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Serum acidic mammalian kinase as a new laboratory test to define subclinical inflammation in Familial Mediterranean fever

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ABSTRACT

Aim: To investigate the relationship between acidic mammalian chitinase (CHIA) level and autoinflammatory diseases, especially in Familial Mediterranean fever.

Methods: We first analyzed CHIA expression, methylation in various autoinflammatory diseases, including, SLE (Systemic Lupus Erythematosus), RA (Rheumatoid Arthritis), SJS (Stevens-Johnson syndrome), SSc (Systemic Sclerosis) and T1D (Type 1 diabetes) patients, case-control and correlation between the MEFV and CHIA genes by using bioinformatics tools. We then measured serum CHIA level in ninety individuals; thirty FMF attacks, thirty FMF remissions and thirty healthy control groups. Statistical analysis was used to evaluate the interaction between clinical parameters and serum CHIA level. The potential of serum CHIA level was tested using AUC and ROC analysis.

Results: According to our ADEx analysis, we observed high CHIA expression in SLE, RA and T1D patients than in the control group. Moreover, we detected that the methylation level decreased in each disease, especially in the cg17143643 and cg7497781 probes. We also observed a correlation between MEFV and CHIA in these autoinflammatory diseases. According to our ELISA results, we also showed an increased CHIA level in FMF attack and remission as compared to the control group in serum (p <0.001, p =0.007; resp). We further observed a relationship between CHIA level and patients with amyloidosis, attack per month, and neutrophil and WBC levels.

Conclusion: Our primary data suggest that CHIA is related to FMF pathogenesis and can be followed in the subclinical period of the disease.

Key words: Familial Mediterranean fever, acidic mammalian chitinase, autoinflammatory diseases, CHIT, TSA1902.

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Introduction

Autoinflammatory diseases are defined by recurrent attacks of localized or systemic inflammation resulting from disorders in the innate immune system. Familial Mediterranean fever (FMF) disease is occurred by pathogenic variants on the MEFV gene which is located on chromosome 16 (16p13.3) [1]. FMF is mostly seen in the Mediterranean and Middle Eastern populations (Jews, Armenians, Arabs, Turks), but it is possible to come across FMF in the rest of the world [2]. Although the clinical findings of the disease are fever, abdominal, chest, or joint

pain and serositis with recurrent attacks, and these findings may vary according to the ethnicity or family history of patients [3]. In addition, these findings are not specific to FMF and they can also be seen in other diseases. Therefore, there is a need for more specific markers of the disease activity/disease attack.

Chitinases are enzymes found in humans and many parasites that break down polysaccharides, called chitin. Chitinase proteins secreted in excess by activated macrophages are included in the innate immunity against chitin-related pathogens [4]. For example, CHIT1 activity is significantly increased in diseases such as diabetic nephropathy, ALS, chronic obstructive pulmonary disease (COPD), alzheimer's, and idiopathic pulmonary fibrosis (IPF). In addition, chitinases can be a useful biomarker for some diseases such as asthma allergic rhinitis, autoimmune, dental, neurological, metabolic or liver-related diseases and autoinflammatory diseases [5,6,7]. CHIA (Acidic mammalian chitinase, AMCASE, CHIT2, TSA1902) one of the chinitase member, is expressed by macrophages and eosinophils, act a role in immunity and take part in Type 2 helper T (Th2)-mediated inflammation [8]. Although chitinases are associated with many diseases, studies about the role of CHIA in FMF disease are very limited. Therefore, in the current study, we examined the serum CHIA levels in autoinflammatory disease, specially, in FMF patients during attack and remission.

Materials and metods

Evaluation of CHIA gene expression and methylation data in various autoinflammatory disease

We used ADEx database (https://adex.genyo.es) and analyzed transcriptomics and methylation profiles from 5609 samples for RA (Rheumatoid Arthritis), SSc (Systemic Sclerosis), SLE (Systemic Lupus Erythematosus), SJS (Stevens-Johnson syndrome) and T1D (Type 1 diabetes) patients and case-control [9]. The methylation and expression of the CHIA gene and its association with MEFV gene were also analyzed in the ADEx databases. The accession codes for datasets that were used in this study are GSE110607 GSE11907, GSE117931, GSE38351, GSE50772, GSE56606, GSE59520, GSE57869 and GSE87095.

