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Are genes encoding proteoglycans really associated with the risk of anterior cruciate ligament rupture?

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ABSTRACT: Proteoglycans are considered integral structural components of tendon and ligament and have been implicated in the resistance of compressive forces, collagen fibrillogenesis, matrix remodelling and cell signalling. Several sequence variants within genes encoding proteoglycans were recently implicated in modulating anterior cruciate ligament ruptures (ACLR). This study aimed to test the previously implicated variants in proteoglycan and vascular epithelial growth factor encoding genes with risk of ACLR in a population from Poland. A case control genetic association study was conducted using DNA samples from 143 healthy participants without a history of ACL injuries (99 male and 44 females) (CON group) and 229 surgically diagnosed ACLR participants (158 males and 71 females). All samples were genotyped for the *ACAN*: rs1516797, *BGN*: rs1042103, rs1126499, *DCN*: rs516115 and *VEGFA*: rs699947 variants. Main findings included the (i) *ACAN* rs1516797 G/T genotype which was underrepresented in the CON group (CON: 36%, n=52, ACLR: 49%, n=112, p=0.017, OR=1.68, 95% CI 1.09 to 2.57) when all participants were investigated and (ii) the *BGN* rs1042103 A allele was significantly under-represented in the male CON group compared to the male ACLR group (CON: 39%, n=78, ACLR: 49%, n=156, p=0.029, OR=1.5, 95% CI 1.05 to 2.15). Furthermore, *BGN* inferred haplotypes were highlighted with altered ACLR susceptibility. Although the study implicated the *ACAN* and *BGN* genes (combination of genotype, allele and haplotype) in modulating ACLR susceptibility, several differences were noted with previous published findings.

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INTRODUCTION

Injuries of ligaments and tendons annually affect 100 million worldwide and are described as the most common structures of musculoskeletal damage. One of the most severe of these injuries include anterior cruciate ligament (ACL) ruptures. The ACL stabilizes and controls the knee joint mobility [1]. During exercise, the ACL lengthens reducing the stiffness of the joint, while after exercise the ACL length returns to baseline due to its viscoelastic properties which protect the knee from excessive slenderness [2]. Dysfunction of the ACL thereby subsequently compromises the knee kinematics.

The function of the ACL can further be ascribed to the arrangement and integrity of the collagen fibril (fibrillogenesis), which is the basic building block of ligaments. The fibril is also composed of proteoglycans such as aggrecan (main proteoglycan in the articular cartilage), fibromodulin (small interstitial proteoglycan), biglycan (affecting muscle development and regeneration, bone growth and collagen fibril assembly in multiple tissues), lumican (distributed in interstitial collagenous matrices throughout the body) and decorin (affecting collagen fibril assembly) which collectively contribute to

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Genetic Association Study

Haplotypes

the structural integrity of the ligament [3]. These components collectively facilitate the dynamic elastic and biomechanical properties of the knee for effective function.

The risk of ACL rupture is increased in sports requiring rapid changes in direction and/or decelerations such in the case of cutting, pivoting and landing [4]. Multiple extrinsic [5] and intrinsic risk factors have been associated with anterior cruciate ligament (ACL) ruptures [6] and more recently, evidence implicating genetic susceptibility as an intrinsic risk factor has been shown [7]. Specifically, roles of the *COL1A1* gene [8], *COL5A1* gene [9,10], *COL12A1* gene [11,12], and the chromosome 11 *MMP* gene cluster [13] have been suggested, to name only a few loci. Although a number of authors claim that greater core stability decreases the risk of ACLR, the search for genetic determinants of susceptibility to ACL rupture continues.

Among such studies, the most promising research which was enthusiastically received by the sporting and physiotherapeutic community implicated genes encoding proteoglycans in the genetic predisposition towards ACL rupture [14]. Furthermore, they suggested that "it is reasonable to propose that genetic sequence variability within the genes encoding proteoglycans may potentially modulate the ligament fibril properties" [14]. This was the first report on associations between genes encoding proteoglycans and ACL injury susceptibility.

Following on from this, we conducted analogous research on a population of Polish athletes. The aim of this study was to verify the first hypothesis in Mannion *et al* that sequence variants in the *ACAN*, *BGN* and *DCN*, as well as *VEGFA* (involved in the angiogenesis-associated signaling pathway) [15] are associated with ACL rupture susceptibility.

