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ORIGINAL ARTICLE

# DOES THE A9285G POLYMORPHISM IN COLLAGEN TYPE XII α1 GENE ASSOCIATE WITH THE RISK OF ANTERIOR CRUCIATE LIGAMENT RUPTURES?

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## ABSTRACT

One of the most severe injuries sustained by athletes is rupture of the anterior cruciate ligament (ACL). Recent investigations suggest that a predisposition for ACL rupture may be the result of specific genetic sequence variants. In light of this, we decided to investigate whether the *COL12A1* A9285G polymorphism was associated with ACL ruptures in Polish football players.

We compared genotypic and allelic frequencies of the *COL12A1* A9285G polymorphism in two groups of athletes: 91 male football players (23 ± 3 years) with surgically diagnosed primary ACL ruptures who qualified for ligament reconstruction (cases) and 143 apparently healthy, male football players of the same ethnicity, a similar age category, and a comparable level of exposure to ACL injury, who were without any self-reported history of ligament or tendon injury (controls). DNA samples extracted from the oral epithelial cells were genotyped by using a real-time polymerase chain reaction (Ri-Ti-PCR) method.

The genotype distribution in the cases were not different from those in controls ($p = 0.70$). The frequency of the G allele was lower in the cases (18.1%) but not statistically significant ($p = 0.40$) when compared with controls (21.3%).

Our results are in contradiction to the hypothesis that the *COL12A1* A9285G polymorphism is associated with a predisposition for ACL injury. However, these conclusions should be supported with more experimental studies on *COL12A1* polymorphisms.

**Keywords:** Anterior cruciate ligament (ACL) rupture; Collagen; *COL12A1* gene; Polymorphism.

## INTRODUCTION

One of the most severe injuries sustained by athletes is rupture of the anterior cruciate ligament (ACL) [1]. The exact etiology of ACL ruptures is poorly understood, but recently conducted investigations indicate that around 70.0% of ACL ruptures are the consequence of forces applied to the knee at the time of injury, which result from the athlete's own movements and do not involve contact with another athlete or object [2]. That seems to be the reason that the risk of ACL rupture is significantly higher in sports requiring change in direction and rapid deceleration during cutting, pivoting and landing [3]. Therefore, it is not surprising that one of the groups of athletes with the highest frequency of ACL rupture are football players [4].

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Analyzing individual cases of ACL injuries in football players raises the question as to why one member of a team (with the same load and movement character) is exposed to ACL injury, while fellow team mates are not. Recent investigations suggest that the risk of ACL rupture results from familial predispositions and specific genetic sequence variants [5]. At this point in time, only a few studies have given evidence of the connection between ACL injury and specific genetic risk factors [6-10], mostly among sequence variants within the *COL5A1* and *COL1A1* genes.

Anterior cruciate ligaments are collagenous structures consisting of water and fibro-cartilaginous specific proteins, which build collagen fibrils [11]. The main structural components of this ligament are collagens type I, III-VI, XII and XIV, but also proteoglycans such as decorin, lumican and versican, and glycoproteins such as elastin, tenascin C and cartilage oligomeric matrix protein (COMP) [12].

In the present state of knowledge, any proteins (and what is more, any genes that encode these proteins) that are functionally associated with ligaments could be potential candidates [13-15]. Hence, any such genes and proteins that have already been implicated with ACL injury should be designated as candidates of priority [7]. In our study, we decided to investigate *COL12A1*, which is one of the less frequently studied genes in the context of predisposition to ACL injury.

The *COL12A1* gene (121 kb; mapped to chromosome 6q12-q13) encodes the α1 chains of the various long (XIIA) and short (XIIB) homotrimeric isoforms of type XII collagen [16,17]. According to the database hosted by the National Center for Biotechnology and Information (NCBI), five single nucleotide polymorphisms (SNPs) are shown in *COL12A1* exons. Only two of them (rs240736 and rs970547) were identified as non synonymous SNPs (*i.e.* SNPs that change the amino acid sequence in the gene product) [13].

In one of the previous studies, it was suggested that especially the A/G transition at position 162 of exon 65 (9285 A/G, S3058G, rs970547) may alter the biomechanical properties of the collagen fibril and thus may increase the risk of ACL ruptures [18]. In light of the facts mentioned above, we decided to investigate whether the *COL12A1* A9285G polymorphism was associated with ACL ruptures in Polish football players.

## MATERIALS AND METHODS

**Study Subjects.** A total of 91 male football players (23 ± 3 years) with surgically diagnosed primary ACL ruptures who qualified for ligament reconstruction, were recruited for this study through Galen Orthopaedics, Bieruń, Poland. The control group comprised 143 apparently healthy, male football players (25 ± 2.6 years), of the same ethnicity, a similar age category, and a comparable level of exposure to ACL injury, who were without any self-reported history of ligament or tendon injury.

