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Title:
No association between risk of anterior cruciate ligament rupture and selected candidate collagen gene variants in female elite athletes from high-risk team sports

Abstract
Background: Several single nucleotide variants (SNVs) in collagen genes have been reported as predisposing factors for anterior cruciate ligament (ACL) tears. However, the evidence is conflicting and does not support a clear association between genetic variants and risk of ACL ruptures.

Purpose: To assess the association of previously identified candidate SNVs in genes encoding for collagen and the risk of ACL injury in a population of elite female athletes from high-risk team sports.
Study Design: Cohort study; Level of evidence, 2.

Methods: 851 female Norwegian and Finnish elite athletes from team sports were included from 2007 to 2011. ACL injuries acquired before inclusion in the cohort were registered by interview. The participants were followed prospectively through 2015 to record new, complete ACL injuries. Six selected SNVs (COL1A1: rs1800012, rs1107946; COL3A1: rs1800255; COL5A1: rs12722, rs13946; COL12A1: rs970547) were genotyped.

Results: No associations were found between ACL rupture and the SNVs tested for.

Conclusion: The study does not support a role of the six selected SNVs in genes encoding for collagen proteins as risk factors for ACL injury.
Clinical Relevance: Genetic profiling to identify athletes at high risk for ACL rupture is not yet feasible.

Keywords: genetic risk; female athlete; anterior cruciate ligament; team sports; gene polymorphisms

What is known about this subject: SNVs in COL1A1, COL3A1, COL5A1 and COL12A1 have been shown to be associated with ACL tears. However, the evidence is conflicting, and several sources of bias have been identified in the published studies.

What this study adds to existing knowledge: This study does not support a role of six selected SNVs in in COL1A1, COL3A1, COL5A1 and COL12A1 as risk factors for ACL injury.

Introduction

An anterior cruciate ligament (ACL) rupture is a common and severe knee injury.[6] The incidence is high among participants in team sports, and female athletes are especially at risk.[1, 8, 14, 20] Studies documenting a familiar predisposition indicate that genetic factors may play a role in the risk profile for ACL rupture. Harner et al. studied athletes with bilateral ACL injuries, and found an increased incidence of ACL injuries among the relatives of those athletes compared to the controls.[9] Flynn et al. showed that athletes with an ACL rupture have greater than twice the risk of having a first, second or third degree relative with an ACL injury than athletes without such injury.[5] When taking into account only first-degree relatives, the risk was even greater.[5] Others have shown that siblings with bilateral ACL injuries share intrinsic risk factors.[10]

Several studies have identified single nucleotide variants, SNVs, as potential risk factors for ACL injury.[3, 7, 12, 13, 15, 16, 23-26, 29] In general, a SNV is a DNA sequence difference between individuals, representing a replacement of one nucleotide by another at a specific location in the genome. The impact of the variant depends on where the SNV is situated relative to the gene of interest, and can vary from no effect to changes in the expression pattern to translation of altered proteins due to substitution of the normal amino acids. Up to now, most studies on ACL injuries have investigated their association with SNVs localized in candidate genes, i.e. genes coding for different musculoskeletal tissue and structural proteins where alteration in composition or levels is assumed to play a role. In ligaments and tendons, collagen constitutes 70-80% of the dry weight.[11] Ligament weakening or strengthening by alterations in its main components could affect the incidence of ligament ruptures. Examining the association between different SNVs in genes encoding collagen chains and the risk of ACL rupture is therefore of interest, and so far known SNVs in COL1A1,[23] COL 3A1,[29] COL5A1,[24, 28] and COL12A1,[25] have been suggested as candidate risk factors.

