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HOW DO THE GENETIC VARIANTS OF *MMP 9* AND *TIMP 1* AND VITAMIN D AFFECT CHRONIC RHINOSINUSITIS WITH NASAL POLYPOSIS?*

MMP 9 VE *TIMP 1* GENETİK VARYANTLARI VE VİTAMİN D NAZAL POLİPOZİSLİ KRONİK SİNÜZİTTE NASIL ETKİLİDİR?

Meltem BOZACI KILIÇOĞLU[1], Barış ERTUĞRUL[2], Göksu KAŞARCI[2], Şenol ÇOMOĞLU[1], Levent AYDEMİR[1], M. Nesil KELEŞ TÜREL[1], Aslı GELİNCİK[3], Bedia ÇAKMAKOĞLU[2]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Otorhinolaryngology, Division of Head and Neck Surgery, Istanbul, Turkiye
[2]Istanbul University, Aziz Sancar Institute of Experimental Medicine, Department of Molecular Medicine, Istanbul, Turkiye
[3]Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine, Division of Allergy and Immunology, Istanbul, Turkiye

ORCID IDs of the authors: M.B.K. 0000-0001-9952-0329; B.E. 0000-0003-3878-1829; G.K. 0000-0001-9766-4361; Ş.Ç. 0000-0003-4632-9218; L.A. 0000-0002-5836-4304; M.N.K.T. 0000-0003-1829-8186; A.G. 0000-0002-3524-9952; B.Ç. 0000-0001-7960-9131

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ABSTRACT

Objective: Chronic rhinosinusitis with nasal polyposis (CRSwNP) is a multifactorial disease in which the sinus and nasal mucosa are affected. We investigated the relationship between the genetic polymorphism of *MMP9* (rs17576) and *TIMP1* (rs48498) and serum levels, and we wanted to investigate the effect of *MMP9* and *TIMP1* genotypes on Vitamin serum levels in CRSwNP with and without Aspirin-Exacerbated Respiratory Disease (AERD).

Materials and Methods: This study consisted of 99 patients with CRSwNP with AERD and CRSwNP without AERD. The RT-PCR method and ELISA were used in this study. ELISA was used for MMP9 and TIMP1 serum levels and Vitamin D levels.

Results: Serum levels of MMP9 were markedly higher in AG genotype than GG genotype in CRSwNP with AERD. Serum levels of MMP9 were statistically higher in carrying of A (+) allele in CRSwNP with AERD than in not carrying of A (+) allele. In addition, serum levels of Vitamin D were discovered significantly lower in TIMP1 CT genotype in comparison to CC and TT genotypes in all of CRSwNP.

Conclusion: The rising of MMP9 serum levels may be a marker of chronic inflammation or they may be a significant factor in the

ÖZET

Amaç: Nazal polipli kronik rinosinüzit sinüs ve nazal mukozanın etkilendiği multifaktöriyel bir hastalıktır. *MMP9* (rs17576) ve *TIMP1* (rs 4898) genetik polimorfizmleri ile serum düzeyi arasındaki ilişki ve *MMP9* ve *TIMP1* genotipleri ve Vitamin D arasındaki bağlantının gösterilmesini araştırdık.

Gereç ve Yöntem: Bu çalışma, aspirinle alevlenen solunum hastalığı olan veya olmayan nazal polipozisli kronik rinosinüzitli 99 hastadan oluşmaktadır. RT-PCR ve ELISA metodu kullanılmıştır. MMP9, TIMP1 ve Vitamin D serum seviyeleri için ELISA metodu kullanılmıştır.

Bulgular: Aspirinle alevlenen solunum hastalığı eşlik eden nazal polipozisli kronik sinüzit hastalarında MMP9 serum seviyesi, *AG* genotipli hastalarda *GG* genotipli hastalara kıyasla daha yüksek seviyededir. Aynı zamanda bu hasta grubunda MMP9 serum seviyesi, A (+) alleli taşıyan grupta A (+) alleli taşımayan gruba göre daha yüksek seviyededir. Buna ek olarak *TIMP1* CT genotipine sahip olanlar, *TIMP1* CC ve TT genotipleri ile kıyaslandığında Vitamin D serum seviyesinin anlamlı olarak düşük olduğu gösterilmiştir.

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Corresponding author/İletişim kurulacak yazar: Meltem BOZACI KILIÇOĞLU – meltembozaci@gmail.com

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resistance to diseases. This study may lead to a further understanding of disease severity in CRSwNP with AERD.

