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ABSTRACT

Purpose: Muscle injury is the most common sports injury. Muscle stiffness, a risk factor for muscle injury, is lower in females than in males, implying that sex-related genetic polymorphisms influence muscle injury associated with muscle stiffness. The present study aimed to clarify the associations between two genetic polymorphisms (rs2234693 and rs9340799) in the estrogen receptor 1 gene (*ESR1*) and muscle injury or muscle stiffness. **Methods:** In study 1, a questionnaire was used to assess the muscle injury history of 1,311 Japanese top-level athletes. In study 2, stiffness of the hamstring muscles was assessed using ultrasound shear wave elastography in 261 physically active young adults. In both studies, rs2234693 C/T and rs9340799 G/A polymorphisms in the *ESR1* were analysed using the TaqMan SNP Genotyping Assay. **Results:** In study 1, genotype frequencies for *ESR1* rs2234693 were significantly different between the injured and non-injured groups in a C-allele dominant (CC+CT vs. TT: odds ratio [OR] = 0.62, 95% confidence interval [CI] = 0.43–0.91) and additive (CC vs. CT vs. TT: OR = 0.70, 95% CI = 0.53–0.91) model in all athletes. In study 2, hamstring muscle stiffness was lower in subjects with the CC+CT genotype than in those with the TT genotype; a significant linear trend was found (r = 0.135, P = 0.029). In contrast, no associations were observed between *ESR1* rs9340799 G/A and muscle injury or stiffness. **Conclusion:** Our results suggest that the *ESR1* rs2234693 C allele, in contrast to the T allele, provides protection against muscle injury by lowering muscle stiffness.

Key Terms: Single nucleotide polymorphism; estrogen receptor; muscle stiffness; injury prediction; athletes

INTRODUCTION

The incidence of sports-related injuries is associated with athletic success in both team and individual sports and may result in termination of an athlete's career in some situations. Muscle injury, especially hamstring strain, is the most common type of injury, and the incidence is increasing in sports involved in sprinting and jumping (1-4). Susceptibility to muscle injury is, at least in part, causally associated with impaired joint flexibility (5, 6), and muscle stiffness is one of the main components of joint flexibility (7, 8). Although it is well known that joint flexibility is influenced by environmental factors, a recent meta-analysis showed that 50% of the variability in joint flexibility is explained by genetic factors (9). Taken together, certain genetic polymorphisms are associated with muscle injury related to joint flexibility and/or muscle stiffness.

There are sex-based differences in joint flexibility and muscle stiffness; females exhibit higher joint flexibility and lower muscle stiffness than males (10). These sex-related differences may be related to circulating levels of sex hormones and their specific receptors such as estrogen and androgen receptors. The effects of estrogen, a female sex hormone, on skeletal muscle are mediated via estrogen receptors in both males and females (11-13). Indeed, previous studies have demonstrated that circulating levels of estrogen are negatively associated with muscle stiffness (14, 15), attributable to suppression of collagen synthesis (16). In addition, estrogen exerts

anti-inflammatory and anti-oxidants effects on skeletal muscles (17-19), which may protect against muscle injury. Thus, it is possible that estrogen receptors functionally influence muscle stiffness and injury.

We focused on two functional polymorphisms, namely, rs2234693 C/T (defined by restriction enzyme *Pvu*II) and rs9340799 G/A (defined by restriction enzyme *Xba*I), in the estrogen receptor 1 gene *(ESR1*). These polymorphisms are in the first intron of *ESR1*, positioned 397 and 351 base pairs upstream of exon 2, respectively. It has been suggested that these polymorphisms influence expression and activation of the ESR1 product and alter the action of estrogen. The rs2234693 C and rs9340799 G alleles in the *ESR1*, for example, are associated with higher gene expression and more favorable estrogen-induced actions (20-22). Based on these considerations, we hypothesized that these alleles provide a protective effect against muscle injury by lowering muscle stiffness. Thus, we aimed to clarify the association of two genetic polymorphisms in the *ESR1* with a history of muscle injury (study 1) and muscle stiffness (study 2).

METHODS

Study 1

We obtained data from a total of 2,181 Japanese athletes from March 2015 through November 2017. We assessed the history of up to 3 sports-related injuries (e.g., hernia, fracture, dislocation,

ligament damage, muscle injury, tendon injury, meniscal injury, concussion, etc.) in descending order of severity using questionnaires, according to a previous study (23). In the present study, we focused on non-contact muscle injuries diagnosed by medical practitioners. This information was collected using a questionnaire and thus relied on athlete recall. In addition, the primary sport, playing years, competition level, and other factors were assessed using questionnaires. We excluded athletes with no Japanese ancestry (n = 29) and a lack of questionnaire data (n = 159). Athletes with regional level (n = 669) and less than 3 years competition in their main sport (n = 13) were also excluded. After exclusion, we used data from 1,311 top-level (those competing on a national or international level) Japanese athletes for further analysis (see Figure, Supplemental Digital Content 1, Flow diagram for the selection of subjects in study 1, http://links.lww.com/MSS/B373). This study was included in The Japanese Human Athlome Project (J-HAP) in "Athlome Project Consortium"(24). Written informed consent was obtained from each athlete in accordance with the tenets of the Declaration of Helsinki. The study was approved by the Ethics Committees of Juntendo University, Nippon Sports Science University, and Tenri University.

