Association between the ACE I/D polymorphism and muscle injuries in Italian and Japanese elite football players

Abstract

ACE I/D polymorphism has been recently associated with the susceptibility to inflammation and muscle damage after exercise. The aim of this study was to understand the association between the *ACE* I/D polymorphism and muscle injuries in a large cohort of elite football players from two different countries. Seven hundred and ten male elite football players from Italy (n=341, age 19.9±5yrs) and Japan (n=369, age 20.8±1.4) were recruited for the study. Genomic DNA was extracted from either the buccal epithelium or saliva using a standard protocol. Structural-mechanical injuries and functional muscle disorders were recorded from 2009 to 2018. A meta-analysis was performed using Review Manager 5.3.5. In the Japanese cohort, the *ACE* I/D polymorphism was significantly associated with muscle injury using the D-dominant model (OR: 0.48, 95% CI: 0.24–0.97, P=0.040). The meta-analysis showed that in the pooled model (Italian and Japanese populations), the frequencies of the DD+ID genotypes were significantly lower in the injured groups than in non-injured groups (OR: 0.61, 95% CI: 0.38–0.98, P=0.040) with a low degree of heterogeneity (I^2 = 0%). Our findings suggest that the *ACE* I/D polymorphism could influence the susceptibility to developing muscle injuries among football players.

Keywords: angiotensin-converting enzyme; gene; muscle disorders; polymorphism; soccer.

Introduction

Athletic muscle injuries are a heterogeneous group of muscle disorders and represent one of the most substantial medical problems in professional football (Ueblacker et al., 2016). Indirect muscle injuries are well described disorders caused in the absence of a direct external trauma (Ekstrand et al., 2011). A football professional level team consists of 25 players can expect 15 muscle injuries each season, with 96% of them occurring in non-contact situations (Ekstrand et al., 2011). About 92% of indirect muscle injuries affect the four major muscle groups of the lower limbs: namely hamstrings (37%), adductors (23%), quadriceps (19%) and calf muscles (13%) (Ekstrand et al., 2011). Identifying the variables that predispose athletes to higher/lower risks of muscle injuries is therefore of interest to coaches, physiologists, and the medical community and may help in identifying the risk factors faced by football players, in identifying different recovery strategies, and in suggesting individualized training methods (Jones et al., 2016).

Despite a strict control of environmental factors, there is a wide inter-individual variability in exercise-induced muscle damage (EIMD) and injuries (Baumert et al., 2016). Considering the members of an elite team, where athletes are usually exposed to the same workout and diet regime, there are footballers that show a higher number and severity of injuries compared to the others who rarely get injured. This inter-individual variability cannot be predicted based on age, ethnicity, body composition, and the fitness level of each subject, suggesting that other factors, such as genetic variations, could be important in determining susceptibility to injury. Specifically, there is growing evidence that genetics contribute to phenotypic responses to EIMD, to the range of increases in plasma creatine phosphokinase (CK) (a marker of muscle damage), and to the degree of muscle damage in response to a given exercise regime (Lindpaintner et al., 1995; Myerson et al., 1999).

Despite the fact that a certain amount of EIMD is necessary for the adaptation process, intense damage or incomplete recovery from EIMD are the factors that can increase injury risk (Baumert et al., 2016).

Recently, we and others have shown an association between several genetic polymorphisms, which are located in the *ACTN3*, *MCT1*, *VDR,* and *ESR1* genes, and the susceptibility to developing muscle damage and musculoskeletal injuries in professional football players (Coelho al., 2019; Massidda et al., 2015a, 2015b, 2019) and other top-level athletes (Kumagai et al., 2019). Among the genetic variants that have a potential influence on the pathogenesis of muscle injuries one variant of interest is the insertion/deletion (I/D) polymorphism (rs1799752) in angiotensin I-converting enzyme (*ACE*), the first gene investigated in the context of human athletic performance (Montgomery et al., 1997). ACE is found both in the circulation and in tissues (Rigat et al., 1990).