Selection of study groups

Sixty randomly selected patients aged 18-60 years diagnosed with FMF and thirty people without any systemic disease were determined as the control group. The FMF patients were determined based on the Tel-Hashomer criteria [10]. All patients were being treated with colchicine (1–2 mg/day), were identified. MEFV analysis results and amyloidosis status of the patients were obtained from the hospital database. Blood samples were collected during attack-free periods (at least 15 days after the last attack) from Physical Therapy department and attack periods (in the first 24 hours) from Emergency department at Tokat Gaziosmanpaşa University.

Patients and control groups, epidemiological data, sex, age, familial history, age at diagnosis and clinical findings were recorded by the doctor. The purpose of the procedure was explained to the patients and the Volunteers Informing Consent form was filled out.

4 ml of blood was collected from each individual in a hemogram tube, centrifuged serum samples were stored at -80 ^{o}C. While collecting blood samples, patients with no diseases that could stimulate or alter CHIA activity were chosen. Main clinical data (fever, symptoms, attack period, amyloidosis status, colchicine uses, or dose) and epidemiologic (age,

sex, familial history) were recorded by the doctor. All participants were informed and their consent was obtained.

This study complied with the Declaration of Helsinki and has been affirmed by the medical ethics committee at Tokat Gaziosmanpasa University (#19-KAEK-164).

Determination of serum CHIA level of FMF patients and control group by ELISA

Evaluation of the serum CHIA level was examined using the ELISA method. The protocol was designed by the manufacturer (Sunred, China). Briefly, the serum samples were centrifuged at room temperature for 10-12 minutes at 2000-3000 rpm. The standard dilution was prepared according to the instructions. Standard (50 µl) and st-HPR (50 µl) were placed in the first five boxes. The sixth well was left empty, and no antibodies and samples were placed. Then, the serum sample (40 µl), the antibody (10 µl) and the HRP (50 µl) were placed in all remaining wells that contain all of the patients and controls samples. The plate was then covered with paraffin and incubated at 37°C for 60 minutes. A washing solution was prepared and appropriate dilution was performed. After removing the samples, the wells were washed five times with a wash buffer and chromogen A/ B (50 µl) solution were added in each well, respectively. The plate was then incubated for 10 minutes at 37°C away from light. 50 µl stop solution was added and a color change from blue to yellow was observed. Optical density was detected at 450 nm by Spectrophotometer (Thermo Scientific, USA). The concentrations of the ELISA samples were determined by preparing a standard curve graph.

Statistical Analysis: The Kolmogorov-Smirnov method was used to detect the normal distribution of the patients. Pearson correlation analysis was used to evaluate the interaction between clinical parameters and serum CHIA level. Statistical significance was analyzed using Student's t-test. In addition, the biomarker capacity of serum CHIA level was tested using AUC analysis. All statistical analyzes were performed with SPSS 23.0 software (SPSS Inc., Chicago, IL, USA) and graphs were symbolized using GraphPad Prism version 8.3.0 (GraphPad Software, Inc., CA, USA). A two-tailed P-value is indicated in the figures and tables as follows: (*: $p < 0.05$; **: $p <= 0.01$, ***: $p <= 0.001$).

Results

CHIA expression, methylation and correlation with MEFV gene in various autoinflammatory diseases: According to our ADEx analysis, we observed a high CHIA expression in SLE (GSE50772), (Figure 1a, 1b), T1D (GSE11907) (Fig 1c), RA (GSE38351; GPL96 and GPL570) (Fig 1d, 1e) patients than the control groups.

We also compared CHIA methylation levels in patients with SLE, RA, T1D and SJS. Although the methylation status changes depending on the probes, we observed that the methylation level decreased for each disease, especially in the cg17143643 and cg7497781 probes. (Figure. 2).

We further examined the correlation between MEFV and CHIA in these autoinflammatory diseases. We observed that there is a positive correlation between MEFV and CHIA in these diseases and this correlation changes depending on the datasets. In addition, the highest association between these genes was observed in patients with SLE and RA (Figure. 3g, 3j).

Clinical and demographic characteristics of FMF attack and remission patients and control group: Sixty FMF patients were divided into two groups; thirty attacks and thirty remissions.

