The *VEGFA* gene and anterior cruciate ligament rupture risk in the Caucasian population

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ABSTRACT: The aim of the present study was to analyse *VEGFA* rs699947, rs1570360, and rs2010963 polymorphisms with susceptibility to anterior cruciate ligament rupture (ACLR) in a Polish population. The study included 412 physically active Caucasian participants. The study group consisted of 222 individuals with surgically diagnosed primary ACLR qualified for ligament reconstruction (ACLR group). The control group consisted of 190 apparently healthy participants without any history of ACLR (CON group). Three polymorphisms within the *VEGFA* (rs699947, rs1570360, and rs2010963) gene were chosen for investigation due to their significance in the angiogenesis signalling pathway and previous associations with risk of ACLRs. Both single-locus and haplotype-based analyses were conducted. No significant differences in the allele and genotype frequency distributions were noted for the rs699947 and rs1570360 polymorphisms. In contrast, rs2010963 was associated with risk of ACLR in the codominant (p=0.047) and recessive model (p=0.017). In the latter, the CC genotype was overrepresented among individuals with ACL rupture (23.4% vs 14.2%, OR=1.85 [1.11-3.08]). Two *VEGFA* haplotypes were associated with ACLR under the additive (global score=11.39, p=0.022) and dominant model (global score=11.61, p=0.020). The [C;G;G] haplotype was underrepresented in the ACLR group (52.2% vs. 60.3%), whereas the [C;G;C] haplotype was overrepresented (2.9% vs 0.5%). The results obtained suggest a potential correlation between the *VEGFA* rs2010963 polymorphism and ACLR risk, suggesting that harbouring this specific C allele may be an unfavourable risk factor for a knee injury in Caucasian participants from Poland.

INTRODUCTION

Angiogenesis is described as the formation of new capillary blood vessels from existing microvessels. It plays a key role in numerous biological processes such as wound healing, embryological development, inflammation, and the pathogenesis of various diseases, e.g. cancer, diabetic retinopathy, and rheumatoid arthritis [1]. Angiogenesis has also been implicated in matrix remodelling following mechanical loading. Its signalling pathway is regulated by numerous growth factors, such as vascular endothelial growth factors (VEGFs) [2]. Studies have shown increased levels of angiogenic cytokines and growth factors after mechanical loading in ruptured tendons and ligaments [3,4], and after cyclic stretching of tendon fibroblasts [5]. These results have suggested that the angiogenesis signalling cascade may be upregulated after loading, to promote matrix remodelling in an effort to maintain homeostasis [2]. However, dysregulation of this process may potentially have negative consequences for tissue capacity [6].

VEGFs, a family of important growth factors, are key signalling proteins involved in both physiological and pathological angiogenesis because they play a role in endothelial cell proliferation and migration. The family contains at least 7 members with a homodimeric structure. In particular, the A isoform, usually referred to as VEGF, shows the highest vasculogenic and angiogenic potency. VEGFA exerts its biologic effect through interaction with cell surface receptors, VEGFR1 and VEGFR2, selectively expressed on vascular

endothelial cells. The protein is encoded by the *VEGFA* gene, which is located on chromosome 6 (6p21.1) and consists of 8 exons [7,8]. At least 30 single nucleotide polymorphisms (SNPs) in this gene have been described [9]. The gene variants may lead to differences in *VEGFA* expression between individuals and may influence the etiology of a variety of pathological conditions with which VEGFA has been associated [10].

Among genetic variants within the *VEGFA* gene which have been described in the context of genetic conditioning for angiogenesis, rs699947 (C/A), rs1570360 (G/A), and rs2010963 (G/C) are the most frequently investigated functional polymorphisms associated with a potential predisposition to anterior cruciate ligament rupture (ACLR) and Achilles tendinopathy (TEN). These associations have been recently replicated in some populations. They were recently implicated with risk of ACLR in a South African Caucasian population [11], a South African Coloured population [2], and also risk of TEN in a study investigating two populations from South Africa and the United Kingdom [12]. Unfortunately, there are currently no data available for the incidence rate of ACLR in an East-European population. It needs to be highlighted that investigation of genetic associations in various populations and related phenotypes to ascertain the important biological pathways underpinning susceptibility to musculoskeletal soft tissue injuries is necessary [13].

