Background: Associations of genetic variants within certain fibril-forming genes have previously been observed with anterior cruciate ligament (ACL) injuries. Evidence suggests a significant role of angiogenesis-associated cytokines in remodeling the ligament fibril matrix after mechanical loading and maintaining structural and functional integrity of the ligament. Functional polymorphisms within the vascular endothelial growth factor A (VEGFA) gene have emerged as plausible candidates owing to their role in the regulation of angiogenic responses.

Hypothesis: VEGFA promoter polymorphisms rs699947 and rs35569394 are associated with ACL injury risk among athletes.

Methods: A total of 90 Indian athletes with radiologically confirmed or surgically proven isolated ACL tears and 76 matched-control athletes were selected for the present cross-sectional genetic association study. Oral mouthwash samples were collected from all the case and control athletes and genotyped for VEGFA rs699947 and rs35569394 using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method.

Results: The A allele (rs699947) was significantly overrepresented in the ACL group (C vs A allele: odds ratio [OR], 1.68 [95% CI, 1.08-2.60]; P = .021) (CC vs CA + AA: OR, 2.69 [95% CI, 1.37-5.26]; P = .004). There was a greater frequency of the AA genotype in the ACL group in comparison with the control group (OR, 3.38 [95% CI, 1.23-9.28]; P = .016) when only male athletes were compared. Likewise, there was a greater frequency of the I allele (rs35569394) in the ACL group (D vs I allele: OR, 1.64 [95% CI, 1.06-2.55]; P = .025) (DD vs DI + II: OR, 2.61 [95% CI, 1.31-5.21]; P = .006). The A-I haplotype was overrepresented in the ACL group compared with the control group (OR, 1.68 [95% CI, 1.08-2.60]; χ² = 5.320; P = .021), and both the polymorphisms were found to be in complete linkage disequilibrium (r² = 0.929; logarithm of the odds score = 63.74; D’ = 1.0). Female athletes did not show any difference in genotype or allele frequency.

Conclusion: This is the first study to investigate the association of VEGFA promoter polymorphisms in ACL tears among Indian athletes. Increased frequencies of the A allele (rs699947) and I allele (rs35569394) were observed in the ACL group. These results suggest that sequence variants in the VEGF gene are associated with ACL injury risk among athletes. Further research with long-term follow-ups measuring VEGF expression levels during recovery is warranted to establish its role in ACL injuries and healing.

Keywords: ACL; athletes; VEGFA; promoter polymorphisms; genotyping; haplotype

Anterior cruciate ligament (ACL) injuries account for almost 50% of all knee injuries occurring in sports. Trauma to the ACL compromises translational and rotational stability of the knee in the anterior joint space in all
Extracellular matrix remodeling via the increased expression of several proangiogenic cytokines has been reported in tendons in response to mechanical stimuli. Angiogenesis-associated signaling pathways have been investigated in relation to certain orthopaedic conditions as well. Recently, genomic sequence variants among genes modulating the angiogenesis-associated signaling pathway have been associated with the susceptibility of ACL injuries. Vascular endothelial growth factor (VEGF), encoded by the VEGF gene, is an essential component in the regulation of angiogenesis-associated signaling pathways. The VEGF gene is located on chromosome 6 (6p21.1), consists of 8 exons, and has at least 30 reported single nucleotide polymorphisms (SNPs) within 5 isoforms: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. The VEGF-A isoform has shown the highest angiogenic potency, and one of its promoter SNPs, rs699947 (-2578 C>A; 6:43768652), has been linked to a number of pathophysiological conditions, including coronary artery disease, cancer, rheumatoid arthritis, and certain tendinopathies. VEGFA rs35569394 (18bp I/D; 6:43736418) is an 18-bp insertion/deletion polymorphism located at the -2549 position in the promoter region of the VEGF gene and has been found to be associated with increased angiogenesis in several conditions such as diabetic nephropathy, diabetic retinopathy, and certain types of cancers. Although a potent angiogenic site, the role of rs35569394 (-2549 18bp I/D) has not been investigated in musculoskeletal soft tissue injuries. Understanding the role of DNA variations in the risk of ACL injuries is paramount for the personalized treatment of athletes to aid healing and the timely return to sports. India, globally the second most populous nation, is also the most diverse genetically. Thus far, limited data are available on the genetic risk factors for an ACL tear in the Indian population, as most of the studies have focused on White populations. Therefore, this study aimed to identify any association between VEGFA SNPs (rs699947 and rs35569394) and ACL injury susceptibility among an Indian athletic population. To the best of our knowledge, this is the first study that reports the association of the rs35569394 polymorphism with ACL injuries.