Study 2

A total of 261 physically active young adults participated in study 2 (males: n = 152, females:

n = 109). We excluded subjects who took part in sports requiring high flexibility of the hip joint such as gymnastics and rhythmic gymnastics. There were no apparent neurological, orthopedic, or neuromuscular problems in any of the subjects. Subjects came to the laboratory at least 20 min before measurements were taken. The laboratory room temperature was maintained at a constant 24 ± 2 °C to minimize temperature-induced effects. The subjects were not allowed to perform warm-up or stretching exercises prior to testing. Written informed consent was obtained from each subject in accordance with the tenets of the Declaration of Helsinki. The study was approved by the Ethics Committee of Juntendo University.

Passive straight-leg raise (SLR) test

All SLR tests were performed on both legs by the same examiner. The subjects lay in a supine position with their legs straight on an examining bed. The pelvis and non-testing leg were secured on the bed with non-elastic straps to avoid posterior pelvic tilt. The examiner held one hand gently on the knee of the testing leg to maintain it straight and raised the leg with the other hand placed near the ankle until tightness was felt by the examiner. The hip flexion angle from the resting position was measured using a digital inclinometer (MLT-100, Sakai Medical, Japan) attached 3 to 4 cm proximal to the lateral malleolus and adopted as the score.

Muscle stiffness

The shear modulus (an index of stiffness; expressed in kPa) of the biceps femoris long head, as well as semitendinosus and semimembranosus of both legs was assessed using an ultrasound shear wave elastography system (Aixplorer ver. 10, Supersonic Imagine, France) with a linear probe (SL10-2, Supersonic Imagine, France) and pre-set for musculoskeletal (MSK) analysis (persistence = med, smoothing = 5). The subjects were seated on a bench with their hip flexed at 70° (0° = anatomical position) and the knee fully extended. This hip flexion angle was chosen according to a recent study (25) that aimed to determine an angle where the hamstring could be stretched to a tensioned state without pain and the shear modulus could be quantified for all subjects. The subjects were requested to fully relax the leg throughout the measurements. Measurements for each muscle were performed at 50% of the thigh length (the distance between the greater trochanter and the lateral epicondyle of the femur). The probe orientation was adjusted to visualize fascicles within the B-mode image for each muscle. Care was taken not to press and deform the muscles while scanning. The images were then acquired after ensuring a stable color distribution for a few seconds. The probe location was slightly adjusted prior to image acquisition when a defocused image with a large variation of shear modulus was observed.

Values for the SLR and hamstring muscle stiffness tests are shown as an average of both legs. The measurement orders for SLR/muscle stiffness and right/left were randomized across subjects.

For each variable, three measurements were performed and averaged. The shear modulus of three muscles was averaged to evaluate passive stiffness of the overall hamstring. The averaged SLR scores and hamstring stiffness values were used for subsequent analyses.

Genotyping analysis

Total DNA was isolated from saliva with an Oragene® DNA collection kit (DNA Genotek, ON, Canada) in accordance with the manufacturer's instructions. The concentration of DNA was quantified using a NanoDrop 8000 UV-Vis spectrophotometer (Thermo Fisher Scientific, DE, USA). DNA samples were stored at 4°C until use. Two *ESR1* polymorphisms (rs2234693 and rs9340799) were genotyped using a real-time thermocycler in the endpoint analysis mode (LightCycler 480, Roche Applied Science, Mannheim, Germany) using the TaqMan® SNP Genotyping Assay (assay ID: C___3163590_10 and C___3163591_10). A genotyping mixture (final volume of 5 µl) containing 2.5 µl TaqMan® GTXpress™ Master Mix (2×), 0.0625 µl TaqMan® SNP Genotyping Assay Mix (40×), 1.4375 µl sterilized water, and 1 µl genomic DNA (10 ng/µl) was used. Two to four negative controls were included on each plate. Genotypes were determined on the basis of the TaqMan® assay results using LightCycler® 480 SW, version 1.5 (Roche Molecular Systems). Approximately 100 samples were genotyped in duplicate for the polymorphisms analyzed in the present study, and we confirmed that the genotyping results

perfectly agreed between duplicates.