Keywords: Nasal polyposis, *MMP9, TIMP1*, genetic polymorphism, chronic rhinosinusitis

Sonuç: MMP9 serum düzeylerinin yükselmesi, kronik inflamasyonun bir belirteci olabilir veya hastalık direncinde önemli bir faktör olabilir. Bu çalışma, aspirinle alevlenen solunum hastalığı olan nazal polipozisli kronik rinosinüzitte hastalık şiddetinin daha iyi anlaşılmasına yardımcı olabilir.

Anahtar Kelimeler: Nazal polip, *MMP9, TIMP1*, genetik polimorfizm, kronik sinüzit

INTRODUCTION

Nasal polyposis is identified as a subgroup of chronic rhinosinusitis in European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012 (1). Nasal polyposis is an inflammatory circumstance in which the sinus and nasal mucosa are affected. There are many factors in the etiology of nasal polyposis, and many theories are mentioned in the pathophysiology of nasal polyposis. A theory that covers all patients has not been defined yet (2). Matrix metalloproteinase (MMP) that has a significant effect in extracellular matrix remodeling is an endopeptidase. MMP7 and MMP9 that were discovered in many tissues have a natural inhibitor named TIMP1. Extracellular matrix deposition occurs due to the instability between MMP and TIMP (3).

Vitamin D has antiproliferative, anti-inflammatory, and immunomodulatory effects. The deficiency of Vitamin D was associated with the severity of disease in CRSwNP (4). The mechanism of how Vitamin D affects the secretion of MMP was not explained fully (5). There are no studies on how genetic polymorphism of *MMP9* and *TIMP1* affects serum levels. In our study, we aimed to research the correlation between genetic polymorphism of MMP9, TIMP1, and their serum levels. Also, the relationship between Vitamin D levels and genotypes of *MMP9* and *TIMP1* was described.

MATERIAL and METHOD

Patients and study design

Ninety nine patients (61 male and 38 female) with chronic rhinosinusitis with nasal polyposis applied to Istanbul University, Istanbul Faculty of Medicine, Otorhinolaryngology-Head, and Neck Surgery Department between August 2018 and April 2019. Their complaints at the time of admission were rhinorrhea, anosmia, nasal obstruction and altered taste perception. Forty one patients were categorized as CRSwNP with AERD, and 58 patients were categorized as CRSwNP without AERD. None of the patients had any history of ciliary dyskinesia or antrochoanal polyp.

According to EPOS 2012 guidelines, the diagnosis of sinus diseases was based on nasal endoscopy, clinical examination, and history. Endoscopic examination was performed with 4 mm 0 and 30-degree rigid endoscopes. To evaluate the endoscopic grading, Meltzer's endoscopic nasal polyp grading was used. Atopy evaluation was performed according to diagnosis of allergic rhinitis, asthma and urticaria. The patients with CRSwNP diagnosis at the time of their visit to asthma and allergic clinic were recruited for the evaluation of AERD. Oral aspirin challenge was used to detect ASA hypersensitivity. The ratio of eosinophil (%) and the level of Vitamin D (ng/ml) were evaluated between these two groups. Vitamin D serum levels examined via ELISA in the routine examinations of patients' files were also evaluated. The quality of life was evaluated with the SNOT-22 questionnaire. The samples were taken into a gel tube for serum analyses and into an EDTA tube for DNA analyses. The study was approved by the Ethics Committee on Istanbul University, Istanbul Faculty of Medicine (Date: 10.08.2018, No: 13). Before sampling and experimental studies, a voluntary informed consent form was taken from all patients.

Real-time PCR analysis

Samples were collected from the peripheral blood, and DNA extraction was performed. TaqMan System (Applied Biosystems, 45 Life Technologies) PrimerProbMix, and qPCR ProbesMaster (Jena Bioscience, Germany, PCR-360) were used for genotyping according to the manufacturer's protocols. A total of two SNPs, i.e. matrix metalloproteinase *MMP9 (*rs17576*)* and tissue inhibitor of metalloproteinase *TIMP1 (*rs4898*)* were selected for this study (Table 1). *TIMP1* rs4898 (372T>C) polymorphism

