Increased serum concentrations of neutrophil-derived protein S100A12 in heterozygous carriers of *MEFV* mutations

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ABSTRACT

Objective. *To assess subclinical inflammation in heterozygous carriers of Mediterranean fever (MEFV) gene mutations, analysis of classical inflammation markers and S100A12 was performed.*

Methods. *Exons 2, 3, and 10 of the* MEFV *gene, C-reactive protein (CRP), serum amyloid A protein (SAA), procalcitonin (PCT), and S100A12 concentrations, erythrocyte sedimentation rate (ESR), and differential blood count were analysed in apparently healthy parents (n=26) of homozygous children with familial Mediterranean fever (FMF). Their general health condition was assessed by a standardised questionnaire. In order to collect data on the disease course, subjects were reevaluated after 5 years by means of telephone interview and/or questionnaire.*

Results. *Twenty-two individuals with one typical mutation in the MEFV gene were included. Mean values (mean±SEM) of classical inflammation markers were within the normal range (ESR of 11.7±1.9 mm/h, SAA 4.7±0.4 mg/l, CRP 0.26±0.04 mg/dl), while PCT was non-detectable in all cases (<0.1 µg/l). Eleven subjects showed elevated S100A12 levels [>140 ng/ml] with a mean concentration of 205±43 ng/ml. Thus, the mean value of S100A12 was 1.5-fold higher than the regular cut-off.*

Conclusion. *50% of the heterozygous MEFV mutation carriers exhibited elevated S100A12 levels, supporting previous observations that S100 molecules are very sensitive biomarkers of subclinical inflammation. Possibly, S100A12 could be a prognostic biomarker to detect individuals at risk of FMF manifestation who might benefit from colchicine therapy.*

Introduction

Familial Mediterranean fever (FMF) is primarily described as an autosomal recessively inherited autoinflammatory disease, although in many cases it behaves like an autosomal dominant disorder with gene dosis effect. Gain-of-function mutations within the *MEFV* gene encoding pyrin lead to overproduction of pro-inflammatory cytokines in an apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC)-dependent process (1).

There is now evidence that in up to 30% of FMF patients only one *MEFV* mutation can be found despite extensive molecular genetic analysis (2). Furthermore, heterozygous mutation carriers might exhibit some degree of inflammatory activity not leading to the full clinical picture of FMF (3), might develop classical FMF in the future, and are at risk for the development of other inflammatory diseases (4, 5). In these subjects, the presence of a persistently enhanced acute phase reaction may on the other hand be a selective survival advantage (6). It is therefore mandatory to establish inflammation markers with the highest possible sensitivity in order to detect inflammatory activity in these individuals.

S100A12 has recently been introduced as a novel biomarker of inflammation in FMF (7). This protein is a member of the damage-associated molecular pattern (DAMP) family of molecules, which are important pro-inflammatory factors of innate immunity. S100A12 is almost exclusively expressed by granulocytes and is released by activated cells in inflamed or damaged tissue (8, 9).

To determine subclinical inflammation in *MEFV* mutation carriers, we quantified S100A12 levels and compared these to conventional biomarkers.

Methods

Study population

Apparently healthy parents (n=26) of FMF-affected homozygous children participated in the study. General health condition was assessed by a standard-

ised questionnaire. Answers were given in terms of clinical presentations of FMF (recurrent fever, abdominal pain or chest pain attacks, joint or skin inflammation), current and previous infections, concomitant diseases of the heart, nervous system, kidney, or other organs, regular medication, history of appendectomy, family history of FMF disease, or kidney failure. Exclusion criteria were clinical and laboratory signs of an acute infection with PCT >0.5 µg/l, fever >38.5°C, or reported infection within the last 14 days. The study was approved by the Ethical Commission of the Medical Faculty of the Charité (EA2/034/09). All patients gave their informed consent.