Statistical analysis

All data are expressed as means ± standard deviations (SD). Hardy-Weinberg equilibrium (HWE) of the *ESR1* rs2234693 and rs9340799 polymorphisms was tested using Chi-square test. In study 1, characteristics of subjects were analyzed using unpaired t-test or Chi-square test between muscle injured and non-muscle injured groups as appropriate. Logistic regression analysis was applied to investigate the associations between *ESR1* genotype and incidence of muscle injury. We adjusted for sex, the main sport (athletics or other), and playing years. Odds ratios (OR) and 95% confidence intervals (CI) were calculated under the dominant, recessive, and additive (allele counting) genetic models to estimate the degree of contribution to muscle injury for optimal genotype. Further, we analyzed the minimum Akaike's information criterion (AIC), which evaluates the best fitting genetic model. In study 2, anthropometric measurements were analyzed by using unpaired t-tests between males and females. A comparison of each parameter among three genotypes (rs2234693: CC vs. CT vs. TT, rs9340799: GG vs. GA vs. AA) was assessed using one-way analysis of variance (ANOVA), and linear trends were assessed using the Spearman correlation coefficient. Dominant and recessive models were assessed using unpaired t-tests (rs2234693: CC+CT vs. TT or CC vs. CT+TT, rs9340799: GG+GA vs. AA).

When there were fewer than 5 subjects (e.g., for the *ESR1* rs9340799 GG genotype), statistical analyses were not conducted in the present study. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using JMP Pro version 12 (SAS Institute).

RESULTS

Study 1

Characteristics of the studied athletes are shown in Table 1. There were no significant differences in age, height, and body mass between the muscle injured and non-muscle injured groups. There were significantly fewer years spent playing a primary sport, on average, in the muscle injured group than in the non-muscle injured group ($P = 0.017$). The rate of genotyping success was 1283/1311 (97.9%) and 1286/1311 (98.1%) for rs2234693 and rs9340799, respectively. Both polymorphisms were in HWE ($P = 0.928$ for rs2234693 and $P = 0.330$ for rs9340799). Results of the logistic regression analyses for rs2234693 and rs9340799 are shown in Table 2 and 3, respectively. Genotype frequencies for the *ESR1* rs2234693 C/T polymorphism were significantly different between the injured group and non-injured group in a C-allele dominant (CC+CT vs. TT: OR = 0.62, 95% CI = 0.43-0.91; AIC = 815.6) and an additive (CC vs. CT vs. TT: OR = 0.70, 95% CI = 0.53-0.91; AIC = 814.5) genetic model for all athletes (Table 2). Based on AIC, the additive model showed the best fit to explain the association between the

rs2234693 polymorphism and muscle injury. Similarly, in subgroup analyses for sex-based differences, genotype frequencies were significantly different between groups in a C-allele dominant genetic model (CC+CT vs. TT: OR = 0.62; 95% CI = 0.39-0.98; AIC = 550.6) in male athletes, and a C-allele recessive (CC vs. CT+TT: OR = 0.30; 95% CI = 0.07-0.88; AIC = 269.7) and an additive (CC vs. CT vs. TT: OR = 0.61; 95% CI = 0.36-0.98; AIC = 270.6) genetic models in female athletes. In contrast, no significant associations were observed for the *ESR1* rs9340799 G/A polymorphism for any group (Table 3).

Study 2

The rate of genotyping success was 261/261 (100%) and 259/261 (99.2%) for the rs2234693 and rs9340799 polymorphisms, respectively. Both polymorphisms were in HWE ($P = 0.574$ for rs2234693 and $P = 0.435$ for rs9340799). Characteristics of the study subjects are shown in Table 4. Male subjects were taller ($P < 0.001$), heavier ($P < 0.001$), had lower SLR scores ($P < 0.001$), and had stiffer hamstring muscle ($P = 0.012$) than female subjects.

Figure 1 shows stiffness of the hamstring muscle for the *ESR1* rs2234693 C/T polymorphism. Subjects with the CC+CT genotype showed significantly lower stiffness of the hamstring muscle than subjects with the TT genotype (28.0 ± 7.2 vs. 30.0 ± 8.6, $P = 0.043$), and the T allele of the *ESR1* rs2234693 polymorphism was positively associated with stiffness of the hamstring muscle

(r = 0.135, *P* = 0.029) (Figure 1-A). In males, stiffness of the hamstring muscle was significantly lower in subjects with the CC+CT genotype than in those with the TT genotype (28.9 ± 7.4 vs. 31.8 ± 9.5, *P* = 0.046) (Figure 1-B). In females, there were no significant differences in muscle stiffness among rs2234693 C/T genotypes (Figure 1-C). Table, Supplemental Digital Content 2 shows the detailed characteristics of subjects with the *ESR1* rs2234693 C/T polymorphism (see Table, Supplemental Digital Content 2, characteristics of subjects with the *ESR1* rs2234693 genotype in accordance with the alleles present, http://links.lww.com/MSS/B374). The T allele of this polymorphism was positively associated with stiffness of the biceps femoris and semimembranosus muscles in all subjects, and with stiffness of the semimembranosus muscle in male subjects.