MATERIALS AND METHODS

Participants

A total of 372 physically active, unrelated, self-reported Caucasian participants were recruited for this case-control genetic association study between the 2009 and 2016. These participants consisted of 229 (158 male) individuals with surgically diagnosed primary ACL rupture (ACLR) who qualified for ligament reconstruction (ACLR group) and 143 (99 male) apparently healthy participants without any history of ACL injuries (CON group). All 229 participants from ACLR group sustained their injury through non-contact mechanisms.

The ACLR participants were soccer players (158 males and 71 females) from the Polish 1st, 2nd and 3rd division soccer league (trained 11-14 hours per week). The control group were healthy, mainly soccer players (99 males and 44 females), who self-reported no history of ligament or tendon injury. All the male participants (ACLR and CON groups) were from the same soccer teams, of the same ethnicity (as self-reported, all Polish, East-Europeans for ≥ 3 generations), of similar age (ACLR group = 26 ± 4 , control group = 25 ± 3), and had a comparable level of exposure to risk of ACL injury (same

volume and intensity of training and match play). The ACLR female participants (age 25 ± 4) were 43 soccer players from Polish 1st division soccer league (trained 10-12 hours per week) and also included amateur skiers ($n=28$). The female participants from CON group (age 29 ± 2) were recruited from sports clubs and wellness centers (physically active for a minimum of 7 hours per week).

Ethics committee

The study was approved by the Pomeranian Medical University Ethics Committee (Poland) and written informed consent was obtained from each participant according to the declaration of Helsinki.

Genetic Analyses

The buccal cells from each participant was collected in resuspension solution (GenElute Mammalian Genomic DNA Miniprep Kit, Sigma, Germany) with the use of sterile foam-tipped applicators (Puritan, USA). DNA was extracted from the buccal cells using a GenElute mammalian genomic DNA miniprep kit (Sigma, Germany) according to the manufacturer's protocol. "All samples were genotyped for the *ACAN*: rs1516797 T>G, *BGN*: rs1042103 G>A, rs1126499 C>T, *DCN*: rs516115 A>G and *VEGFA*: rs699947 A>C variants." Genotyping was conducted in duplicate using an allelic discrimination assay on a CFX96 Touch™ Real-Time Polymerase Chain Reaction (RT-PCR) instrument (BIO-RAD, USA).

All laboratory work, including DNA extraction and sample genotyping, took place at the Centre for Human Structural and Functional Research Faculty of Physical Culture and Health Promotion, University of Szczecin.

Statistical analysis

Genotyping results were analysed using the programming environment R [16] and R package, SNPAssoc (version 1.9.2) [17]. Differences between genotype and allele frequencies were determined using Pearson's chi-squared (χ^2) and Fisher's exact test. Post hoc analysis of the genotype frequencies considering co-dominant, dominant and recessive inheritance models was performed to identify the over represented genotype. Hardy-Weinberg equilibrium for each group was determined using SNPAssoc. A Bonferroni correction was not applied to the significance level in this pilot study. This study aimed to replicate the associations found in previous studies and Bonferroni correction would mask any statistically significant effects or trends [14, 15]. A significance level of $p < 0.05$ was set for all statistical analyses.

Genotype data for the *BGN* rs1042103 and rs1126499 was used to generate allele constructs using the haplo.stats package (version 1.7.6) [18]. This package was further used to compare the frequency distribution of the inferred allele constructs between the control and case participants using Haplo.score [18]. Haplo.score is a score statistic based on the strength of the association of a haplotype (allele construct) with a given phenotype. A positive value indicates increased susceptibility to an ACLR while a negative value

Proteoglycan gene sequence profiles and ACLR susceptibility

TABLE 1. Genotype and minor allele frequency distributions and p-values for the Hardy-Weinberg Exact test of the *ACAN* rs1516797, *BGN* rs1042103, *BGN* rs1126499, *DCN* rs516115 and *VEGFA* rs699947 in All, Male and Female participants between the control (CON) group and non-contact mechanism of anterior cruciate ligament rupture (ACLR) group in a polish Caucasian cohort.