Determination of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), procalcitonin (PCT), serum amyloid A protein (SAA), and differential blood count

ESR was determined directly in the outpatient department by means of the Westergren method (normal cut-off: <20mm/h). CRP (<0.5mg/dl) and PCT (<0.5 µg/l) concentrations as well as the differential blood count were analysed in the hospital laboratory of the Charité. Samples for the measurement of SAA (<10 mg/l) were sent to an external reference laboratory (Limbach, Heidelberg, Germany). Concentrations were determined by a latex-enhanced nephelometric immunoassay (N Latex SAA; Dade Boehring Diagnostic, Schwalbach, Germany).

Determination of S100A12

Serum samples were centrifuged within 2 h after blood drawing and frozen at -20°C until measurement. Concentrations of S100A12 were determined in patient sera by a double sandwich ELISA system established at the University Hospital Muenster, as described previously (10). Samples were diluted, and measured blinded to the researchers in duplicates of at least three different dilutions within the linear range of the ELISA. Normal S100A12 cut-off [140 ng/ml] was determined as mean+2SD in 50 healthy, age-matched Caucasian control subjects (Table I). A present infection was excluded by measuring ESR und CRP levels (6±3 mm/h and <0.5 mg/dl, respectively).

MEFV *gene sequencing*

Sequence analysis of MEFV exons 2, 3, and 10 was performed blinded at an external reference laboratory (Department of Clinical Chemistry – Großhadern, University of Munich) as previously described (11).

Statistical analysis

SPSS V.19.0 (SPSS; Chicago, Illinois, USA) and Graph PadPrism 6 (Software, Inc. San Diego, California, USA) for Windows were used for statistical analyses. Data are expressed as mean±SEM (standard error of the mean) except where stated otherwise. There was no missing test result, and no indeterminate or outliers were excluded. The Mann-Whitney U-Test for two independent samples was performed to determine significant differences between distinct categories. We did not adjust the *p*-values for multiple testing because of the exploratory study design. *p* values ≤0.05 were considered to be statistically significant.

Results

Patients

Between July and October 2009, 26 apparently healthy parents, whose children attended the outpatient clinic for paediatric rheumatology at the Children's Hospital of the Charité, were recruited. Thereof, 22 patients finally formed the study group composed of 12 females and 10 males; two were excluded due to compound heterozygosity (M694V/A744S and E148Q/M680I) and two due to previous infections.

Direct interviews revealed that six individuals (27%) suffered of occasional recurrent chest and/or abdominal pain attacks. All of them carried a p.M694V mutation in heterozygous form.

In a final contact in November 2014, 12 of the 22 individuals (54%) were interviewed once again either directly or by the standardised questionnaire. Within the group of six parents initially suffering from recurrent pain attacks, one female patient reported of colchicine treatment since the end of 2009 whereby pain attacks resolved, indicating FMF. Another four were diagnosed as having gastritis, meteorism, musculoskeletal pain or intercostal neuralgias as cause for recurrent symptoms. Thus, in five patients of this group, only one minor criterion of the FMF criteria by Livneh *et al*. was met and no definite diagnosis of FMF was established (12). Detailed patients characteristics are summarised in Table I.

Serum concentrations of inflammation markers

The leukocyte and neutrophil concentrations were 7.19±0.4 /nl (median, IQR: 6.82, 5.83–7.78) and 4.17±0.3 /nl (median, IQR: 3.84, 3.20–5.01), respectively. Only one subject showed an elevated leukocyte count without neutrophilia. Similarly, mean concentrations of CRP (0.26±0.04 mg/dl, median, IQR: 0.20, 0.13–0.42) and SAA (4.7±0.4 mg/l, median, IQR: 4.95, 3.5–5.9) as well as ESR (11.7±1.9 mm/h, median, IQR: 8, 5–17) were within the normal range (Table I). Three subjects had discretely elevated CRP levels (maximum 0.63 mg/dl), while five others showed an ESR elevation (maximum 30 mm/h). Not a single SAA level above the cut-off was found, and the PCT values were below the detection limit [0.1 µg/l] in all cases.