METHODS

Participants
A total of 90 athletic patients (75 male and 15 female) with either radiologically confirmed or surgically proven ACL tears participated as cases in the study. Patients were included if they met the following criteria: (1) participation in any contact or noncontact sports, (2) age between 18 and 35 years, (3) isolated ACL injuries with no multiligament or meniscal injury or signs of osteoarthritis in the knee, (4) an ACL tear during training or competition, and (5) not suffering from any chronic disease. In addition, 76 unrelated, healthy, physically active athletes (59 male and 17 female) matched to ACL athletic patients in terms of age, sex, nature of sports played, and training regimen, with no self-reported history of knee ligament/tendon injuries, were recruited as controls. All the athletes were recruited from various sports injury clinics, orthopaedic rehabilitation centers, and prominent sports training academies in India from September 2018 to May 2019. After providing informed consent, every case and control athlete underwent a thorough clinical evaluation and completed a questionnaire consisting of general details (name, age, sex, state of domicile) as well as details about their sporting career, mechanism of injury and treatment, and personal and family history of such trauma. Athletes with an ACL injury were stratified according to mechanism of injury into contact (n = 28) versus noncontact (n = 62) groups in accordance with the classification system of the American Orthopaedic Society for Sports Medicine. This study was approved by an institutional human ethics committee and was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki (2013).

DNA Extraction
To extract genomic DNA, oral mouthwash samples were obtained from athletes in a sterile container containing 2 mL sodium chloride–tris–EDTA buffer (100 mM NaCl; 10 mM Tris-Cl; pH 8.0; 1 mM EDTA). Genomic DNA was extracted from the oral epithelial cells using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s protocol. The eluted DNA was quantified using NanoPhotometer P330 (Implen), electrophoresed in 1x TAE 0.8% agarose gel, and visualized by ethidium bromide staining.
VEGFA Genotyping

Standard genotyping protocols including polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis were employed. The manufacturer’s instructions were followed for all the reactions, with minor modifications. Details about primers and reaction conditions are provided in Appendix Table A1. For VEGFA rs699947 screening, PCR amplifiers were digested with FastDigest BglII (Thermo Fisher Scientific). The PCR amplifiers of rs35569394 and digested products of rs699947 were electrophoresed in 2.5% agarose gel and visualized by ethidium bromide staining. Figure 1A shows a schematic view of a VEGF gene fragment targeted for PCR-RFLP. The lengths of PCR- and PCR-RFLP digested products are shown in Figure 1B and 1C. Genotypes were determined based on the length of the DNA fragments calculated by electrophoresis of the DNA ladder (Figure 1, B and C). Additionally, PCR products of 9 random samples were sequenced by the BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific) to further confirm the RFLP results (Figure 1, B and D). Multiple attempts to sequence the 476-bp amplicon of the rs699947 CA genotype (homozygous) were unsuccessful because of the significant association of the ID-CA genotype (multiple sequence alignment) (Figure 1E), which perhaps hampered capillary electrophoresis of the BigDye Terminator–labeled DNA samples in the DNA sequencer. Thus, amplicons of 214 bp and 196 bp were eluted separately and sequenced to confirm the CA genotype of randomly selected samples (data not shown).

Statistical Analysis

Association analysis was performed using SPSS 22.0 software (IBM) and GraphPad InStat Version 3 (GraphPad Software). Continuous variables were reported as the mean ± SD. Data normality was assessed using the Shapiro-Wilk test, and the independent-samples t test or Mann-Whitney U test was performed to investigate any difference between the control, ACL, and noncontact ACL groups accordingly. The genotype and allele frequencies and other between-group categorical comparisons were performed using the chi-square test or Fisher exact test. The association analysis in the present study was performed in 2 ways: (1) general association with a 2 × 2 contingency table and (2) association analysis of 3 different genetic models: additive model, dominant inheritance model for minor alleles, and recessive inheritance model for minor alleles. Allele-based odds ratios (ORs) and 95% CIs were calculated for the ACL injury risk. Haplotypes were calculated from genotyped data, and haplotypes and linkage disequilibrium (LD) were calculated using Haploview 4.2 software (Broad Institute, Cambridge, Massachusetts). The Hardy-Weinberg equilibrium was determined using the online calculator from the Online Encyclopedia for Genetic Epidemiology Studies (https://www.oegge.org/software).24 P values <.05 were considered statistically significant.

RESULTS

Participant Characteristics

Because sampling was conducted previously and several extracted DNA samples failed in PCR amplification afterward, the remaining samples were genotyped for polymorphisms. Failure of PCR reactions when oral mouthwash DNA is used as a template is a common occurrence. Also, some participants were initially included but later excluded from analysis, as they did not meet all the inclusion criteria. Thus, overall, 76 control athletes and 90 ACL athletic patients were analyzed for 2 promoter polymorphisms within VEGFA: rs699947 and rs35569394. We performed a sex-based comparison for continuous characteristics. Demographic details of these participants are presented in Table 1.