	All participants		global p-value*	Male		global p-value#	Female		global p-value&
	CON	ACLR		CON	ACLR		CON	ACLR	
<i>ACAN</i> rs1516797									
N	143	229		99	158		44	71	
T/T	47 (67)	40 (92)	0.041	41 (41)	36 (57)	0.055	59 (26)	49 (35)	0.571
G/T	36 (52)	49 (112)		37 (37)	51 (81)		34 (15)	44 (31)	
G/G	17 (24)	11 (25)		21 (21)	13 (20)		7 (3)	7 (5)	
G allele	35 (100)	35 (162)	0.937	40 (79)	38 (121)	0.780	24 (21)	29 (41)	0.447
HWE	0.017	0.316		0.035	0.318		0.683	0.774	
<i>BGN</i> rs1042103									
N				99	158		44	71	
G/G				61 (60)	51 (80)	0.117	41 (18)	44 (31)	0.279
A/G				-	-		39 (17)	46 (33)	
A/A				40 (39)	49 (78)		20 (9)	10 (7)	
A allele				39 (78)	49 (156)	0.029	40 (35)	33 (47)	0.324
HWE				-	-		0.540	0.031	
<i>BGN</i> rs1126499									
N				99	158		44	71	
C/C				55 (54)	49 (77)	0.364	34 (15)	27 (19)	0.106
C/T				-	-		43 (19)	62 (44)	
T/T				46 (45)	51 (81)		23 (10)	11 (8)	
T allele				46 (90)	51 (162)	0.206	44 (39)	42 (60)	0.785
HWE				-	-		0.241	0.793	
<i>DCN</i> rs516115									
N	143	229		99	158		44	71	
A/A	40 (57)	51 (117)	0.104	42 (42)	49 (78)	0.477	34 (15)	55 (39)	0.082
A/G	50 (72)	42 (95)		49 (48)	44 (70)		55 (24)	35 (25)	
G/G	10 (14)	7 (17)		9 (9)	6 (10)		11 (5)	10 (7)	
G allele	35 (100)	28 (129)	0.060	33 (66)	28 (90)	0.278	39 (34)	28 (39)	0.082
HWE	0.269	0.87		0.497	0.332		0.523	0.371	
<i>VEGFA</i> rs699947									
N	143	229		99	158		44	71	
A/A	23 (33)	24 (55)	0.144	19 (19)	28 (44)	0.183	27 (12)	25 (18)	0.682
A/C	59 (84)	50 (114)		60 (59)	49 (77)		57 (25)	52 (37)	
C/C	18 (26)	26 (60)		21 (21)	23 (37)		16 (7)	23 (16)	
C allele	48 (136)	51 (234)	0.366	49 (97)	52 (165)	0.526	44 (39)	49 (69)	0.587
HWE	0.045	1.000		0.072	0.332		0.378	0.815	

Genotype and allele frequencies were expressed as a percentage with the number of participants (n) in parenthesis. P-values for the exact test of Hardy-Weinberg equilibrium (HWE) for each of the groups are included in the table. p values in bold typeset indicate significance ($p < 0.05$).

* CON versus ACLR in All participants

CON versus ACLR (unadjusted p values) in male participants

& CON versus ACLR (unadjusted p values) in female participants

indicates reduced risk. Due to the previous sex-specific associations observed all analyses were stratified by sex [9,14].