Taken together, the aforementioned findings suggest that *VEGFA* polymorphisms are associated with risk of ACLR in various populations. The aim of the present study was to analyse *VEGFA* rs699947, rs1570360, and rs2010963 polymorphisms with susceptibility to ACLRs in a Polish population.

MATERIALS AND METHODS

Ethics committee
The procedures followed in the study were conducted ethically according to the principles of the World Medical Association Declaration of Helsinki and were approved by the Ethics Committee of the Pomeranian Medical University in Szczecin (approval number 09/KB/IV/2011).

Participants
A total of 412 physically active, unrelated, self-reported Caucasian participants were recruited for the study between 2009 and 2016. The study group consisted of 222 (66 females and 156 males) individuals with surgically diagnosed primary ACLR who qualified for ligament reconstruction (ACLR group). All 222 participants from the ACLR group sustained their injury through non-contact mechanisms [14]. The control group consisted of 190 (83 females and 107 males) apparently healthy participants without any history of ACLR (CON group).

The ACLR participants were soccer players from the Polish 1st, 2nd, and 3rd division soccer league (training 11-14 hours per week). The control group comprised healthy, physically active individuals with the majority playing soccer as their main sport, 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 (all self-reported Polish, East-Europeans for ≥3 generations), of similar age (ACLR group=26±4 years, CON group=25±3 years), and had a comparable level of exposure to risk of ACLR (same volume and intensity of training and match play). The ACLR female participants (mean age: 25±4 years) consisted of 41 soccer players from the Polish 1st division soccer league (training 10-12 hours per week) and also included amateur skiers (n=25). The female control participants from the CON group (age 29±2 years) were recruited from sports clubs and wellness centres and self-reported as being physically active for a minimum of 7 hours week.

Genetic analyses
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 in duplicate using an allelic discrimination assay on a StepOne Real-Time Polymerase Chain Reaction instrument (Applied Biosystems, USA) with TaqMan probes. To discriminate *VEGFA* rs699947, rs1570360, and rs2010963 alleles, TaqMan Pre-Designed SNP Genotyping Assays were used (Applied Biosystems, USA) (assay ID: C___8311602_10, C___1647379_10, and C___8311614_10 respectively), including primers and fluorescently labelled (FAM and VIC) MGB probes to detect alleles.

Statistical methods
Statistical analysis was conducted with the SNPassoc package for R (version 3.4.0, The R Foundation for Statistical Computing,

TABLE 1. Minor allele frequencies (MAF) and the probabilities that the genotype frequencies do not differ from Hardy-Weinberg expectations for the rs699947, rs1570360 and rs2010963 *VEGFA* polymorphisms

SNP	MAF (%)	ACLR+CON (n=412)	CON (n=190)	ACLR (n=222)
rs699947	A (42.2)	0.069	0.226	0.174
rs1570360	A (27.2)	1.0	1.0	0.873
rs2010963	C (43.9)	1.0	0.449	0.686

Note: ACLR – anterior cruciate ligament rupture, CON – control group.

https://cran.r-project.org). Genotype models were constructed with respect to the minor allele. Haplotype analysis was conducted with R (https://cran-r.project.org, version 3.1.0) using the haplo.stats package and haplo.glm regression function. Percentage change over training was used as the dependent variable, while the PPARD haplotypes were used as the independent variables. P values <0.05 were considered statistically significant.

RESULTS

The genotype frequencies did not differ significantly from the expectations of Hardy-Weinberg equilibrium in the control group, in the ACL group or in the pooled case-control sample (Table 1).