Higher levels of ACE activity are associated with the deletion form of this variant (the D-allele) in both European (Rigat et al., 1990) and East Asians (Yamamoto et al., 1997). The *ACE* I/D polymorphism has also been associated with a variety of exercise-related phenotypes, including muscle strength (Pescatello et al., 2006), muscle metabolism (Vaughan et al., 2016), muscle volume (Charbonneau et al., 2008), cardiac growth response to exercise (Montgomery et al., 1997), differences in skeletal muscle fibre distribution, composition, and capillarization (Flück et al., 2019), and fatigue resistance in response to physical training (Kang et al., 2012). Although there is some inconsistency in the literature (Massidda et al., 2011; Papadimitriou et al., 2018), numerous studies have found an association between the *ACE* polymorphism and elite athlete status (Ma et al., 2013). This association is more consistent for the deleted form of the variant (the D allele), which seems to provide an advantage in sports requiring short bursts of power or sprinting (Papadimitriou et al., 2016).

The association between the *ACE* I/D polymorphism and elite athlete status might also be explained by a genotype link with the susceptibility to exertional muscle damage and injury (Baumert et al., 2016). Different concentrations of circulatory creatine kinase (CK), which is a marker of EIMD, have also been observed among the different *ACE* I/D genotypes after eccentric exercise (Yamin et al., 2007). Specifically, individuals with EIMD harbouring one or two copies of the D-allele had lower increases and lower peak CK values than individuals with the II genotype. Furthermore, there was an association of the D-allele with a lower CK response after triathlon (Del Coso et al., 2017a) and marathon (Del Coso et al., 2017b) races.

Moreover, EIMD susceptibility may be related to the inflammatory process modulated by renin-angiotensin system (RAS) and kallikrein-kinin system (KKS). A level of bradykinin (which induces local inflammation, vasodilation, and extravasation of myocellular proteins) is suppressed by a higher ACE activity. A recent study by Sierra et al. (2019) reported that DD genotype (high ACE activity) of the ACE I/D and the +9/+9 genotype (low bradykinin activity) of the bradykinin B2 receptor gene −9/+9 polymorphisms are associated with low inflammatory response as a result of muscle damage induced by exercise. Thus, difference ACE activity by the I/D polymorphism in the ACE might influence EIMD susceptibility via inflammatory responsiveness.

These findings suggest that the D-allele of the *ACE* I/D polymorphism is associated with a lower susceptibility to muscle damage.

We hypothesized that the *ACE* D-allele could also be associated with a lower susceptibility to muscle injuries in elite football players, because, compared to the *ACE*-I allele, it is possible that the lower muscle damage seen following strenuous exercise could also protect from muscle injuries

such as muscle strain. The aim of this study was to clarify the association between the *ACE* I/D polymorphism and muscle injuries in a large cohort of football players from two different countries.

Materials and Methods

Participants

Seven hundred and ten male elite football players participated in the study. Participants were all Italian for ≥ 3 generations (n=341, age 19.9±5years; height 178.1±7.1cm; weight 69.7±9.1kg) or Japanese for ≥ 2 generations (n=369, age 20.8±1.4 years; height 174.1±6.2cm; weight 69.1±6.8 kg). The Italian football players participated in the Official National Football Championship (Serie A, Primavera, Allievi, Giovanissimi) and most of them were members of the Italian national team. The athletes trained for 8 weeks (25.0 ± 10.3 h/week) in the preseason period and for 38 weeks during the competitive season (10.0 ± 7.4 h/week). The players who joined/left the team were included/excluded from the date when they joined/left. The Japanese football players were the participants in the Japanese Human Athlome Project (J-HAP) in the "Athlome Project Consortium" (Pitsiladis et al., 2016); and players with national or international level performances and with more than 3 years of competition in football were included in the present study.