RESULTS

Genotype and allele frequencies

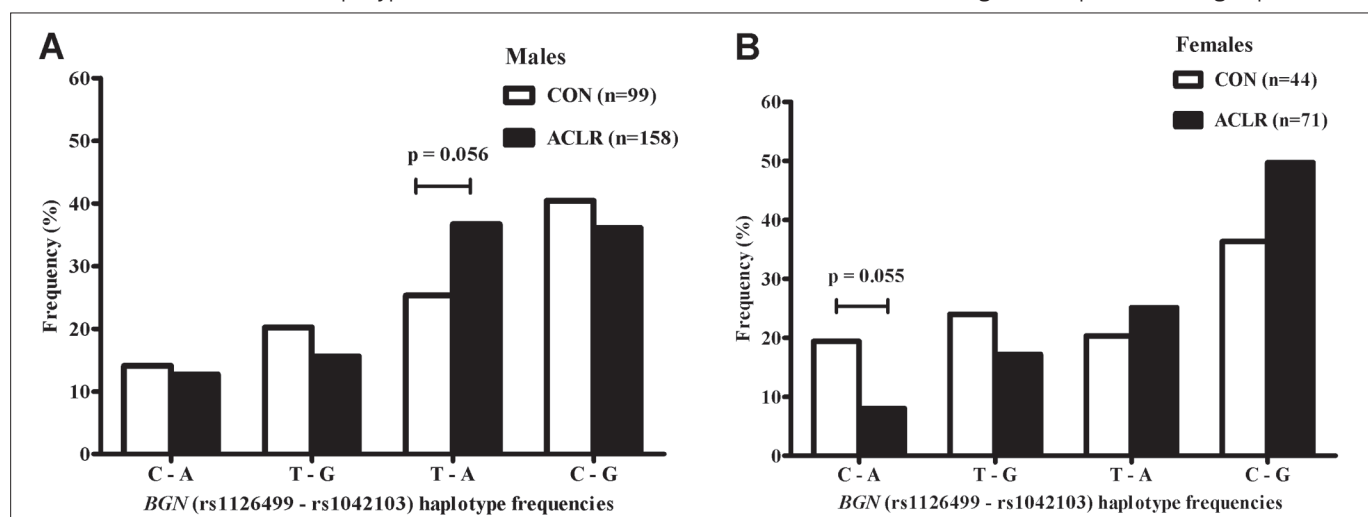
When all participants were analysed there was a significant difference in the distribution of *ACAN* rs1516797 genotype frequencies between the CON and ACLR groups ($p=0.041$) (Table I). The G/T genotype was underrepresented in the CON group (CON: 36%, $n=52$, ACLR: 49%, $n=112$, $p=0.017$, OR=1.68, 95% CI 1.09 to 2.57). No significant difference in the allele frequencies for rs1516797 was noted ($p=0.937$). No significant differences in genotype ($p=0.05$) or allele frequency ($p=0.780$) distributions were noted when only male participants were evaluated. Although, a trend was noted for the genotype frequency distribution to be underrepresented in the CON group (CON: 37%, $n=37$, ACLR: 51%, $n=81$, $p=0.029$, OR=1.76, 95% CI 1.06 to 2.94). No significant differences in the genotype ($p=0.571$) and allele frequencies ($p=0.447$) were noted when only females were evaluated. Deviations in the *ACAN* rs1516797 HWE was noted for the CON group when all ($p=0.017$) participants were analysed and when only male ($p=0.035$) participants were evaluated. Despite not being in HWE the genotype proportional distribution between the Polish CON group and the CEU CON group was comparable (CEU CON: G/G 9%, T/G 36% and T/T 55%; Polish CON: G/G 17%, T/G 36% and T/T 47%). To address the deviation in HWE the Polish ACLR group was compared to a HapMap Central European (CEU) CON group using data available on the National Center for Biotechnology Information database of single nucleotide polymorphisms (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1516797). The CEU CON group is comprised of 226 individuals with northern and western European ancestry living in Utah (United States). When the CEU CON group was compared to the Pol-

ish ACLR group there were significant genotype and allele frequency differences for the rs1516797 polymorphism. The G/T genotype was significantly underrepresented in the CEU CON group compared to the Polish ACLR group (CON: 36%, $n=82$, ACLR: 49%, $n=112$, $p=0.017$, OR=1.72, 95% CI 1.12 to 2.63). Additionally, the T/T genotype was significantly overrepresented in the CEU CON group compared to the Polish ACLR group (CON: 55%, $n=124$, ACLR: 40%, $n=92$, $p=0.006$, OR=1.83, 95% CI 1.20 to 2.81). When the allele frequency differences for the CEU CON group were compared to the Polish ACLR group the G allele was significantly underrepresented in the CEU CON group (CON: 27%, $n=122$, ACLR: 35%, $n=162$, $p=0.008$, OR=1.47, 95% CI 1.11 to 1.95).

When only the male participants were analysed there was no significant difference in the frequency distribution of *BGN* rs1042103 genotypes (Table I). Conversely, the A allele of rs1042103 was significantly under-represented in the male CON group compared to the ACLR group (CON: 39%, $n=78$, ACLR: 49%, $n=156$, $p=0.029$, OR=1.5, 95% CI 1.05 to 2.15). No other significant differences were noted for the genotype and allele frequency distributions for *BGN* rs1042103. Deviations in the *BGN* rs1042103 HWE ($p=0.031$) was noted for the ACLR group when female participants were analysed. Similarly, no significant differences in the genotype and allele frequencies were noted for *BGN* rs1126499 between ACLR group and CON group. No deviations for the *BGN* rs1126499 HWE were noted for any of the groups.