The results of single-locus association analysis are summarized in Tables 2-4. No associations were found between the rs699947 and rs1570360 SNPs and ACL status in all models (Table 2 and 3). However, there was a significant association of the rs2010963 with ACL rupture in the codominant (p=0.047) and recessive model (p=0.017). In the latter, the CC genotype was overrepresented among individuals with ACL rupture (23.4% vs 14.2%, OR=1.85 [1.11-3.08]). In addition, a haplotypic association between the *VEGFA* gene and ACL rupture was detected (Table 5). Four *VEGFA* haplotypes ([rs699947; rs1570360; rs2010963]) were inferred with frequencies >1% ranging from 1.8% ([C;G;C]) to 55.9% ([C;G;G]). The global score test indicated the association between *VEGFA* haplotypes

TABLE 2. Association analysis of *VEGFA* gene rs699947 polymorphism with anterior cruciate ligament rupture.

Model	CON (n=190)	%	ACLR (n=222)	%	OR	95% CI		p*
Codominant								
C/C	66	34.7	62	27.9	1.00			
A/C	99	52.1	121	54.5	1.30	0.84	2.01	0.231
A/A	25	13.2	39	17.6	1.66	0.90	3.06	
Dominant								
C/C	66	34.7	62	27.9	1.00			0.137
A/C-A/A	124	65.3	160	72.1	1.37	0.90	2.09	
Recessive								
C/C-A/C	165	86.8	183	82.4	1.00			0.216
A/A	25	13.2	39	17.6	1.41	0.82	2.42	
Overdominant								
C/C-A/A	91	47.9	101	45.5	1.00			0.627
A/C	99	52.1	121	54.5	1.10	0.75	1.62	

TABLE 3. Association analysis of *VEGFA* gene rs1570360 polymorphism with anterior cruciate ligament rupture

Model	CON (n=190)	%	ACLR (n=222)	%	OR	95% CI		p*
Codominant								
G/G	110	57.9	108	48.6	1.00			
A/G	69	36.3	95	42.8	1.40	0.93	2.11	0.147
A/A	11	5.8	19	8.6	1.76	0.80	3.87	
Dominant								
G/G	110	57.9	108	48.6	1.00			0.061
A/G-A/A	80	42.1	114	51.4	1.45	0.98	2.14	
Recessive								
G/G-A/G	179	94.2	203	91.4	1.00			0.277
G/G	11	5.8	19	8.6	1.52	0.71	3.29	
Overdominant								
G/G-A/A	121	63.7	127	57.2	1.00			
A/G	69	36.3	95	42.8	1.31	0.88	1.95	0.180

TABLE 4. Association analysis of *VEGFA* gene rs2010963 polymorphism with anterior cruciate ligament rupture.

Model	CON (n=190)	%	ACLR (n=222)	%	OR	95% CI		p*
Codominant								
G/G	66	34.7	63	28.4	1.00			
C/G	97	51.1	107	48.2	1.16	0.74	1.80	**0.047**
C/C	27	14.2	52	23.4	2.02	1.13	3.60	
Dominant								
G/G	66	34.7	63	28.4	1.00			0.166
C/G-C/C	124	65.3	159	71.6	1.34	0.88	2.04	
Recessive								
G/G-C/G	163	85.8	170	76.6	1.00			**0.017**
C/C	27	14.2	52	23.4	1.85	1.11	3.08	
Overdominant								
G/G-C/C	93	48.9	115	51.8	1.00			
C/G	97	51.1	107	48.2	0.89	0.61	1.31	0.563

TABLE 5. Haplotype-based association of *VEGFA* gene polymorphisms (rs699947, rs1570360, rs2010963) with anterior cruciate ligament rupture