In the present cross-sectional study, the athletes in the control and ACL groups were matched for age, height, weight, and body mass index (BMI) (Table 1). However, the ACL group was significantly heavier (P < .01) and also had a significantly higher BMI than the control group (P < .01) (Table 1). The athletes belonged to different sporting disciplines, and most ACL injuries occurred in contact games such as wrestling, judo, and throwing events in track and field. Also, patients in the ACL group were contacted during the rehabilitation phase at clinics and training facilities; complete ACL rehabilitation took place between 6 and 9 months21 after surgical reconstruction/nonoperative management, and we required patients to avoid any kind of vigorous activity, as it might lead to increases in weight and BMI in the ACL group compared with the control group. Further, most of the patients in the ACL group underwent delayed ACL reconstructive surgery, thus adding to inactivity and subsequent increases in weight and BMI. No ACL cases or controls reported any injury to other knee joint structures or any chronic illness. Hardy-Weinberg equilibrium principles were met for the genotype distribution in all the groups (Table 2).

Genotype and Allele Frequency Analysis

The distribution of genotype and allele frequencies of both the polymorphisms is presented in Tables 2 and 3, respectively (Figure 2). Male athletes in the control and ACL groups showed a significant difference in the genotype and allele frequency distribution, as presented in Appendix Tables A2 and A3. Because the sample size of female athletes was smaller across all the groups, no meaningful conclusion could be drawn toward a phenotype-by-genotype association analysis of either polymorphism between the control, ACL, and noncontact ACL groups.

Haplotype Analysis and LD

Results of haplotype analysis and LD between VEGFA polymorphisms (rs699947 and rs35569394) are presented in Table 4. There were 3 common haplotypes for VEGFA rs699947 C/A and VEGFA rs35569394 18-bp I/D (C-D, C-I, A-I) that were calculated for 2 polymorphisms, and the C-D haplotype was significantly overrepresented in the control group (OR, 1.64 [95% CI, 1.06-2.55]; χ² = 5.024; P = .025)
Figure 1. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and sequencing of the vascular endothelial growth factor A (VEGFA) promoter. (A) Schematic view of a VEGF gene fragment targeted by PCR, followed by restriction digestion with BglII for PCR-RFLP assay. (B) Agarose gel electrophoresis of genomic DNA, PCR amplification of rs699947, BglII restriction digestion, and Sanger sequencing electropherogram for rs699947 wild-type and homozygous samples. (C) Agarose gel electrophoresis and PCR amplification pattern for rs35569394. (D) Sanger sequencing electropherogram showing the association of the A, I, C, and D alleles. Asterisk indicates the C/A allele, and block arrows show the I/D genotype location. (E) Multiple sequence alignment showing the I/D allele and association with the C/A allele.
VEGFA (rs699947 and rs35569394) Genotype and Allele Association

The results of our study revealed that there was a significantly greater frequency of the VEGFA rs699947 (-2578 C>T) polymorphism among the ACL injury group compared with the control group (OR, 2.56 (1.03-6.37) vs 1.08 (0.98-1.18); P = .051). The r² value was 0.929, which is considered to be high LD (range, 0.70-0.98), with a logarithm of the odds score of 63.74 and a D’ of 1.0 (polymorphisms have the strongest LD) (Appendix Figure A1).

**DISCUSSION**

Angiogenesis-associated signaling is considered a key modulator of fibroblast cytoskeletal matrix remodeling after cyclic mechanical loading during complex sporting maneuvers, with VEGFA having the highest angiogenic potency. Conflicting observations for genomic sequence variants within the VEGF gene have previously been reported for the ACL injury risk and tendinopathies in multiple White populations. Thus, we hypothesized that polymorphisms within the VEGFA promoter region (rs699947 and rs35569394) could be positively associated with the ACL rupture risk in the Indian athletic population.