In contrast, eleven subjects (50%) showed elevated S100A12 levels. The mean S100A12 concentration was 205±43 ng/ml (median, IQR: 130, 67–260) with a range between 33 ng/ml and 760 ng/ml. Thus, the mean value of S100A12 was 1.5-fold higher than the regular cut-off (Fig. 1).

Interestingly, in both individuals exhibiting the highest S100A12 levels (760 ng/ml and 750 ng/ml), no increase of other inflammation markers or an abnormal differential blood count was detected. One of them was the female patient with the later established FMF diagnosis who had a S100A12 level of 750 ng/ml. Comparing the group with pain attacks to that without clinical symptoms, mean S100A12 levels were significantly higher (402±119 ng/ml to 131±22 ng/ml; median, IQR: 330, 150–750 to 96.5, 63.5–205; p=0.015) (Fig. 2).There was no other distinguishing feature between those with elevated and those with normal S100A12 levels.

Table I. Patient characteristics.

	Age, years median (range)	Sex, m/f	Mutation	CRP, mg/dl	SAA, mg/l	ESR, mm/h	S100A12, ng/ml
All heterozygous carriers	40.3 (29.2 – 51.7)	10/12	16 x M694V, 3 x V726A, 2 x M680I, 1 x M694I	0.26±0.04	4.7±0.4	11.7±1.9	205±43
Subjects with pain attacks	41.3 (30.3 - 47)	2/4	6 x M694V	0.31±0.07	4.9±0.8	11.8±4.0	402±119
Subjects without pain attacks	39.3 (29.2 - 51.7)	8/8	10 x M694V, 3 x V726A, 2 x M680I, 1 x M694I	0.24±0.05	4.6±0.5	11.7±2.3	131±22
Healthy Controls	31 (18 – 57)	32/18	n.d.	all < 0.5	n.d.	6±0.4	71±5

Detailed patient characteristics

Ethnicity, sex, age (years)	Mutation	Family history for FMF	Symptoms / Diseases (in bold: symptomatic patients)	CRP, mg/dl	SAA, mg/l	ESR, mm/h	S100A12, ng/ml	Leuko/ Neutro/nl
Turkish, f, 39	**M680I**	**pos.**	**hypothyreosis, diabetes, nervous disease**	**0.13**	**3.7**	**25**	**220**	**4.4 / 2**
Iranian, f, 51	**M680I**	**neg.**	**none**	**0.06**	**5.2**	**2**	**260**	**7.8 / 6**
Turkish, f, 30	**M694V**	**neg.**	**rec. chest pain (intercostal neuralgia)*, rec. abdominal pain; (1 minor criterion)**	**0.46**	**3.5**	**5**	**390**	**7.3 / 3.9**
Turkish, m, 45	**M694V**	**uk**	**sporadic abdominal pain (1 minor criterion)**	**0.2**	**2.8**	**12**	**760**	**6.2 / 3.7**
Turkish, f, 40	**M694V**	**neg.**	**rec. chest pain (musculoskeletal)*, rec. strong abdominal pain;(1 minor criterion)**	**0.17**	**5.4**	**30**	**150**	**5.4 / 2.6**
Turkish, m, 41	**M694V**	**neg.**	**gastritis*, rec. chest pain; (1 minor criterion)**	**0.46**	**6.1**	**6**	**270**	**6.3 / 3.2**
Turkish, f, 47	**M694V**	**neg.**	**sporadic abdominal pain (meteorism)*, rec. chest pain, hyperthyreosis, art. Hypertonia, diabetes; (1 minor criterion)**	**0.14**	**8.1**	**4**	**94**	**5.2 / 2.9**
Turkish, f, 41	**M694V**	**pos.**	**FMF diagnosed 2009, diabetes, hyperthyreosis, art. hypertonia**	**0.41**	**3.6**	**14**	**750**	**9.9 / 6.2**
Turkish, f,45	M694V	uk	appendectomy	0.42	3.3	22	220	5 / 2.9
Turkish, m, 46	M694V	neg.	none	0.13	7.8	3	37	6.5 / 3.8
Turkish, m, 29	M694V	neg.	none	0.44	5.6	3	330	6.5 / 3.9
Turkish, m, 35	M694V	pos.	none	0.26	4.9	2	179	10.7 / 6.8
Turkish, f, 34	M694V	uk	back pain (herniated disc)	0.63	7.7	17	84	11.2 / 7.6
Armenian, m, 44	M694V	neg.	none	0.2	4.5	8	110	5.8 / 3.3
Armenian, f, 34	M694V	neg.	none	0.03	3.7	7	190	7.5 / 5
Turkish, m, 40	M694V	neg.	none	0.09	1.6	5	93	7.5 / 4.2
Turkish, f, 37	M694V	pos.	none	0.32	6.6	15	62	5.8 / 3.5
Turkish, m, 44	M694V	neg.	appendectomy	0.08	1.7	12	33	10.5 / 5
Syrian, f, 33	M694I	neg.	appendectomy	0.07	1.7	8	67	7.7 / 4.7
Turkish, m, 37	V726A	pos.	none	0.16	5	30	52	5.8 / 0.5
Turkish, m, 41	V726A	neg.	appendectomy	0.21	5.9	6	100	7.1 / 2.5
Turkish, f, 39	V726A	neg.	none	0.55	5.2	22	65	8 / 4.8