Haplotype [rs699947; rs1570360; rs2010963]	Frequency (%)			Additive (Score=11.39, p=0.022)		Dominant (Score=11.61, p=0.020)		Recessive (Score=3.05, p=0.384)	
	ACLR +CON	ACLR	CON	Score	p	Score	p	Score	p
[C;G;G]	55.9	52.2	60.3	-2.32	0.020	-2.37	0.018	-1.49	0.137
[A;G;C]	15.0	14.9	15.3	-0.13	0.895	-0.60	0.551	0.60	0.546
[A;A;C]	27.1	29.7	23.9	1.86	0.062	1.78	0.074	1.08	0.281
[C;G;C]	1.8	2.9	0.5	2.59	0.009	2.60	0.009	NA	NA

Note: NA – Not applicable.

and ACL rupture under the additive (p=0.022) and dominant (p=0.020) model. In each model, the [C;G;G] haplotype was associated with reduced risk of ACL rupture (score=-2.32, p=0.020; score=-2.37, p=0.018, for the additive and dominant model, respectively), whereas the [C;G;C] haplotype conferred enhanced risk of ACL rupture (score=2.59, p=0.009; score=2.60, p=0.009, for the additive and dominant model, respectively).

DISCUSSION

Recently, numerous studies have investigated the associations between ACLRs and various genetic markers, highlighting the importance of genetic predisposition as a risk factor in ACLRs [11,15-18]. Due to their regulatory role in the angiogenesis signalling pathway, the genes encoding VEGFA growth factors are promising candidates for the regulation of risk of musculoskeletal soft tissue injuries. Considering previous studies, *VEGFA* polymorphisms, tested individu-

ally or in haplotype combination, have been associated with increased or reduced risk of non-contact ACLRs [12,18]. We hypothesized that the three functional SNPs within the *VEGFA* gene would modulate the risk of ACLRs in Caucasian participants from Poland.

The main finding of this study was the observation that harbouring a specific *VEGFA* genotype may be associated with different risk of non-contact ACLRs in the recruited Polish population. When tested individually, no significant differences in allele and genotype frequency distributions were noted for two of three investigated *VEGFA* SNPs, rs699947 and rs1570360. However, our statistical analyses revealed that the third one, rs2010963, was linked with risk of ACLRs in the studied population. We described the overrepresentation of the rs2010963 CC genotype in the ACLR group compared to the CON group, suggesting that harbouring this specific C allele may be an unfavourable risk factor for a knee injury. To the best of our knowledge, our research group is the first team to explore

the association of *VEGFA* rs2010963 polymorphism with ACLR risk. Moreover, when the results obtained in the present study were incorporated in the complex haplotype analysis, the novel important finding was that carriers of the *VEGFA* (rs699947/rs1570360/rs2010963) [C;G;G] haplotype was found to exhibit lower risk of ACL rupture, suggesting that harbouring this specific [C;G;G] haplotype has a protective effect. On the other hand, the [C;G;C] haplotype was associated with higher risk of ACL rupture. This observation constitutes the second important finding of the current study, implying that some individuals may benefit from carrying the [C;G;G] haplotype. It needs to be highlighted that haplotype analysis confirmed the association between the rs2010963 C allele and risk of ACL ruptures as both protective and risk haplotype differed at only one locus corresponding to rs2010963 variant.

The *VEGFA* functional polymorphisms are associated with differentiated gene expression and protein production. The *VEGFA* rs2010963 CC genotype was previously linked with enhanced VEGFA protein expression and its higher levels in blood plasma [19]. Previously, some studies have revealed that an increase in *VEGFA* expression may upregulate the expression levels of matrix metalloproteinases (MMPs), which may ultimately compromise extracellular matrix (ECM) homeostasis, thereby altering the ligament biomechanical properties [20]. Nell et al. [21] reported that too much or too little expression of the protein may have negative effects. The conservation and timing of the balance between inhibitors and stimulators during matrix remodelling are essential to maintaining homeostasis [22].