**Ethics Committee.** The study was conducted in accordance with the ethical standards as described by Kruk [19]. Additionally, the Pomeranian Medical University (Szczecin, Poland) Ethics Committee approved the details of this study and all related informational and consent documentation before any data collection. In accordance with the Pomeranian Medical University Ethics Committee's guidelines, the investigator informed all the subjects as to the benefits and possible risks associated with participation in the study, and all subjects signed a written informed consent document indicating their voluntary participation.

**Genotyping.** Genomic DNA was extracted from the oral epithelial cells using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich Chemie, Steinheim, Germany) according to manufacturer's protocol. Allelic discrimination of the A9285G *COL12A1* (rs970547) polymorphic site was performed using a TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA), including primers and fluorescently labelled (FAM and VIC) MGB probes for the detection of the alleles. All samples were genotyped on a Rotor-Gene real-time polymerase chain reaction (Re-Ti-PCR) instrument (Corbett Research, Sydney, NSW, Australia). Thermal cycler conditions were as follows: an initial step at 95 °C for 5 min., followed by 45 cycles of denaturation at 94 °C for 15 seconds and annealing/extension at 60 °C for 1 min.

**Statistical Analysis.** Any differences in genotype and allele frequency were analyzed using $\chi^2$ tests (or Fisher exact tests). Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated. All calculations were performed using Statistica (StatSoft Inc., Tulsa, OK, USA; 2011). STATISTICA (data analysis software system, version 10,

www.statsoft.com) was used for computing statistics, except Hardy-Weinberg equilibrium which was tested with the programming language and environment R (http://www.r-project.org) and the test for linear trend, which was performed using the STATCALC module in Epi Info (http://wwwn.cdc.gov/epiinfo). The $p$ <0.05 values were considered to be statistically significant.

## RESULTS

Genotype distributions met Hardy-Weinberg proportions in the control group ($p$ = 0.81) and in the cases ($p$ = 1.0). The distributions of the A9285G *COL12A1* genotypes and alleles are given in Table 1. The genotype distribution in the cases were not different from those in controls ($p$ = 0.70). Underrepresentation of the GG genotype in the ACL rupture group was not statistically significant ($p$ = 0.744, Fisher's exact test, recessive mode: GG *vs.* GA+ AA). The frequency of the G allele was lower in the cases (18.1%), but not statistically significant ($p$ = 0.40) when compared with controls (21.3%). Given the G allele, the likelihood of ACL injury was 0.82 times higher (95% CI: 0.50-1.34; $p$ <0.00001; $p_{ad}$ <0.0001) than in the control group.

## DISCUSSION

The role of genetics in sport research increases with every passing year [20,21]. Knowledge of the role of individual genes in the processes occurring in the human body can also be used in sport rehabilitation and injury prevention [22]. Precise determination of genotypes at risk for acute or chronic diseases related to sport will probably enable adjustments in individual training plans to greatly minimize the risk of injury.

Approximately two-thirds of ACL tissue consists of water. The rest is made up of tightly packed parallel collagen fibril bundles consisting predominately of type I collagen fibrils (60.0-80.0% dry mass of ligament) [11].

Collagen XII belongs to the subfamily of fibril-associated collagens with interrupted triple helices (FACIT) [23,24] that are believed to form interfibrillar connections and mediate fibril interaction with other extracellular and cell surface molecules within tendons and other tissues [24,25]. On the basis of this fact, we suspect that collagen XII may be considered an influential component in ligament and tendon strength and flexibility.

This assumption seems to be indirectly confirmed by the fact that the key elements of tendons' stretch-responsiveness have been identified in regulatory regions of the *COL12A1* gene. Additionally, earlier studies indicated that collagen XII significantly promotes the contraction of collagen gels (such as tenascin C) and consequently modulates the cellular response of tissue to mechanical stress [26-28]. Furthermore, September *et al.* [8] suggested collagen XII may be involved in similar biological processes as both tenascin C and type V collagen, *i.e.,* regulation of the assembly of fibrils (fibrillogenesis).

**Table 1.** Genotype and allele frequencies of the A9285G *COL12A1* gene.

| Subjects | HWE | Genotype *n* (%) | *p* Values | Allele (%) | OR (95% CI) | Allele *p* Value |
|---|---|---|---|---|---|---|
| Cases ($n$ = 91) | 1.0 | AA: 61 (67.0)<br>AG: 27 (29.7)<br>GG: 3 (3.3) | 0.701[a]<br>$p_D$ 0.487[b]<br>$p_R$ 0.744[b] | A (81.9)<br>G (18.1) | 0.82<br>(0.50-1.34) | 0.400[a] |
| Controls ($n$ = 143) | 0.808 | AA: 89 (62.2)<br>AG: 47 (32.9)<br>GG: 7 (4.9) | | A (78.7)<br>G (21.3) | | |

HWE: Hardy-Weinberg equilibrium.

