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$\alpha_9\beta_1$ integrin & its ligands as new potential biomarkers in FMF

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***Background & objectives*: Familial Mediterranean Fever (FMF) manifests as a hereditary condition characterized by repeated bouts of fever, abdominal, chest, and joint discomfort, and swelling. Colchicine is the most common form of treatment, but it does not eliminate the disease. The underlying causes of the inflammatory mechanism are still not fully known.**

***Methods*: A total of 20 healthy controls, 16 individuals with FMF in the attack period, and 14 in the remission period participated in the study. ITGA9, ITGB1, OPN, TNC, VEGF, VCAM-1, TGM2, TSP-1, Emilin-1, and vWF levels were measured by ELISA by obtaining serum from blood samples of individuals. In addition, gene expressions of $\alpha_9\beta_1$ (*ITGA9, ITGB1*) and its best known ligands (*TNC, SPP1*) were analyzed by quantitative real-time PCR (qPCR).**

***Results*: The findings of this study showed that serum levels of $\alpha_9\beta_1$ and its ligands were higher in individuals with FMF in the attack period than in the healthy controls and the FMF group in the remission period ($P<0.05$). The marker levels of the healthy group were also higher than those in the remission period ($p<0.05$). In addition, when the gene expressions were compared between the healthy controls and FMF group, no significant difference was found for *ITGA9*, *ITGB1*, *TNC*, and *SPP1* genes.**

***Interpretation & conclusions*: The function of $\alpha_9\beta_1$ and its ligands in FMF disease was investigated for the first time in this study as per our knowledge. Serum levels of these biomarkers may help identify potential new targets for FMF disease diagnosis and treatment approaches.**

Key words $\alpha_9\beta_1$ - attack - FMF - ligands - remission

Familial Mediterranean Fever (FMF) is an ethnicity-related disease transmitted hereditarily and progresses with acute fever attack and serosal inflammation[1]. FMF is mainly seen in individuals in the geography close to the Mediterranean region and occurs due to point mutations in the *MEFV* gene[2].This gene encodes the pyrin molecule, a regulatory protein with limited tissue expression, especially in neutrophils, and suppresses inflammation due to neutrophil activation. The pyrin protein, formed due to a mutation in FMF, disrupts the order in neutrophil activation[3]. Neutrophil activation is a defining phenomenon in the inflammatory response. The interactions between the integrins released from the surface of neutrophils and their ligands, which

have crucial tasks in the development of inflammation, take an active role in critical situations such as cell activation, adhesion, and migration[4].

The integrin protein family consists of alpha and beta subtypes that form transmembrane heterodimers[5,6]. $\alpha_9\beta_1$ integrin is one of the members of the integrin family that has been emphasized in recent years. Different studies have shown that $\alpha_9\beta_1$ integrin plays an essential role in refractory diseases (cancer, autoimmune diseases, nerve damage, thrombosis, *etc*.), and it highlights the possibility of $\alpha_9\beta_1$ which is considered as a potential therapeutic target[7]. In addition, ligands for $\alpha_9\beta_1$ integrin, including tenascin-C (TNC) and osteopontin (OPN), are well established as critical regulators of inflammatory conditions. However, further studies are needed on other $\alpha_9\beta_1$ integrin ligands such as vascular endothelial growth factor (VEGF), vascular cell adhesion molecule (VCAM-1), thrombospondin (TSP), transglutaminase (TGM), elastin microfibril interfacer (Emilin-1), von Willebrand factor (vWF). These ligands have multiple functions, and the cell types that release these vary widely. Therefore, these contribute significantly to the functioning of the biological and chemical pathways in different body parts during the inflammatory process.

FMF is an inflammatory disease that affects all systems in the body, so it is believed that these neutrophil-related markers could potentially impact the disorder's progression. Hence, this study was undertaken to investigate the efficacy of $\alpha_9\beta_1$ integrin and its ligands in FMF disease.

Material & Methods

The study was undertaken at the department of Medical Pharmacology, Faculty of Medicine, Bursa Uludag University. The study was initiated after obtaining the approval from the Ethics Committee. and patient's data and blood samples were provided by the department of Rheumatology of the Faculty of Medicine, Bursa Uludag University.