The study protocol conformed to the Declaration of Helsinki for Human Research of 1974 (last modified in 2000) and was approved by the Local Ethical Committee of Cagliari University (Comitato Etico dell'Azienda Ospedaliero-Universitaria di Cagliari) and the Ethics Committee of Juntendo University. Written informed consent was obtained from all participants.

Data Collection

An indirect muscle injury was defined as any muscle disorder, caused in the absence of a direct external trauma, occurring during practice that prevented a player from participating in training or match play for at least one day after the day of onset (Fuller et al., 2006). Among the indirect muscle injury, the present study focused on structural-mechanical injuries and functional muscle disorders assessed by medical doctors/practitioners.

In the Italian cohort, the study design mirrored the consensus on definitions and data collection procedures in studies involving football injuries outlined in the consensus document (Fuller et al., 2006) and by UEFA (Hagglund et al., 2005). Data were collected from 2009 to 2018 and the follow-up time for injuries was between 1 and 8 years. The injury data for players who left the team during the season, for example due to a trade, were included only for the period they were on the roster. Players with an existing injury were not excluded from the study; however, their existing

injuries were not included in the study. Registration of a muscle injury was based on a clinical examination by the team's medical staff. Time loss due to injury was recorded on a weekly basis by the team's medical staff (physicians and coaches) using a standardized injury report form (FIFA F-Marc - http://www.f-marc.com/) during the preseason and during the regular season. Ultrasound and magnetic resonance imaging scans were used to morphologically classify the injuries. Muscle injuries included in the study were classified according to a scheme devised by Muller-Wohlfarth (Muller-Wohlfarth et al., 2013) derived from a consensus statement for sports injuries. In the Italian sample, training exposure was defined as any team-based or individual physical activity conducted under the control or guidance of the team's coaching and fitness staff that was aimed at maintaining or improving players' football skills or physical condition. Matches between teams were considered as training exposure. Any match activity that was part of a player's rehabilitation from injury was not recorded as a match exposure.

In the Japanese cohort, the history of up to three sports-related injuries in descending order of severity was assessed by questionnaire, as previously described (Kumagai et al., 2019; Miyamoto-Mikami et al., 2019). Briefly, for each injury, name of the injury, injured body part, type of the injury, injury cause (overuse or trauma and contact or not), when the injury occurred, time loss due to the injury, whether medical practitioner has diagnosed the injury or not, and number of injury of the same type at the same site were asked in the questionnaire. In the present study, we focused only on indirect muscle injuries diagnosed by medical practitioners.

In both the cohorts, injuries were categorized into four degrees of severity based on the number of days of absence: minimal (1–3 days), mild (4–7 days), moderate (8–28 days), and severe (>28 days) (Fuller et al., 2006). If a player had multiple muscle injuries, data relating to the most severe injury were used. Participants with direct muscle injuries (contusions and lacerations) were excluded from the study.

DNA Analyses

Italian cohort. A buccal swab was collected for each participant and stored in a tube with 1ml of ethanol. Genomic DNA was extracted using a buccal swab according to the manufacturer's directions provided with a commercially available kit (Qiagen, Hilden, Germany). The concentration of extracted DNA was determined using a fluorometric method (through Qubit by Invitrogen, Waltham, MA, USA). On average, the concentration of the DNA obtained was 20 mg/mL, which is sufficient for performing PCR. The polymorphic region of the *ACE* gene was amplified using the following primers: 5′-CTGGAGACCACTCCCATCCTTTCT-3′ and 5′-ATGTGGCCATCACATTCGTCAGAT-3′(Rigat et al., 1990). The amplified PCR fragment was

subjected to electrophoresis in a 2% agarose gel to allow for identification of the three different genotypes: DD, ID, and II. Identification of the bands was made using comparison to a marker that allows for the exact recognition of the size of each band (I allele: 490bp; D allele: 190 bp).