However, the genetic association studies published so far are case-control studies, where several sources of bias have been identified.[12, 15] The participants were athletes from mixed sports and with varying physical activity levels. In one study the cases were professional soccer players,[3] whereas other studies included only recreational athletes.[23, 27] In most studies, the cases with ACL ruptures are patients with surgically diagnosed ACL rupture.[3, 23, 27] The sampling method for cases was in some cases specified as convenience sampling,[23] and in some not further specified.[28, 29] Usually, the sampling method for controls was not described at all. The studies are mainly restricted to Polish and South African populations [12] and several have included men only.[27-29] A recent ACL-study used a genome wide approach (GWAS), and not a candidate gene approach. The authors screened for an association between several hundreds of thousands SNVs scattered throughout the entire genome and ACL injury. No SNV variant was found to be significantly associated with ACL injury at the genome level. The study included 598 cases with ACL injury and 98 744 controls.[17]

Our hypothesis was that SNVs localized in genes coding for collagen proteins represent potential risk factors for ACL injury, and the aim of the current prospective cohort study was to assess the association of previously identified candidate SNVs in genes encoding for collagen and the risk of ACL injury in a population of elite female athletes from high-risk team sports. This population is relatively homogenous with regard to age, fitness level and athletic performance and has a high frequency of ACL ruptures.

Material and Methods

Study participants

The participants were female athletes from team sports playing at the elite level in Norway and Finland. The cohorts have been described in detail previously.[18, 22] The Norwegian athletes were team handball and football players, expecting to play in the premier league, that were invited to participate in a pre-season screening examination from 2007 (handball) or 2009 (football) through 2014. Complete ACL tears were registered prospectively from June 2007 through May 2015. The Finnish athletes were recruited from 22 teams playing floorball, ice hockey, volleyball or basketball. The teams were either from the two highest youth league levels or adult elite teams. The Finnish athletes were screened in May 2011 and ACL tears were recorded through December 2015. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the XXXXXX (ETL-code R10169), the Regional Committee for Medical Research Ethics, the South-Eastern Norway Regional Health Authority, and the Norwegian Social Science Data Services, Norway.

Previous injury history

Prior to inclusion, previous ACL injuries were registered by interview with the participants..

Genetic testing

Based on previous case-control studies,[3, 4, 21, 23-25, 28, 29] six candidate SNVs of four collagen genes, COL1A1, COL3A1, COL5A1, and COL12A1, were selected.

DNA isolation

EDTA whole blood was collected from all 851 participants and stored at -20 °C until analyzed. In Norway, genomic DNA was extracted using a MagNA Pure LC DNA Isolation kit I (cat# 03 003990 001, Roche, Basel, Switzerland) on a MagNAPure LC Instrument (Roche). DNA yield and purity were controlled using Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, USA) before storage at +4 °C prior to downstream analysis.
In Finland, genomic DNA was extracted from peripheral blood leukocytes by using QIAamp®DNA Blood Minikit and automated biorobot M48 extraction (Qiagen, Hilden, Germany).

Genotyping

All the selected SNVs (COL1A1: rs1800012, rs1107946; COL3A1: rs1800255; COL5A1: rs12722, rs13946; COL12A1: rs970547) were genotyped by PCR

using allelic discrimination with predesigned, specific TaqMan®SNP Genotyping Assays (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA), essentially as recommended by the manufacturer. In Norway, analyses were executed on a Viia7 Real-Time PCR Instrument, and in Finland genotyping was performed by ABI Prism 7900HT Sequence Detection System (both Applied Biosystems, Thermo Fisher). A non-template control and longitudinal controls, representing the three different genotypes for each SNV, were included in each run.

Injury registration

During the follow-up period, complete ACL injuries were recorded primarily through regular consultations with the participating teams.[18, 19, 22] Injured players were interviewed and detailed descriptions of the injury situations were obtained. All complete ACL tears were verified by MRI or through arthroscopic examination. Only non-contact injuries, defined as no direct contact to the involved leg in the injury situation were included.[18, 19]

Statistical analyses

Statistical analyses were performed using R version 3.3.1 (http://www.R-project.org/). Categorical variables (SNV- and allele frequencies and baseline characteristics) were compared using Pearson chi-square tests or Fisher exact tests, as appropriate. Adjusted analyses were performed using a logistic regression model. Adjustments were made on type of sport and country. Means were compared using Student's t-test. A p value <0.05 was regarded significant. Each individual SNV was tested for Hardy–Weinberg Equilibrium (HWE) using the R package SNPassoc.[7]

Results

The present study included a final sample of 851 participants, comprising 569 Norwegian and 282 Finnish athletes recruited from different sports (Table 1).

