Submitted: 12 March, 2024 Accepted: 24 May, 2024 Published: 30 October, 2024 DOI:10.22514/jomh.2024.177

ORIGINAL RESEARCH

JOMH Journal of Men's Health

Genetic susceptibility of *MMP3* (rs3025058) variant allele in male football players with multiple ACL surgeries: a PCR-RFLP analysis

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Abstract

This study aimed to investigate the genetic susceptibility of the Matriks Metalloproteinaz-3 (*MMP3*) (rs3025058) gene variant allele and genotype distribution in male football players who actively participate in professional leagues and have undergone a minimum of two anterior cruciate ligament (ACL) surgeries. The study comprised 44 male football players who had undergone a minimum of two ACL surgeries and 61 male football players who had not undergone any ACL surgery. Participants' blood samples were collected and DNA was extracted. Allele types were determined by analyzing the *MMP3* (rs3025058) variant gene polymorphism using the restriction fragment length polymorphism (PCR-RFLP) method with appropriate primers and restriction enzymes. The results revealed no statistical significant differences in genotype distribution for 6A/6A, 5A/6A and 5A/5A ($p = 0.736$) or in 6A-allele frequency distribution between the control (CON) and ACL groups (Odds Ratio (OR) = 1.119, 95% Confidence Interval (CI): 0.637–1.965; $p = 0.318$). The analysis of both dominant (6A/6A *vs.* 5A/6A + 5A/5A) and recessive (6A/6A + 5A/6A *vs.* 5A/5A) models for the inheritance of the minor allele did not yield statistically significant difference, with odds ratios (OR) of 0.647 (95% CI: 0.283–1.478; $p = 0.408$) and 1.472 (95% CI: 0.506–4.281; $p = 0.584$), respectively. This study investigated the impact of *MMP3* gene polymorphisms in ACL injuries, uncovering an intricate interplay between genetic and environmental factors that contribute to injury susceptibility. While some studies suggest potential links between *MMP3* variants and ACL risks, the available information is inconclusive and necessitates additional confirmation in other demographics. Continued research is crucial to develop effective, personalized injury prevention strategies based on robust genetic markers.

Keywords

ACL injuries; Matrix metalloproteinase-3; Gene polymorphisms; Male football player

1. Introduction

Football, widely recognized as the sport with the largest and most varied participant base, encompasses individuals ranging from passive spectators to active participants, at both professional and amateur levels. The sport's dynamic nature, characterized by quick variations in speed, agility and abrupt stops during training and competitive play, intrinsically increases the likelihood of sports-related injuries [1]. The physical encounters, which frequently vary in intensity, mostly during offensive and defensive maneuvers, further heighten the susceptibility to injuries and trauma. In the light of this, it becomes imperative for football clubs to establish robust sports medicine infrastructure and assemble comprehensive medical teams capable of addressing the full spectrum of health issues that football players may encounter [2]. A review of the existing literature highlights the prevalence of football-related injuries, specifically focusing on the lower extremities and limbs in studies on football and injuries [3, 4]. Numerous studies have highlighted the association between football and injuries, with a recurring emphasis on the lower extremities, particularly the thigh and knee regions [5, 6]. This trend is also exemplified in a study conducted on the Turkish National Team, which reported that football injuries predominantly afflict the lower extremities of the body.

Multiple studies have repeatedly found that the knee region is the most frequent site of injury in football, with the anterior cruciate ligament (ACL) being particularly vulnerable. ACL injuries pose a significant risk for football players, frequently impeding their ability to resume playing and potentially leading to the termination of their careers [6–8]. Given that ACL is one of the most common knee ligament injuries and often

requires surgical reconstruction, it is crucial to comprehend the elements that impact these injuries [9]. Although athletes may resume sporting activities within 6–12 months following ACL reconstruction, long-term follow-up studies spanning 2–7 years post-reconstruction have shown that fewer than 50% of players regain their previous levels of athleticism. The substantial decline in performance highlights the necessity for more targeted prevention and treatment strategies. Investigating matrix metalloproteinase (MMP) gene variants is crucial in this context, as these genes are involved in collagen breakdown and tissue remodeling—key processes in ligament repair and healing. An investigation into MMP gene variants may uncover genetic tendencies towards ACL injuries, which could result in enhanced screening methods, personalized injury prevention programs and more efficient post-injury rehabilitation regimens tailored to the genetic profiles of individual athletes [10–12].