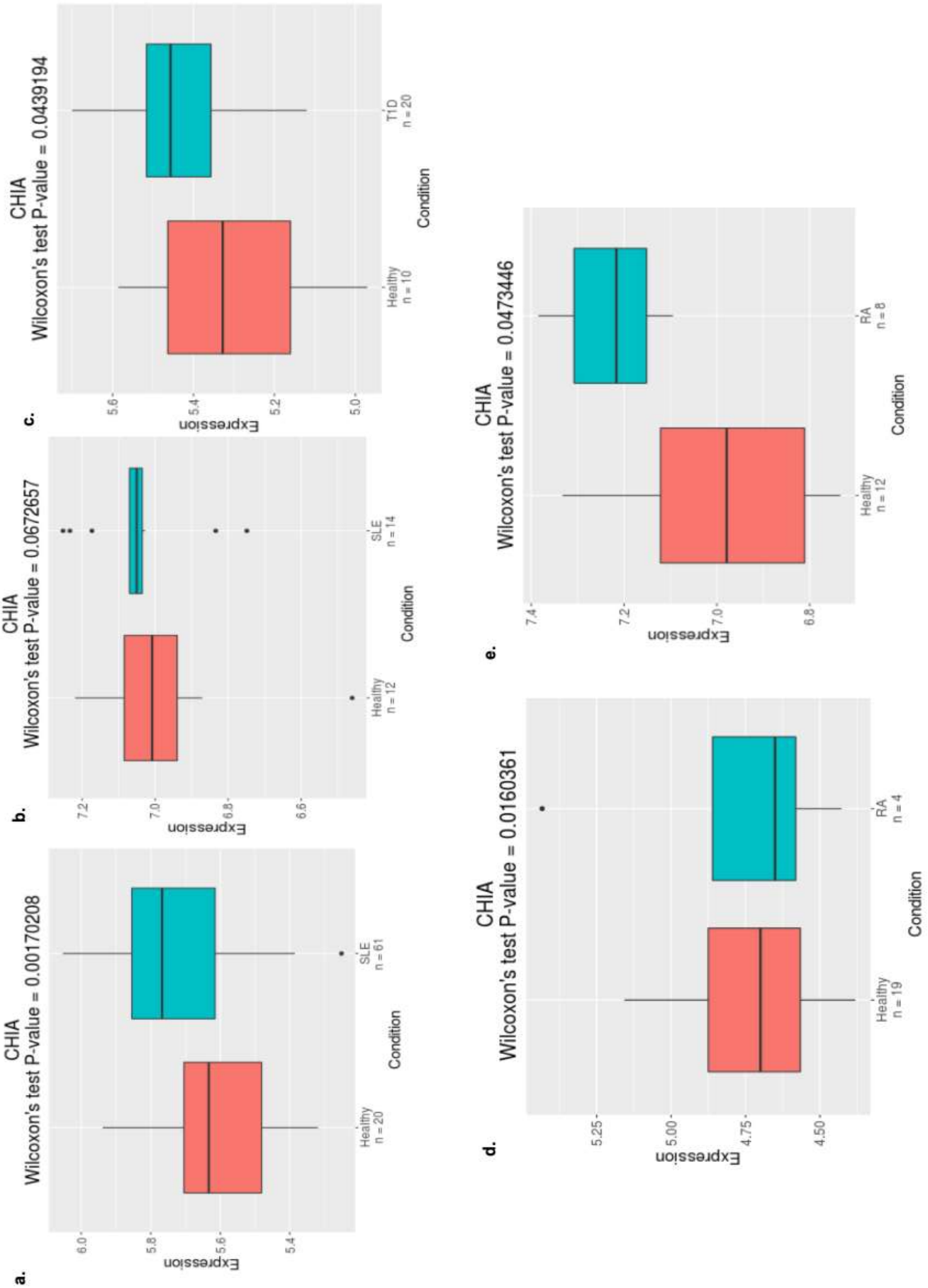

Figure 1. Gene expression of CHIA in various autoinflammatory diseases. (a) GSE50772 (b) GSE38351-GPL96 (c) GSE11907-GPL96 (d) GSE38351-GPL570 (e) GSE38351-GPL96.

