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Research article

Are *IL1B, IL6* and *IL6R* Gene Variants Associated with Anterior Cruciate Ligament Rupture Susceptibility?

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Abstract

Cytokines, such as interleukins, are crucial in regulating critical cell signaling pathways as well as being major contributors to inflammatory response and are upregulated during ligament and tendon injuries. The genes encoding key interleukins, such as *IL1B* and *IL6* as well as interleukin receptor *IL6R*, were chosen as candidate genes for association with soft tissue injuries. The aim of the case-control study was to verify the hypothesis that sequence variants rs1143627, rs16944, rs1800795, rs2228145 in the *IL1B*, *IL6* and *IL6R* genes are associated with ACL rupture susceptibility in a Polish population. Among four analyzed SNPs, the rs1800795 *IL6* gene polymorphism was found to be the only one significantly associated with ACL rupture ($p = 0.010$, $p = 0.022$, $p = 0.004$ for codominant, recessive and overdominant models, respectively; odds ratio = 1.74, 95% CI 1.08-2.81, sex adjusted $p = 0.032$ for recessive model). With reference to the other analyzed polymorphisms, we failed to show significant differences in the genotype and allele frequencies for *IL6R* rs2228145as well as *IL1B* rs16944 and rs1143627 (analyzed alone or in haplotype combination) between the ACL rupture group and the healthy control group among Polish participants. Due to the nature of case-control studies, the results of this study need to be confirmed in independent studies with larger sample sizes.

Key words: Soft tissue injuries, ACL rupture, *IL1B*, *IL6*, *IL6R*.

Introduction

The cellular and molecular mechanisms underlying the development of musculoskeletal soft tissue injuries (such as anterior cruciate ligament ruptures or tendinopathy) are complicated, but inflammatory mediators produced by connective tissue cells in response to repetitive mechanical loading may be an important factor for this pathology. Cytokines, such as interleukins, are crucial in regulating critical cell signaling pathways as well as being major contributors to inflammatory response which is upregulated during ligament and tendon injuries (Tsuzaki et al., 2003).

The inflammation processes involved during anterior cruciate ligament (ACL) injuries are accompanied by upregulation of interleukin-1β (IL-1β, encoded by the *IL1B* human gene) production. IL-1β is a potent pro-inflammatory cytokine produced mainly by macrophages in injured tissues and upregulates the expression of other inflammatory mediators (Newton and Covington, 1987), including interleukin-6 (IL-6, encoded by the *IL6* gene), which is secreted after tendon and ligament injuries by human fibroblasts (Benazzo et al., 2008). IL-6 is a pleiotropic cytokine whose expression levels correlate with the severity of inflammation. IL-6 plays a crucial role in bone resorption (Ferrari et al., 2003) as well as apoptosis of cells (Legerlotz et al., 2012). In the context of ACL injuries, it is worth observing that *IL6* expression increases after cyclic stretching of human tendon fibroblasts (Skutek et al., 2001) as well as in pathological conditions in tendons (Legerlotz et al., 2012; Millar et al., 2009) or ligaments (Cameron et al., 1994). IL-6 exerts its biological effect by binding to the interleukin-6 receptor (IL-6R), encoded by the *IL6R* gene (Galicia et al., 2004).

Taking these many factors into account, the genes encoding key interleukins, such as *IL1B* and *IL6* as well as interleukin receptor *IL6R*, were chosen as candidate genes for association with soft tissues injuries. Thus, we have decided to test the hypothesis that sequence variants rs1143627, rs16944, rs1800795, rs2228145 in the *IL1B*, *IL6* and *IL6R* genes are associated with ACL rupture susceptibility in the Polish population in a case study comparing ACL patients and healthy controls. Additionally, this study allows us to further explore the role of *IL1B*, *IL6*, *IL6R* polymorphisms in a risk model for ACL rupture.