[a] $\chi^2$: $p$ value.

[b] $p_D$ and $p_R$ are two-sided Fisher's exact test probabilities with dominant (GG+AG *vs.* AA) and recessive (GG *vs.* AG+AA) modes of inheritance of minor alleles (G), respectively.

The A9285G *COL12A1* polymorphism within exon 65, is a non synonymous coding variant, which changes the amino acid at position 3058 from a serine to a glycine. Although the wild type serine amino acid is a neutral polar amino acid with a larger side chain than the substituted non polar neutral glycine amino acid, some investigators speculate that this change in amino acid sequence may alter the biomechanical properties of the collagen fibril [29]. On the other hand, it is not proven that this SNP has any effect on protein expression or function.

The first report concerning the possible importance of *COL12A1*variants for achilles tendon injuries did not identify a statistically significant difference in the genotype or allele distribution in the A9285G *COL12A1* polymorphism [29]. On the other hand, the same investigators showed that the A9285G *COL12A1* polymorphism is associated with ACL ruptures in females [29]. The obtained results suggested that females with an AA genotype are at increased risk for ACL ruptures (AA *vs.* GT+GG; OR = 2.4; 95% CI 1.0-5.5; $p$ <0.05). Additionally, September *et al.* [13] observed a trend for the AA genotype to be overrepresented (AA *vs.* GA+GG; $p$ = 0.08) in female participants with a family history of ligament injury. The ambiguity of the results obtained by September *et al.* [13] and Posthumus *et al.* [29] and a lack of other studies concerning the role A9285G *COL12A1* in ACL injures may be considered as one of the most important reasons to conduct repeated investigations in order to identify the genetic background of individuals predisposed to tendon and ligament injury.

Our results were contrary to the hypothesis that the A9285G *COL12A1* polymorphism is associated with ACL injuries. We did not find any statistical difference in the A9285G genotype and allele frequencies in male football players with surgically confirmed primary ACL ruptures compared to injury-free athletes. To summarize, considering we only investigated male subjects, we reached a similar conclusion as September *et al.* [13].

In our investigation, the ACL injury group closely resembled the control group of injury-free athletes in numerous aspects, being of similar ages and ethnicities and identical athletic disciplines. The last of these is a key component due to the variability of inciting events among different disciplines. Participants from the control group in our study had the similar internal and external risk factors relating to the examined phenotype, overcoming a known limitation of case-control studies.

We investigated male football players with surgically confirmed primary ACL rupture, who were qualified for an ACL reconstruction procedure. The control group comprised of only males of the same ethnicity, similar in age, participating in the same sport, their knee joints being exposed to comparable forces and movements, controlling the many internal and external risk factors. Thanks to this solution in our investigations cases and controls are similar in variables that may be related to the phenotype that is under examination, as well as the inciting events is very difficult, a known limitation of case-control studies.

The homogeneity of the investigated groups seems to be a strength of this study, however, this fact may be considered as a limitation, because we did not manage to confirm or deny the important role of the investigated polymorphism with regard to the risk of ACL injury in women. We included only male participants, but it should be noted that women are 2-3 times more likely to sustain an ACL injury than men [18]. A number of intrinsic risk factors classified as anatomical, hormonal, or neuromuscular, have been linked to this observed phenomenon. In the case of female patients, each of the aforementioned factors may be more likely to affect the gene environmental interaction, causing significantly higher incidence of this polymorphism's association with ACL injuries [30,31], a relationship that is not seen as clearly in men.

In conclusion, this study found that there is no association between the A9285G *COL12A1* polymorphism and ACL ruptures in men. On the other hand, the lack of statistical significance in genotype and allele distribution of A9285G *COL12A1* shown in our investigation, does not necessarily mean that the investigated polymorphism has no effect on ACL injuries. Tendon and ligament injuries are complex, multifactorial conditions, caused by interactions of a number of different proteins, encoded by different genes on different chromosomes (gene-gene interactions), and the interactions of these genetic components with different environmental factors (gene-environment interactions) [13]. Thus, our findings should be supported with more experimental studies on *COL12A1* polymorphisms including their inter-

action with other genes. Additionally, our results need to be confirmed in a larger sample of subjects. Lastly, it should be noted that genetic association studies must always be interpreted with caution.

**Declaration of Interest.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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