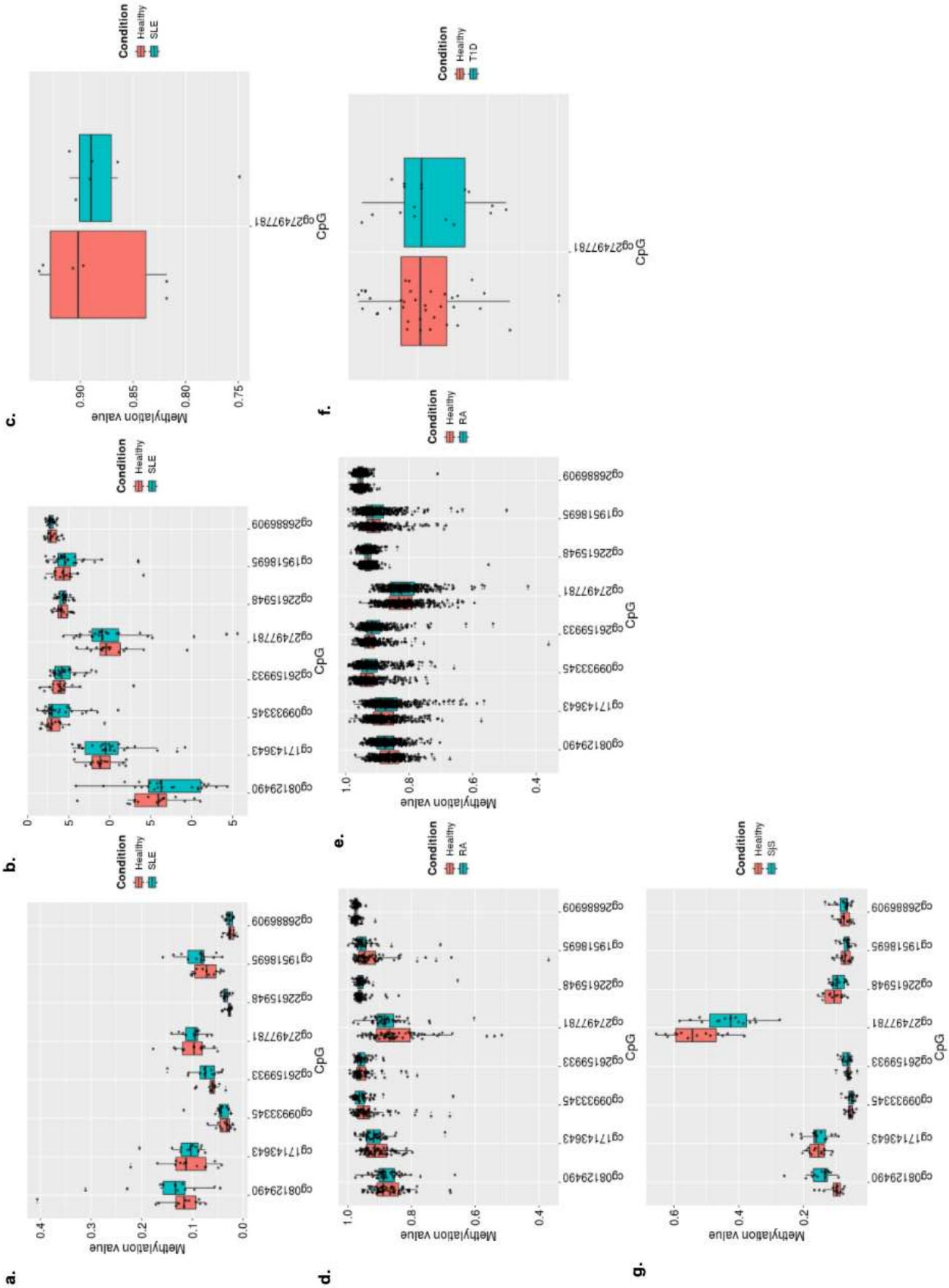

Figure 2. The boxplot demonstrates the related status from the body and promoters for every CpG site of CHIA in ADEx. (a) GSE110607 and (b) GSE59520 and (c) GSE57869 and (d) GSE87095 (e) GSE42861 (f) GSE117931 (g) GSE110007 (h) GSE56606.