Figure 2 shows stiffness of the hamstring muscle for the *ESR1* rs9340799 G/A polymorphism in all subjects. There were no significant differences in hamstring muscle stiffness among G/A genotypes for any group (Figure 2-A, B, C). Table, Supplemental Digital Content 3 shows the detailed characteristics of subjects with the *ESR1* rs9340799 G/A polymorphism (see Table, Supplemental Digital Content 3, characteristics of subjects with the *ESR1* rs9340799 genotype in accordance with the alleles present, http://links.lww.com/MSS/B375). The A allele of this polymorphism was positively associated with stiffness of the semitendinosus muscle in female subjects.

DISCUSSION

Preventing muscle injury is crucial in both professional and amateur sports, and muscle injury occurs as a consequence of intrinsic (i.e., age, sex, genetic polymorphisms, etc.) and extrinsic factors (i.e., exercise intensity, exposure time to practice and play matches, practice environments such as surface, etc.). Several previous studies have indicated the importance of intrinsic factors for preventing sports-related injuries (26, 27). Indeed, regarding musculoskeletal soft tissue injuries such as anterior cruciate ligament injury and Achilles tendon injury, several studies have reported that the association between genetic polymorphisms and injuries in case-control association analysis (28-32). In addition to these case-control association studies, other studies have investigated the functional role of injury-related genetic polymorphisms (33-35). On the other hand, although the details of specific genetic polymorphisms as determinants for muscle injury risk are gradually being elucidated (36-44); the sample sizes of previous studies have been relatively small (54–257 athletes). In addition, there are no studies on the effect of muscle injury-related genetic polymorphisms on physiological functions (e.g., muscle stiffness). Therefore, it is difficult to draw firm conclusions regarding the associations between genetic polymorphisms and muscle injury. It is important to conduct replication and/or functional studies in addition to a case-control association study in large cohort to avoid false positive results in Sports Genetics. In study 1 of the present report, we examined associations

between two *ESR1* genetic polymorphisms and the prevalence of muscle injuries in 1,311 top-level Japanese athletes. In study 2 of the present report, we investigated physiological functions such as muscle elasticity in relation to two *ESR1* genetic polymorphisms in physically active subjects. We found that the *ESR1* rs2234693 polymorphism was associated with muscle injury as well as muscle stiffness. Specifically, individuals with the C allele showed resistance against muscle injury and lower muscle stiffness than those with the T allele. As such, the present design, i.e., the combined investigation of large case-control association and functional studies, will make a substantial contribution toward uncovering the associations between genetic polymorphisms and sports-related injuries.

A lack of joint flexibility and high muscle stiffness are risk factors for muscle injury (5, 6) and can be reduced via the actions of estrogen (14, 15). Estrogenic activity on muscles is mediated by means of estrogen-receptors in skeletal muscle tissue in both males and females (11-13). Moreover, previous studies have suggested that the *ESR1* rs2234693 C/T polymorphism alters its own gene expression, with the C allele being associated with higher gene expression and more favorable estrogen-induced actions (20-22). In study 1 of the present report, the *ESR1* rs2234693 C allele showed greater protective effect against muscle injury than the T allele in athletes. In study 2, there was significantly lower muscle stiffness in individuals with the *ESR1* rs2234693 C allele than in individuals with the T allele in physically active subjects. Considering the previous

and present findings, it is possible that the *ESR1* rs2234693 polymorphism results in increased estrogenic activity and lower muscle stiffness, which is a possible physiological mechanism underlying the relation between the *ESR1* rs2234693 polymorphism and muscle injury.

Muscle stiffness is mainly influenced by collagenous connective tissue (45), and it is well known that estrogen suppresses collagen synthesis (16). In the human α1 collagen gene, activator protein 1 (AP-1) binding sites are present on the first intron (46), and estrogen signaling regulates AP-1 binding sites (47). Collectively, these data suggest that estrogen lowers muscle stiffness by suppressing collagen synthesis. Indeed, in study 2 of the present report, we found that the subjects with the *ESR1* rs2234693 C allele, which is reported to be associated with higher *ESR1* gene expression and increased estrogen-induced actions (20-22), showed significantly lower muscle stiffness compared to the subjects with the TT genotype. Taken together, these data suggest that estrogen-associated suppression of collagen synthesis is a possible explanation for the relation between the *ESR1* rs2234693 polymorphisms and muscle stiffness.