**TABLE 1**

| Characteristics of Study Participants² | Control | ACL | P Value: Control vs ACL | Noncontact ACL | P Value: Control vs Noncontact ACL |
|---------------------------------------|---------|-----|-------------------------|----------------|-------------------------------|
| All participants, n (%)               | 76 (100) | 90 (100) |                         | 62 (100)       |                                |
| Age, y                                | 24.2 ± 4.1 | 26.6 ± 6.2 | .02a                    | 26.4 ± 6.3 | .08                           |
| Height, cm                            | 169.4 ± 9.4 | 171.2 ± 7.2 | .18                     | 171.2 ± 7.3 | .24                           |
| Weight, kg                            | 63.9 ± 10.0 | 74.1 ± 11.3 | <.01c                   | 73.6 ± 11.9 | <.01c                         |
| BMI, kg/m²                            | 22.2 ± 2.2 | 25.2 ± 3.1 | <.01c                   | 25.0 ± 3.4 | <.01c                         |
| Female participants, n (%)            | 17 (22.4) | 15 (16.7) |                         | 7 (11.3)      |                                |
| Age, y                                | 22.6 ± 1.8 | 23.4 ± 2.9 | .34                     | 22.8 ± 2.2 | .75                           |
| Height, cm                            | 159.6 ± 6.4 | 162.8 ± 6.6 | .18                     | 159.6 ± 5.7 | .98                           |
| Weight, kg                            | 53.4 ± 5.8 | 65.6 ± 12.2 | .01b                    | 63.7 ± 16.3 | .03b                          |
| BMI, kg/m²                            | 20.9 ± 1.8 | 24.7 ± 4.6 | <.01c                   | 25.0 ± 6.5 | .03b                          |
| Male participants, n (%)              | 59 (77.6) | 75 (83.3) |                         | 55 (88.7)     |                                |
| Age, y                                | 24.7 ± 4.5 | 27.2 ± 6.5 | .03b                    | 26.8 ± 6.6 | .13                           |
| Height, cm                            | 172.3 ± 8.1 | 172.8 ± 6.1 | .29                     | 172.6 ± 6.0 | .37                           |
| Weight, kg                            | 66.9 ± 8.8 | 75.7 ± 10.4 | <.01c                   | 74.8 ± 10.7 | <.01c                         |
| BMI, kg/m²                            | 22.5 ± 2.1 | 25.3 ± 2.7 | <.01c                   | 25.0 ± 2.8 | <.01c                         |

²Data are reported as mean ± SD unless otherwise indicated. Data sets among groups were normally distributed except for age and BMI (P < .05). Bolded P values indicate statistical significance (P < .05). ACL, anterior cruciate ligament; BMI, body mass index.

³Mann-Whitney U test.

¹Student t test.

**TABLE 2**

| 2 × 3 Phenotype-by-Genotype Association Analyses of VEGFA Polymorphisms With ACL Injury Risk² | Control (n = 76) | ACL (n = 90) | Control vs ACL | Noncontact ACL (n = 62) | Control vs Noncontact ACL |
|-------------------------------------------------------------------------------------------------|-----------------|--------------|----------------|------------------------|--------------------------|
| VEGFA rs699947                                                                                   |                 |              |                |                        |                          |
| CC                                               | 33 (43)         | 20 (22)     | >.99          | 14 (23)          | >.99                    | >.99                     |
| CA                                               | 30 (40)         | 51 (57)     | .004          | 2.80 (1.37-5.74) | 35 (56)          | .011                     | 2.75 (1.24-6.08)         |
| AA                                               | 13 (17)         | 19 (21)     | .052          | 2.41 (0.98-5.92) | 13 (21)          | .087                     | 2.36 (0.88-6.35)         |
| Χ² value (P value)                               | 8.64 (.013)     |              |                |                        |                          |
| Hardy-Weinberg equilibrium                       | 0.18            | 0.21         |                |                        |                          |
| VEGFA rs35569394                                                                                   |                 |              |                |                        |                          |
| DD                                               | 30 (40)         | 18 (20)     | >.99          | 12 (19)          | >.99                    | >.99                     |
| ID                                               | 33 (43)         | 52 (58)     | .009          | 2.63 (1.27-5.45) | 36 (58)          | .015                     | 2.73 (1.20-6.19)         |
| II                                               | 13 (17)         | 20 (22)     | .041          | 2.56 (1.03-6.37) | 14 (23)          | .051                     | 2.69 (0.98-7.38)         |
| Χ² value (P value)                               | 7.61 (.022)     |              |                |                        |                          |
| Hardy-Weinberg equilibrium                       | 0.45            | 0.14         |                |                        |                          |

²Data are reported as n (%) unless otherwise indicated. P values are unadjusted. Bolded P values indicate statistical significance (P < .05). ACL, anterior cruciate ligament; OR, odds ratio; VEGFA, vascular endothelial growth factor A.

compared with the ACL group, and the A-I haplotype was found overrepresented in the ACL group compared with the control group (OR, 1.68 [95% CI, 1.08-2.60]; Χ² = 5.320; P = .021). The r² value was 0.929, which is considered to be high LD (range, 0.70-0.98), with a logarithm of the odds score of 63.74 and a D’ of 1.0 (polymorphisms have the strongest LD) (Appendix Figure A1).
C>A) CA genotype in the ACL group and noncontact ACL subgroup, with a 2.80- and 2.75-fold more risk of ACL rupture compared with the asymptomatic control group, respectively (Table 2 and Figure 2). The C allele has been reported to enhance VEGF expression,19 which might assist in matrix remodeling after cyclic loading, thus restoring the structural integrity and functional properties of the ligament.3 Rahim et al19 reported that the frequency of the C allele was notably higher in the ACL rupture group, with the C allele thus being the risk allele. However, our results demonstrated that the frequency of the A allele was high in the ACL group, thus opposing the findings of Rahim et al. Our study concentrated specifically on Indian athletes, and the observation of Rahim et al is based on an African population, thus racial/genetic variations between Africans and Indians may be a reason for the difference in findings.