Most ACL injuries in football are categorized as non-contact injuries, which happens when a player lands from a jump, decelerates or abruptly changes direction. The anterior tibial translation and lower extremity valgus, caused by force applied to one leg near full extension and displacing the foot from the body's center of mass, are critical risk factors [13]. Significantly, the occurrence of anterior cruciate ligament injuries is typically higher during actual football matches than during training sessions, underscoring the dynamic and unpredictable nature of match conditions [14, 15]. In addition to studying genes that affect athletic ability and inclination to athletic performance, sports genetics research has also begun to gain prominence in the realm of frequently occurring sports-related injuries. It is believed that profiling genomic DNA related to athletic performance and sports injuries will serve as a gateway to understanding genetic advantages as well as genetic limitations that athletes may encounter. The investigation of MMP gene variations is particularly important since these genes produce enzymes that are essential for collagen degradation and tissue remodeling—processes integral to ligament repair and recovery following an injury. By examining these variants, researchers aim to identify genetic markers that may predispose athletes to ACL injuries or affect their recovery, potentially leading to targeted interventions that enhance preventive measures and optimize rehabilitation strategies [16].

The cause of anterior cruciate ligament (ACL) injuries is not fully understood; nonetheless, numerous intrinsic and extrinsic risk factors have been identified [17]. These factors encompass anatomical, hormonal, biomechanical and neuromuscular elements, alongside considerations of familial predisposition and specific genetic sequences, all of which contribute to the risk profile for ACL injuries [18]. Genetic variables mostly center around structural proteins, such as collagen, genes involved in the repair process like matrix metalloproteinases (MMPs), and elements of the apoptotic pathway. For instance, there has been an association between ACL tears and genetic variables like type V collagen A1 (COL5A1), while *MMP3* has been linked to arthritis [19]. Moreover, genetic differences in many genes that code for collagens, which are essential for controlling the formation of collagen fibrils, the basic structural units of ligaments, have been associated with the development of ACL ruptures [20].

It is well-established that genes responsible for encoding extracellular matrix proteins, along with genes controlling the complex processes of tendons and biological signalling molecules, including adaptation, healing, and remodeling, are closely linked to injury susceptibility. An important field of research focuses on investigating the functions of matrix metalloproteinase proteins (MMPs) in the etiology of tendinopathy [21]. Research has shown that matrix metalloproteinases (MMPs) play a crucial regulatory role in preserving the balance of the extracellular matrix (ECM). Matrix metalloproteinases, are a heterogeneous group consisting of 20 distinct endopeptidases. They have the ability to catalyze a wide range of compounds, regardless of whether they are connected to the extracellular matrix or not. Within this family, *MMP3* stands out as it possesses the enzymatic capability to cleave multiple substrates, including but not limited to type II, IV, V, IX and X collagens, laminin, fibronectin, proteoglycan and decorin.

The relationship between genotypes and non-contact ACL injuries appears to involve multiple genetic factors, although the evidence for specific gene correlations might be intricate and differs by populations. Studies have identified significant associations between certain genotypes and the susceptibility to non-contact ACL injuries [22–25]. For instance, polymorphisms within the genes encoding inflammatory interleukins such as Interleukin 1 Beta (IL1B) rs16944 and Interleukin-6 receptor (IL6R) rs2228145 have been found to have different frequencies in individuals with non-contact ACL injuries compared to controls. In one study, certain genotypes of these interleukins were either underrepresented or overrepresented in groups with non-contact ACL injuries, suggesting a potential genetic predisposition related to inflammatory responses [25].