It has been suggested that estrogen has muscle protective effects, including anti-inflammatory and anti-oxidant properties (17-19). It is well known that strenuous and repeated contractions are associated with exercise-induced muscle damage and soreness (48, 49). Williams et al. reported that high levels of estrogen suppress the increases in creatine kinase levels observed after

prolonged aerobic exercise in young females (18). Additionally, Feng et al. suggested that estrogen increases antioxidant capacity, resulting in reduced muscle damage and accelerated muscle regeneration after muscle strain injury in rats (19). Thus, the biological activity of estrogen is associated with muscle injury. Taken together, these data suggest that variations in estrogen activity attributable to the *ESR1* rs2234693 polymorphism may be associated with muscle injury through anti-inflammatory and anti-oxidant effects.

The present study has several limitations, including a cross-sectional study design; follow-up studies are needed to confirm these findings. In addition, the sample size of female subjects in study 1 was relatively small. In rs2234693 polymorphism in the *ESR1*, the statistical power to detect an association with an odds ratio of 2.0 and minor allele frequency of 0.43 in a group with 133 muscle injured and 1150 non-muscle injured is 0.966. When subjects were divided into two groups, namely, males and females, statistical powers dropped to lower values of 0.874 for males and 0.570 for females. Thus, further replication and functional studies with larger sample size are necessary to confirm the present findings, especially in females because of the lack of statistical power. Further, we measured muscle stiffness in hamstrings under resting conditions; muscle injuries typically occur in situations where muscles are contracting. Thus, we need to investigate active muscle stiffness in addition to passive muscle stiffness in the future.

In study 1 of the present report, we found that the *ESR1* rs2234693 C allele has more of a protective effect against muscle injury than the T allele in top-level Japanese athletes. In study 2 of the present report, the *ESR1* rs2234693 C allele resulted in significantly less muscle stiffness than the T allele in physically active subjects. Our results suggest that the *ESR1* rs2234693 C allele, in contrast to the T allele, provides protection against muscle injury by lowering muscle stiffness.

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Conflicts of interest

No conflicts of interest, financial or otherwise, are declared by the authors. The results of the present study do not constitute endorsement by ACSM. All authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

References

1. Orchard J, Marsden J, Lord S, Garlick D. Preseason hamstring muscle weakness associated with hamstring muscle injury in Australian footballers. Am J Sports Med. 1997;25(1):81-5.

2. Opar DA, Williams MD, Shield AJ. Hamstring strain injuries: factors that lead to injury and re-injury. Sports Med. 2012;42(3):209-26.

3. Ekstrand J, Walden M, Hagglund M. Hamstring injuries have increased by 4% annually in men's professional football, since 2001: a 13-year longitudinal analysis of the UEFA Elite Club injury study. Br J Sports Med. 2016;50(12):731-7.

4. Ekstrand J, Hagglund M, Kristenson K, Magnusson H, Walden M. Fewer ligament injuries but no preventive effect on muscle injuries and severe injuries: an 11-year follow-up of the UEFA Champions League injury study. Br J Sports Med. 2013;47(12):732-7.

5. Watsford ML, Murphy AJ, McLachlan KA, et al. A prospective study of the relationship between lower body stiffness and hamstring injury in professional Australian rules footballers. Am J Sports Med. 2010;38(10):2058-64.

6. Witvrouw E, Danneels L, Asselman P, D'Have T, Cambier D. Muscle flexibility as a risk factor for developing muscle injuries in male professional soccer players. A prospective study. Am J Sports Med. 2003;31(1):41-6.

7. Miyamoto N, Hirata K, Kanehisa H. Effects of hamstring stretching on passive muscle stiffness vary between hip flexion and knee extension maneuvers. Scand J Med Sci Sports. 2017;27(1):99-106.

8. Magnusson SP, Simonsen EB, Aagaard P, Boesen J, Johannsen F, Kjaer M. Determinants of musculoskeletal flexibility: viscoelastic properties, cross-sectional area, EMG and stretch tolerance. Scand J Med Sci Sports. 1997;7(4):195-202.

9. Schutte NM, Nederend I, Hudziak JJ, de Geus EJ, Bartels M. Differences in Adolescent Physical Fitness: A Multivariate Approach and Meta-analysis. Behav Genet. 2016;46(2):217-27.

10. Morse CI. Gender differences in the passive stiffness of the human gastrocnemius muscle during stretch. Eur J Appl Physiol. 2011;111(9):2149-54.

11. Wiik A, Ekman M, Johansson O, Jansson E, Esbjornsson M. Expression of both oestrogen receptor alpha and beta in human skeletal muscle tissue. Histochem Cell Biol. 2009;131(2):181-9.

12. Lemoine S, Granier P, Tiffoche C, Rannou-Bekono F, Thieulant ML, Delamarche P. Estrogen receptor alpha mRNA in human skeletal muscles. Med Sci Sports Exerc. 2003;35(3):439-43.