Additionally, the dominant model for minor alleles (CC vs CA + AA) again suggested the A allele was overrepresented in the ACL and noncontact ACL groups, with a 2.69- and 2.63-fold more risk of sustaining ACL injuries than the asymptomatic control group (Table 3). We also noted an independent association of genotype and allele frequency of rs699947 with ACL injury susceptibility among male patients, and the A allele was overrepresented in the ACL and noncontact ACL groups, with a 3.01- and 3.23-fold more risk of ACL injuries, respectively (Appendix Table A2).

VEGFA rs699947 was in complete LD with another promoter polymorphism VEGFA rs35569394.4,12 A patient having the A allele at the −2578 position (rs699947) also had an 18-bp insertion at the −2549 position in the promoter region of the VEGF gene, while a patient with the C allele had an 18-bp deletion at the same location.12 In our study, the frequencies of the ID and II genotypes and the I allele (rs35569394) were significantly higher in the ACL group, with a 1.64-fold more risk of ACL rupture than the control group, and because a noncontact mechanism is an important subcategory for the internal locus of causality of ACL ruptures, it is essential to report the OR associated with this category as well (Table 2 and Figure 2). Kapahi et al12 suggested that the D allele of rs35569394 leads to enhanced transcriptional activity of the VEGF gene, resulting in an increased angiogenic response. Because the ACL is a hypovascular structure,26,31 Cook and Docking6 suggested that any significant increase in angiogenesis might lead to increased healing responses but compromised ACL strength. Also, Takayama et al29 found that a low dose of VEGF (25% VEGF) was most effective in promoting healing and restoring biomechanical strength of ACL-reconstructed knees compared with a high dose of VEGF (100%), and blocking angiogenesis with anti-VEGF sFLT1 (soluble fms-like tyrosine kinase 1) significantly reduced the biomechanical strength and healing capacity of the ACL in a rat model. Results from our study suggest that optimally enhanced VEGF activity might be protective against musculoskeletal soft tissue injuries such as ACL ruptures. Ethnic differences in the genomic distribution may explain the variability in the findings of phenotype-by-genotype association studies on multiple populations.

### TABLE 3

| Analysis of Selected Polymorphisms Based on 3 Genetic Models for ACL Injury Risk<sup>a</sup> |
|---------------------------------|-----------------|-----------------|-----------------|
| VEGFA rs699947 | Control vs ACL | Control vs Noncontact ACL |
| Additive | Control | ACL | χ² Value (P Value) | OR (95% CI) | Control | ACL | χ² Value (P Value) | OR (95% CI) |
| C | 96 (63) | 91 (51) | 5.32 (.021) | 1.68 (1.08-2.60) | 63 (51) |
| A | 56 (37) | 89 (49) | 8.52 (.004) | 2.69 (1.37-5.26) | 61 (49) |
| Dominant (CC vs CA + AA) | C | 33 (43) | 20 (22) | 0.43 (.514) | 1.30 (0.59-2.84) | 49 (79) |
| | A | 63 (83) | 71 (79) | 0.68 (.410) | 1.38 (0.64-3.01) | 14 (22) |
| | C | 30 (39) | 18 (20) | 7.60 (.006) | 2.61 (1.31-5.21) | 50 (81) |
| | I | 13 (17) | 19 (22) | 5.02 (.025) | 1.64 (1.06-2.55) | 14 (23) |

### TABLE 3<sup>a</sup>

Data are reported as n (%) unless otherwise indicated. P values are unadjusted. Bolded P values indicate statistical significance (P < .05). ACL, anterior cruciate ligament; OR, odds ratio; VEGFA, vascular endothelial growth factor A.

Shukla et al The Orthopaedic Journal of Sports Medicine
VEGFA (rs699947 C/A and rs35569394 18bp I/D)

Inferred haplotype analysis for VEGFA polymorphisms (rs699947 C/A and rs35569394 18bp I/D) revealed perfect LD between VEGFA rs699947 (-2578 C/A) and VEGFA rs35569394 (-2549 18bp I/D) (Table 4 and Appendix Figure A1). Patients who possess the C allele at position -2578 inside the VEGFA promoter (rs699947) have an 18-bp deletion at the -2549 position, resulting in enhanced VEGF protein expression.4,7,12,19,27 We found that the C-D haplotype was significantly overrepresented in the control group, which suggests that enhanced VEGF protein expression helps to improve the healing capacity of musculoskeletal soft tissues after trauma and restores the biomechanical strength of the ACL.29,30 The A-I haplotype was overrepresented in the ACL group.