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 ethical standards in sport and exercise science research. The procedures followed in the study were approved by the Ethics Committee of the Pomeranian Medical University in Szczecin (approval number 09/KB/IV/2011). All participants were

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given a consent form and a written information sheet concerning the study, providing all pertinent information (purpose, procedures, risks, and benefits of participation). The potential participant had time to read the information sheet and the consent form. After ensuring that the participant had understood the information, every participant gave written informed consent (signed consent form) to genotyping with the understanding that it was anonymous and that the obtained results would be confidential. The experimental procedures were conducted in accordance with the set of guiding principles for reporting the results of genetic association studies defined by the Strengthening the Reporting of Genetic Association studies (STREGA) Statement (Little et al., 2009).

Participants

A total of 423 physically active, unrelated, self-reported Caucasian participants were recruited for the study between 2009 and 2016. The study group consisted of 229 (65 females and 164 males) individuals with surgically diagnosed primary ACL rupture who qualified for ligament reconstruction (ACLR group). All 229 participants from the ACLR group sustained their injury through non-contact mechanisms. The control group: 194 (85 females and 109 males) seemingly healthy participants without any history of ACL injuries (CON group).

The ACLR participants were soccer players from the 1st, 2nd, and 3rd division Polish soccer leagues (trained 11-14 hours per week). The control group were healthy, physically-active individuals, with the majority playing soccer as their main sport with no self-reported 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, Eastern-Europeans for ≥3 generations), of similar age (ACLR group = 26 ± 4 years, CON group = 25 ± 3 years), with 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 soccer players from the 1st division Polish soccer league (trained 10-12 hours per week). The female control participants from the CON group (age 29±2 years) were recruited from sports clubs and wellness centers and were self-reported as being physically active for a minimum of 7 hours per week.

Genetic analyses

The buccal cells donated by the subjects were 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 in duplicate using an allelic discrimination assay on a StepOne Real-Time Polymerase Chain Reaction (RT-PCR) instrument (Applied Biosystems, USA) with Taqman probes. To discriminate *IL1B*rs16944 and rs1143627 as well as *IL6* rs1800795 and *IL6R* rs2228145alleles, a TaqMan Pre-Designed SNP Genotyping Assay (Applied Biosystems, USA) (assay ID: C___1839943_10, C___1839944_10, C___1839697_20, C__16170664_10, respectively) were used, including primers and fluorescently labeled (FAM and VIC) MGB probes to detect both alleles. Every genotyping was carried out together with a no-template negative control, i.e. sample of appropriate Genotyping Assay mixed with distilled water without DNA, showing there is no contaminant in the reagents and that the fluorescence is not due to probe degradation. The positive controls containing genomic DNA samples of known genotypes (homozygous major and minor for each SNP) were used for all experiments to test that both assay probes function. The same genomic DNA samples have been used as positive controls for all PCR plates to check for consistency in genotype calls. Genotypes were assigned using all of the data from the study simultaneously.

Statistical analyses

Statistical analysis was conducted with *SNPassoc* package for R (version 3.4.0, The R Foundation for Statistical Computing, https://cran.r-project.org). A single locus analysis including pairwise SNP x SNP interaction was conducted using the *SNPassoc* (version 1.9.2) under the assumption of the four genetic models (codominant, dominant, recessive and overdominant). The models were constructed with respect to the minor allele. The p values for SNP x SNP interactions (epistasis) were computed using log-likelihood ratio test. The power was calculated using QUANTO (version 1.2.4, http://biostats.usc.edu) assuming a dominant genetic model, the incidence of ACL tears of 68 per 100,000 person-years (Sanders et al., 2016) and the risk allele frequencies taken from the HapMap in CEU population. The power for odds ratio ranging from 1.2 to 2.0 varied between 14% (OR=1.2) to 92% (OR=2.0). P values <0.05 were considered statistically significant. The level of statistical significance was set at $p < 0.05$.

Results

The measured genotype frequencies were not significantly different from the expectations of Hardy-Weinberg equilibrium in the control sample (p values range 0.083 to 0.859) as well as the pooled case-control sample (p values range 0.114 to 1.0). However, in the case sample, the observed rs1800795 (*IL6*) genotype frequencies differed significantly from expectations (p = 0.023) (Table 1).