ACL injuries are particularly prevalent among individuals aged 15 to 45, which corresponds with higher sports activity levels. Women are disproportionately affected due to anatomical and biomechanical differences, such as wider hips and variations in neuromuscular control, which heighten their risk [26, 27]. The type of sports also significantly influences ACL injury risks; activities involving sudden stops, jumps, or direction changes—like soccer, basketball and skiing—are known to have higher incidences. This is particularly evident in sports that require pivot movements, exerting additional strain on the ACL [26]. Moreover, environmental factors like the type of playing surface and the prevailing weather conditions have significant impacts. For example, artificial turf and dry weather can enhance ground traction, thereby raising the likelihood of ACL injuries during dynamic movements such as pivoting or rapid directional changes [28]. The hypothesis of this study was that there is no significant difference in the distribution of *MMP3* (rs3025058) genotypes between soccer players with multiple ACL surgeries and those without.

Previous investigations [29, 30] into *MMP3* polymorphisms have primarily focused on comparing individuals who have suffered ACL injuries with control groups comprising the general population, typically non-exercisers. In particular, the *MMP3* (rs3025058) variant, along with the relative scarcity of research related to ACL injuries, underscores the significance of this study. Another important outcome of this research is that both the control group and the case group consist of professional male football players with a minimum of 10

years of football experience. This study aims to delve into the complex interaction between the *MMP3* (rs3025058) gene variant and anterior cruciate ligament injuries, addressing a significant gap in the literature that has hindered our comprehensive understanding of injury susceptibility to date.

2. Materials and methods

2.1 Study participants

The study was conducted with two different groups of football players. Football players who have had two or more ACL surgeries may show a more pronounced effect of genetic predisposition. Therefore, 41 football players aged 18–35 years who had at least two anterior cruciate ligament surgeries and were actively participating in different teams in the Turkish professional football leagues in the 2020–2021 season were included in the study. The control group consisted of male football players between the ages of 18–35 years, with at least 5 years of football experience and actively participating in different teams in the Turkish professional football leagues in the 2020–2021 season. Importantly, the control group had not previously undergone surgical procedures due to injuries. Blood samples used for the study were obtained as remnants from routine screenings, with the full permission and informed consent of the participants.

2.2 DNA isolation

DNA isolation was carried out utilizing a DNA isolation kit (GeneAll, Exgene™ Clinic SV, 29021X, Seoul, South Korea). The allele types were determined by analyzing the *MMP3* (rs3025058) variant gene polymorphism using the restriction fragment length polymorphism (PCR-RFLP) method. This analysis was performed using appropriate primers and restriction enzymes (specific enzymes provided by Thermo Fisher Scientific, 67890, Waltham, MA, USA).

2.3 Genomic DNA extraction

Genomic DNA was extracted from ethylenediaminetetraacetate (EDTA)-treated whole venous blood samples using a commercial DNA isolation kit (GeneAll, South Korea). For genotyping a 129 bp fragment of the *MMP3* gene polymorphism (rs3025058), a polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay was employed using primers designed by Primer3 software and restriction HpaII enzymes supplied by Thermo Fisher Scientific (01007395, Waltham, MA, USA).

2.4 PCR primers

The sense oligonucleotide primer for *MMP3* (rs3025058) polymorphism was 5′-GGTTCTCCATTCCTTTGATGGGGGGAAAGA-3′, and the antisense primer was 5′-CTTCCTGGAATTCACATCACTGCCACCACT-3′.