Table 1
Characteristics of the 569 Norwegian and 282 Finnish participants. Age is shown as average ± SD and categorical variables are shown in percentages.

	Norwegian Cohort	Finnish Cohort	All participants
	n=569	n=282	n=851
Age	21.2 ± 4.0	17.3 ± 4.0	19.9 ± 4.4
- Basketball		36.5%	12.1%
- Floorball		36.5%	12.1%
- Volleyball		6.4%	2.1%
- Ice hockey		20.6%	6.8%
- Football	65.7%		44.0%
- Handball	34.3%		22.9%
ACL status			
- History of ACL injury at	9.3% (n=53/569)	2.8% (n=8/282)	7.2% (n=61/851)

inclusion			
- ACL injury during follow-up period	7.7% (n=44/569)	8.9% (n=25/282)	8.1% (n=69/851)
- ACL injury during follow up among players with no previous ACL injury	6.8% (n=35/516)	8.4% (n=23/274)	7.3% (n=58/790)
- At least one ACL injury (past or current)	15.5% (n=88/569)	11.0% (n=31/282)	14.0% (n=119/851)
- No ACL injury	84.5% (n=481/569)	89.0% (n=251/282)	86.0% (n=732/851)

The Finnish athletes were younger and had fewer ACL injuries prior to inclusion compared to the Norwegians. Originally the Finnish cohort included both men and women, but only data from the female participants were included in this study. From the original Norwegian cohort, athletes with insufficient data on previous ACL injury history (n=37) were excluded. Otherwise all athletes whose genotype could be defined were included. Genotype and history of previous ACL injury were available from 851 athletes. Overall, 119 of the participants (14.0%) had at least one ACL injury, while 58 (7.3%) out of 790 participants with no prior ACL injury suffered an ACL injury during the follow-up period.

The allelic distribution of the six SNVs, localized in the COL1A1, COL3A1, COL5A1 and COL12A1 genes, followed the HWE, both in the total sample as well as in the Norwegian and Finnish cohorts. Genotype and minor allele frequencies (MAF) of all sequence variants in the total sample and in the Norwegian and Finnish subgroups are shown in Table 2. The distribution pattern was almost similar in the two groups, with the exception of the SNV in the COL12A1 gene, where both the genotypes and the C allele differed between the Norwegian and Finnish cohorts ($p<0.001$). In addition, the A allele of the rs1800012 in the COL1A1 gene was more abundant in the Norwegian cohort than in the Finnish study (0.18 vs 0.14, respectively, $p<0.03$).

Table 2
Genotype distribution and Minor Allele Frequencies (MAF) of 6 SNVs in four candidate genes within the Norwegian and Finnish cohort. * Pearson chi-square tests were used to compare SNV genotype frequencies and MAF between Norwegian and Finnish athletes.

Gene SNV	Geno-type	Norwegian Cohort n=569	Finnish Cohort n=282	p-values*		MAF All participants
COL1A1						
rs1800012	C/C	0.67	0.74			
C>A	A/C	0.30	0.25			
	A/A	0.03	0.01	0.09		
	MAF(A)	0.18	0.14		0.03	0.17
rs1107946	C/C	0.75	0.73			
A>C	A/C	0.23	0.25			
	A/A	0.02	0.02	0.87		
	MAF(A)	0.14	0.15		0.65	0.14
COL3A1						
rs1800255	G/G	0.55	0.58			
G>A	A/G	0.39	0.34			
	A/A	0.06	0.09	0.26		
	MAF(A)	0.26	0.26		0.97	0.26
COL5A1						
rs12722	T/T	0.35	0.38			
C>T	C/T	0.48	0.49			
	C/C	0.18	0.13	0.18		
	MAF(C)	0.42	0.37		0.10	0.40
rs13946	T/T	0.55	0.59			
C>T	C/T	0.38	0.38			
	C/C	0.07	0.04	0.15		
	MAF(C)	0.26	0.23		0.14	0.25
COL12A1						
rs970547	T/T	0.59	0.47			
C>T	C/T	0.36	0.41			
	C/C	0.05	0.12	<0.001		
	MAF(C)	0.23	0.32		<0.001	0.26