Table 1. Minor allele frequencies (MAF) and the probabilities that the genotype frequencies do not differ from Hardy-Weinberg expectations.

SNP	MAF (%)	ACLR+CON	ACLR	CON
***IL1B* (rs16944)**	allele A (32.4)	0.823	0.664	0.859
***IL1B* (rs1143627)**	allele G (32.4)	1.0	0.664	0.605
***IL6* (rs1800795)**	allele C (45.9)	0.625	0.023	0.083
***IL6R* (rs2228145)**	allele C (36.2)	0.114	0.088	0.640

MAF – minor allele frequency

Table 2. Association analysis of the *IL1B* gene rs16944 polymorphism with ACL rupture.

Model		ACLR (n=229)	%	CON (n=181)	%	OR	95% CI		p*
Codominant	G/G	99	43.2	89	49.2	1.00			0.262/0.469
	A/G	101	44.1	77	42.5	0.85	0.56	1.28	
	A/A	29	12.7	15	8.3	0.58	0.29	1.14	
Dominant	G/G	99	43.2	89	49.2	1.00			0.231/0.334
	A/G-A/A	130	56.8	92	50.8	0.79	0.53	1.16	
Recessive	G/G-A/G	200	87.3	166	91.7	1.00			0.151/0.306
	A/A	29	12.7	15	8.3	0.62	0.32	1.20	
Overdominant	G/G-A/A	128	55.9	104	57.5	1.00			0.751/0.731
	A/G	101	44.1	77	42.5	0.94	0.63	1.39	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals

Table 3. Association analysis of the *IL1B* gene rs1143627 polymorphism with ACL rupture.

Model		ACLR (n=229)	%	CON (n=194)	%	OR	95% CI		p*
Codominant	A/A	99	43.2	94	48.5	1.00			0.208/0.377
	A/G	101	44.1	85	43.8	0.89	0.59	1.33	
	G/G	29	12.7	15	7.7	0.54	0.27	1.08	
Dominant	A/A	99	43.2	94	48.5	1.00			0.283/0.366
	A/G-G/G	130	56.8	100	51.5	0.81	0.55	1.19	
Recessive	A/A-A/G	200	87.3	179	92.3	1.00			0.094/0.197
	G/G	29	12.7	15	7.7	0.58	0.30	1.11	
Overdominant	A/A-G/G	128	55.9	109	56.2	1.00			0.952/0.898
	A/G	101	44.1	85	43.8	0.99	0.67	1.45	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals

Table 4. Association analysis of the *IL6* gene rs1800795 polymorphism with ACL rupture.

Model		ACLR (n=228)	%	CON (n=194)	%	OR	95% CI		p*
Codominant	G/G	60	26.3	61	31.4	1.00			**0.010/0.018**
	C/G	131	57.5	84	43.3	0.63	0.40	0.99	
	C/C	37	16.2	49	25.3	1.30	0.75	2.27	
Dominant	G/G	60	26.3	61	31.4	1.00			0.246/0.286
	C/G-C/C	168	73.7	133	68.6	0.78	0.51	1.19	
Recessive	G/G-C/G	191	83.8	145	74.7	1.00			**0.022/0.032**
	C/C	37	16.2	49	25.3	1.74	1.08	2.81	
Overdominant	G/G-C/C	97	42.5	110	56.7	1.00			**0.004/0.007**
	C/G	131	57.5	84	43.3	0.57	0.38	0.83	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; bold p values - statistically significant differences ($p < 0.05$)

Table 5. Association analysis of the *IL6R* gene rs2228145 polymorphism with ACL rupture.