13. Brown M, Ning J, Ferreira JA, Bogener JL, Lubahn DB. Estrogen receptor-alpha and

-beta and aromatase knockout effects on lower limb muscle mass and contractile function in female mice. Am J Physiol Endocrinol Metab. 2009;296(4):E854-61.

14. Eiling E, Bryant AL, Petersen W, Murphy A, Hohmann E. Effects of menstrual-cycle hormone fluctuations on musculotendinous stiffness and knee joint laxity. Knee Surg Sports Traumatol Arthrosc. 2007;15(2):126-32.

15. Bell DR, Myrick MP, Blackburn JT, Shultz SJ, Guskiewicz KM, Padua DA. The effect of menstrual-cycle phase on hamstring extensibility and muscle stiffness. J Sport Rehabil. 2009;18(4):553-63.

16. Kwan G, Neugarten J, Sherman M, et al. Effects of sex hormones on mesangial cell proliferation and collagen synthesis. Kidney Int. 1996;50(4):1173-9.

17. Tiidus PM. Can oestrogen influence skeletal muscle damage, inflammation, and repair? Br J Sports Med. 2005;39(5):251-3.

18. Williams T, Walz E, Lane AR, Pebole M, Hackney AC. The effect of estrogen on muscle damage biomarkers following prolonged aerobic exercise in eumenorrheic women. Biol Sport. 2015;32(3):193-8.

19. Feng X, Li GZ, Wang S. Effects of estrogen on gastrocnemius muscle strain injury and regeneration in female rats. Acta Pharmacol Sin. 2004;25(11):1489-94.

20. Herrington DM, Howard TD, Brosnihan KB, et al. Common estrogen receptor

polymorphism augments effects of hormone replacement therapy on E-selectin but not C-reactive protein. Circulation. 2002;105(16):1879-82.

21. Nordstrom P, Glader CA, Dahlen G, et al. Oestrogen receptor alpha gene polymorphism is related to aortic valve sclerosis in postmenopausal women. J Intern Med. 2003;254(2):140-6.

22. Ryan J, Canonico M, Carcaillon L, et al. Hormone treatment, estrogen receptor polymorphisms and mortality: a prospective cohort study. PLoS One. 2012;7(3):e34112.

23. Fuller CW, Ekstrand J, Junge A, et al. Consensus statement on injury definitions and data collection procedures in studies of football (soccer) injuries. Br J Sports Med. 2006;40(3):193-201.

24. Pitsiladis YP, Tanaka M, Eynon N, et al. Athlome Project Consortium: a concerted effort to discover genomic and other "omic" markers of athletic performance. Physiol Genomics. 2016;48(3):183-90.

25. Miyamoto N, Hirata K, Kimura N, Miyamoto-Mikami E. Contributions of Hamstring Stiffness to Straight-Leg-Raise and Sit-and-Reach Test Scores. Int J Sports Med. 2018;39(2):110-114.

26. Vlahovich N, Fricker PA, Brown MA, Hughes D. Ethics of genetic testing and research in sport: a position statement from the Australian Institute of Sport. Br J Sports Med. 2017;51(1):5-11.

27. Collins M. Genetic risk factors for soft-tissue injuries 101: a practical summary to help clinicians understand the role of genetics and 'personalised medicine'. Br J Sports Med. 2010;44(13):915-7.

28. Abrahams Y, Laguette MJ, Prince S, Collins M. Polymorphisms within the COL5A1 3'-UTR that alters mRNA structure and the MIR608 gene are associated with Achilles tendinopathy. Ann Hum Genet. 2013;77(3):204-14.

29. Mokone GG, Schwellnus MP, Noakes TD, Collins M. The COL5A1 gene and Achilles tendon pathology. Scand J Med Sci Sports. 2006;16(1):19-26.

30. Posthumus M, September AV, Keegan M, et al. Genetic risk factors for anterior cruciate ligament ruptures: COL1A1 gene variant. Br J Sports Med. 2009;43(5):352-6.

31. Posthumus M, September AV, O'Cuinneagain D, van der Merwe W, Schwellnus MP, Collins M. The COL5A1 gene is associated with increased risk of anterior cruciate ligament ruptures in female participants. Am J Sports Med. 2009;37(11):2234-40.

32. September AV, Cook J, Handley CJ, van der Merwe L, Schwellnus MP, Collins M. Variants within the COL5A1 gene are associated with Achilles tendinopathy in two populations. Br J Sports Med. 2009;43(5):357-65.

33. Abrahams S, Posthumus M, Collins M. A polymorphism in a functional region of the COL5A1 gene: association with ultraendurance-running performance and joint range of motion.

Int J Sports Physiol Perform. 2014;9(3):583-90.

34. Foster BP, Morse CI, Onambele GL, Williams AG. Human COL5A1 rs12722 gene polymorphism and tendon properties in vivo in an asymptomatic population. Eur J Appl Physiol. 2014;114(7):1393-402.