Limitations

Although our study presents promising results for screening ACL injury-prone athletes and has scope in the individualization of a training program, there remain certain limitations of this study. In our experience, most athletes and their coaches did not agree with providing blood or oral mouthwash samples, or complying with other invasive sample collection methods, thus resulting in a limited number of participants for this study owing to administrative constraints and the fear of disclosure of their identity or doping sanctions. The limited number of female athletes
in the study may be attributed to the cultural issue of sports participation in India and to higher dropout rates among female athletes after ACL tears and other injuries, as female patients are considered to be at an increased risk of ACL ruptures; several studies have investigated sex-specific genetic associations with ACL injuries.\textsuperscript{2,3,13,18,28,35} Also, patients were categorized into the contact or noncontact ACL group according to their self-reported mechanism of injury. As there is no current clinical tool to confirm the mechanism of ACL injuries, this remains a limitation of the study.

CONCLUSION

This was the first study to investigate the association of VEGF promoter polymorphisms with ACL injuries among Indian athletes. Multifactorial analyses of ACL injuries incorporating molecular profiles of angiogenesis-promoting, collagen matrix–remodeling, and repair-associated genes; gene-gene interactions; and linkage disequilibrium should be performed to fully elucidate the pathophysiology of sports injuries in general and ACL injuries in particular.

ACKNOWLEDGMENT

The authors acknowledge the contributions of the sports training and technical support staff of the Army Sports Institute (Pune, India), Netaji Subhas National Institute of Sports (Patiala, India), Sports Injury Centre (New Delhi, India), and Lakshmibai National Institute of Physical Education (Gwaltoli, India) during sample collection. The Central Instrumentation Facility at Jiwaji University is acknowledged for providing the DNA sequencing infrastructure.

REFERENCES

1. Amle D, Mir R, Khaneja A, et al. Association of 18bp insertion/deletion polymorphism, at \textasciitilde 2549 position of VEGF gene, with diabetes nephropathy in type 2 diabetes mellitus patients of North Indian population. J Diabetes Metab Disord. 2015;14:19.
2. Anderson A, Dome DC, Gautam S, et al. Correlation of anthropometric measurements, strength, anterior cruciate ligament size, and intercondylar notch characteristics to sex differences in anterior cruciate ligament tear rates. Am J Sports Med. 2001;29(1):58-66.
3. Bray RC, Leonard CA, Salo PT. Correlation of healing capacity with vascular response in the anterior cruciate and medial collateral ligaments of the rabbit. J Orthop Res. 2003;21(6):1118-1123.
4. Brogan U, Khan N, Isaac K, et al. Novel polymorphisms in the promoter and 5’UTR regions of the human vascular endothelial growth factor gene. Hum Immunol. 1999;60:1245-1269.
5. Buraczynska M, Książek P, Baranowicz-Gaszczyk I, et al. Association of the VEGF gene polymorphism with diabetic retinopathy in type 2 diabetes patients. Nephrol Dial Transplant. 2007;22:827-832.
6. Cook J, Docking S. “Rehabilitation will increase the ‘capacity’ of your…insert musculoskeletal tissue here…” Defining “tissue capacity”: a core concept for clinicians. Br J Sports Med. 2015;49:1484-1485.
7. Cooper ME, Vranes D, Youssef S, et al. Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes. Diabetes. 1999;48:2229-2239.
8. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9:669-676.
9. Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. Science. 2002;296(5576):2225-2229.
10. Han SW, Kim GW, Seo JS, et al. VEGF gene polymorphisms and susceptibility to rheumatoid arthritis. Rheumatology. 2004;43:1173-1177.
11. Joseph AM, Collins CL, Henke NM, et al. Multisport epidemiologic comparison of anterior cruciate ligament injuries in high school athletics. J Athl Train. 2013;48(6):810-817.
12. Kapahi R, Manjari M, Uppal MS, et al. Association of \textasciitilde 2549 insertion/deletion polymorphism of vascular endothelial growth factor with breast cancer in North Indian patients. Genet Test Mol Biomarkers. 2013;17:242-248.
13. Lohmander LS, Englund PM, Dahl LL, et al. The long-term consequence of anterior cruciate ligament and meniscus injuries: osteoarthritis. Am J Sports Med. 2007;35(10):1756-1769.
14. Lušińska-Kuklik E, Leźnicka K, Humirska-Lisowska K, et al. The VEGFA gene and anterior cruciate ligament rupture risk in the Caucausian population. Biol Sport. 2019;36(1):3-8.
15. Marshall SW, Padua D, McGrath M. Incidence of ACL injuries. In: Hewett TE, Schultz SJ, Griffin LY, eds. Understanding and Preventing Noncontact ACL Injuries. Champaign, Illinois: Human Kinetics; 2007:5-30.
16. Noyes FR, Barber-Westin S. The ACL: anatomy, biomechanics, mechanics of injury, and the gender disparity. In: Noyes F, Barber-Westin S, eds. ACL Injuries in the Female Athlete. Berlin: Springer; 2018:3-32.
17. Petersen W, Varoga D, Zantop T, et al. Cyclic strain influences vascular endothelial growth factor (VEGF) and the hypoxia inducible factor 1 alpha (HIF-1a) in tendon fibroblasts. J Orthop Res. 2004;22:847-853.
18. Rahim M, El Khoury LY, Raleigh SM, et al. Human genetic variation, sport and exercise medicine, and Achilles tendinopathy: role for angiogenesis-associated genes. OMICS. 2016;20(9):520-527.
19. Rahim M, Gibbon A, Hobbs H, 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):1612-1618.
20. Rahim M, Hobbs H, van der Merwe W, et al. Investigation of angiogenesis genes with anterior cruciate ligament rupture risk in a South African population. J Sports Sci. 2018;36(5):551-557.
21. Rambaud AJM, Semay B, Samozino P, et al. Criteria for return to sport after anterior cruciate ligament reconstruction with lower reinjury risk (CR’STAL study): protocol for a prospective observational study in France. BMJ Open. 2017;7:e015087.
22. Reich D, Thangaraj K, Patterson N, et al. Reconstructing Indian population history. Nature. 2009;461(7263):489-494.
23. Rodriguez S, Gaunt TR, Day INM. Hardy-Weinberg equilibrium testing of biological ascertainment for Mendelian randomization studies. Am J Epidemiol. 2009;169(4):505-514.
24. Salles JI, Duarte MEL, Guimarães JM, et al. Vascular endothelial growth factor receptor-2 polymorphisms have protective effect against the development of tendinopathy in volleyball athletes. PLoS One. 2016;11(12):e0167717.
25. Sanders TL, Maradit Kremers H, Bryan AJ, et al. Incidence of anterior cruciate ligament tears and reconstruction: a 21-year population-based study. Am J Sports Med. 2016;44(6):1502-1507.
26. Scapinelli R. Studies on the vasculature of the human knee joint. Acta Anat. 1968;70(3):305-331.
27. Shahbazi M, Fryer AA, Pravica V, et al. Vascular endothelial growth factor gene polymorphisms are associated with acute renal allograft rejection. J Am Soc Nephrol. 2002;13:260-264.
28. Sivertsen EA, Haug KBF, Kristianslund EK, et al. No association between risk of anterior cruciate ligament rupture and selected candidate collagen gene variants in female elite athletes from high-risk team sports. Am J Sports Med. 2019;47(1):52-58.
29. Takayama K, Kawakami Y, Mifune Y, et al. The effect of blocking angiogenesis on anterior cruciate ligament healing following stem cell transplantation. Biomaterials. 2015;60:9-19.