Twenty healthy controls (5 male, 15 female) and 30 (10 male, 20 female) individuals with FMF presenting to Rheumatology outpatient clinic, Bursa Uludag University Hospital, between March 16, 2022, and December 20, 2022, were included in this study. An informed, and a signed consent form was obtained from all those willing to participate in this study. The determination of FMF was conducted using Tel Hashomer criteria[7]. Accordingly, it was considered suitable for two major or one major and two minor criteria for a definitive diagnosis. The number of FMF attacks the participants had in the last six months was also considered. FMF attacks and remission period distinctions were determined by biological parameters (such as sedimentation, haemogram, and CRP values) with genetic and clinical findings. The distribution of the MEFV genotype interims of numbers of study participants as follows: M694V/-11; M680I/-4; E148Q/ -2; M694V/R761H-1; R202Q/ M694V- 1; M694V/E148Q -1; M694V/V726A-1; M680I/R761H-1; E148Q/R202Q-1; no mutation-7s .All FMF patients received colchicine treatment with 0.5 to 2 mg in one or two (maximum 4) divided doses daily. Individuals in the healthy group did not receive any treatment. The study excluded people with organ failure, anemia, leukopenia, acute infection, or autoimmune/inflammatory disease. Table I shows the diagnosis criteria of FMF patients according to Tel Hashomer[7].

Table I. Clinical features of participants with FMF according to Tel Hashomer criteria[7]

	Total (n)	%
Major criteria		
Recurrent febrile episodes with serositis	16	53.3
Amyloidosis of AA type without a predisposing disease	18	60
Favorable response to regular colchicine treatment	27	90
Minor criteria		
Recurrent febrile episodes	20	66.7
Erysipelas-like erythema	8	26.7
FMF-in a first-degree relative	22	73.3

Measurement of markers: Blood samples (~20 ml) from healthy individuals and individuals with FMF were centrifuged at 3000xg for 10 min. The obtained sera were separated for each sample, moved to Eppendorf tubes, and kept at -80°C.

Concentrations of $\alpha_9\beta_1$ and its ligands were measured using commercial ELISA kits (BT Lab, Zhejiang, China) as per prescribed manufacturer's protocol. Each assay was duplicated, and the results were derived using the standard curve. The sensitivity of the assay for markers was, 0.14 ng/ml for ITGA9; 14.49 ng/L for ITB1;10.57 ng/L for TNC; 0.15 ng/ml for OPN; 10.42 ng/L for VEGF; 0.23 ng/ml for VCAM-

Table II. Serum (mean±SD) levels of biological markers in healthy control, attack, and remission period of participants with FMF

Study parameters	Healthy control	Attack period in participants with FMF	Remission period of participants with FMF
ITGA9 (ng/ml)	5.6±1.1	7.9±1	3.2±1.4
ITGB1 (ng/l)	1242.6±286.4	2367.5±1064.2	506.8±254.6
TNC (ng/l)	1176.1±338.4	2265.5±1394.4	709.8±186.3
OPN (ng/ml)	10.1±3.3	17.8±7.1	5±2
VEGF (ng/l)	992.6±507	1367.2±441.3	536.6±229.3
VCAM-1 (ng/ml)	47.2±13	84.9±41.9	24.2±6.8
TSP-1 (ng/ml)	119.2±45.6	218.3±110.9	61.5±29.3
TGM2 (ng/l)	167±92.3	342.3±199.3	53.1±16.6
Emilin-1 (ng/ml)	9.6±2.9	18.7±10.3	5.5±2.5
vWF (ng/ml)	30.2±8	59.5±20.4	11.6±4.1

1; 2.39 ng/ml for TSP1; 2.74 ng/L for TGM2; 0.048 ng/ml for Emilin-1; 0.23 ng/ml for vWF, respectively.

Quantitative real-time PCR: RNAeasy®Mini Kit (Qiagen, Germany) and RT^2 First Strand Kit (Qiagen, Germany) were used for isolating RNA from the serum samples and cDNA synthesis, respectively, as previously described[9]. qRT-PCR assays were conducted in duplicate using RT^2 SYBR Green Mastermix (Qiagen, Germany). The primer sets from commercial sources that were used in this study are detailed in Supplementary Table. Actin-β (ACTB) was utilized as the housekeeping gene. As part of the data standardization process, all C_t values were adjusted against the mean C_t values of a housekeeping gene (ACTB) present on the array using the $2^{-\Delta\Delta Ct}$ method[7].