Table 1: Detailed information on the studied SNPs Sequels

SNP ID	rs17576	rs4898
Gene name	MMP matrix metallopeptidase 9	TIMP metallopeptidase inhibitor 1
Polymorphism	A/G, Transition Substitution	C/T, Transition Substitution
Context Sequence [VIC/FAM]	*CTCCTCGCCCCAGGACTCTACACCC[A/G]GGACGGCAATGCTGATGGGAAACC*	*TCTTGCACATCACTACCTGCAGTTT[C/T]GTGGCTCCCTGGAACAGCCTGAGT*

and *MMP9* rs17576 (Gln279Arg) polymorphism were investigated. Allelic discrimination of two SNPs was performed with ABI StepOne RT PCR System (Applied Biosystems, 45 Life Technologies). The TaqMan assay was used. The total reaction mix volume was 20 µL. 2 µL of DNA, 10 µL of qPCR ProbesMaster, 7 µL PCR Grade water, and 1 µL of TaqMan probe mix. For real-time PCR reaction, a 2-minute cycle was used performed as the first denaturation step at 95 °C. Immediately followed were 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C each. After fluorescence level measurement, allele frequencies were evaluated with the TaqMan assay with the aid of SDS v 3.0 (Applied Biosystems). All experimental studies, including PCR analysis and determination of serum protein levels, were carried out in the laboratory of Aziz Sancar Experimental Medicine Research Institute, Molecular Medicine Department.

ELISA test

MMP9 and TIMP1 serum levels were determined in supernatants of patients with each nasal polyposis using the MMP-9 Human ELISA Kit (Invitrogen, Vienna, Austria) and the TIMP1 Human ELISA Kit (ENZO LIFE, Farmingdale, NY, USA). The plate was incubated for 2 hours at room temperature. After washing, 100 µL of the streptoavidin-HRP solution was added and incubated for 30 minutes at room temperature. After the second washing step, 100 µL of chromogen was added to each well. After 30 minutes of incubation at room temperature, enzymatic activity was stopped with a stop solution. Optical density (OD) was measured with a Tecan ELISA plate reader (Tecan, Switzerland). After the standard graph was created, MMP9 and TIMP1 concentrations were calculated.

Statistical analysis

SPSS ver.22.0 (IBM Corp., Armonk, NY, USA) program was used for data analysis. The statistical significance limit was taken as $p<0.05$. Student's t-test and Chi-square, Fischer and Annova tests were chosen to evaluate the effects of genes on activity.

RESULTS

Fifty eight patients (58.6%) were included in CRSwNP without AERD, and forty one patients (41.4%) were included in CRSwNP with AERD. Concerning the eosinophil ratio, it was found to be higher in CRSwNP with AERD. The presence of atopy was 82.9% in CRSwNP with AERD, and 10.3% in CRSwNP without AERD. It was observed that the presence of atopy increased 42-fold in CRSwNP with AERD. When smoking was evaluated in the overall study group, it was statistically lower in CRSwNP with AERD ($p=0.033$) (Table 2).

According to the genotypes and alleles of *MMP9* and *TIMP1*, no statistical difference was found between CRSwNP with AERD and without AERD ($p>0.05$) (Tables 3 and 4).

There was no statistical difference in serum levels of MMP9 according to genotypes and alleles in CRSwNP without AERD. When serum levels of MMP9 were compared in CRSwNP with AERD, they were statistically higher in AG genotype than GG genotype ($p=0.012$). In addition, they were statistically higher in carrying of A (+) allele than in not carrying of A (+) allele in CRSwNP with AERD ($p=0.028$) (Table 5).

According to genotypes and alleles of *TIMP1*, serum levels of TIMP1 were compared between CRSwNP with AERD and CRSwNP without AERD. When these parameters were examined, there was no statistical difference in this study ($p>0.05$).

The ratio is 75% for undergoing two or more surgeries, while the ratio is 58% for undergoing less than two

Table 2: Demographic and clinical characteristics in CRSwNP with AERD and without AERD

	CRSwNP without AERD	CRSwNP with AERD	P
Age (year) (mean±SD)	46.31±14.06	45.27±12.93	0.708
Gender (female/male)	16/42	22/19	0.009
BMI (kg/m²) (mean±SD)	26.80±4.93	26.72±4.0	0.927
Smoking (no/yes) (%)	60.3/39.7	80.5/19.5	0.033
Alcohol (no/yes) (%)	94.8/5.2	100/0	0.197
Right polyp grade (mean±SD)	2.64±0.85	2.32±1.15	0.114
Left polyp grade (mean±SD)	2.60±0.81	2.49±1.05	0.557
Eosinophil ratio (mean±SD)	3.75±2.93	5.41±3.53	0.012
Atopy (no/yes) (%)	89.7/10.3	17.1/82.9	0.000*
Family history (no/yes) (%)	84.5/15.5	78.0/22.0	0.414