30. Takayama K, Ying T, Bing W, et al. Effect of angiogenesis on the regenerative capacity of ACL-derived CD34+ cells in ACL reconstruction. Mol Ther. 2013;21(suppl 1):S234.

31. Toy BJ, Yeasting RA, Morse DE, et al. Arterial supply to the human anterior cruciate ligament. J Athl Train. 1995;30(2):149-152.

32. Wang JHC. Mechanobiology of tendon. J Biomech. 2006;39(9):1563-1582.

33. Wang Y, Huang Q, Liu J, et al. Vascular endothelial growth factor A polymorphisms are associated with increased risk of coronary heart disease: a meta-analysis. Oncotarget. 2017;8(18):30539-30551.

34. Watson CJ, Webb NJ, Bottomley MJ, et al. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. Cytokine. 2000;12:1232-1235.

35. Zhao M, Hu Y, Yu Y, et al. Involvement of IL-37 in the pathogenesis of proliferative diabetic retinopathy. Invest Ophthalmol Vis Sci. 2016;57:2955-2962.

APPENDIX

TABLE A1
Standard Genotyping Protocols Used for Screening Selected Polymorphisms

| Polymorphism       | Screening Method | Primer Sequence | PCR Conditions                        |
|--------------------|------------------|-----------------|---------------------------------------|
| VEGFA rs699947     | PCR-RFLP         | Forward: 5′-ATAAGGGCCCTAGGACACCA-3′<br>Reverse: 5′-GCTACTTCTCCAGGCTCACA-3′ | 1. Initial denaturation: 94°C for 5 min<br>2. Denaturation: 95°C for 45 s<br>3. Annealing: 61°C for 30 s<br>4. Extension: 72°C for 45 s<br>5. Final extension: 72°C for 5 min<br>Steps 2-4 repeated for 35 cycles |
| VEGFA rs35569394   | PCR              | Forward: 5′-CATTCCCCATTCTCAGTCCAT-3′<br>Reverse: 5′-CCCATCCCATCTTGCATA-3′ | 1. Initial denaturation: 94°C for 5 min<br>2. Denaturation: 95°C for 45 s<br>3. Annealing: 58°C for 30 s<br>4. Extension: 72°C for 45 s<br>5. Final extension: 72°C for 5 min<br>Steps 2-4 repeated for 35 cycles |

aPCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; VEGFA, vascular endothelial growth factor A.