35. Foster BP, Morse CI, Onambele GL, Williams AG. Variants within the MMP3 gene and patellar tendon properties in vivo in an asymptomatic population. Eur J Appl Physiol. 2014;114(12):2625-34.

36. Myosotis M, Sarah V, Claudia C, et al. ACTN3 R577X Polymorphism Is Associated With the Incidence and Severity of Injuries in Professional Football Players. Clin J Sport Med. 2017; doi: 10.1097/JSM.0000000000000487.

37. Massidda M, Eynon N, Bachis V, et al. Influence of the MCT1 rs1049434 on Indirect Muscle Disorders/Injuries in Elite Football Players. Sports Med Open. 2015;1(1):33.

38. Massidda M, Corrias L, Bachis V, et al. Vitamin D receptor gene polymorphisms and musculoskeletal injuries in professional football players. Exp Ther Med. 2015;9(5):1974-8.

39. Massidda M, Bachis V, Corrias L, Piras F, Scorcu M, Calo CM. Influence of the COL5A1 rs12722 on musculoskeletal injuries in professional soccer players. J Sports Med Phys Fitness. 2015;55(11):1348-53.

40. Larruskain J, Celorrio D, Barrio I, et al. Genetic Variants and Hamstring Injury in

Soccer: An Association and Validation Study. Med Sci Sports Exerc. 2018;50(2):361-8.

41. Pruna R, Ribas J, Montoro JB, Artells R. The impact of single nucleotide polymorphisms on patterns of non-contact musculoskeletal soft tissue injuries in a football player population according to ethnicity. Med Clin (Barc). 2015;144(3):105-10.

42. Pruna R, Artells R, Ribas J, et al. Single nucleotide polymorphisms associated with non-contact soft tissue injuries in elite professional soccer players: influence on degree of injury and recovery time. BMC Musculoskelet Disord. 2013;14:221.

43. Pruna R, Artells R, Lundblad M, Maffulli N. Genetic biomarkers in non-contact muscle injuries in elite soccer players. Knee Surg Sports Traumatol Arthrosc. 2017;25(10):3311-8.

44. Miyamoto N, Miyamoto-Mikami E, Hirata K, Kimura N, Fuku N. Association analysis of the ACTN3 R577X polymorphism with passive muscle stiffness and muscle strain injury. Scand J Med Sci Sports. 2018;28(3):1209-14.

45. Gajdosik RL. Passive extensibility of skeletal muscle: review of the literature with clinical implications. Clin Biomech (Bristol, Avon). 2001;16(2):87-101.

46. Slack JL, Liska DJ, Bornstein P. Regulation of expression of the type I collagen genes. Am J Med Genet. 1993;45(2):140-51.

47. Weisz A, Bresciani F. Estrogen regulation of proto-oncogenes coding for nuclear

proteins. Crit Rev Oncog. 1993;4(4):361-88.

48. Newham DJ, Mills KR, Quigley BM, Edwards RH. Pain and fatigue after concentric and eccentric muscle contractions. Clin Sci (Lond). 1983;64(1):55-62.

49. Armstrong RB. Mechanisms of exercise-induced delayed onset muscular soreness: a brief review. Med Sci Sports Exerc. 1984;16(6):529-38.

Figure legends

Figure 1. Stiffness of hamstring muscles in all subjects (**A**), male subjects (**B**), and female subjects (**C**) with the *ESR1* rs2234693 genotype in accordance with the alleles present (i.e., CC, CT, or TT).

Figure 2. Stiffness of hamstring muscles in all subjects (**A**), male subjects (**B**), and female subjects (**C**) with the *ESR1* rs9340799 genotype in accordance with the alleles present (i.e., GG, GA, or AA).

Table 1. Characteristics of athletes recruited in study 1

	Muscle injured (n = 133)	Non-muscle injured (n = 1178)	*P*-value
Sex			0.736
Male, n (%)	90 (67.7)	780 (66.2)	
Female, n (%)	43 (32.3)	398 (33.8)	
Age, years	20.2 ± 1.7	20.6 ± 2.9	0.128
Height, cm	170.0 ± 8.2	169.5 ± 8.3	0.670
Body mass, kg	64.7 ± 11.4	65.3 ± 12.2	0.526
Main sport			**< 0.001**
Athletics, n (%)	63 (47.4)	243 (20.6)	
Soccer, n (%)	43 (32.3)	437 (37.1)	
Baseball, n (%)	10 (7.5)	48 (4.1)	
Basketball, n (%)	1 (0.8)	34 (2.9)	
Martial arts, n (%)	3 (2.2)	157 (13.3)	
Other, n (%)	13 (9.8)	259 (22.0)	
Years playing main sport, years	10.7 ± 3.7	11.6 ± 3.9	**0.017**

Data are shown as the mean ± SD. Bold: $P < 0.05$.