CRSwNP without AERD: chronic rhinosinusitis with nasal polyposis without Aspirin –Exacerbated Respiratory Disease, CRSwNP with AERD: chronic rhinosinusitis with nasal polyposis with Aspirin –Exacerbated Respiratory Disease, SD: Standard deviation, BMI: Body mass index

Table 3: *MMP9* genotypes and A/G alleles relationships in CRSwNP with AERD and without AERD

	CRSwNP without AERD n=58	CRSwNP with AERD n=41	OR and CI
***MMP9* genotypes**			
AA	24 (41.4%)	18 (43.9%)	reference
GG	8 (13.8%)	6 (14.6%)	1.0 (0.295-3.395)
AG	26 (44.8%)	17 (41.5%)	0.872 (0.367-2.069)
Alleles			
A	74 (63.7%)	53 (64.6%)	reference
G	42 (36.2%)	29 (35.3%)	0.902 (0.402-2.024)

CRSwNP without AERD: chronic rhinosinusitis with nasal polyposis without Aspirin –Exacerbated Respiratory Disease, CRSwNP with AERD: chronic rhinosinusitis with nasal polyposis with Aspirin –Exacerbated Respiratory Disease, MMP9: Matrix Metalloproteinase 9 p>0.05

Table 4: *TIMP 1* genotypes and C/T alleles relationships in CRSwNP with AERD and without AERD

	CRSwNP without AERD n=58	CRSwNP with AERD n=41	OR and CI
***TIMP1* genotypes**			
CC	23 (39.7%)	13 (31.7%)	reference
TT	28 (48.3%)	17 (41.5%)	1.074 (0.433-2.665)
CT	7 (12.1%)	11 (26.8%)	2.78 (0.866-8.92)
Alleles			
C	53 (45.68%)	37 (45.12%)	reference
T	63 (54.31%)	45 (54.87%)	1.415 (0.610-3.28)

CRSwNP without AERD: chronic rhinosinusitis with nasal polyposis without Aspirin –Exacerbated Respiratory Disease, CRSwNP with AERD: chronic rhinosinusitis with nasal polyposis with Aspirin –Exacerbated Respiratory Disease, TIMP1: tissue inhibitor of metalloproteinase 1 p>0.05

Table 5: MMP9 serum levels according to AA, AG and GG genotypes and A/G alleles

	CRSwNP without AERD	P	CRSwNP with AERD	P
***MMP 9* genotypes**	MMP9 serum levels		MMP9 serum levels	
AA	858.39±382.46	ns	795.56±239.75	
GG	565.32±283.60	ns	605.00±250.72	***0.012*** *vs AG
AG	713.66±409.74	ns	908.12±241.03	
A-	760.18±400.00		605.00±250.72	
A+	565.32±283.60	ns	853.55±243.42	***0.028***
G-	858.39±382.46		795.56±239.75	
G+	669.70±377.69	ns	829.05±273.97	ns

CRSwNP without AERD: chronic rhinosinusitis with nasal polyposis without Aspirin –Exacerbated Respiratory Disease, CRSwNP with AERD: chronic rhinosinusitis with nasal polyposis with Aspirin –Exacerbated Respiratory Disease, MMP9: Matrix Metalloproteinase 9 ns: p>0.05; Serum levels are expressed as mean (±SD).

surgeries in patients who had *TIMP1* in carrying of T (+) allele in CRSwNP without AERD. Likewise, the ratio is 75% for undergoing two or more surgeries and it is 44% for undergoing less than two surgeries in patients who had *TIMP1* TT genotype. (Table 6). Although not reaching statistical significance, the risk of having more than two surgeries increased approximately 2-fold in CRSwNP without AERD with *TIMP1* in carrying of T (+) allele and approximately 4-fold in CRSwNP without AERD with the *TIMP* TT genotype. (p>0.05, OR: 2.17, 95 % CI: 0.39-11.84; p>0.05, OR: 3.81, 95 % CI: 0.70-20.79).

The ratio is 91% for undergoing two or more surgeries, while it is 77% for undergoing less than two surgeries in patients who had *MMP9* carrying of A (+) allele in CRSwNP

with AERD. Likewise, the ratio is 52.2% for undergoing two or more surgeries and it is 33% for undergoing less than two surgeries in patients who had *MMP9* AA genotype. Although not reaching statistical significance, the risk of undergoing more than two surgeries increased approximately 3 times in CRSwNP with AERD with *MMP9* in carrying of A (+) allele and approximately 2 times in CRSwNP with AERD with *MMP9 AA* genotype (p>0.05, OR: 3.00, %95 CI: 0.48-18.64 for A (+) allele; p>0.05, OR: 2.18, 95% CI: 0.60-7.81 for *MMP9* AA genotype).