2.5 PCR reaction

PCR reactions were carried out in a total volume of 25 μL, comprising 25–50 ng of genomic DNA, 0.8 nmol/μL of each primer, 1.5 μL $MgCl_2$ (25 mM), 2.5 μL 10$\times$ PCR buffer, 0.3 μL dNTP (25 mM) and 1 Unit of Taq polymerase (01007395, Thermo Fisher Scientific, Waltham, MA, USA). The amplification process involved the following conditions: an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 66 °C for 45 seconds, extension at 72 °C for 45 seconds, and a final extension step at 72 °C for 15 minutes.

2.6 RFLP analysis

The 129 bp PCR products were subsequently subjected to digestion using the ThtIII1 restriction enzyme. This enzymatic digestion resulted in two distinct fragments: 129 bp for the 6A allele and 97 bp and 32 bp for the 5A allele. The digested PCR products were then separated by electrophoresis on a 3% nusieve-agarose gel, stained with ethidium bromide, and the genotypes were determined under ultraviolet (UV) illumination (Fig. 1).

2.7 Statistical analysis

Statistical analyses were conducted using Open Epi Info Software Version 3.2.2 (CDC, Atlanta, GA, USA). To compare the results between the patient and control groups, we employed either Chi-square or Fisher's exact test. In instances where Chi-square or Fisher's exact test yielded statistically significant difference, odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) were computed. Additionally, the Chi-square test was employed to assess the goodness of fit for genotypic distributions, and the assessment of Hardy-Weinberg equilibrium was assessed using Arlequin Software Version 2000 (University of Geneva, Geneva, Switzerland). The significance level was evaluated as $p < 0.05$ in the analyses.

As a result of the literature study [20], the test power was found to be 0.74. The study group was found to be 40 people per group. Since there were two different groups in the study, it was determined as $40 \times 2 = 80$. In this study, 105 people participated.

3. Results

The study sought to examine the genetic susceptibility profiles for injury in professional male football players who are actively involved in professional leagues and have undergone at least two anterior cruciate ligament (ACL) surgeries. The study attempted to gain a comprehensive understanding of these profiles from multiple viewpoints. The results are displayed in the below tables:

Male professional football players in both the CON and ACL groups were matched in terms of age, height, weight and sport experience (Table 1). These results indicate that there are differences between the ACL group and the control group in terms of age and sports experience, but had similarities in terms of height and body weight.

According to the results in Table 2, there was no statistically significant difference in the genotype distribution between the control group and the ACL group. The *p*-values for the 6A/6A, 5A/6A and 5A/5A genotypes were 0.736, 0.408 and 0.584, respectively. In addition, no statistically significant difference