*reasons for symptoms were evaluated in direct telephone interview; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; FMF: familial Mediterranean fever; SAA: serum amyloid A protein; n.d.: not determined; pos.: positive; neg.: negative; uk: unknown; rec.: recurrent.

Discussion

Our results obtained in a cohort of heterozygous carriers of *MEFV* mutations confirm previous findings of subclinical inflammation in these subjects (7,13). Despite normal levels of classical inflammation markers, some degree of inflammatory activity was detected, as shown by the elevated S100A12 levels in 50% of the subjects. S100A12 thus might be a more sensitive marker in *MEFV* mutation carriers than classical inflammation markers, as shown in FMF patients before (7).

Several previous publications demonstrated that the clinical picture of heterozygotes can resemble that of homozygous patients. In the cohort of Koné-Paut *et al*., disease in heterozygous patients was nearly as severe as in homozygous patients, and 82% required colchicine treatment (2).

In our study group, FMF diagnosis was established after study inclusion in one subject, and colchicine resolved pain attacks. Five other individuals reported recurrent chest and/or abdominal pain attacks, diagnosed as gastritis, musculoskeletal pain, intercostal neuralgia, and meteorism in four of them. According to the FMF criteria of Livneh *et al*., atypical attacks may occur without concomitant fever (12). Therefore, we considered five of these patients as suffering from an incomplete (1 minor criterion) disease (Table I) although precise classification of symptoms were hampered by the fact that clinical data were collected retrospectively by a questionnaire (*e.g*. probably elevated body temperature). But interestingly, mean S100A12 levels in this group were still significantly higher than in the remaining subjects without pain attacks, suggesting autoinflammation with mild clinical features.

As previously demonstrated, the degree of (sub-)clinical inflammation determined by the S100A12 level depends on the disease activity, *e.g*. 440±80 ng/ml in well controlled, 6,260±2,120 ng/ml in poorly controlled FMF, and 33,500