The relationship between the mean of SNOT 22 scores and genotypes of *MMP9* and *TIMP1* was investigated, but no significant differences were found.

Serum levels of Vitamin D were researched concerning the genotypes of *MMP9* and *TIMP1*. When Vitamin D serum levels were evaluated according to *TIMP1* genotypes, it was observed that they were statistically lower in *TIMP1* CT genotype in CRSwNP with AERD and without AERD (p=0.030) (Table 7).

DISCUSSION

Chronic rhinosinusitis with nasal polyposis which reduces the quality of life and productivity-working capacity were examined (6) in this study. MMP9 and TIMP1 are endopeptidases that are significant factors in the pathophysiology of nasal polyposis and lower and upper respiratory tract remodeling (7). At the same time, the deficiency of Vitamin D is relevant to CRSwNP, but it is unclear how Vitamin D affects MMP secretion (5).

The atopy rate was found 85% in CRSwNP with AERD, and 66% in CRSwNP without AERD by Stevens and colleagues (8). The atopy rate was significantly different between CRSwNP with AERD and CRSwNP without AERD (8). In this study, the atopy rate was discovered at 82.9% in CRSwNP with AERD, 10.3% in CRSwNP without AERD. Furthermore, the frequency of atopy was 42-fold higher in CRSwNP with AERD.

When the literature was reviewed, we couldn't find any studies on why patients with CRSwNP with AERD don't smoke. Since respiratory system diseases such as asthma are seen in patients with CRSwNP with AERD, we think that these patients avoid smoking.

MMP9 single nucleotide polymorphism (SNP) rs3787268, rs2664538 (including rs17576), rs2274756 (including rs17577), and rs3918242 were examined in CRSwNP and the control group. According to subset decomposition, none of the SNPs had a significant p value *rs 2664538* under the recessive model in recurrent nasal polyposis. Statistical differences were not analyzed between recurrent

Table 6: The relationship between *TIMP1* TT genotype, T (+) allele and the number of surgeries in CRSwNP without AERD (P>0.05, OR:3.81, 95 % CI: 0.70-20.79 for *TIMP1* TT genotype) (p>0.05, OR: 2.17, %95 CI: 0.39-11.84 for T(+) allele)

CRSwNP without AERD	**TT-**	**TT+**	**Total**
S<2 Number (n)	28	22	50
Ratio (%)	56	44	100
S≥2 Number (n)	2	6	8
Ratio (%)	25	75	100
CRSwNP without AERD	**T -**	**T+**	**Total**
S<2 Number (n)	21	29	50
Ratio (%)	42	58	100
S≥2 Number (n)	2	6	8
Ratio (%)	25	75	100

CRSwNP without AERD: chronic rhinosinusitis with nasal polyposis without Aspirin –Exacerbated Respiratory Disease. S: surgery

Table 7: Vitamin D serum levels according to *MMP9* and *TIMP1* genotypes in the whole group

	TIMP1			**MMP9**		
	CC	**CT**	**TT**	**AA**	**AG**	**GG**
SNOT 22	47.34±16.30	46.17±17.96	43.98±18.71	46.49±18.68	45.05±18.61	43.38±10.85
D Vitamin (ng/ml)	19.51±6.61	15.40±6.44	19.19±6.35	18.40±6.28	18.67±7.26	19.09±5.53

MMP9: Matrix Metalloproteinase 9, TIMP 1: tissue inhibitor of metalloproteinase 1, SNOT: sinonasal outcome test (p=0.030 Vitamin D levels in *TIMP1* CT genotype were compared to *TIMP1* CC and TT genotype). Values are expressed as mean (±SD)

and non-recurrent CRSwNP in other genetic polymorphisms. GG, AG, and AA genotype were 56.3%, 28.1%, and 10.9% respectively in recurrent nasal polyposis (9). The distribution of genotype in our study is different from the previously mentioned study. When *MMP9* rs17576 polymorphism was checked in CRSwNP with AERD and CRSwNP without AERD, there was no significant difference according to MMP9 genotypes. GG, AG and AA genotype was 14.6%, 41.5%, and 43.9% respectively in CRSwNP with AERD. Since the dispersion of genotype differed between populations, genotypes were different in this study. The hazard of undergoing more than two operations was discovered nearly 3-fold in *MMP9* in carrying of A (+) allele; however, it was examined roughly 2-fold in the *MMP9* AA genotype in CRSwNP with AERD. This information proves that the patients with CRSwNP AERD are more resistent to treatment and necessitate recurrent surgeries in this study.