Statistical analysis: The ITGA9 variable exhibited an effect size d=50. G power software (V 3.1.9.7; Heinrich-Heine-Universität Düsseldorf, Germany) was employed to derive α=0.005 and ß=0.20 values for sample size determination[31]. It was concluded that each group should consist of at least 14 individuals, totaling at least 42 participants for the study.

The student's t-test was employed for comparing parameters between the groups. The mean±standard deviation was computed for the variables, and the mRNA expression analysis was conducted using RT^2 Profiles PCR Array Data Analysis version 3.5 (Qiagen, Germany). ΔCt values of the difference between test and control groups was determined using One-way ANOVA. A *P* value below 0.05 was considered statistically significance. Statistical analysis was conducted utilizing Graph Pad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

No significant difference was observed for the mean age value (P=0.937) between the control group (38±4.5) and FMF group (37.9±11.05). In terms of gender, the number of females was significantly (P<0.05) higher than males in each group.

Serum levels of $\alpha_9\beta_1$ and its ligands: The expression of ITGA9 and ITGB1, markers of $\alpha_9\beta_1$, were higher in individuals with FMF in the attack period than in the healthy controls and remission groups (P<0.05). In addition, the ITGA9 and ITGB1 levels of the healthy group were higher than the FMF group in remission (P<0.05) (Supplementary Figure). Serum levels of $\alpha_9\beta_1$ ligands were also similar to $\alpha_9\beta_1$. TNC, OPN, VEGF, VCAM-1, TSP-1, TGM2, Emilin-1, and vWF concentrations were highest in individuals with FMF, with an attack period. Their levels in the healthy group were higher than in the FMF group in remission (P<0.05) (Table II; Supplementary Figure).

Gene expressions of ITGA9, ITGB1 and TNC, SPP1: *ITGA9* and *ITGB1*, as the $\alpha_9\beta_1$ subunits with *TNC* and *OPN* (*SPP1*) genes, which are the best-defined $\alpha_9\beta_1$ ligands, were analyzed on the mRNA expression profile. Supplementary Table provides details regarding the primers used in this study. According to the results, it was concluded that there was no disparity in gene expression levels between the groups. However, the fold change values of the individuals with FMF in the remission period were higher for the ITGB1 (0.68 fold) and SPP1 (0.64 fold) genes compared to the healthy control. When participants in the attack and remission periods were compared, the fold change values of *ITGA9* (1.57 fold) and *TNC* (1.47 fold) were

Table III. Comparison of attack and remission period FMF patients with healthy controls in terms of 2^(-Avg.(ΔCt)), fold change, and *P*-value

Genes	Healthy control	Attack period in participants with FMF			Remission period in participants with FMF		
	2^(-Avg.(ΔCt))	2^(-Avg.(ΔCt))	Fold change	*P* value	2^(-Avg.(ΔCt))	Fold change	*P* value
ACTB	1	1	1	0	1	1	0
ITGA9	1.03	0.09	0.09	0.13	0.08	0.06	0.15
ITGB1	0.08	0.01	0.2	0.09	0.02	0.68	0.46
TNC	0.48	0.08	0.18	0.22	0.09	0.12	0.25
SPP1	0.09	0.01	0.17	0.08	0.04	0.64	0.15

*P**<0.05

Table IV. Comparison of the gene expression between participants with FMF with attack and remission period

Genes	2^(-Avg.(ΔCt))	Attack period vs remission period in participants with FMF	*P* value
		Fold change	
ACTB	1	1	0
ITGA9	0.09	1.57	0.25
ITGB1	0.01	0.29	0.35
TNC	0.08	1.47	0.36
SPP1	0.01	0.29	0.38

*P**<0.05

higher in the attack group than in remission, but this was not statistically significant. The mRNA expression profile for *ITGA9*, *ITGB1*, *OPN*, and *SPP1* is shown in Table III and IV.

Discussion

FMF is an autoinflammatory disease (AID) characterized by recurrent fever attacks and polyserositis. It develops depending on different mechanisms, including genetic and geographical conditions. In addition to the existing information about the disease, there are current approaches to diagnosis and treatment[7].This study proposed to figure out the function of $\alpha_9\beta_1$, one of the frequently emphasized markers and its ligands in FMF disease. Furthermore, this study identified notable distinctions in the release of $\alpha_9\beta_1$ and its ligands between the healthy controls and individuals with FMF.