Japanese cohort. Genomic DNA was isolated from the saliva of all participants using an Oragene® DNA Collection Kit (DNA Genotek, ON, Canada) and quantified using a NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, DE, USA). Genotyping of the *ACE* I/D polymorphism was performed using the rs4341 polymorphism, which is in perfect LD with the *ACE* I/D polymorphisms (Glenn et al., 2009; Tanaka et al., 2003). The DNA samples were analysed for the rs4341 polymorphism using a TaqMan® SNP Genotyping Assay (Assay ID: C__29403047_10) and a Light Cycler® 480 System (Roche Molecular Systems, Mannheim, Germany). PCR was performed in a 5μL genotyping mixture containing 2.5 μL of TaqMan® GTX press™ Master Mix (2×), 0.0625 μL of TaqMan® SNP Genotyping Assay mix (40×), 1.4375 μL of sterilized water, and 1 μL of genomic DNA (10 ng/μL). Two to four negative controls were included in each plate. All genotypes were called based on TaqMan® assay results using LightCycler® 480 SW (version 1.5, Roche Molecular Systems).

Statistical analyses.

Data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using JMP Pro version 12 (SAS Institute, USA) and STATISTICA (Statsoft, version 13.0). The Hardy-Weinberg equilibrium of the *ACE* I/D polymorphism was assessed using a χ^2 test. A comparison between the injured and non-injured groups was conducted using an unpaired Student's t-test. A logistic regression analysis was applied to investigate the associations between the *ACE* I/D polymorphism and muscle injury. Age and training exposure (in the Italian cohort) or playing years (in the Japanese cohort) were adjusted for in the analysis. Odds ratios (OR) and 95% confidence intervals (CI) were calculated under the D-dominant, D-recessive, and D-additive models. The association of the *ACE*I/D polymorphism with the severity of muscle injury was assessed using an ordinal regression analysis.

A meta-analysis was performed using Review Manager 5.3.5 (http://tech.cochrane.org/revman). The Mantel-Haenszel test for random effects was used to calculate OR. Heterogeneity among the study results was assessed using the I^2 statistic. Statistical significance was set at $P< 0.05$.

Results

Table 1 shows the characteristics of the participants in each ethnic cohort. In the Italian cohort, significant differences were observed in age and training exposure between the injured and non-

injured groups (P<0.05). Injured football players were older and had higher levels of training exposure than non-injured players. In the Japanese cohort, there were no significant differences in age, height, weight, and playing years between the injured and non-injured groups (P>0.05).

The *ACE* I/D genotype frequencies in each cohort did not deviate from Hardy-Weinberg equilibrium (Italy, P=0.79; Japan, P=0.46). A logistic regression analysis showed that there was no significant association of the *ACE* I/D polymorphism with muscle injury in the Italian cohort (Table 2), while the *ACE* I/D genotype frequency was significantly different between the injured and non-injured groups in the Japanese cohort under the D-dominant model (OR: 0.49, 95% CI: 0.24–0.97, P=0.04, Table 2). A meta-analysis showed that in the pooled model (Italian + Japanese cohorts), the frequency of DD+ID genotype was significantly lower in the injured group than in the non-injured group (OR: 0.61, 95% CI: 0.38- 0.98, P=0.04, Figure 1). There was no heterogeneity between the studies (I^2=0%, P=0.34, Figure 1).

Figure 2 shows the distribution of muscle injury severity for each *ACE* I/D genotype. Although there was no significant difference in the distribution of injury severity between the genotypes (Italy, D-dominant: P=0.95, D-recessive: P=0.92, D-additive: P=0.99; Japan, D-dominant: P=0.29, D-recessive: P=0.61, D-additive: P=0.57), the proportion of severe injuries (>28 days) were lower in the DD genotype compared with the ID and II genotypes in both cohorts (Italy, DD: 23.1% < ID: 28.4% < II: 31.8%; Japan, DD: 50.0% < ID: 54.6% < II: 68.4%).

