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Abstract

Introduction: This study investigated the differences in ACTN3, ACE and ADRB3 variants in top-level soccer players who entered the Croatian National Team and the ones who did not but played for two best Croatian teams. **Material and Methods:** The buccal swabs were collected from 56 soccer players playing for the Croatian National Team (N = 31) and/or for one of the two most prestigious Croatian soccer clubs (N = 25). Each participant's genotype was determined by analyzing the single-nucleotide polymorphism. The ACTN3 gene (rs1815739) on chromosome 11 and the ACE (rs1799752) gene on chromosome 17 were determined. **Results:** No significant differences between the players who entered the national team and the ones who did not were found in ACTN3 R577X ($p = 0.437$) and ADRB3 ($p = 0.202$) polymorphism distribution, while the differences existed in ACE ($p = 0.044$). The significant differences were determined in the "Athletic index" between the national-team and non-national-team players ($p = 0.023$). Regarding the position, the "Athletic index" was significantly higher only in national team midfielders (2.50 ± 1.08 points vs. 1.38 ± 1.06 ; $p = 0.034$). **Conclusion:** It seems that the soccer players with a favorable genetic combination on the ACTN3 gene and ACE gene might have had a better chance to enter the National Team.

Keywords

CTN3, ACE, ADRB3, soccer players, National Team

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Cover Page Footnote

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Article

Differences in *ACTN3*, *ACE*, and *ADRB3* polymorphisms between Croatian National Team and non-national team elite soccer players

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Abstract: Introduction: This study investigated the differences in *ACTN3*, *ACE* and *ADRB3* variants in top-level soccer players who entered the Croatian National Team and the ones who did not but played for two best Croatian teams. Material and Methods: The buccal swabs were collected from 56 soccer players playing for the Croatian National Team (N = 31) and/or for one of the two most prestigious Croatian soccer clubs (N = 25). Each participant's genotype was determined by analyzing the single-nucleotide polymorphism. The *ACTN3* gene (rs1815739) on chromosome 11 and the *ACE* (rs1799752) gene on chromosome 17 were determined. Results: No significant differences between the players who entered the national team and the ones who did not were found in *ACTN3* R577X ($p = 0.437$) and *ADRB3* ($p = 0.202$) polymorphism distribution, while the differences existed in *ACE* ($p = 0.044$). The significant differences were determined in the "Athletic index" between the national-team and non-national-team players ($p = 0.023$). Regarding the position, the "Athletic index" was significantly higher only in national team midfielders (2.50 ± 1.08 points vs. 1.38 ± 1.06 ; $p = 0.034$). Conclusion: It seems that the soccer players with a favorable genetic combination on the *ACTN3* gene and *ACE* gene might have had a better chance to enter the National Team.

Keywords: *CTN3*, *ACE*, *ADRB3*, soccer players, National Team.

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1. Introduction

The worldwide interest in soccer is rising with every world cup. The determinants of success in soccer are multiple, and physical demands for the highest level of soccer players include both endurance and strength. Elite soccer players cover from 9 to 13 km during the game, but the overall amount of running does not necessarily discriminate between players of different levels. The ranges are rather wide, which is certainly linked to the differences between players' position and different game styles. A better description of the load the players are exposed to demands division of this total distance according to movement intensity. Around 80% of the distance is covered by walking, jogging or running, while 20% remains high-speed running or sprinting, often involved in determinant phases of the game. It is described that soccer players change their activities almost every 4 to 6 seconds, including changes in the intensity of activities such as acceleration, deceleration, jumps and direction changes [1].

Apart from a wide range of generic and specific tests used to evaluate endurance, strength and power abilities in soccer players, scientists try to find the determinates of