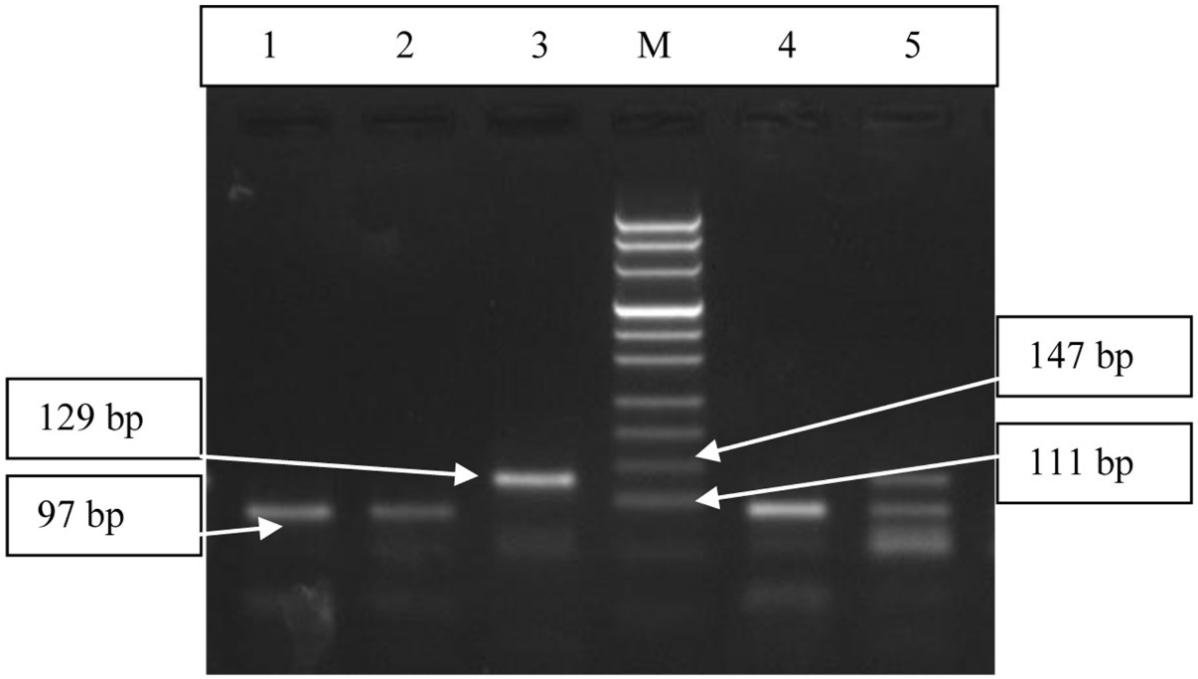

FIGURE 1. Gel image for *MMP3* polymorphism.

TABLE 1. Characteristics of Turkish professional male football players in the control (CON) and anterior cruciate ligament rupture (ACL) groups.

Groups	n	Age (yr)	Experience (yr)	Height (cm)	Body Weight (kg)
ACL	44	27.06 ± 3.79	13.94 ± 3.43	177.23 ± 4.36	75.44 ± 5.59
CON	61	29.07 ± 5.67	16.66 ± 4.44	176.65 ± 5.18	76.66 ± 4.66

TABLE 2. Genotype and minor allele frequency distribution of the *MMP3* (rs3025058) 5A > 6A variant in the control and anterior cruciate ligament rupture groups of Turkish professional male football players.

		ACL	CON	*p*	OR (95% CI)
	n	44	61		
Genotype, Allele					
	6A/6A	29.5 (13)	39.3 (24)	0.736	
	5A/6A	52.3 (23)	47.5 (29)	0.408[d]	0.647 (0.283–1.478)
Genes (SNP ID)	*MMP3*				
Genotype, Allele					
	5A/5A	18.2 (8)	13.1 (8)	0.584[r]	1.472 (0.506–4.281)
	6A	55.7 (49)	63.1 (77)	0.318	1.119 (0.637–1.965)
Genes SNP ID	HWE *p*	0.873	0.270		

[d]: Dominant model; [r]: Recessive mode; HWE: Hardy-Weinberg equilibrium; OR: Odd rate; ACL: Anterior Cruciate Ligament; CON: Control; CI: Confidence Interval; SNP ID: Single Nucleotide Polymorphism Identifier; MMP3: Matriks Metalloproteinaz-3.

was found between the 6A and 5A allele frequencies (*p*-value: 0.318). The analyses for dominant and recessive models also showed no statistically significant difference. For Hardy-Weinberg equilibrium (HWE), no statistically significant difference was found between the control and ACL groups (*p*-values: 0.873 and 0.27, respectively). These results indicate that the 5A > 6A variant in the *MMP3* gene (rs3025058) does not show a different distribution between the control and ACL groups.

4. Discussion

Upon examining the literature, it becomes clear that the anterior cruciate ligament damage is one of the most common non-contact injuries in sports [2]. Multiple studies have emphasized that these injuries, especially when they happen often, can be partially related to insufficient teamwork among coaches, athletes and healthcare practitioners [31–33]. The idea of utilizing genetic testing to identify athletes predisposed to

sports-related injuries holds significant appeal. These tests have the potential to identify athletes who are at risk and enable the implementation of preventive measures. Upon reviewing literature, it becomes evident that anterior cruciate ligament injury is one of the most common non-contact injuries in sports [2].