Table 2. Associations of rs2234693 genotype with odds ratio of muscle injury with several adjustments for candidate confounding risk factors

	Genotype	n (%)		OR [95% CI], AIC					
		Muscle injured	Non-muscle injured	Dominant CC+CT vs. TT	*P* value	Recessive CC vs. CT+TT	*P* value	Additive CC vs. CT vs. TT	*P* value
All	CC	18 (13.5)	219 (19.0)	**0.62 [0.43-0.91]**	**0.016**	0.63 [0.36-1.04]	0.069	**0.70 [0.53-0.91]**	**0.008**
	CT	61 (45.9)	566 (49.2)	**815.6**		818.2		**814.5**	
	TT	54 (40.6)	365 (31.7)						
Male	CC	15 (16.7)	142 (18.6)	**0.62 [0.39-0.98]**	**0.043**	0.80 [0.42-1.41]	0.458	0.74 [0.53-1.03]	0.073
	CT	38 (42.2)	374 (49.1)	**550.6**		554.2		551.6	
	TT	37 (41.1)	246 (32.3)						
Female	CC	3 (7.0)	77 (19.9)	0.64 [0.33-1.27]	0.203	**0.30 [0.07-0.88]**	**0.026**	**0.61 [0.36-0.98]**	**0.042**
	CT	23 (53.5)	192 (49.5)	273.1		**269.7**		**270.6**	
	TT	17 (39.5)	119 (30.7)						

OR: odds ratio, CI: confidence intervals, AIC: Akaike's information criterion, Bold: $P < 0.05$.

In all subjects, values were adjusted by sex, main sport and playing years.

In male and female, values were adjusted by main sport and playing years.

TT genotype, CT+TT genotype and TT genotype were the references (OR = 1.00) in C-allele dominant, C-allele recessive and C-allele additive models, respectively.

The odds ratio of the additive model is shown based on the allele counts, i.e., homozygous genotype CC has an odds ratio twice that of heterozygous genotype CT.

Table 3. Associations of rs9340799 genotype with odds ratio of muscle injury with several adjustments for candidate confounding risk factors

	Genotype	n (%)		OR [95% CI], AIC					
		Muscle injured	Non-muscle injured	Dominant GG+GA vs. AA	*P* value	Recessive GG vs. GA+AA	*P* value	Additive GG vs. GA vs. AA	*P* value
All	GG	3 (2.3)	33 (2.9)	0.72 [0.47-1.07]	0.104	0.72 [0.17-2.11]	0.590	0.75 [0.51-1.07]	0.110
	GA	33 (25.0)	355 (30.8)	816.6		819.0		816.7	
	AA	96 (72.7)	766 (66.4)						
Male	GG	3 (3.4)	24 (3.1)	0.71 [0.42-1.16]	0.179	1.07 [0.25-3.26]	0.915	0.78 [0.49-1.19]	0.257
	GA	21 (23.6)	230 (30.1)	550.6		552.4		551.1	
	AA	65 (73.0)	511 (66.8)						
Female	GG	0 (0)	9 (2.3)	0.73 [0.34-1.45]	0.370	0.00 [0.00-NA]	0.120	0.68 [0.34-1.28]	0.245
	GA	12 (27.9)	125 (32.1)	274.1		272.3		273.5	
	AA	31 (72.1)	255 (65.5)						

OR: odds ratio, CI: confidence intervals, AIC: Akaike's information criterion, Bold: $P < 0.05$.

In all subjects, values were adjusted by sex, main sport and playing years.

In male and female, values were adjusted by main sport and playing years.

AA genotype, GA+AA genotype and AA genotype were the references (OR = 1.00) in G-allele dominant, G-allele recessive and G-allele additive models, respectively.

The odds ratio of the additive model is shown based on the allele counts, i.e., homozygous genotype GG has an odds ratio twice that of heterozygous genotype GA.

Table 4. Characteristics of subjects recruited in study 2

	All (n = 261)	Male (n = 152)	Female (n = 109)	*P*-value (Male vs. Female)
Age, year	20.4 ± 2.0	20.4 ± 1.4	20.4 ± 2.6	0.856
Height, cm	169.3 ± 9.0	174.5 ± 6.8	162.1 ± 6.3	**< 0.001**
Body mass, kg	63.4 ± 10.8	67.9 ± 11.1	57.3 ± 6.3	**< 0.001**
SLR score, degree[#]	69.5 ± 10.2	65.3 ± 8.3	75.4 ± 9.7	**< 0.001**
Hamstring muscle stiffness, kPa	28.7 ± 7.8	29.7 ± 8.2	27.3 ± 7.0	**0.012**

Data are shown as the mean ± SD. Bold: $P < 0.05$.

SLR: passive straight-leg raise.

[#]Data are available 151 and 108 subjects in male and female, respectively.