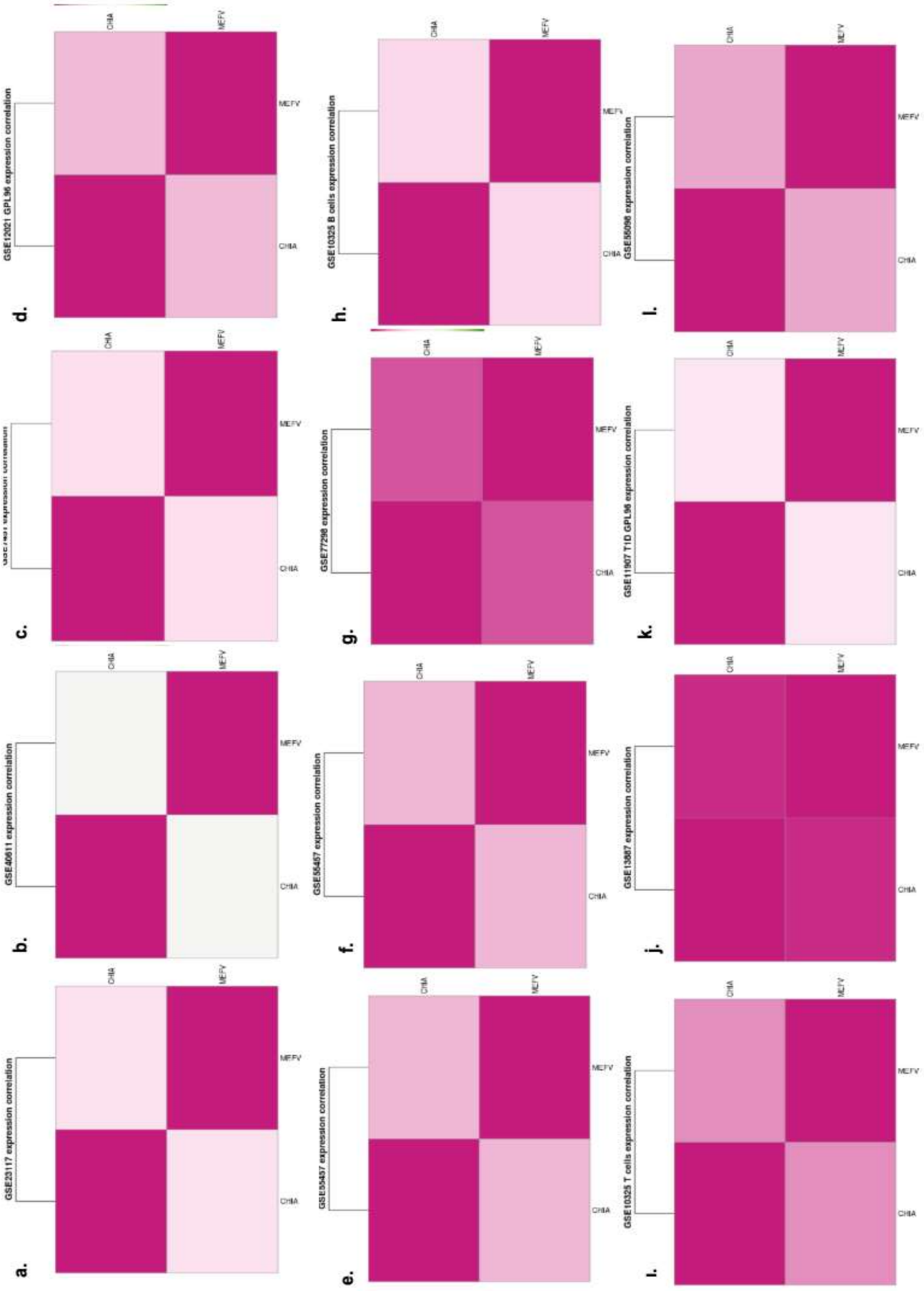

Figure 3. The correlation between MEFV and CHIA.

Demographic data of FMF attack, remission patient and control group are presented in Table 1. There were 14 males and 16 females in the attack period. The mean age of the patients was calculated as 30.4±10.3 years. There were 16 males and 14 females in the FMF remission group. The mean age of the patients in the remission period was 39.8±11.9 years. There were 11 men and 19 women in the control group. No difference was observed in terms of age and gender of FMF attack and remission patients and the control group. While homozygous MEFV mutation was observed most frequently among FMF attack patients, heterozygous MEFV mutation was observed in remission patients. While amyloidosis was detected in 76.6% of FMF attack patients, it was detected in 13.3% of the remission group (Table 1).

Table 1. Demographic data of FMF attack, remission and control group.