Although, no significant differences in the genotype and allele distributions were noted for *DCN* rs516115, a trend was noted when only female participants were investigated (Table I). When the individual genotypes were investigated the A/A genotype was underrepresented in the CON group compared to ACLR group (CON: 34%, $n=15$, ACLR: 55%, $n=39$, $p=0.029$, OR=0.42, 95% CI 0.19 to 0.93) while the A/G genotype was over-represented in the CON

FIG. 1. Frequency distribution for the: (A) Male *BGN* (rs1126499 C>T – rs1042103 A>G); (B) Female *BGN* (rs1126499 C>T – rs1042103 A>G) inferred haplotypes in the control (CON) and non-contact anterior cruciate ligament rupture (ACLR) groups.



group (CON: 55%, n=24, ACLR: 35%, n=25, p=0.042, OR=0.45, 95% CI 0.21 to 0.98). There was also a trend for the *DCN* rs516115 G allele to be over-represented in the CON group compared to the ACLR group in all the participants (CON: 35%, n=100, ACLR: 28%, n=129, p=0.060, OR=0.729, 95% CI 0.53 to 1.00). Similarly, there was a trend for the G allele to be over-represented in the CON group when only female participants were analysed (CON: 39%, n=34, ACLR: 28%, n=39, p=0.082, OR= 0.60, 95% CI 0.34 to 1.06). No deviations for *DCN* rs516115 HWE was noted for any of the groups.

Furthermore, no significant differences in genotype and allele frequencies were noted between any of the groups for *VEGFA* rs69947 (Table 1). A deviation from HWE was noted for the CON group when all participants (p=0.045) were analysed.

Inferred haplotypes

Inferred haplotypes were constructed for the *BGN* gene using the genotype data (rs1042103 A>G and rs1126499 C>T) for the male and female participants separately (Figure 1). All of the haplotypes had a frequency greater than 5%. When the frequency distribution of the haplotypes between the male and female participants were analysed there were no significant differences. The *BGN* (rs1042103 – rs1126499) C-G haplotype was most frequent in both males and females while the A-C haplotype was the least frequent. There was a difference in the frequency of the T-A haplotype between males and females (males: 32%, n= 83; females: 23%, n= 27; p=0.234). When only the male participants were analysed the T-A haplotype was under-represented in the CON group (CON: 25.3%, ACLR: 36.7%, p=0.056, Hap-score=1.911). When only the female participants were analysed the *BGN* (rs1042103 – rs1126499) C-A haplotype was over-represented in the CON group (CON: 19.4%, ACLR: 8%, p=0.055, Hap-score=-1.922).

DISCUSSION

Proteoglycans are proteins, which have been implicated in an array of functional roles within tendon and ligament. They are considered integral structural components of the extracellular matrix and have been implicated in the resistance of compressive forces, collagen fibrillogenesis, matrix remodelling and cell signalling [19,20]. In addition altered gene (i) expression levels [21,22] and (ii) sequence profiling [14] have been noted between ruptured and non ruptured ACL, tissue and DNA, samples respectively.

This study aimed to test the previously implicated variants in proteoglycan and vascular epithelial growth factor encoding genes with risk of ACLR in a population from Poland. The novel findings of this study included the association of the (i) *ACAN* rs1516797 G/T polymorphism and the (ii) *BGN* rs1042103 A allele to be associated with increased susceptibility to ACLR. Furthermore, *BGN* inferred haplotypes were highlighted with altered ACLR susceptibility.

Although the study implicated the *ACAN* and *BGN* genes (through a combination of genotype, allele and haplotypes) in altered ACLR risk susceptibility, several differences were noted between our findings

and previous literature. The *ACAN* gene codes for the chondroitin sulphate attachment domain. The *ACAN* rs1516797 G/T genotype was associated with a 1.68 fold increased risk of ACLR when the polish CON group was compared to the polish ACLR group. The *ACAN* rs1516797 CON group was not in HWE therefore the Polish ACLR group was compared to the HapMap central European (CEU) CON group. The *ACAN* rs1516797 G/T genotype was also significantly associated with a 1.7 fold increased risk of ACLR (Polish CON vs ACLR). Moreover, the rs1516797 T/T genotype was associated with a 1.83 fold reduction in ACLR risk when the CEU CON group was used (CEU CON vs Polish ACLR). Collectively these comparisons are suggesting the *ACAN* locus requires further investigation. Interestingly, Mannion *et al* also found a trend for the *ACAN* rs1516797 T/T genotype to be associated with increased protection [14]. It was further noted that the genotype distribution for the Polish ACLR group and the South African ACL group are similar (Polish ACLR: G/G 11%, T/G 49% and T/T 40%; South African ACL: G/G 11%, T/G 47% and T/T 42%). No association was noted between the rs1516797 allele frequencies when the Polish CON and ACLR groups were compared, however, the G allele was associated with a 1.47 fold increased risk of ACLR when the CEU CON group was used (CEU CON vs Polish ACLR). The association of the G allele with increased risk is in alignment with the results of Mannion *et al* [14] where the rs1516797 G allele was implicated with an increased risk of ACLR injuries (p=0.024, OR=1.38, 95% CI 1.04 to 1.83). The results for the *ACAN* locus were similar to the study by Mannion *et al* which provides preliminary evidence that this gene requires further investigation in a larger sample size within the Polish population.