TABLE A2
2 × 3 Phenotype-by-Genotype Association Analyses of Selected VEGFA Polymorphisms With ACL Injury Risk Among Male Participants

| Polymorphism | Control (n = 59) | ACL (n = 75) | Control vs ACL | Noncontact ACL (n = 55) | Control vs Noncontact ACL |
|--------------|------------------|--------------|----------------|-------------------------|---------------------------|
|              | P Value | OR (95% CI)    | P Value | OR (95% CI)    | P Value | OR (95% CI) |
| VEGFA rs699947 |        |                |        |                |        |                |
| CC           | 27 (46) | 16 (21) | >.99  | >0.99 | 12 (22) | >.99  | >0.99 |
| CA           | 23 (39) | 41 (55) | .006  | 3.01 (1.34-6.71) | 33 (60) | .007  | 3.23 (1.36-7.66) |
| AA           | 9 (15)  | 18 (24) | .016  | 3.38 (1.23-9.28) | 10 (18) | .107  | 2.50 (0.81-7.73) |
| χ² value (P value) | 9.09 (.011) | 7.48 (.024) |

VEGFA rs35569394

| DD           | 24 (41) | 14 (19) | >.99  | >0.99 | 11 (20) | >.99  | >0.99 |
| ID           | 26 (44) | 42 (56) | .014  | 2.77 (1.22-6.29) | 31 (56) | .032  | 2.60 (1.08-6.30) |
| II           | 9 (15)  | 19 (25) | .013  | 3.62 (1.29-10.15) | 13 (24) | .039  | 3.15 (1.04-9.56) |
| χ² value (P value) | 8.17 (.017) | 5.86 (.053) |

aData are reported as n (%) unless otherwise indicated. P values are unadjusted. Bolded P values indicate statistical significance (P < .05). ACL, anterior cruciate ligament; OR, odds ratio; VEGFA, vascular endothelial growth factor A.
### Table A3
Analysis of Selected Polymorphisms Based on 3 Genetic Models for ACL Injury Risk Among Male Participants

| Polymorphism       | Control vs ACL | Noncontact ACL |
|--------------------|----------------|----------------|
|                    | $\chi^2$ Value (P Value) | OR (95% CI) | $\chi^2$ Value (P Value) | OR (95% CI) |
| VEGFA rs699947     |                |               |                            |
| Additive           | 7.37 (.007)    | 1.98 (1.21-3.25) | 4.24 (.039) | 1.75 (1.03-2.97) |
|                     | C 77 (65) 73 (49) |                | 57 (52) |               |
|                     | A 41 (35) 77 (51) |                | 53 (48) |               |
| Dominant (CC vs CA + AA) | 9.04 (.003)    | 3.11 (1.46-6.61) | 7.25 (.007) | 3.02 (1.33-6.86) |
|                     | C 27 (46) 16 (21) |                | 12 (22) |               |
|                     | A 32 (54) 59 (79) |                | 43 (78) |               |
| Recessive (CC + CA vs AA) | 1.57 (.210)    | 1.75 (0.72-4.25) | 0.18 (.675) | 1.23 (0.46-3.31) |
|                     | C 50 (85) 57 (76) |                | 45 (82) |               |
|                     | A 9 (15) 18 (24) |                | 10 (18) |               |
| VEGFA rs35569394   | 6.84 (.009)    | 1.92 (1.17-3.14) | 4.87 (.027) | 1.80 (1.07-3.07) |
| Additive           |                |               |                            |
|                     | D 74 (63) 70 (47) |                | 53 (48) |               |
|                     | I 44 (37) 80 (53) |                | 57 (52) |               |
| Dominant (DD vs ID + II) | 7.86 (.005)    | 2.99 (1.37-6.51) | 5.72 (.017) | 2.74 (1.18-6.36) |
|                     | D 24 (41) 14 (19) |                | 11 (20) |               |
|                     | I 35 (59) 61 (81) |                | 44 (80) |               |
| Recessive (DD + ID vs II) | 2.03 (.154)    | 1.88 (0.78-4.54) | 1.28 (.257) | 1.71 (0.67-4.42) |
|                     | D 50 (85) 56 (75) |                | 42 (76) |               |
|                     | I 9 (15) 19 (25) |                | 13 (24) |               |

*Data are reported as n (%) unless otherwise indicated. P values are unadjusted. Bolded P values indicate statistical significance (P < .05). ACL, anterior cruciate ligament; OR, odds ratio; VEGFA, vascular endothelial growth factor A.*

**Figure A1.** Pairwise linkage disequilibrium plot between vascular endothelial growth factor A polymorphisms rs699947 (single nucleotide polymorphism [SNP] 1) and rs35569394 (SNP 2). The red color shows a high $r^2$ value.