In this study, exclusively male professional football players were examined, and the findings indicate that there is no statistically significant difference in the distribution of the *MMP3* (rs3025058) variant alleles between professional male football players with anterior cruciate ligament injuries and the control group. For *MMP3* (rs3025058), the frequency distributions of the genotypes in the dominant model and recessive model were found as follows: for the dominant model ACL 13 CON 13 for genotype 6A/6A, ACL 41 CON 37 for genotype 5A/6A + 5A/5A, and for the recessive model ACL 8 CON 8 for genotype 5A, ACL 36 CON 53 for genotype 5A/6A + 6A/6A. The findings regarding the lack of statistically significant differences in the genotype and allele frequencies between the ACL injury group and the control group, as well as the adherence to Hardy-Weinberg equilibrium, suggest that the 5A > 6A variant of the *MMP3* gene (rs3025058) is not associated with an increased risk of ACL injuries in the studied population. The lack of statistically significant differences in genotype and allele frequencies between the ACL and control groups suggests that the *MMP3* (rs3025058) 5A > 6A variant alone may not be a major determinant of ACL injury susceptibility in this population. However, comprehending the function of *MMP3* in the alteration of the matrix and tissues repair might offer valuable understanding into the underlying biological processes.

Anterior cruciate ligament (ACL) tears are widely recognized as one of the most severe articular injuries in sports. However, the exact factors behind ACL injury are still not well understood. To date, several studies have investigated *MMP3* polymorphisms and their potential influence on susceptibility to tendon-ligament injuries, yielding diverse and inconclusive outcomes. More recently, there has been emerging evidence linking the gene responsible for encoding matrix metalloproteinase-3 (*MMP3*, also known as stromelysin-1) with anterior cruciate ligament ruptures [34]. A groundbreaking study examining *MMP3* variations found a significant association between certain genotypes, such as the GG genotype of rs679620, the CC genotype of rs591058, and the AA genotype of rs650108, with an increased susceptibility to developing Achilles tendinopathy in a Caucasian population [35]. In contrast, Waldén *et al.* [15], conducted a study that precisely investigated the association between gender and the occurrence of anterior cruciate ligament injuries. Their research revealed that female elite football players were more than twice as likely to experience anterior cruciate ligament injuries compared to their male counterparts. Variations in hormone levels, particularly estrogen, can affect the ligament's strength and stiffness. Estrogen has been linked to increased ligament laxity, making the ACL more prone to tears during high-stress activities. Additionally, differences in lower limb alignment and the relative strength of the muscles around the knee can also contribute to higher rates of ACL injuries in women. Females frequently exhibit a greater quadriceps-to-hamstring strength ratio, which can may undermine knee stability during exertion.

Raleigh *et al.* [21], reported that individuals with the GG genotype of rs679620 in the *MMP3* gene are at an increased risk of Achilles tendinopathy, particularly among Caucasians. Several studies have highlighted the significant association between *MMP3* polymorphisms and susceptibility to tendon-ligament injuries. In a study conducted by Posthumus *et al.* [36], they investigated four DNA variants (MMP1 rs1799750, *MMP3* rs679620, MMP10 rs486055, MMP12 rs2276109) located on the 11q22 chromosomal region in a South African cohort to assess their potential link to the risk of ACL ruptures. Their findings revealed that the MMP12 rs2276109 G allele was less common among participants with non-contact ACL ruptures compared with the control group. Furthermore, while the *MMP3* rs679620 locus did not independently exhibit a significant association with the risk of ACL injury, the GG genotype showed a trend towards statistical significance, occurring more frequently in the control group compared to the ACL rupture group. Interestingly, the authors observed that the *MMP3* rs679620 G allele consistently appeared in higher proportions among controls when quadvariate, trivariate and bivariate haplotypes were considered. This led the authors to conclude that low-risk haplotype combinations may be linked to the presence of G alleles for both the *MMP3* rs679620 and MMP12 rs2276109 variants.