OPN and TNC are two common ligands with similar structures and properties for $\alpha_9\beta_1$ integrin, and binding of $\alpha_9\beta_1$ integrin is primarily induced by inflammation. In the RA study 2006, it was reported that $\alpha_9\beta_1$ integrin is released from osteoclasts and participates in the functionality of these cells[10]. Expression of α_9 integrin mRNA is elevated in mouse bone marrow cells during osteoclast differentiation. In addition, impaired bone resorption has also been observed in osteoclasts from α_9 integrin-deficient mice[10]. Like mouse RA models, α_9 integrin is released from human synovial fibroblasts and macrophages[11]. Besides, there are essential differences between the arthritic joint regions of mice and humans. While OPN production was observed to be higher than TNC production in mouse synovial tissues during inflammatory arthritis, TNC expression in individuals with RA was also found to be considerably higher than OPN[11]. In addition, TNC levels in synovial cells and serum were reported to have increased in individuals with RA, and TNC levels were correlated with joint erosion. Based on these findings, it has been suggested that the interaction between $\alpha_9\beta_1$ integrin and TNC may be a functional target for RA, and OPN blockade may also benefit individuals RA[12,13]. Besides, it has been reported that ECM proteins TNC and OPN are upregulated in various pathological foci in diseases such as autoimmune arthritis, experimental allergic encephalomyelitis (EAE), and inflammatory bowel disease (IBD). In a collagen antibody-induced arthritis model of mice, it was observed that macrophages and fibroblasts in pathological foci primarily expressed α_9 integrin, and inhibition of the interaction between α_9 and TNC/OPN decreased the production of inflammatory cytokines in the arthritic region[14]. Chabas *et al*[15] demonstrated overexpression of OPN transcripts in the spinal cord of rats with EAE and brain tissues of individuals with MS in microarray analysis. In addition, another study found that the induction of EAE in TNC-deficient mice was impaired through T cell down-regulation that produces IFN-γ and IL-17[15].

$\alpha_9\beta_1$ integrin expression is upregulated in various cancers, influencing tumor progression through different mechanisms. ITGA9 has been identified as a potential biomarker for prostate tumors and linked to drug-resistant colon cancer, as well as a diagnostic

marker for hepatocellular carcinoma. Additionally, studies show that while the α_9 subunit is absent in adult neurons, forced expression of α_9 in certain cells promotes axonal regeneration, suggesting a significant role for the $\alpha_9\beta_1$-TNC interaction in this process[16,17,18].

VCAM-1, an $\alpha_9\beta_1$ ligand released by activated endothelium in response to inflammation, binds to $\alpha_9\beta_1$ integrin and plays a crucial role in neutrophil survival and function. Research indicates that this interaction impacts neutrophil pathophysiology and helps regulate inflammation. Additionally, studies suggest that the $\alpha_9\beta_1$/VCAM-1 interaction may facilitate neutrophil migration during acute inflammation[19,20,21].

A study on VEGF reported that $\alpha_9\beta_1$ directly binds to VEGF-A and supports VEGF-A-induced angiogenesis[22]. However, previous experiments of the same study group proved that VEGF-C and D also support lymphangiogenesis by directly binding to $\alpha_9\beta_1$ integrin[23]. Studies evaluating the relationship between VEGF and $\alpha_9\beta_1$ are limited, so more studies are needed.

It has been suggested that many integrins may be associated with TGM2. The experiment conducted in collagen-induced mice determined that TGM2 activity was associated with cartilage degradation in the joints[24]. Different studies related to TGM2, such as cancer, vascular inflammation, tissue fibrosis, and autoimmunity, demonstrated that this marker can control the level of integrin on the cell surface. Nevertheless, the precise molecular mechanism governing this regulation remains unidentified[25].