success also in individual genetic profiles. The aforementioned activities that involve speed, strength and power had previously been associated with the gene responsible for alpha-actinin-3 protein (*ACTN3* gene) which plays a role in crosslinking actin to Z-disks [2, 3]. Athletes with polymorphisms *ACTN3 RR* and even the *RX* genotype were perceived to adapt better to strength training, making them more inclined to success in sports or disciplines depending on those physical characteristics [4, 5]. By contrast, athletes in sports demanding high aerobic endurance might benefit from a special polymorphism principally in angiotensin-converting enzyme (*ACE* gene) and likely in adrenoceptor beta 3 (*ADRB3*). The *ACE* gene is a major component of the renin-angiotensin system responsible for control of blood pressure and regulating the body fluids. The Ins/Ins homozygous genotype (of the *ACE* gene) was found to be related to higher aerobic capacity and better adaptations to an aerobic type of training [6, 7, 8]. The Ins and Del allele are related to performance in endurance sports and power sports, respectively [9, 10, 11]. In the study of Akhmetov et al. [12], the *ACE* Ins genotype was found to be a possible genetic marker associated with enhanced aerobic performance and, together with some other alleles, suggested as a possible diagnostic tool for prognosis of human physical performance. In addition to the documented role of *ACE* gene in predicting aerobic performance, it seems that the cardiac function and metabolism regulating gene *ADRB3* might also have implications in endurance variables. In particular, heterozygotes for rs4994 on the *ADRB3* gene showed better adaptations to endurance training due to a higher Trp/Arg genotype frequency in professional endurance athletes compared to non-athletes [13]. However, the *ADRB3* gene was investigated more in relation to obesity, diabetes mellitus and its metabolic influences [14, 15, 16], while its relation to athletic performance is yet to be investigated.

Considering the role of the above three genes and the proteins encoded by them in building athletic abilities, which are perceived as important for soccer, it seems rational to suppose they could individually or as a combination affect long-term development of soccer players. Although some researchers showed that specific gene variants are associated with performance in soccer [17, 18, 19], stronger evidence of the influence of these genes on predicting a possible reach in soccer are lacking. Hence, the objective of this study was to investigate the possible differences in genotypes *ACTN3* R577X (RR, RX, and XX), *ACE* (Ins/Ins, Ins/Del and Del/Del) and *ADRB3* (Trp/Trp, Trp/Arg and Arg/Arg) between the top-level Croatian soccer players – those that made it to the national soccer team and those that did not. In addition, the secondary aim was to search for differences between two groups depending on the playing position. We hypothesized that either *ACE*, *ACTN3* or *ADRB3* have separate power to discriminate between the groups of national team players and non-national team players.

2. Materials and Methods

2.1. Participants

In spring of 2014, buccal swabs were collected from 56 soccer players (age 18–35) playing at that time for the Croatian national team and/or for one of the two most prestigious soccer clubs in First Croatian League (GNK Dinamo Zagreb and HNK Hajduk Split). The subjects signed informed consent forms, and the study was approved by the Research and Ethics Committee of the Faculty of Kinesiology, University of Zagreb (2014/3 and 2018/56). The data analysis for this study was performed after the 2018 FIFA World Cup in Russia. The subjects were divided into two groups: the group of players that played for the Croatian national team at any point in the period from the 2014 FIFA World Cup to (and including) the 2018 FIFA World Cup (N = 31) and the control group of elite players who were never selected for the national team (N = 25).

2.2. Laboratory Analysis

Each participant's genotype was determined by analyzing the single-nucleotide polymorphism (SNP) on a sample of the buccal mucosa cells in a certified clinical laboratory. The Chelex 100 method (Bio-Rad Laboratories Inc, Hercules, CA) was used to extract DNA. The *ACTN3* R577X gene (rs1815739) on chromosome 11 and the *ACE* (rs1799752) gene on chromosome 17 were amplified by the polymerase chain reaction (PCR) using PyroMark PCR Kit (Qiagen NV, Venlo, Netherlands). Pyrosequencing was performed using a PyroMark Q24 (Qiagen NV, Venlo, Netherlands), while the capillary electrophoresis was used for the separation of amplified fragments for the *ACE* gene using a Genetic Analyzer 3131 (Applied Biosystems Inc, Foster City, CA). The size of the DNA fragments was determined using GeneMapper ID-X (Applied Biosystems Inc, Foster City, CA) [20, 21].