The results of the genetic association analyses are presented in Table 3. The genotype and minor allele frequencies of all the SNVs in athletes with no ACL injuries (n=732) were first compared to the frequencies in athletes with ACL injury either before or after inclusion (n=119). There were no significant associations, neither in the unadjusted analyses nor when adjusting for cohort and sport. Then we compared athletes with no past history of ACL injuries (n=790) to athletes with ACL injury only after inclusion in the study (n=58). There were no associations in the unadjusted analyses. When adjusting for country and sport, the genotype frequencies of rs13946 in the COL5A1 gene showed a significant association (p=0.019). There were no athletes that suffered an ACL injury in the follow-up period that had the C/C genotype.

Table 3
Genotype distribution and Minor Allele Frequencies (MAF) of 6 SNVs in four candidate genes in the whole study group of 851 athletes without ACL injury, with ACL injury at any time and with ACL injury in the follow-up period.* Pearson chi-square test, or Fisher Exact test where appropriate, were used to compare SNV genotype frequencies and MAF.

Gene SNV	Geno-type	No ACL injury n=732	ACL injury n=119	p-values*		ACL injury in follow up only n=58	p-values*	
COL1A1								
rs1800012	C/C	0.70	0.66			0.71		
C>A	A/C	0.28	0.32			0.28		
	A/A	0.03	0.02	0.65		0.02	1	
	MAF(A)	0.16	0.18		0.59	0.16		0.84
rs1107946	C/C	0.75	0.69			0.69		
A>C	A/C	0.23	0.29			0.26		
	A/A	0.02	0.03	0.33		0.05	0.17	
	MAF(A)	0.14	0.17		0.17	0.18		0.16
COL3A1								
rs1800255	G/G	0.56	0.52			0.47		
G>A	A/G	0.37	0.41			0.45		
	A/A	0.07	0.07	0.63		0.09	0.34	
	MAF(A)	0.25	0.27		0.53	0.31		0.18
COL5A1								
rs12722	T/T	0.35	0.40			0.36		
C>T	C/T	0.48	0.46			0.47		
	C/C	0.17	0.13	0.46		0.17	0.97	
	MAF(C)	0.41	0.37		0.21	0.41		0.94
rs13946	T/T	0.56	0.59			0.53		
C>T	C/T	0.38	0.38			0.47		
	C/C	0.07	0.03	0.39		0.00	0.06	
	MAF(C)	0.25	0.22		0.30	0.23		0.61
COL12A1								
rs970547	T/T	0.56	0.50			0.52		
C>T	C/T	0.37	0.43			0.41		
	C/C	0.07	0.06	0.35		0.07	0.79	
	MAF(C)	0.26	0.28		0.50	0.28		0.65

Separate analysis of the two SNVs with different distributions between the two national cohorts (rs1800012 and rs970547) did not reveal any associations with ACL injury risk.

Comparing athletes having had an ACL injury before inclusion (n=61) to those with no history of ACL injury (n=790), the risk ratio for suffering a new ACL injury during follow-up was 2.5 (95% CI 1.4 to 4.6, $p<0.01$).

Discussion

This first prospective cohort study on the association between collagen gene variants and risk of ACL injury does not support previous findings from case-control studies that SNVs localized in genes coding for collagen proteins represent risk factors for ACL injury. Several factors could have influenced this. First, our study was designed as a cohort study with better control of confounding factors and less risk of bias than previously published case-control studies. Second, the present study was performed in Norwegian and Finnish athlete populations that may differ genetically from South African and Polish athlete populations, where most of the previous ACL association studies have been carried out. Third, the participants in this study were all female elite athletes, whereas several of the previous studies have included men only.[27-29] Last, differences in recruitment strategy between the published studies may have contributed to differences in the study populations. The participating athletes in the present study were all from high-risk team sports in contrast to previous studies that have recruited convenience samples of both recreational athletes and participants from the general population. Even though contact injuries were excluded in the analyses, there is a possibility that the energy causing the injuries in the current study were greater than in comparable studies. If so, intrinsic factors could play a smaller role the risk profile.