TSP-1 was previously identified as an angiogenesis modulator[25]. This protein was shown to directly inhibit endothelial cell functions such as proliferation and migration, but some N-module fragments stimulate similar endothelial responses. In Stanizewska *et al*[25] study, the N-terminal domain [NoC1] binding to cells expressing $\alpha_9\beta_1$activated the signaling of proteins such as Erk1/2 and paxillin. Subsequently, the proangiogenic activity of NoC1 was inhibited by $\alpha_9\beta_1$ inhibitors. As a result, it was revealed that $\alpha_9\beta_1$released from the microvascular endothelial surface interacts with TSP-1, which is associated with the modulation of angiogenesis[26].

In a 2013 study[26] on Emilin-1 protein, another $\alpha_9\beta_1$ligand, it was suggested that the Emilin1/ $\alpha_9\beta_1$integrin interaction may play an essential role in lymphatic valve formation and maintenance. Donussi *et al*[26] reported that Emilin1-a_4/a_9 integrin binding restrains dermal fibroblast and keratinocyte production. Accordingly, it was thought that Emilin1 and keratinocyte $\alpha_9\beta_1$ might play a protective role in skin homeostasis. Findings regarding the involvement of homeostatic molecules in the inflammatory process highlight the link between inflammation and homeostasis[27].

In addition, the link between vWF and inflammation has also taken its place among current issues. Reportedly due to the acute release of vWF from the activated endothelium, it was defined as an inflammation marker only in some pathologies. However, vWF was later found to be directly related to inflammation, suggesting it has a more extensive assignment. The ability of vWF to recruit leukocytes either by direct binding to leukocytes or via platelets has been attributed as the most crucial evidence in this regard[28].There are various studies in the literature on whether the vWF level is a remarkable parameter in evaluating the course of the disease in cases of acute systemic inflammation. Still, the findings need to be more consistent. Despite some studies reporting that individuals with high vWF levels are more likely to survive than others, studies have not confirmed it[29,30]. Mourik *et al*[30] showed that both vWF and propeptide levels increase during the acute response of the endothelium (septicemia). Conversely, vWFpp concentrations were only slightly elevated throughout the chronic stimulation (*eg*., diabetes mellitus), although vWF levels were elevated[31].

Despite many studies in which systemic inflammatory diseases and various immune and genetic parameters were conducted, any study showing the relationship between FMF and $\alpha_9\beta_1$ integrin molecule was not found in the literature. In this direction, we obtained significant differences in ITGA9 and ITGB1 levels of $\alpha_9\beta_1$ integrin markers between healthy individuals and those with FMF. $\alpha_9\beta_1$ release was highest in individuals with FMF during the attack period and more than a healthy control group in those with remission. Similarly, $\alpha_9\beta_1$ligands TCN, OPN, VCAM-1, VEGF, TGM2, TSP-1, Emilin-1, and vWF levels varied significantly between groups. Based on the overall results, the expression of these parameters in individuals with FMF may provide significant benefits regarding the detection and follow up of the disease. The fact that the levels of $\alpha_9\beta_1$ and ligands showed a relation with each other suggests that these factors should be handled together in the pathogenesis of FMF. In addition, the $\alpha_9\beta_1$ integrin subunits ITGA9 and ITGB1 levels the gene expression of their the best known ligands, TNC and SPP1 vary between groups

in terms of two genes. ITGA9 and ITGB1's expression levels in the individuals with FMF were higher than in the healthy individuals. Accordingly, the higher expression of the $\alpha_9\beta_1$ subunit ITGA9 and the SPP1 ligand in FMF individuals compared to the healthy group may provide an alternative to familiar genetic markers. It can also be evaluated genetically as an inflammation marker that can be used to diagnose FMF disease.

The main limitation of this study is the low sample size in both the study groups. In addition, some of the $\alpha_9\beta_1$ ligands were examined, and the mRNA profile was studied only for the best known ligands. Examining the mRNA expression of all markers will be beneficial by adding more ligands and individuals to future studies. Additionally, further elaborate studies undertaken at the molecular level, encompassing different methods such as protein analysis in the upcoming stages, will be beneficial.

In conclusion, the present study aimed to identify the relationship between the $\alpha_9\beta_1$ and FMF disease. However, the utility of $\alpha_9\beta_1$ integrin as a therapeutic agent for treating inflammatory disorders needs to be further explored. A better understanding of how $\alpha_9\beta_1$ integrin interacts in autoimmune/autoinflammatory diseases will contribute to developing integrin-targeted therapy approaches in this area.

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