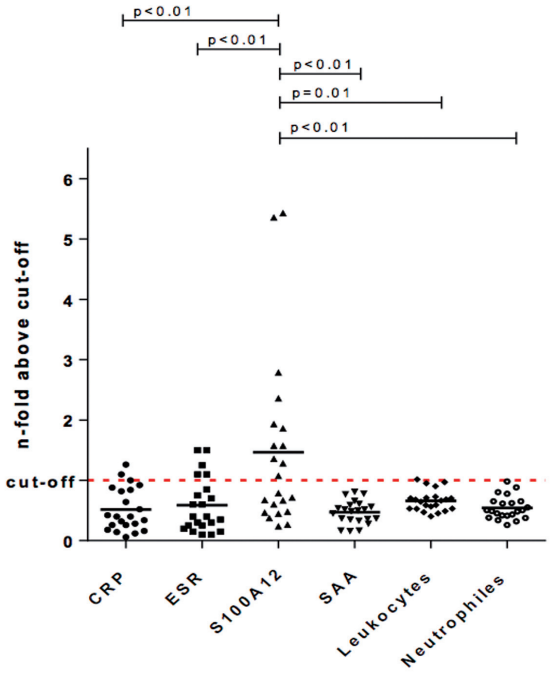

Fig. 1. Inflammatory markers of all patients presented in comparison to the normal cut-off (dashed line).
Mean S100A12 concentration was 1.5-fold higher than the regular cut-off and significantly higher than mean concentrations of C-reactive protein (CRP), ESR, serum amyloid A protein (SAA), leucocytes and neutrophils, respectively. Mann-Whitney U-Test for two independent samples was used to test the differences between the groups. *p*-values ≤0.05 were considered to be significant.

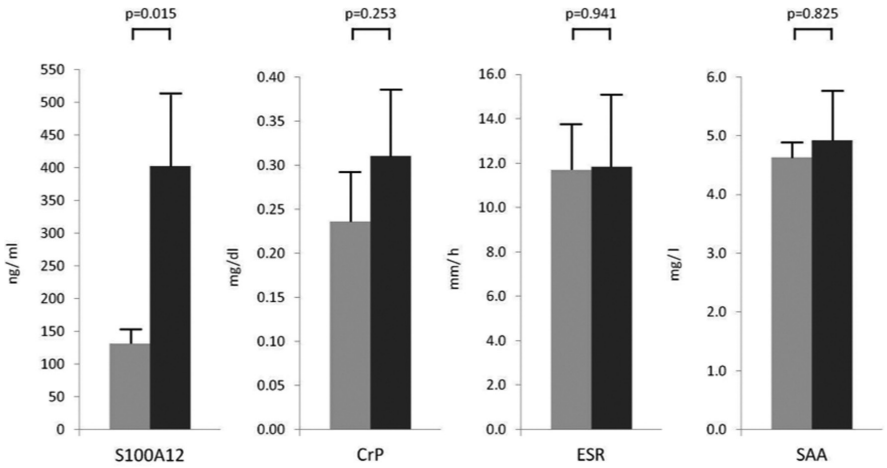

Fig. 2. Inflammatory markers analysed in patients with and without pain attacks.
Serum concentrations of S100A12, C-reactive protein (CRP), ESR and Serum Amyloid A (SAA) are depicted. Y-axes differ in ranges and concentrations. Grey bars: patients without pain attacks (n=16); black bars: patients with pain attacks (n=6). Mann-Whitney U-Test for two independent samples was used to test the differences between the groups. *p*-values ≤0.05 were considered to be significant.

± 22,200 ng/ml during a flare (7). The current study also demonstrates a correlation between the clinical phenotype and S100A12 levels with 131±22 ng/ml in those without any clinical complaints and with 402 ± 119 ng/ml in heterozygous carriers exhibiting some pain symptoms. Thus, the level of this serum marker mirrors the different clinical disease expressions (no mutation < heterozygous without complaints < heterozygous with some complaints < well controlled FMF patients < poorly controlled FMF patients < flare).

In our cohort, a heterozygous p.M694V status was the most frequently found phenotype. This mutation is known to be associated with a severe disease in homozygous patients and is also the most frequent alteration in Turkish cohorts. All of the subjects with reported pain attacks were p.M694V heterozygotes. Within the group with elevated S100A12 levels, only p.M680I – known to be a another severe mutation – occured besides p.M694V (14).
Due to the structure of supply, the study has the limitation that we could only analyse one single S100A12 level instead of serial measurements.
Despite the small cohort size we propose that S100A12 is a valuable biomarker for the identification and follow-up of the subgroup of heterozygous *MEFV* carriers at risk of developing overt clinical disease.

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