Model		ACLR (n=228)	%	CON (n=194)	%	OR	95% CI		p*
Codominant	A/A	99	43.2	81	41.8	1.00			0.760/0.714
	A/C	94	41.0	86	44.3	1.12	0.74	1.69	
	C/C	36	15.7	27	13.9	0.92	0.51	1.64	
Dominant	A/A	99	43.2	81	41.8	1.00			0.759/0.677
	A/C-C/C	130	56.8	113	58.2	1.06	0.72	1.56	
Recessive	A/A-A/C	193	84.3	167	86.1	1.00			0.603/0.610
	C/C	36	15.7	27	13.9	0.87	0.50	1.49	
Overdominant	A/A-C/C	135	59.0	108	55.7	1.00			0.496/0.433
	A/C	94	41.0	86	44.3	1.14	0.78	1.68	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals

Tables 2-5 summarize the results of association analysis between the single nucleotide polymorphism within the *IL1B* (Tables 2, 3), *IL6* (Table 4), *IL6R* (Table 5) genes and predisposition to ACL rupture (ACLR). The *IL6* gene polymorphism (rs1800795) was found to be the only SNP significantly associated with ACLR (Table 4). The associations were demonstrated for all genetic models except the dominant model (G/G vs C/G-C/C). The highest odds ratio (1.74, 95% CI 1.08-2.81, sex adjusted p = 0.032) was found for the recessive model (G/G-C/G vs C/C). *IL6* rs1800795 heterozygosity was associated with an odds ratio for ACLR of 0.57 (95% CI 0.38-0.83, sex adjusted p = 0.007).

Gene-gene (only pairwise interactions were considered) interactions were investigated under the assumption of the same genetic models as for the single-gene analyses (except for the overdominant model). Only one *IL1B* SNP was used in the interaction analysis due to strong linkage disequilibrium between the two *IL1B* SNPs (D' = 0.98, r^2 = 0.96, $p < 0.0001$). No association between SNP-SNP interaction and ACL rupture was revealed for the codominant (Tables 6-8), dominant (Tables 9-11) or recessive (Tables 12-14) models.

Table 6. Association analysis of the *IL1B* x *IL6* interaction with ACL rupture (codominant model).

IL1B x *IL6*		*IL6* (rs1800795)															p *vs* CON*
		G/G					C/G					C/C					
		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		
IL1B **(rs16944)**	G/G	29	32	1.00	NA	NA	54	37	0.62	0.32	1.19	16	20	1.13	0.50	2.59	0.661/ 0.558
	A/G	22	24	0.99	0.46	2.13	60	32	0.48	0.25	0.94	19	21	1.00	0.45	2.23	
	A/A	9	3	0.30	0.07	1.22	17	10	0.53	0.21	1.35	2	2	0.91	0.12	6.85	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; NA – not applicable.

Table 7. Association analysis of the *IL1B* x *IL6R* interaction with ACL rupture (codominant model).

IL1B x *ILR*		*IL6R* (rs2228145)															p *vs* CON*
		A/A					A/C					C/C					
		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		
IL1B **(rs16944)**	G/G	46	39	1.00	NA	NA	36	37	1.21	0.65	2.27	17	13	0.90	0.39	2.09	0.987/ 0.991
	A/G	39	31	0.94	0.50	1.77	48	37	0.91	0.50	1.66	14	9	0.76	0.30	1.94	
	A/A	14	8	0.67	0.26	1.77	10	5	0.59	0.19	1.87	5	2	0.47	0.09	2.57	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; NA – not applicable

Table 8. Association analysis of the *IL6* x *IL6R* interaction with ACL rupture (codominant model).

IL6 x *IL6R*		*IL6R* (rs2228145)															p *vs* CON*
		A/A					A/C					C/C					
		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		
IL6 **(rs1800795)**	G/G	29	30	1.00	NA	NA	23	26	1.09	0.51	2.33	8	5	0.60	0.18	2.06	0.818/ 0.936
	C/G	51	33	0.63	0.32	1.23	59	39	0.64	0.33	1.23	21	12	0.55	0.23	1.32	
	C/C	18	18	0.97	0.42	2.21	12	21	1.69	0.71	4.05	7	10	1.38	0.46	4.12	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; NA – not applicable

Table 9. Association analysis of the *IL1B* x *IL6* interaction with ACL rupture (dominant model).