Discussion

The aim of this study was to analyse the role of the *ACE* I/D polymorphism in the development of indirect muscle injury in elite football players from two different ethnicities. We found, for the first time, an association between the *ACE* I/D polymorphism and the prevalence of muscle injury for carriers of the D-allele (ID+DD genotypes) which were lower in the injured group with respect to the non-injured group. Regarding the severity of muscle injuries, although we did not find any significant difference between genotypes, the proportion of severe injuries were lower in the DD genotype compared with the ID and II genotypes in both cohorts. These results suggest that D allele of the *ACE* I/D polymorphism is associated with protective effect against the incidence and exacerbation of muscle injury.

Muscle injuries are a heterogeneous group of multifactorial disorders that present with differences in type, location, severity, and size which makes it hard to define and categorize them (Ekstrand et al., 2012). In professional football, injuries to the hamstring, quadriceps femoris, adductors, and soleus-gastrocnemius account for 80–90% of all muscle injuries. Many studies have shown that teams that minimize time-loss injuries often achieve greater league success (Eirale et al., 2013).

Although muscle injuries are one of the most frequent injury types in football (Ekstrand et al., 2012), our understanding of the pathophysiology and the factors that predispose players to muscle injuries is limited. In fact, it is still not completely understood why some athletes are at higher risk than others under non-contact conditions, hypothesizing that genetic factors may play a role. In recent studies, an association between some polymorphisms and the incidence/severity of muscle injuries in professional football players has been shown (Massidda et al., 2015a, 2015b, 2019).

The present study partially supports the findings of Yamin et al. (2007), which observed different levels of circulatory CK between the different *ACE* genotypes after eccentric exercise, suggesting that the D-allele could have a protective effect against muscle damage after eccentric exercise. Because the D allele of the *ACE* I/D polymorphism is linked to higher ACE activity in serum and tissue (Rigat et al., 1990) compared to the I allele, a higher level of ACE activity may play a role in protection against muscle damage and injury. Indeed, it has been demonstrated that inhibition of ACE increases muscle damage in a rabbit muscle overuse model induced by electrical stimulation (Song et al., 2014).

ACE plays a key role in the renin–angiotensin system (RAS) and the tissue kallikrein–kinin system (TKKS). In RAS, ACE catalyzes the conversion of angiotensin I into angiotensin II, and in TKKS ACE catalyzes the degradation of bradykinin to biologically inactive fragments. Therefore, higher ACE activity, associated with the D allele of the *ACE* I/D polymorphism, will result in greater production of angiotensin II and a decreased half-life for bradykinin. It is known that angiotensin II and bradykinin are both involved in the inflammatory processes that occur following muscle damage (Baumert et al., 2016). Recently, Sierra et al. (2019) reported an association between ACE related polymorphisms and inflammation and muscle damage in Brazilian male runners after a marathon race. This study showed that inflammatory and muscle damage markers in the blood were lower in runners with the DD genotype compared to those with the II genotype, suggesting that *ACE* DD genotype decreases the susceptibility to inflammation and muscle damage after exercise. The low inflammatory response and muscle damage in D allele carriers may explain the association between the *ACE* I/D polymorphism and muscle injury in elite football players in the present study.

While the direction of the association between *ACE* D-allele and muscle injuries was the same in both groups of athletes (Italian and Japanese), in the Japanese football players this association was significant and more marked than in the Italian cohort. There are several factors that might contribute to this difference between ethnicities. Different genetic backgrounds may account for the different results. In this context it is important to highlight that, according to previous studies, the *ACE* D allele is commonly associated with sprint/power performance in European populations (Maciejewska-Skrendoa et al., 2019) whereas, conversely, the I allele is associated with

sprint/power performance in Asians population (Wang et al., 2013). Moreover, the different living conditions between the Asians and Caucasians may contribute to the different genetic effects. Also, interactions with other predisposed gene polymorphisms in the different ethnicities may also be an influencing factor.