2.3. Pondering the Polymorphisms

In the next step, the pondering of the polymorphisms was performed in a way that credits were appointed to the polymorphisms for endurance or sprint ability. The number of credits was determined according to the current body of knowledge published in the studies that previously researched the relations of those genes and performance. The *ACTN3* R577X gene was appointed with the "Strength credits" (RR-2 points, RX-1 point, and XX-0 points) while the *ACE* gene received "Endurance credits" (Ins/Ins = 2 points, Ins/Del = 1 point and Del/Del = 0). For this study, the sum of the credits was named the "Athletic index".

2.4. Statistical Analysis

The descriptive data are presented as percentages and counts regarding single genes while the endurance, strength and overall athletic credits are presented as median values. The differences in occurrence of the gene variants in groups were tested with the Chi-square test, and the differences between the groups with the Mann Whitney U test (Median test) of Student t-test for independent samples. The significance level was set at $p < 0.05$. Statistica for Windows, version 13.3., was used to perform data analysis.

3. Results

According to the playing position, there were 8 goalkeepers (14%), 20 defenders (34%), 18 midfielders (36%) and 10 forwards (16%). There was no difference in distribution of the playing positions between those that made the national team and those that did not (chi-square = 1.36; $p = 0.715$).

Then the 2×3 or 2×2 Chi-square contingency tables were used to test the polymorphism proportions in the *ACTN3* R577X, *ACE* and *ADRB3* genes. As indicated in Table 1, no significant differences were found between the group that made the national team and the one that did not in the *ACTN3* R577X and *ADRB3* polymorphism distribution. By contrast, the analysis confirmed meaningful differences between the compared groups in the polymorphism distribution in the *ACE* gene.

Table 1. Results of the 2×3 chi-square statistics for proportion testing.

<i>ACTN3</i> R577X	RR	RX	XX	
National team	13	12	5	Chi-square= 1.656 $p = 0.437$
Non-national team	7	13	6	
<i>ACE</i>	Ins/Ins	Ins/Del	Del/Del	
National team	7	16	7	Chi-square= 6.242 $p = 0.044^*$
Non-national team	2	10	14	
<i>ADRB3</i>	Trp/Trp	Trp/Arg	Arg/Arg	
National team	26	4	-	Chi-square= 1.630 $p = 0.202$
Non-national team	19	7	-	

* significant at $p < 0.05$

When the groups were compared differently, e.g. Ins/Del polymorphism was also considered to be to endurance related (all Ins/Del and Ins/Ins polymorphisms were summed up), the differences were even more significant. The national team group had 23 “endurance prone” subjects out of 30, while the non-national team group had only 12 out of 24 (Fisher exact $p = 0.018$).

That was not the case in the *ACTN3* R577X gene, and when all RX genotypes were added to the RR genotypes and represented “strength prone” subjects, the number of subjects with the RR and RX genotypes was higher in the national team group but the difference was insignificant.

In the next step, solely for the purpose of this study, the ponderers were added to the *ACTN3* R577X and *ACE* polymorphisms (as described in the Methods section) so that the “Athletic index” could be calculated. The values of the “Athletic index” ranged from 0 credits (for a player that had Del/Del on the *ACE* gene and XX on *ACTN3* R577X, as he got 0 points for both) to a maximum of 4 credits (for a player that had Ins/Ins on the *ACE* gene and RR on *ACTN3* R577X, as he got 2 points for both). The non-parametric median test confirmed differences between the groups (median national team 1.50 vs. median non-national team 2.0; $p = 0.023$) but as there were no outliers. To provide more clarity, the means \pm SE are presented in Figure 1.