IL1B x *IL6*		*IL6* (rs1800795)										p *vs* CON*
		G/G					C/G-C/C					
		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		
IL1B **(rs16944)**	G/G	29	32	1.00	NA	NA	70	57	0.74	0.40	1.36	0.943/0.878
	A/G-A/A	31	27	0.79	0.38	1.62	98	65	0.60	0.33	1.09	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; NA – not applicable

Table 10. Association analysis of the *IL1B* x *IL6R* interaction with ACL rupture (dominant model).

IL1B x *IL6R*		*IL6R* (rs2228145)										p *vs* CON*
		A/A					A/C-C/C					
		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		
IL1B **(rs16944)**	G/G	46	39	1.00	NA	NA	53	50	1.11	0.63	1.98	0.667/0.724
	A/G-A/A	53	39	0.87	0.48	1.57	77	53	0.81	0.47	1.41	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; NA – not applicable

Table 11. Association analysis of the *IL6* x *IL6R* interaction with ACL rupture (dominant model).

IL6 x *IL6R*		*IL6R* (rs2228145)										p *vs* CON*
		A/A					A/C-C/C					
		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		
IL6 **(rs1800795)**	G/G	29	30	1.00	NA	NA	31	31	0.97	0.47	1.97	0.734/0.822
	C/G-C/C	69	51	0.71	0.38	1.34	99	82	0.80	0.44	1.44	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; NA – not applicable

Table 12. Association analysis of the *IL1B* x *IL6* interaction with ACL rupture (recessive model).

IL1B x *IL6*		*IL6* (rs1800795)										p *vs* CON*
		G/G-C/G					C/C					
		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		
IL1B **(rs16944)**	G/G-A/G	165	125	1.00	NA	NA	35	41	1.55	0.93	2.57	0.813/0.822
	A/A	26	13	0.66	0.33	1.34	2	2	1.32	0.18	9.50	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; NA – not applicable

Table 13. Association analysis of the *IL1B* x *IL6R* interaction with ACL rupture (recessive model).

IL1B x *IL6R*		*IL6R* (rs2228145)										p *vs* CON*
		G/G-C/G					C/C					
		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		
IL1B **(rs16944)**	G/G-A/G	169	144	1.00	NA	NA	31	22	0.83	0.46	1.50	0.899/0.950
	A/A	24	13	0.64	0.31	1.29	5	2	0.47	0.09	2.46	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; NA – not applicable

Table 14. Association analysis of the *IL6* x *IL6R* interaction with ACL rupture (recessive model).

IL6 x *IL6R*		*IL6R* (rs2228145)										p *vs* CON*
		G/G-C/G					C/C					
		ACLR	CON	OR	95% CI		ACLR	CON	OR	95% CI		
***IL6* (rs1800795)**	G/G-C/G	162	128	1.00	NA	NA	29	17	0.74	0.39	1.41	0.537/0.693
	C/C	30	39	1.65	0.97	2.79	7	10	1.81	0.67	4.88	

* after a slash p value adjusted for sex; OR – odds ratio, 95% CI – confidence intervals; NA – not applicable.

Discussion

Correlation studies of selected gene variants that can be included in the group of genetic risk factors for musculoskeletal soft tissues, due to their ability to affect the structural development, function and mechanical properties of tendons or ligaments, are very common. In recent years, there have been a number of studies suggesting associations between ACL rupture and polymorphisms in the structural genes, possibly suggesting that genetic predisposition is a factor of importance in ACL rupture (Ficek et al., 2014; Khoschnau et al., 2008; Posthumus et al., 2012; 2009; 2010; Stępień-Słodkowska et al., 2015). However, it is worth remembering that such injuries are always accompanied by inflammation, which is characterized by an upregulation of interleukin and its receptor production (Tsuzaki et al., 2003). Inflammation may be a beneficial occurrence that leads not only to the removal of offending factors, but also to restoration of tissue structure and physiological function. Moreover, interleukins (together with growth factors, signaling molecules and other cytokines) may be included among modulators of the matrix remodeling pathway (Akama and Chun, 2018; Bailey et al., 2012; Catalán et al., 2016; Cox and Erler, 2011; Du et al., 2017; Liu et al., 2018; Millar et al., 2009; September et al., 2011; Thampatty et al., 2007; Yang et al., 2005). The process of matrix remodeling is essential to maintain homeostasis in ligaments withstanding mechanical loads (Yang et al., 2005), as keeping the balance between degradation and synthesis of extracellular matrix (ECM) components is the basis for adaptive ligament response, crucial to withstanding increases in mechanical loads during physical activity (Cox and Erler, 2011; Rahim et al., 2017).