Malila *et al.* [34], conducted a study to investigate the relationship between the *MMP3*-1612 variant 5A/6A polymorphism and anterior cruciate ligament (ACL) ruptures. They compared 86 individuals with ACL rupture to 100 healthy controls who had no history of ligament or tendon damage. Their findings revealed that the 5A/5A and 5A/6A genotypes, as well as the 5A allele frequencies, were significantly higher in individuals with ACL rupture in comparison to the control group. Based on these results, the researchers suggested that the *MMP3* 5A/6A polymorphism can serve as a valuable predictor for anterior cruciate ligament injuries. They also acknowledged that the outcomes of such studies may not necessarily generalize to the broader population, as the cases examined may not be representative of the general population. Lulińska *et al.* [37], found no statistically significant difference in genetic frequencies between 229 cases with surgical anterior cruciate ligament rupture and 192 control male athletes in their study, although they concluded that the *MMP3* rs679620 G and rs591058C alleles were significantly higher in cases compared to controls.

Within the setting of ACL injuries, an increased level of *MMP3* activity (associated with the 5A allele) can lead to excessive degradation of the matrix. This degradation can weaken the structural integrity of the ligament and the tissues around it, making them more prone to injury. Nevertheless, the results of the study indicate that there is no significant variation in the occurrence of 5A and 6A alleles between injured and non-injured players, suggesting that other genetic, environmental or biomechanical factors may contribute more prominently to ACL injury risk.

In conclusion, while the *MMP3* (rs3025058) 5A > 6A variant does not appear to significantly influence ACL injury risk in the studied population, its role in tissue remodeling

highlights the complexity of genetic contributions to injury susceptibility. Further research is necessary to unravel the multifactorial nature of ACL injuries and devise more effective prevention strategies.

5. Conclusions

Anterior cruciate ligament (ACL) injuries pose significant challenges in sports medicine, affecting athletes' careers and long-term health. This study explored the role of *MMP3* gene polymorphisms in ACL injuries, revealing a complex interaction between genetic and environmental factors that contribute to injury susceptibility. Although many research studies indicate that possible connections *MMP3* variants and ACL risks, the evidence is inconclusive and necessitates additional validation in other groups. Continued research is crucial to develop effective, personalized injury prevention strategies based on robust genetic markers.

The study focused on a very specific subgroup professional male football players which inherently limits the pool of available participants. The specificity is crucial for maintaining relevant and applicable results to this particular demographic, but it naturally restricts the number of potential study participants.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

AB and ÇD—concept; AB, MeE and NB—data acquisition; MuE and KŞ—data analysis and interpretation; MeE—drafting manuscript; DŞT and YEG—critical revision of manuscript; MuE—final approval and accountability; YEG—technical or material support; DŞT—supervision.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

I have been informed about the nature, purpose, and potential risks of this study on genetic injury susceptibility profiles in professional male soccer players. Participation is voluntary, may be withdrawn at any time without penalty and all personal data will be kept confidential. They agreed to provide a blood sample for DNA analysis and to complete a questionnaire about my medical and sports history. The study protocol was approved by Tokat Gaziosmanpaşa University Faculty of Medicine Clinical Research Ethics Committee (28 January 2021 and numbered 21-KAEK-028).

ACKNOWLEDGMENT

Not applicable.

FUNDING

This research received research and study support from Tokat Gaziosmanpaşa University Scientific Research Projects Unit (Project number: 2021/23).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Alpay Bülbül, Çağatay Dereceli, Meriç Eraslan, Nihan Bozkurt, Kübra Şahin, Murat Eliöz, *et al.* Genetic susceptibility of *MMP3* (rs3025058) variant allele in male football players with multiple ACL surgeries: a PCR-RFLP analysis. Journal of Men's Health. 2024; 20(10): 183-189. doi: 10.22514/jomh.2024.177.