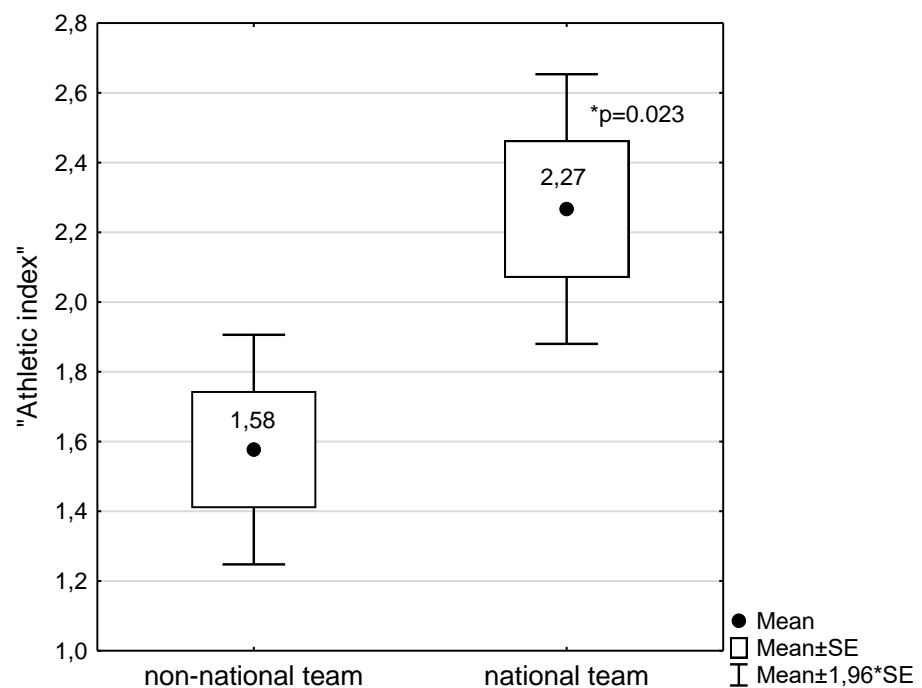


Figure 1. Means \pm SE of the Athletic index

The post hoc power and effect sizes were calculated for the main findings regarding the differences only between the national team and the non-national team groups in GPower free software. For the “Athletic index,” the effect size was found to be larger than expected (noncentrality parameter $\delta = 2.649$; critical $t = 1.673$; effect size $d = 0.710$ meaning medium to large; $\alpha = 0.050$, and the final achieved power was satisfactory (Power ($1 - \beta$ err prob) = 0.834).

The significant differences in national team and non-national team players according to the playing position were found only in midfielders, as the “Athletic index” was shown to be significantly higher in midfielders that were playing in the national team, while in all other positions that was not the case. The midfielders who played for the national team ($N = 12$) scored 2.50 ± 1.08 , while the ones who played only for club teams ($N = 8$) scored

1.38±1.06; $p = 0.034$. Even though this was a relatively small sample, the post hoc power analysis showed a very large effect size ($d = 1.05$) with power of 0.713.

4. Discussion

This study aimed to evaluate the strength and power ability associated gene *ACTN3* and endurance associated with genes *ACE* and *ADRB3* to discriminate on an individual and combined basis between two groups of elite soccer players: national team players vs. non-national team players and according to the players' team positions. The main finding is that the groups of national team players and non-national team players have different distribution of the polymorphisms in the endurance associated *ACE* gene. In particular, the group of national team players have been found to carry more Ins/Ins and Ins/Del genotypes in comparison to the group of national league players. In contrast, our result did not support the presumption that either *ACTN3* or *ADRB3* have separate power to discriminate between the groups of national team players and non-national team players. However, once the strength and power associated genotypes RR and RX of the gene *ACTN3* were joined to endurance favorable genotypes Ins/Ins and Ins/Del of the gene *ACE* to calculate so-called "Athletic index", this combination of the above polymorphisms showed further ability to positively discriminate between players selected for the national team. The above difference between groups when the polymorphisms of *ACTN3* and *ACE* were combined was additionally confirmed among midfielders, while no differences were observed among defenders and forwards.