Taking into account that the molecular mechanism underlying matrix remodeling and the signaling molecules, such as cytokines and interleukins, involved in these processes, are key contributors in determining ligament capacity, it might be speculated that variation in the interleukin genes may contribute to the inter-individual variation in response to mechanical loading and potentially contribute to injury susceptibility as a factor of an individual's inherent risk of injury. Thus, the variants of genes encoding inflammatory factors may be regarded as a promising risk modulators of soft tissue injuries. The analyses of these genes may be helpful for better understanding the progression of the healing process to develop new therapeutic strategies. To date, several sequence variants in the genes encoding interleukins and their receptors have been studied and polymorphisms within the *IL1B*, *IL6* and *IL6R* genes has been implicated in numerous diseases or musculoskeletal soft tissues dysfunctions in humans (Ferrari et al., 2003; 2001; Fishman et al., 1998; Kelempisioti et al., 2011; Rahim et al., 2017; September et al., 2011).

The presented study explores four polymorphisms (*IL1B* rs16944 and rs1143627, *IL6* rs1800795 and *IL6R* rs2228145) within three candidate genes encoding key inflammatory factors IL-1β, IL-6, IL-6R for association with ACL injury risk. Among the four analyzed SNPs, the rs1800795 *IL6* gene polymorphism was found to be the only one significantly associated with ACL rupture in our study. With regard to rs1800795 SNP, this is a G/C polymorphism located at position -174 upstream of the *IL6* promoter is a functional polymorphism, influencing the *IL6* mRNA level. Fishman et al. (1998) demonstrated lower expression of a construct containing the C allele, while the G allele carriers were characterized by increased transcription. The rs1800795 CC genotype has been recognized as being potentially protective – its reduced frequency in young patients with systemic onset juvenile rheumatoid arthritis was thought to contribute to its pathogenesis (Fishman et al., 1998). It was also confirmed that *IL6* polymorphisms are able to influence the risk of osteoporosis as well as other chronic disorders involving IL-6 activity. It has been demonstrated that postmenopausal women homozygous for the rs1800795 C allele have low serum levels of C-terminal cross-linking of type I collagen (sCTx), a marker of bone resorption (Ferrari et al., 2001). Moreover, the rs1800795 polymorphism, together with other *IL6* promoter -572 G/C variants (rs1800796) influence C-reactive protein (CRP), serum and urinary sCTx as well as osteocalcin (a marker of bone formation) serum levels. *IL6* rs1800796/ rs1800795 haplotypes (G/C, G/G, and C/G) were significantly associated with all biochemical markers, and additive effects of the two polymorphic loci were found. Thus, there was a significant increase in the level of CRP and bone resorption markers with a decreasing number (from four to one) of *IL6* protective alleles: rs1800796 G allele and rs1800795 C allele. In addition, there was a trend for lower age-adjusted bone mineral density in the lumbar vertebrae in subjects with fewer *IL6* protective alleles (Ferrari et al., 2003). Another study indicated that rs1800795 is in the group of candidate gene polymorphisms that may be associated with intervertebral disc degeneration progressing with aging (Kelempisioti et al., 2011). Moreover, the *IL6* rs1800795 as well as *IL1B* rs16944 interacted together with the previously described *COL5A1* rs12722 polymorphism (Mokone et al., 2006) as risk variants of Achilles tendinopathy (September et al., 2011).