Parameters	**Attack (n = 30)**	**Remission (n = 30)**	**Control (n=30)**
Age (years)	30.4±10.3*	39.8±11.9	37.7±1.3
Age of diagnosis (years)	18.9±7.8	28.1±13.5	-
Disease duration (years)	11.5±8.8	11.6±6.8	-
BMI	25.0±3.9	27.1±4.0	25.4±7.3
Dosage of colchicine (mg/day)	1.3 ± 0.8	2.0±1.0	-
Family history, (n, %)	10.33, 3%	13, 43.3%	-
Attack, per month	2.3±0.9	1.7±1.3	-
Fever, n (%)	17, 56%	15, 50%	-
Abdominal pain, n (%)	21, 70%	19, 63.3%	-
Chest pain, n (%)	10, 33.3%	10, 33.3%	-
Joint pain	17, 56%	14, 46.6%	-
Arthritis / Arthralgia, n (%)	7, 23.3	11, 36.6%	-
Myalgia, n (%)	4, 13.3%	7, 23.3%	-
Amyloidosis, n (%)	23, 76.6%	4, 13.3%	-
Type of mutation			
Homozygote, n (%)	11 (36.6)	4 (13.3)	-
Heterozygote, n (%)	6 (20.0)	11 (36.6)	-
Compound heterozygote, n (%)	9 (30)	10 (33.3)	-
No mutation, n (%)	4 (13.3)	5 (16.6)	-

Mean ± standard deviation. n: number of people.

The clinical data of the FMF attack, remission patient and control group are presented in Table 2. We observed that WBC (p<0.001, p=0.002), neutrophil (p<0.001, p=0.001, resp.), monocyte (p=0.031, p=0.018, resp.) values of the FMF attack patients were significantly higher than the patients in remission and the healthy control group. Basophil (p = 0.015) level was higher in patients in the remission period compared to the control period. There was an increase in CRP (p<0.001, p=0.006, resp.) values in both the attack and remission period patient groups compared to the control group.

Table 2. Clinical data of FMF attack, remission and control group.

Parameters	**Attack (n = 30)**	**Remission (n = 30)**	**Control (n=30)**
WBC, 10^3/mL	10.5±3.5[ł***]	6.9±2.2	6.6±2.0
Neu, %	8.4±4.1[łł***]	4.0±1.8	4.3±1.4
Monocyte, %	0.6±0.2 [*]	0.4±0.2	0.4±0.1
Basophil, %	0.04±0.02	0.03±0.02[ł]	0.05±0.05
CRP, mg/L	28.0±26.9 [ł]	15.0±13.7 [ł]	3.1±2.0
CHIA	2.8±0.5	2.0±0.5	1.7±0.2

*ł: comparison with the control group. *: comparison with the remission group, mean ± standard deviation of the data. SD: standard deviation, WBC: white blood cell, NEU: neutrophil, CRP: C-reactive protein.*

Table 3. Correlation of serum CHIA level with demographic or clinical parameters in FMF patients.

Variables	**Correlation with CHIA**	
	r	***p***
Amyloidosis	-0.337	0.038
Attack, per month	0.316	0.047
WBC, 10^3/mL	0.333	0.037
Neu, %	0.312	0.043

Table 4. Regression analysis of serum CHIA level in the FMF attack and remission patients.

Variables	**AUC (95% CI)**	**Cut off**	***p***	**Sensitivity (%)**	**Specificity (%9**
Attack	0.98 (0.95-1.00)	2.03	<0.001	86.67	93.33
Remission	0.69(0.56-0.82)	1.74	0.009	76.67	50.00

The relationship between serum CHIA levels and clinical parameters of FMF attack and remission patients and control group individuals

We then compared the serum CHIA levels of FMF attack and remission patients and the control group. We observed that the serum CHIA level of the control group was lower than both FMF attack and remission (p <0.001, p =0.007; resp). In addition, a higher serum level was found in the attack period compared to remission (p <0.001), (Table 2).

Correlation analysis was performed to examine if there is a relationship between clinical and demographic data of FMF patients and CHIA levels. In this analysis, FMF attack and remission groups were evaluated together. As a result, we detected that serum CHIA level was correlated with amyloidosis, attacks per month, white blood cells and neutrophils (Table 3).

AUC analysis was performed to understand whether CHIA was also a predictable value between groups. The area under the curve for the CHIA value in attack patients was 0.98 (p<0.001). In remission patients, the area under the curve was 0.69 (p<0.009). Accordingly, the cut-off value was determined according to the Youden index and found 2.03 ng/ml for attack and 1.74 ng/ml for remission patients (Table 4).