The main novelty of this study is the comparison of already selected elite soccer players among which some competed at the highest level, meaning playing for the national team in international competitions. Previous investigations on genetic profiles favorable in soccer mostly relied on case-control studies, comparing different samples of soccer players to ethnically related non-athletic population [18, 19, 22, 23, 24]. To the best of authors' knowledge, there were only several studies that collected data on the *ACE* and the *ACTN3* allelic frequency in samples of soccer players comprising groups different according to the competition level. Initial evidence on the association of the allelic frequency in genes *ACTN3* and *ACE* with soccer players' status was established in two studies comparing Spanish professional soccer players to endurance athletes and sedentary controls [13, 25]. Both above studies comprised a sample of players competing in the first and the second national soccer division. Unfortunately, authors reported no data concerning a possible association of the allelic frequency in the above genes and the players' competition level. Subsequently, a study including three European cohorts searched for an association of the *ACTN3* allelic frequency and the competitive status in team-sport athletes including a high portion of soccer players [26]. While comparing athletes that competed at various international events and athletes that competed at national level events only, authors found no association for the genotype and the competition level. A study comprising soccer players of different playing status compared the first league players to the second league and the youth category players in genotype and allele frequencies of 8 gene variants and in the total genotype score [27]. However, authors did not report differences either in the genotype and allele frequencies of 8 gene variants or in the total genotype score among three competitive groups. Hence, it is interesting to find in this study for the first time that soccer players that made the national team have different genetic profiles in comparison to the group of elite non-national team players. Finally, the most recent review and meta-analysis of McAuley et al. [28] explored the association of the *ACTN3* R577X and *ACE* Ins/Del polymorphisms with the athlete status in soccer. They concluded that the *ACTN3* R577X polymorphism was related to professional players vs. non-athletic group and that the R allele is a likely contributing factor to gaining professional status in soccer. The *ACE* Ins/Del polymorphism did not show significant contribution to the differences between professional and non-professional soccer players.

The main source of difference between national team players and non-national team players in this study seems to be the higher frequency of both the *ACE* Ins/Ins and Ins/Del

genotypes, while trivial differences between the groups were observed in the distribution of the *ADRB3* and *ACTN3* R577X genotypes. The *ACE* homozygous Ins/Ins and heterozygous Ins/Del genotypes were previously associated with better endurance and aerobic performance [8, 29, 30, 31]. It is common that elite soccer requires extremely high aerobic capacity and that the maximal oxygen uptake in soccer players is often as high as in some endurance athletic disciplines [32, 33, 34]. Thus, it was no surprise that the difference in the allelic frequency in the endurance related *ACE* gene might at least slightly contribute to the predisposition to enter a national team and reach a very high level of competition. In line with the present finding, Gineviciene et al. [18] and Contro et al. [24] reported that the *ACE* Ins/Del and, even more so, the *ACE* Ins/Ins genotypes were associated with a soccer player phenotype when players were compared to the general population. However, authors also reported slightly different and/or opposite results regarding the association of the *ACE* allelic distribution to a soccer player phenotype. Specifically, Ergorova et al. [27] and Ulucan et al. [34] noticed that soccer players are likely to have overrepresented Ins/Del and Del/Del genotype in comparison to non-athletes. This agrees only partially with the present results, since the Del/Del genotype is found to be underrepresented in national team members in comparison to non-national team players. It should be stressed that numerous investigations found no differences in the *ACE* gene allele distribution between soccer players and non-athletic population [17, 19, 22, 25, 36]. The discrepancy in results of this study and the above findings might be explained by a different study design and sampling methodology, since this is one of the first studies that compared two groups of thoroughly selected players. In addition, differences in playing styles among several studied populations possibly cause differences in the training approach and by extent of different selection criteria in soccer academies. Overall, results of this study suggested that the *ACE* Ins/Ins and Ins/Del polymorphisms might have separate power to distinguish not only between the general population and soccer players, but also between the two groups of elite players competing at different levels.

Interestingly, the studied groups differed insignificantly in the distribution of the R and X *ACTN3* genotypes, but results indicated both a higher rate of the homozygous RR and heterozygous RX genotypes and nearly double prevalence of the RR genotype in national team members. A similar result was presented in the investigation from Italy [23]. Male soccer players from a team of Italian National League (2nd Division) showed a greater prevalence of the *ACTN3* RR genotype, but there was not a significant difference between soccer players and non-athletes in *ACTN3* RR and RX distribution. Using a similar case-control design, studies by Santiago et al. [13] and Ergorova et al. [27] both found a higher frequency of the *ACTN3* RR genotype in elite and sub-elite soccer players while comparing them to the general population. Results of Ergorova et al. [27] partly agree with the present finding, as authors have shown a higher frequency of the *ACTN3* RR genotype in elite players in comparison to sub-elite players (approx. 81 % vs. 65%), but also including a lack of statistical significance. Based on their overall findings, both groups of authors concluded that soccer players are primarily prone to strength and power-oriented activities [13, 25, 27]. The former authors explained this by the fact that the early selection of soccer players might have been done most effectively by means of sprint and strength tests. Generally, it seems that the allelic frequency in strength and power associated gene *ACTN3* could be a source of difference between soccer players and non-athletes, but it possess no single power to discriminate between the groups of selected players, as confirmed in this study.