With reference to the other polymorphisms analyzed in our study, we failed to show significant differences in the genotype and allele frequencies for *IL6R* rs2228145, including *IL1B* rs16944 and rs1143627 (analyzed alone or in haplotype combination) between the ACL rupture group

and healthy control group among Polish participants. Although no independent associations were noted for the variants investigated in the *IL1B* nor were there any in the *IL6R* gene, these remain plausible candidate loci requiring further investigation. The polymorphism rs1143627 is in the group of SNPs associated with interindividual differences in *IL1B* expression (Auron and Webb, 1994). It was described as a C/T substitution at position -31 from the transcription start site that involves the TATA sequence in the *IL1B* gene promoter (El-Omar et al., 2000). The qPCR mRNA measurements showed that rs1143627 T allele carriers expressed significantly higher *IL1B* mRNA levels compared with those with a C allele (Landvik et al., 2009). *IL1B* promoter assays confirmed that for the rs1143627 T allele, the promoter had a 10-fold increase in activity compared to the C allele, which is linked with increased *IL1B* mRNA expression for T allele carriers, resulting in less secretion of IL-1β in CC genotype (Chakravorty et al., 2006; Chen et al., 2006). Moreover, in position -511 from the *IL1B* transcription start site, there is also another T/C polymorphism (rs16944). Similar to the rs1143627 T allele, the rs16944 C allele is also associated with increased mRNA expression and in this way may influence the IL-1β levels (Landvik et al., 2009). On the other hand, Chen (2006) described an opposite trend indicating a modest increase in transcriptional activity for rs16944T allele when compared with allele C (Chen et al., 2006). In the *IL6R* gene, the non-synonymous Asp358Ala polymorphism (rs2228145; resulting from A/C transversion) occurs at the proteolytic cleavage site of IL-6R and probably acts via affecting cleavage efficiency – in consequence, it could affect the level of the circulating soluble IL-6 receptor (IL-6SR), with increases in IL-6SR levels in C allele carriers (Galicia et al., 2004; Reich et al., 2007). In the context of soft tissue injuries, the rs2228145 polymorphism was positively correlated with a reduced risk of carpal tunnel syndrome (CTS) (Burger et al., 2015a). It was also revealed that the *IL6R* rs2228145 polymorphism can interact with the previously described *COL5A1* (Burger et al., 2015b) and *BGN* (Burger et al., 2014) risk variants of CTS (Burger et al., 2015a).

As mentioned earlier, the polymorphisms in the *IL1B*, *IL6*, and *IL6R* genes were collectively associated with the risk of musculoskeletal soft tissue injuries, such as Achilles tendinopathy or carpal tunnel syndrome, as part of an inferred allele combination with the previously associated variants in collagen genes. Therefore, it is not surprising that the study followed a pathway-based approach in order to explore the aforementioned polymorphisms as well as other candidate genes for association with ACL injury risk has been performed (Rahim et al., 2017). This study was performed in South African participants and revealed that *IL1B* rs16944 variants are associated with the risk of ACL injury in sex-specific manner – specifically: the rs16944 TT genotype was associated with a 3.1-fold increased risk of non-contact ACL ruptures in female participants (Rahim et al., 2017). Moreover, this study explored possible gene–gene interactions between *COL5A1* rs12722 T/C, *IL1B* rs16944 C/T, *IL6* rs1800795 G/C, *IL6R* rs2228145 A/C polymorphisms and described a specific haplotype allele combination that plays a role in the etiology of ACL injuries in South African Caucasian participants: the T-C-G-A inferred allele combination was significantly associated with increased risk of injury (Rahim et al., 2017). Looking into respective alleles of this haplotype, it is worth observing that the *COL5A1* rs12722 T allele was previously associated with increased risk of ACL ruptures and non-contact ACL ruptures in female participants (Posthumus et al., 2009). The rs16944 C allele was linked with increased *IL1B* mRNA expression (Landvik et al., 2009), which may be associated with higher secretion of IL-1β and upregulation of signaling cascades activated by this interleukin (Thampatty et al., 2007; Yang et al., 2005). The studies in patients revealed that the rs1800795 G allele is associated with increased transcription of the IL-6 which may lead to excessive apoptotic cell death (Fishman et al., 1998). The last element in the described by Rahim et al. (Rahim et al., 2017) T-C-G-A risk haplotype is the A allele of *IL6R* rs2228145 SNP. This polymorphism is considered to affect cleavage efficiency of the receptor precursor protein: the rs2228145 C allele carriers are characterized by increased shedding of the membrane-bound IL-6 receptor and an increase in the soluble form of the receptor (Galicia et al., 2004). Furthermore, in a previous study by September et al. the T-C-G-A2 inferred allele combination (*COL5A1* rs12722 T/C, *IL1B* rs16944C/T, *IL6* rs1800795 G/C and *IL1RN* rs2234663 VNTR) was significantly associated with risk of Achilles tendinopathy (September et al., 2011). These two risk haplotypes (T-C-G-A for ACL injuries and T-C-G-A2 for Achilles tendinopathy) are clearly very similar and only differ at the last position for the *IL1RN* rs2234663 VNTR locus, which additionally strengthens the case that the indicated alleles have roles as risk factors in both acute and chronic musculoskeletal soft tissue injuries (Rahim et al., 2017).