Previously, Rahim et al. [2] did not find independent or haplotype associations for the three functional *VEGFA* polymorphisms in a South African Coloured population. Another study revealed that the rs699947 CC genotype was associated with increased risk of non-contact ACL ruptures and the rs1570360 GA genotype was associated with a reduced risk of ACL ruptures in a Caucasian population. Additionally, inferred haplotype associations were linked to altered risk of ACL ruptures [11]. Similarly, a *VEGFA* haplotype analysis was associated with increased risk of TEN in a South African Caucasian group and a combined South African and British study group [2]. So far, *VEGFA* rs2010963 polymorphism has not been described in the context of higher risk of knee injury. To the best of our knowledge, our research group is the first team to explore the association of *VEGFA* rs2010963 polymorphism with ACLR risk. The carriers of the *VEGFA* C allele displayed higher risk of ACL ruptures in the Polish population. However, more experimental studies are needed to confirm the association described in this study. The differences and similarities observed between the above-mentioned results highlight the role of hypothesis testing in various groups to reduce confounding due to population stratification. Thus the association of a genetic marker in numerous studies supports the evidence for a potential biological role rather than this being a statistical association due to heterogeneity in allele frequencies [18].

The main limitation of the genetic association study is the relatively small sample size; therefore the results should be repeated in bigger, independent groups. The insufficient number of female cases included in the study is in part due to limited numbers of women who reported to the hospital for ACL reconstructive surgery. Soccer is also more popular amongst males [23]. Moreover, sports participation data were self-reported and the mechanism of ACLR was categorised based on injury details reported by the participants to the investigator. As there is no clinical tool to confirm the mechanism by which ACL rupture occurred, this constitutes the next limitation of our study. On the other hand, a strength of the present study was the inclusion of surgically diagnosed primary ACLR patients who sustained their injury through non-contact mechanisms, as these injuries are considered to have a larger genetic component.

In conclusion, this is the first study to investigate a potential correlation between the *VEGFA* rs2010963 polymorphism and ACLR risk, suggesting that harbouring this specific C allele may be an unfavourable risk factor for a knee injury in Caucasian participants from Poland. Although other associations between rs699947 as well as rs1570360 and ACLR risk were not confirmed in this study, *VEGFA* remains a significant candidate gene requiring further exploration due to its key role in angiogenesis.

REFERENCES

1. Zhao M, Hu Y, Yu Y, Ling Q, Yang J, Su SB, et al. Involvement of IL-37 in the pathogenesis of proliferative diabetic retinopathy. Invest Ophthalmol Vis Sci. 2016;57:2955–62.
2. Rahim M, Hobbs W, van der Merwe W, Posthumus M. Collins M, September AV, Investigation of angiogenesis genes with anterior cruciate ligament rupture risk in a South African population. J Sports Sci. 2018;36(5):551-7.
3. Pufe T, Petersen W, Tillmann B, Mentlein, R. The angiogenic peptide vascular endothelial growth factor is expressed in foetal and ruptured tendons. Virchows Archiv. 2001; 439(4):579–85.
4. Beye JA, Hart, DA, Bray RC, McDougall JJ, Salo PT. Injury-induced changes in mRNA levels differ widely between anterior cruciate ligament and medial collateral ligament. The Am J Sports Med. 2008; 36(7), 1337–46.
5. Petersen W, Varoga D, Zantop T, Hassenpflug J, Mentlein, R, Pufe T. Cyclic strain influences vascular endothelial growth factor (VEGF) and the hypoxia inducible factor 1 alpha (HIF-la) in tendon fibroblasts. J Orthop Res. 2004;22(4):847-53.
6. Cook J, Docking S. "Rehabilitation will increase the 'capacity' of your …insert musculoskeletal tissue here…" Defining "tissue capacity":A core concept for clians. Br J Sports Med 2015; 49,1484–5. doi:10.1136/bjsports-2015-094849
7. Ferrara N, Gerber, HP, LeCouter, J. The biology of VEGF and its receptors. Nature Medicine. 2003; 9(6):669–76.
8. Zhao T, Zhao W, Chen Y, Ahokas RA, Sun Y. Vascular endothelial growth factor (VEGF)-A: role on cardiac angiogenesis following myocardial infarction. Microvasc Res. 2010; 80(2):188-94.

