus	**Genotype**	**Observed number(%)**	**Expected number[a]**	***p* value**
SAA1 -13C/T	C/C	67(33.5)	69.6	$\chi^2=0.384$ $p=0.535$
	C/T	102(51.0)	96.8	
	T/T	31(15.5)	33.6	
SAA2	A/A	163(81.5)	162.9	$\chi^2=0.104$ $p=0.747$
	A/G	35(17.5)	35.2	
	G/G	2(1.0)	1.9	
IL1β -511	C/C	59(29.5)	59.4	$\chi^2=0.001$ $p=0.978$
	C/T	100(50.0)	99.2	
	T/T	41(20.5)	41.4	

[a]Expected genotype frequencies based on observed allele frequencies and assuming Hardy-Weinberg equilibrium.

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In occlusion, this study shows a significant prevalence of the -13T allele in Japanese patients with FMF. This comparative case-control study demonstrated that the *SAA1* gene polymorphisms might affect susceptibility to FMF, which is presumed to be a monogenic disease. Further studies are required to determine the impact of *SAA1* gene polymorphisms and the occurrence of FMF in large studies in different geographic areas.

Ethics approval

This study was conducted with the approval of the ethical committees of Nagasaki Medical Center.

Author Contributions

Conceived and designed the experiments: KM KA JM HI AK RU YN . Performed the experiments: YJ YM MY. Analyzed the data: KM M. Nakamura YM. Contributed reagents/materials/analysis tools: SH YI TK M. Nakashima YF FN KE HF TN. Wrote the paper: KM M. Nakamura YM.

References

1. Ben-Chetrit E, Levy M (1998) Familial Mediterranean fever. Lancet 351: 659–64.
2. Chae JJ, Aksentijevich I, Kastner DL (2009) Advances in the understanding of familial Mediterranean fever and possibilities for targeted therapy. Br J Haematol 146: 467–78.
3. The International FMF Consortium (1997) Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. Cell 90: 797–807.
4. Marek-Yagel D, Berkun Y, Padeh S, Abu A, Reznik-Wolf H, et al (2009) Clinical disease among patients heterozygous for familial Mediterranean fever. Arthritis Rheum 60: 1862–6.
5. Booty MG, Chae JJ, Masters SL, Remmers EF, Barham B, et al (2009) Familial Mediterranean fever with a single MEFV mutation: where is the second hit? Arthritis Rheum 60: 1851–61.
6. Franchi L, Warner N, Viani K, Nuñez G (2009) Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev 227: 106–28.
7. Savic S, Dickie LJ, Battellino M, McDermott MF (2012) Familial Mediterranean fever and related periodic fever syndromes/autoinflammatory diseases. Curr Opin Rheumatol 24: 103–12.
8. Franchi L, Eigenbrod T, Muñoz-Planillo R, Nuñez G (2009) The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol 10: 241–7.

9. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, et al (2008) The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol 9: 857–65.
10. Niemi K, Teirilä L, Lappalainen J, Rajamäki K, Baumann MH, et al (2011) Serum amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway. J Immunol 186: 6119–28.
11. Marhaug G, Dowton SB (1994) Serum amyloid A: an acute phase apolipoprotein and precursor of AA amyloid. Baillieres Clin Rheumatol 8: 553–73.
12. Nakamura T, Higashi S, Tomoda K, Tsukano M, Baba S, et al (2006) Significance of SAA1.3 allele genotype in Japanese patients with amyloidosis secondary to rheumatoid arthritis. Rheumatology (Oxford) 45: 43–9.
13. Livneh A, Langevitz P, Zemer D, Zaks N, Kees S, et al (1997) Criteria for the diagnosis of familial Mediterranean fever. Arthritis Rheum 40: 1879–85.
14. Tanaka M, Migita K, Miyashita T, Maeda Y, Nakamura M, et al (2007) Coexistence of familial Mediterranean fever and Sjögren's syndrome in a Japanese patient. Clin Exp Rheumatol 25: 792.
15. Ajiro J, Narita I, Sato F, Saga D, Hasegawa H, et al (2006) SAA1 gene polymorphisms and the risk of AA amyloidosis in Japanese patients with rheumatoid arthritis. Mod Rheumatol 16: 294–9.
16. Migita K, Maeda Y, Abiru S, Nakamura M, Komori A, et al (2007) Polymorphisms of interleukin-1beta in Japanese patients with hepatitis B virus infection. J Hepatol 46: 381–6.
17. Moriguchi M, Terai C, Kaneko H, Koseki Y, Kajiyama H, et al (2001) A novel single-nucleotide polymorphism at the 5′-flanking region of SAA1 associated with risk of type AA amyloidosis secondary to rheumatoid arthritis. Arthritis Rheum 44: 1266–72.
18. El-Shanti H, Majeed HA, El-Khateeb M (2006) Familial mediterranean fever in Arabs. Lancet 367: 1016–24.
19. Ting JP, Kastner DL, Hoffman HM (2006) CATERPILLERs, pyrin and hereditary immunological disorders. Nat Rev Immunol 6: 183–95.
20. Ozen S (2009) Changing concepts in familial Mediterranean fever: is it possible to have an autosomal-recessive disease with only one mutation? Arthritis Rheum 60: 1575–7.
21. Chae JJ, Cho YH, Lee GS, Cheng J, Liu PP, et al. (2011) Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1ß activation and severe autoinflammation in mice. Immunity 34: 755–68.
22. Ozen S, Bakkaloglu A, Yilmaz E, Duzova A, Balci B, et al (2003) Mutations in the gene for familial Mediterranean fever: do they predispose to inflammation? J Rheumatol 30: 2014–8.
23. Ozen S, Berdeli A, Türel B, Kutlay S, Yalcinkaya F, et al (2006) Arg753Gln TLR-2 polymorphism in familial mediterranean fever: linking the environment to the phenotype in a monogenic inflammatory disease. J Rheumatol 33: 2498–500.
24. Speletas M, Kalala F, Mitroulis I, Papadopoulos V, Merentiti V, et al (2009) TLR2 and TLR4 polymorphisms in familial Mediterranean fever. Hum Immunol 70: 750–3.
25. Balci-Peynircioglu B, Taskiran ZE, Türel B, Arici M, Bakkaloglu A, et al. (2008) The analysis of interleukin-1 receptor antagonist and interleukin-1beta gene polymorphisms in Turkish FMF patients: do they predispose to secondary amyloidosis? Clin Exp Rheumatol 26: S99–102.
26. Nakamura T (2011) Amyloid A amyloidosis secondary to rheumatoid arthritis: pathophysiology and treatments. Clin Exp Rheumatol 29: 850–7.
27. Yamada T, Okuda Y, Takasugi K, Wang L, Marks D, et al (2003) An allele of serum amyloid A1 associated with amyloidosis in both Japanese and Caucasians. Amyloid 10: 7–11.
28. Booth DR, Booth SE, Gillmore JD, Hawkins PN, Pepys MB (1998) SAA1 alleles as risk factors in reactive systemic AA amyloidosis. Amyloid 5: 262–5.
29. Yilmaz E, Balci B, Kutlay S, Ozen S, Ertürk S, et al. (2003) Analysis of the modifying effects of SAA1, SAA2 and TNF-alpha gene polymorphisms on development of amyloidosis in FMF patients. Turk J Pediatr 45: 198–202.
30. Akar N, Hasipek M, Akar E, Ekim M, Yalçinkaya F, et al. (2003) Serum amyloid A1 and tumor necrosis factor-alpha alleles in Turkish familial Mediterranean fever patients with and without amyloidosis. Amyloid 10: 12–6.
31. Cazeneuve C, Ajrapetyan H, Papin S, Roudot-Thoraval F, Geneviève D, et al (2000) Identification of MEFV-independent modifying genetic factors for familial Mediterranean fever. Am J Hum Genet 67: 1136–43.
32. Gershoni-Baruch R, Brik R, Zacks N, Shinawi M, Lidar M, et al (2003) The contribution of genotypes at the MEFV and SAA1 loci to amyloidosis and disease severity in patients with familial Mediterranean fever. Arthritis Rheum 48: 1149–55.
33. Bakkaloglu A, Duzova A, Ozen S, Balci B, Besbas N, et al (2004) Influence of Serum Amyloid A (SAA1) and SAA2 gene polymorphisms on renal amyloidosis, and on SAA/C-reactive protein values in patients with familial mediterranean fever in the Turkish population. J Rheumatol 31: 1139–42.
34. Akar N, Hasipek M, Oztürk A, Akar E, Tekin M (2006) Serum amyloid A1 -13 T/C alleles in Turkish familial Mediterranean fever patients with and without amyloidosis. J Nephrol 19: 318–21.
35. Mavragani CP, Yiannakouris N, Zintzaras E, Melistas L, Ritis K, et al (2007) Analysis of SAA1 gene polymorphisms in the Greek population: rheumatoid arthritis and FMF patients relative to normal controls. Homogeneous distribution and low incidence of AA amyloidosis. Amyloid 14: 271–5.
36. Moriguchi M, Kaneko H, Terai C, Koseki Y, Kajiyama H, et al (2005) Relative transcriptional activities of SAA1 promoters polymorphic at position -13(T/C): potential association between increased transcription and amyloidosis. Amyloid 12: 26–32.
37. van der Hilst JC, Yamada T, Op den Camp HJ, van der Meer JW, Drenth JP, et al (2008) Increased susceptibility of serum amyloid A 1.1 to degradation by MMP-1: potential explanation for higher risk of type AA amyloidosis. Rheumatology 47: 1651–4.
38. Yakar S, Livneh A, Kaplan B, Pras M (1995) The molecular basis of reactive amyloidosis. Semin Arthritis Rheum 24: 255–61.
39. Jéru I, Hayrapetyan H, Duquesnoy P, Cochet E, Serre JL, et al (2009) Involvement of the modifier gene of a human Mendelian disorder in a negative selection process. PLoS One 4: e7676.
40. Gershoni-Baruch R, Brik R, Zacks N, Shinawi M, Lidar M, et al. (2003) The contribution of genotypes at the MEFV and SAA1 loci to amyloidosis and disease severity in patients with familial Mediterranean fever. Arthritis Rheum 48: 1149–55.
41. Gershoni-Baruch R, Brik R, Lidar M, Shinawi M, Livneh A (2003) Male sex coupled with articular manifestations cause a 4-fold increase in susceptibility to amyloidosis in patients with familial Mediterranean fever homozygous for the M694V-MEFV mutation. J Rheumatol 30: 308–12.