Our study in a Polish sample may be regarded as a replication analysis of the studies presented above. The current study was focused on the *IL1B*, *IL6*, and *IL6R* among Polish participants and represents an additional attempt to identify risk associated *loci* of biological importance across different populations. Among the obtained results in our study, there are significant differences in the genotype and allele frequencies for *IL6* rs1800795 between the ACL rupture group and the healthy control group. With reference to the *IL6R* rs2228145 as well as the *IL1B* rs16944 and rs1143627 variants, our analysis did not reveal any significant associations between these sequence variants (analyzed alone or in haplotype combination) and risk of ACL rupture among Polish participants. It should be emphasized that sample size is a major limitation for all genetic association studies, particularly for haplotype analyses. This is also a limitation of our current study: the same group of 423 physically active, unrelated, participants were recruited for this study. Therefore, due to the nature of case-control studies, the results of this study need to be confirmed in independent studies with larger sample sizes. Further studies in a larger group of participants are required to replicate these findings. Future analyses should also consider investigating other variants within the *IL1B*, *IL6* and *IL6R* genes for a more comprehensive risk profiling analyses of soft tissue injuries. Moreover, it is worth noting that genetic variants included in this study account for only a

small number of the genes involved in the biology of the ligament. It is highly unlikely that only the *IL1B*, *IL6* and *IL6R* genes are exclusively associated with ACL rupture susceptibility, since numerous interleukins and their receptors are involved in the pathology as well as healing processes and regeneration of tendons, ligaments and other connective tissue structures (Collins and Raleigh, 2009). This supports the hypothesis that not a single, but rather multiple genetic variants are associated with risk of ACL injuries. Other candidate genetic variants within the inflammatory factors and other genes should therefore be further investigated. Another important aspect of musculoskeletal sports injury may also be connected with epigenetic factors that can affect transcription levels and might significantly modify the effect of genotype upon phenotype (El Khoury et al., 2018). Therefore, in the future studies the epigenetic factors should be considered as a potential modifier of genotype when considering risk of ACL injuries.

Conclusion

Among four analyzed SNPs, the rs1800795 *IL6* gene polymorphism was found to be the only one significantly associated with ACL rupture. No significant differences were found in the genotype and allele frequencies for *IL6R* rs2228145as well as *IL1B* rs16944 and rs1143627 (analyzed alone or in haplotype combination) between the ACL rupture group and the healthy control group among Polish participants. It seems that not a single, but rather multiple genetic variants are associated with risk of ACL injuries.

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Key points

- A number of studies have suggested associations between ACL rupture and polymorphisms in the structural genes, possibly proposing that genetic predisposition is a factor of importance in ACL rupture.
- It is highly unlikely that only the *IL1B*, *IL6* and *IL6R* genes are exclusively associated with ACL rupture susceptibility.
- This study supports the hypothesis that not a single, but rather multiple genetic variants are associated with risk of ACL injuries.

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