Guerra et al investigated the relationship with the concentrations of MMP2, MMP7 and MMP9, and TIMP1 and 2. In this study, increased concentrations of MMP9, MMP7 and MMP2 and decreased concentrations of TIMP1 and TIMP2 were demonstrated in nasal polypoid tissue. Also, MMP/TIMP ratio was found significantly higher in nasal polypoid tissue compared to the control group. (10) Kyung-Yeo et al. studied *MMP9* mRNA expression, Wang et al. examined *MMP9* single nucleotide polymorphism, and Mudd et al. also checked *MMP9* expression in CRSwNP (9,11,12). Wang et al. observed polymorphism of *MMP9* (9). However, the influence of these polymorphisms on serum levels was not examined. In this study, serum levels of MMP9 and TIMP1 were researched according to genotypes and alleles. According to genotypes and alleles of *TIMP1*, no significant difference was found in the comparison of TIMP1 serum levels. MMP9 serum levels were statistically higher in AG genotype compared to GG genotype in CRSwNP with AERD. Furthermore, MMP9 serum levels were higher in *MMP9* in carrying of A (+) allele compared to *MMP9* in not carrying of A (+) allele in CRSwNP with AERD. That is why the recurrence of disease increases. This study supports that the patients carrying A (+) allele in CRSwNP with AERD had an approximately 3-fold risk of having more than two surgeries.

TIMP1 genetic polymorphism was investigated in malignancies such as lung and breast cancers, vascular pathologies like intracerebral hemorrhage, senile aortic stenosis (16), and diseases such as systemic lupus erythematosus in the literature (13-17). There is no article on the examination of the genetic polymorphism of *TIMP1* in CRSwNP. We aimed to evaluate *TIMP1* rs4898 C/T polymorphism in all chronic rhinosinusitis with nasal polyposis in this study. Following genotypes and alleles, no significant difference was found between the two groups including CRSwNP with and without AERD.

Vitamin D is capable of modulating pro-inflammatory cytokines, thus having an important position in the pathogenesis of many allergic disorders (18). In addition, it inhibits the proliferation of B lymphocytes and their differentiation into anti-body secretin cells (19). Wang et al. stated MMP2 and MMP9 serum levels raised in proinflammatory with TNFα fibroblast cell cultures, and it was shown that their biological effects were significantly suppressed as a result of calcitriol administration. How Vitamin D influence the secretion of MMP9 is not known. Vitamin D may affect intracellular production, translation, or transcription (20). We aimed to evaluate how the genotypes of *MMP9* and *TIMP1* affect Vitamin D serum levels. Vitamin D serum levels were investigated according to MMP9 and TIMP1 genotypes. Serum levels of Vitamin D were significantly lower in CRSwNP who had *TIMP1* CT genotype in all patients. We did not find any articles that were about the relationship between the genotypes of *TIMP1* and *MMP9* and Vitamin D serum levels.

In this study, the influence of these polymorphisms on serum levels was studied. MMP9 serum levels were significantly higher in AG genotype and A (+) allele in CRSwNP with AERD. According to the genetic polymorphism of *MMP9* and *TIMP1*, serum levels of Vitamin D were investigated. Serum levels of Vitamin D were found statistically lower in *TIMP1* CT genotype in all CRSwNP.

Our study design has limitations. Our sample size is small, that may cause a decrease in statistical power.

We investigated the correlations between the genetic polymorphisms of *MMP9* and *TIMP1* and their serum levels. Also, the relationship between Vitamin D serum levels and genotypes of MMP9 and TIMP1 was described. The rising of MMP9 serum levels may be a marker to chronic inflammation or they may be a significant factor in the resistance of disease. It will be possible to obtain information about the prognosis of disease according to alleles and genotypes. More studies are needed to show possible differences.

CONCLUSION

We found that MMP9 serum levels were significantly higher in AG genotype than GG genotype in CRSwNP with AERD. The rising of MMP9 serum levels may be a marker to chronic inflammation or they may be a significant factor in the resistance of disease. More studies are needed to show possible differences.

Ethics Committee Approval: This study was approved by Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 10.08.2018, No: 13).

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