Taken together with previous findings, it appears that success in soccer depends on both endurance capacity and strength/power abilities during the game. From the above perspective, the "Athletic index" calculated for the purpose of this study might be explanatory. Both, the approach of pondering and combining performance enhancing polymorphisms and the outcomes are comparable to those reported by Egorova et al. [27]. Authors also found the overall favorable accumulated polygenetic combination to be soccer related when several genes were combined in order to compare the scores between soccer players and non-athletes in Russia. In this study, when the *ACTN3* R577X polymorphisms were

given the credits, and those credits added to *ACE* gene values, the difference between the groups became even larger than while looking only into a single gene. Noteworthy is that Gineviciene et al. [18] also drew a conclusion that the *ACE* Ins/Del and Ins/Ins genotypes are “preferable genotypes” for soccer players and that they are associated with the players’ status. The differences were significant for all players, but especially for midfield players and defenders. In this study, the “Athletic index” that combined the frequency of the *ACE* Ins/Ins and Ins/Del genotype with *ACTN3* RR and RX genotype was not sensitive to distinguish national team forwards and defenders from their non-national team counterparts. Yet, it revealed a higher frequency of the *ACE* Ins/Ins and Ins/Del genotype and the *ACTN3* RR and RX genotype in the national team midfielders in comparison to the top non-national team midfield players. Indeed, during the world cup tournament in Russia 2018, the Croatian national team seemed to have an advantage especially in its midfielders. The Croatian team had top three players (all midfielders) of the tournament in the distance covered during the game and five players of the top ten in most sprints performed (FIFA 2018). Hence, it is interesting to confirm their dominance on the genetic profile as the “Athletic index” was shown to be significantly higher in national team midfielders in comparison to non-national team players. The midfielders often cover a distance up to 13 km per game but including more than 3.5 km being covered during both highly intensive offensive and defensive actions [37, 38, 39, 40], which involve sprints, high jumps and direction changes. It seems, therefore, unsurprising that the combination of the *ACE* Ins/Ins and Ins/Del genotypes with the *ACTN3* RR and RX genotypes makes a favorable genotype to distinguish national team players from top non-national team counterparts.

Among the limiting factors that may affect the conclusions, the small sample size of the two soccer players’ cohorts is certainly the main weakness. This size seems, however, understandable given the high representability of both groups. Another issue is the uncertainty that individuals forming the group of non-national team players will not be selected for the national team in the future. To minimize this possibility and to attenuate the bias, the analysis was performed with a delay of 5 years following the collection of data. Finally, all players that collected at least one cap for the national team were included in the analysis regardless of the fact if they appeared in national team games continuously during the observed period. This approach seems rational because the selection for national team was the main criteria that homogenizes the highest competition-level group and discriminates among two groups of elite players participating in the study.

5. Conclusions

It seems that soccer players with a combination of a favorable genetic profile for strength and endurance on the *ACTN3* gene (RR or RX) and the *ACE* gene (Ins/Ins or Ins/Del) might have a better chance to enter the national team (at least in the studied population). It might be especially true for midfielders who are extremely important in creating the modern soccer game. The polygenic approach will probably be the future of genetic analysis in sport, but much more research is needed to find the ideal set of genes for a specific sport or discipline and to test the pondering system for each candidate gene.

6. Practical Application

The practical applications of this study are diverse and still rather imprecise. The role of genetic profiles in sport is still not researched enough, but it seems that its contribution to sport success might be larger than it is supposed. Still, the genetic profiling should not be used to exclude younger athletes from selections. Similarly, the decision whether to pursue a sports carrier should not be based solely on the genetic profile, even though in future the indices involving more genes might be quite precise, as other factors influencing the performance may overcome genetic disadvantage. Nevertheless, genetic profiling might help coaches, especially conditioning coaches, to identify favorable and non-favorable profiles and to focus on training and developing specific abilities more.

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