Several studies have suggested that the homozygous A/A genotype of the rs1800012 SNV in the COL1A1gene is protective against ACL injury as well as other soft tissue injuries.[2, 16, 23] This could not be verified in the present study. Two out of 119 (1.7 %) participants with ACL injury were carriers of the A/A genotype. However, the carrier frequency was only 2.5% among the controls in our study, compared to 4.6% in the study by Posthumus et al.[23] In a Polish study, the distribution of this genotype was 2.5 % in the control group, and no protective effect of this genotype was found.[3] This is similar to our findings. In the same study, Ficek and coworkers reported no association between the rs1107946 variant of the COL1A1 and ACL injury, which is in line with our results.[3] Two studies from the Polish population have identified the A/A genotype of the rs1800255 SNV in the COL3A1 gene as a risk factor for ACL injury,[21, 29] whereas no association was found in the South African population.[21] The latter finding is in line with our results. An association has earlier been reported between the C/C genotype of the rs12722 variant in the COL5A1 gene and reduced risk for ACL injuries in female South Africans.[24] In this study, the authors found a substantial difference in the C/C genotype between females with (27.4%) and without (5.6%) ACL rupture. Thus, the results were highly significant even though not more than 122 athletes were included in the study. The results were not confirmed in a study on male Polish skiers,[28] nor in our study. Neither in the Polish nor in the South African populations was an association found between the rs13946 of COL5A1 and ACL injury.[24, 28] In our study, we found a protective effect of the C/C genotype in the adjusted, but not in the unadjusted analyses. It is noteworthy that the Fisher exact test used in the unadjusted analysis of this genotype resulted in a p value of 0.06, which is near the level of significance. In the adjusted analyses, we had to use logistic regression, which resulted in a p-value below the level. If we were to use logistic regression in the unadjusted analysis, the

result would have been statistically significant as well (0.015). All in all, this indicates it might be a protective effect of the C/C genotype, as no ACL injury in the follow-up period was registered in athletes with this genotype (Table 3). However, the possible protective effect is probably not strong as 4 ACL injuries in athletes with this genotype was registered before start of the prospective registration. The most common genotype of the rs970547 variant in the COL12A1 gene is the homozygous T/T. In one study, this genotype has been shown to be overrepresented in female athletes with ACL rupture.[25] However, in the same study, this was not the case for male participants. In contrast to this, an Indian study that contained mainly male patients found that the C/T and C/C alleles were underrepresented in patients with ACL tear.[13] However, neither in another study of Polish footballers,[4] nor in the present study was an association found.

In recent years opportunities to screen the entire genome for SNVs have emerged. A recent case-control study used a genome wide approach (GWAS) to study the association of SNVs with ACL injury.[17] The study included 598 patients with ACL ruptures and 98 744 controls. After variance analysis, covering all SNVs registered, no variant was found to be significantly associated with ACL injury at the genome level. Furthermore, none of the SNVs previously reported to be associated with ACL injury, was found to be significantly associated in this study.

One limitation of our study is the limited number of ACL cases. However, the absolute differences in genotype distribution and minor allele frequency between the athletes with and without ACL injury are small, and are unlikely to be clinically significant. The follow-up period is also limited. However, the incidence of ACL injuries in the cohort is much higher than in the general population. Although athletes are likely to enjoy a relatively active lifestyle even after the end of their elite career, their risk of acquiring an ACL injury then is probably similar to the general population.

In conclusion, our study does not support a role of the six selected SNVs in genes encoding for collagen proteins as risk factors for ACL injury. Although a genetic predisposition may exist, the importance of this, and thus the clinical significance, is unclear. Theoretically, measures to prevent ACL injuries could be targeted to individuals with a specific genetic make-up, but currently there is no evidence supporting such an approach.

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