Discussion

FMF is an autoinflammatory disease caused by mutation in the MEFV gene encoding pyrin. The gain or loss mutations in pyrin causes an excessive inflammatory response and the release of cytokines such as IL-1β, IL-18 and IL-16 [11]. Chitotriosidases are mainly produced by activated macrophages in both normal and inflammatory conditions [6-12]. Chitinases are involved in various pathological conditions such as Gaucher disease (CHIT1), obesity, diabetes, asthma (CHIA), and cardiovascular diseases [13,14,15,16]. In human macrophages, CHIT1 and CHIA are known to be highly expressed [14]. It is also known that chitinases are involved in the proinflammatory response by producing chitin fragments that contribute to the development of innate and adaptive immunity against the invading pathogen [16,17]. On the other hand, there is no information about the effect of FMF disease. For this purpose, we investigated the CHIA levels in FMF patients and the healthy control group.

We first examined the regulation of the CHIA gene in other autoinflammatory diseases using a bioinformatics database. We observed high CHIA expression in SLE, RA and T1D patients than in the control group. In addition, we thought that changes in the CHIA expression could be due to DNA methylation, thus we analyzed the methylation status of CHIA in SLE, RA, SSc, SJS and RA diseases and observed that CHIA is methylated especially at the cg17143643 and cg7497781 probes. These data suggest that the increase in CHIA transcription in autoinflammatory diseases may be due to methylation in the CpG Island. Our results also show that epigenetic factors may play a role in the regulation of CHIA in autoinflammatory diseases. Moreover, we observed a correlation between MEFV and CHIA in SLE, RA, SSc, SJS and RA diseases. This suggests that any change in MEFV gene expression and transcription in FMF disease may alter the profile of CHIA. Regardless, further studies are needed to elucidate the relationship between CHIA in FMF disease.

When we compared the demographic data of FMF attack and remission patients, the frequency of attacks per month was higher in the attack patients than in remission. Amyloidosis status serves as an important prognosis factor in the subclinical period of FMF disease [18]. In the

current study, we observed amyloidosis in both FMF groups. In addition, it is known that ESR and CRP levels increase in the acute period of FMF disease and this situation reflects chronic inflammation [19]. In our study, an increase in acute phase proteins was observed in FMF patients in the attack and remission period compared to the control. Moreover, we observed higher macrophages, neutrophils and white blood cell numbers, in patients with attack, compared to both remission patients and controls. Considering that pyrin is expressed through neutrophils and mutant pyrin induced an excessive inflammatory response, our results were consistent with other studies [20-21].

Two studies on the role of chitinases in FMF focused on the serum chitotriosidase levels of patients, and two different results were obtained. Taylan et al. showed that there were increased human chitinolytic enzyme concentrations in FMF patients and showed a correlation with inflammation markers such as hs-CRP, SAA and S100A12 [22]. In another study, it was observed that chitotriosidase (CHIT1) concentrations and activity were decreased in FMF patients compared to healthy controls, but there was no relationship between the clinical parameters of the patients [23]. In our study, we observed that serum CHIA level increased in FMF attack and remission patients. Unlike CHIA, CHIT1 is expressed in macrophages. Colchicine reduces the release of molecules such as 5-lipoxygenase and arachidonate in macrophages. When the colchicine use of the patients in this study was evaluated, colchicine may be the main reason for the decrease in the CHIT1 level. According to our correlation analysis, we also showed that CHIA correlated with amyloidosis, monthly attack frequency, white blood cells and neutrophils. Moreover, we determined that CHIA would be useful for follow-up in the subclinical period of FMF by AUC analysis. As a result, the correlation between the mentioned above that are important in the diagnosis of the disease. In addition, the significant increase of CHIA in the FMF attack compared to the remission period may also help to control the effectiveness of the treatment by detecting the inflammation in the subclinical period, to separate from attack period.

Conclusions

In summary, CHIA may be useful as a new laboratory test for defining subclinical inflammation and/or identification of FMF. CHIA correlates well with the presence of amyloidosis and inflammatory markers in FMF patients and may be useful for restraining inflammation and aiding in the management of FMF patients. Although this study is a cross-sectional study which may limit to make a general conclusion, but a longitudinal study can be designed by expanding the number of patients in a future study.

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Conflict of interest: *The authors declare that they have no conflict of interest.*

Ethical statement: *This study complied with the Declaration of Helsinki and has been affirmed by the medical ethics committee at Tokat Gaziosmanpasa University (#19-KAEK-164).*

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