9. Watson CJ, Webb NJ, Bottomley MJ, Brenchley PE. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. Cytokine.2000; 12:1232-5.

10. Bhanoori M, Arvind BK, Pavankumar Reddy NG, Lakshmi RK, et al. The vascular endothelial growth factor (VEGF) +405G>C 5'-untranslated region polymorphism and increased risk of endometriosis in South Indian women: a case control study. Hum. Reprod. 2005;20:1844-9.

11. Rahim M, Gibbon A, Hobbs H, van der Merwe W, Posthumus M, Collins M, et al. The association of genes involved in the angiogenesis-associated signaling pathway with risk of anterior cruciate ligament rupture. J Orthop Res. 2014;32(12):1612–8.

12. Rahim M, El Khoury LY, Raleigh SM, Ribbans WJ, Posthumus M, Collins M, et al. Human genetic variation, sport and exercise medicine, and achilles tendinopathy: Role for angiogenesis-associated genes. OMICS 2016; 20(9),520–7.

13. Rahim M., Collins M, September A. V. Genes and musculoskeletal soft-tissue injuries. In M. Posthumus, M. Collins (Eds.), Medicine and sport science. 2016; (2nd ed., Vol. 61, pp. 68–91). Basel: Karger.

14. Marshall S, Padua D, McGrath M. Incidence of ACL injuries. In Hewett T, Shultz S, Griffin L (eds): Understanding and preventing noncontact ACL injuries. Champaign, IL: Human Kinetics. 2007; 5–30.

15. Posthumus M, September A V, O'Cuinneagain D, van der Merwe W, Schwellnus MP, Collins M. The association between the COL12A1 gene and anterior cruciate ligament ruptures. Br J Sports Med. 2010; 44(16):1160-1165.

16. Posthumus M, Collins M, van der Merwe L, O'Cuinneagain D, van der Merwe W, Ribbans WJ, Schwellnus MP, Raleigh SM. Matrix metalloproteinase genes on chromosome 11q22 and the risk of anterior cruciate ligament (ACL) rupture. Scand J Med Sci Sports. 2012;22(4): 523-33.

17. Ficek K, Stepien-Slodkowska M, Kaczmarczyk M, Maciejewska-Karlowska A, Sawczuk M, Cholewinski J, et al. Does the A9285G Polymorphism in Collagen Type XII α1 Gene Associate with the Risk of Anterior Cruciate Ligament Ruptures? Balkan J Med Genet 2014; 17(1):41-6.

18. Rahim M, Mannion S, Klug B, Hobbs H, van der Merwe W, Posthumus M, et al. Modulators of the extracellular matrix and risk of anterior cruciate ligament ruptures. J Sci Med Sport. 2017;20(2):152-8.

19. Schneider B, Radovich M, Sledge G, Robarge JD, Li L, Storniolo AM, et al. Association of polymorphisms of angiogenesis genes with breast cancer. Breast Cancer Res Treat. 2008; 111: 157–63.

20. Wang H, Keiser JA. Vascular endothelial growthfactor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. Circ Res. 1998; 83:832–40.

21. Nell EM, van der Merwe L, Cook J, Handley C J, Collins M, September AV.The apoptosis pathway and the genetic predisposition to Achilles tendinopathy. J Orthop Res. 2012; 30(11):1719–24.

22. Pasternak B, Aspenberg P. Metalloproteinases and their inhibitors-diagnostic and therapeutic opportunities in orthopedics. Acta Orthop. 2009;80(6):693-703.

23. Gianotti SM., Marshal SW., Hume PA., Bunt L. Incidence of anterior cruciate ligament injury and other knee ligament injuries: A national population-based study. J Sci Med in Sport.2009;12: 622–7. doi:10.1016/j. jsams.2008.07.005