The limitation of the present study is the different method used in Japanese and Italian cohorts to assess the muscle injuries. This is evident by the differences in the ratio of injured to non-injured players between the Italian and Japanese cohorts (Table 1). However, we believe that this limitation is overcome by our choice to use a meta-analysis to analyze the data, as evidenced by the low heterogeneity obtained as result (χ^2:0.91; l^2=0%). Moreover, it is important to highlight that multiple genetic variants (polygenic in nature) are thought to influence exercise-related phenotypes (Hughes et al. 2011; William & Folland 2008; Massidda et al. 2011) and that muscle injuries are a complex multifactorial trait influenced by genetic factors including a number of nucleotide variants, environmental factors, and the interaction among them. In the present study, we focused on one polymorphism, being well conscious that it represents a part of a complex picture, and it could affect partially the predisposition to develop muscle injuries in football players.

In conclusion, our findings suggest that the *ACE* I/D polymorphism is one of the genetic variants that could influence the susceptibility to developing muscle injuries among football players. While further studies are needed to confirm these findings in other professional football player cohorts, our data could be added to the other genetic variants already associated with muscle injuries in football with the aim to build a genotype score useful to predict the risk to develop muscle injuries among footballers.

This study showed that, among a large population of football players, the *ACE* I/D polymorphism influences the susceptibility to develop muscle injuries. Interestingly, the direction of the association was not related to the ethnicity, as has been found in studies on *ACE* and physical performance, but rather was independent from it. These results suggest that football players with the D allele could be more protected against the risk of developing muscle injuries caused by football practice, and this should be considered when planning the training workout or when developing injury rehabilitation and injury prevention strategies. Therefore, in this context we would highlight the importance of including an analysis of the *ACE* gene in future research aimed to build a polygenic score for the predisposition to muscle injuries.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 1. Characteristics of the study participants.

	Injured	**Non-injured**	**P value**
Italy			
N (%)	154 (45.2)	187 (54.8)	
Age, years	21.0 ± 5.5	19.3 ± 5.5	**0.005**
Height, cm	179.9 ± 8.2	179.7 ± 7.2	0.888
Weight, kg	73.2 ± 9.0	73.0 ± 8.7	0.895
Training Exposure, h	1115.1 ± 996.1	624.5 ± 470.9	**0.001**
Japan			
N (%)	37 (10.0)	332 (90.0)	
Age, years	20.5±1.0	20.8±1.5	0.358
Height, cm	173.8±7.0	174.1±6.1	0.781
Weight, kg	68.6±9.3	69.1±6.5	0.663
Playing years	14.3±2.3	14.4±2.4	0.844

Values are presented as the mean ± SD unless noted otherwise. Bold emphasis: $P < 0.05$.

Table 2. Associations of *ACE* I/D genotypes with muscle injury

	Genotype	N(%)		Dominant		Recessive		Additive	
		Injured	Non-injured	OR [95% CI] AIC	P value	OR [95% CI] AIC	P value	OR [95% CI] AIC	P value
				DD+ID vs. II		DD vs. ID+II		DD vs. ID vs. II	
Italy	II	22 (14.3)	21 (11.2)	0.71 [0.37-1.39]	0.322	0.96 [0.61-1.51]	0.856	0.90 [0.65-1.25]	0.541
	ID	67 (43.5)	86 (46.0)	438.6		439.5		439.2	
	DD	65 (42.2)	80 (42.8)						
Japan	II	20 (54.1)	120 (36.1)	**0.48 [0.24-0.97]**	**0.040**	1.32 [0.47-3.18]	0.567	0.72 [0.42-1.21]	0.219
	ID	11 (29.7)	169 (50.9)	243.2		247.1		245.9	
	DD	6 (16.2)	43 (13.0)						

Bold emphasis: $P < 0.05$.

Figure legends

Figure 1. Results of the meta-analysis for the association between the *ACE* I/D polymorphisms and muscle injury.

Figure 2. Proportion of severity of muscle injury by *ACE* I/D genotype in the Italian (a) and Japanese (b) cohorts.