Biglycan, a small leucine-rich proteoglycan is involved in collagen fibrillogenesis, regulation of bone formation [20] and has also been implicated in the inflammatory pathway, specifically modulating several growth factor and cytokine functions such TGF- β [23]. No independent genotype or allele associations were noted in the South African cohort, besides the inferred haplotype associations. The current study however noted a 1.5 fold increased risk of sustaining an ACLR associated with the *BGN* rs1042103 A allele in the male participants. In addition, several inferred haplotypes associated with risk modulation were noted in both studies. Specifically, in the South African cohort the *BGN* (rs1042103 – rs1126499) inferred C-G haplotype was implicated with decreased risk of ACLR in female participants while no significant associations were noted when only male participants were evaluated [14]. In contrast, the current Polish study implicated the inferred C-A haplotype with reduced ACLR susceptibility when only female participants were evaluated and the inferred T-A haplotype with increased ACLR susceptibility when males were evaluated. It is interesting to note, that the alternate alleles of the inferred haplotypes have been implicated with opposite risk susceptibility in the female (C-G; increased) and male (T-A; decreased) participants between the South African and Polish studies respectively. However, the authors do note that the samples size in both studies is a major limitation for such haplotypes analyses. It is there-

fore recommended that the samples numbers be increased for a more comprehensive risk profiling analyses for ACLR susceptibility.

Decorin is the most abundant proteoglycan. The results in this study did not replicate the finding of Mannion *et al* [14], more specifically we did not observe the *DCN* rs516115 G/G genotype to be over-represented in the female control group compared to the ACLR group. Although, a trend was noted in the current study suggesting that the A/G genotype may be associated with reduced ACLR susceptibility and similarly the G allele may be associated with reduced ACLR susceptibility. Therefore, although the findings for the *DCN* genetic profile were not reproduced in the two studies; there are similarities suggesting that this particular genetic region within the *DCN* gene requires further interrogation with injury susceptibility.

Functional variants within the *VEGFA* gene, encoding vascular endothelial growth factor A (a critical regulator of angiogenesis) were recently associated with increased risk of ACLR susceptibility, specifically the rs699947 C/C genotype and C allele [15]. This study did not note any independent associations with this polymorphism with ACLR susceptibility and the frequency distributions were similar to Rahim *et al* (2014) [15].

This study in a Polish sample, represents a pilot study to facilitate mapping the genomic region around the *ACAN*, *BGN*, *DCN* and *VEGFA* loci in an attempt to identify risk associated loci of biological importance across different populations. The results suggest that polymorphisms within the *ACAN* and *BGN* genes are associated with altered susceptibility to ACLR. However, one of the limitations to this study is that the small sample size means Bonferroni correction for multiple testing cannot be applied without removing all statistical

effects and the results should be interpreted with this in mind. To fully characterize the *ACAN*, *DCN* and *VEGFA* genes with ACLR susceptibility in a Polish population, additional polymorphisms would have to be investigated to facilitate a more comprehensive haplotype analysis. Furthermore, the sample size should be increased to enable a Bonferroni correction without masking statistically significant effects.

CONCLUSIONS

In conclusion, we emphasize that ACL rupture cases were properly investigated on the basis of surgically diagnosed primary ACL ruptures who qualified for ligament reconstruction (ACLR group). It is important because manual palpation based on hypermobility (ACL length) is not 100% effective as some patients with long ACLs may feature a wide range of joint motion, which may be mistaken for an ACL rupture. The results of studies by Mannion 2014 and Rahim 2014 and the present study, differed mostly. However, the genotype, allele and haplotype associations identified in this study does provide evidence suggesting that these genetic loci require more comprehensive investigation in larger cohorts.

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