

An innovative way to highlight the power of each polymorphism on elite athletes phenotype expression

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Abstract

The purpose of this study was to determine the probability of soccer players having the best genetic background that could increase performance, evaluating the polymorphism that are considered Performance Enhancing Polymorphism (PEPs) distributed on five genes: PPAR α , PPARGC1A, NRF2, ACE e CKMM. Particularly, we investigated how each polymorphism works directly or through another polymorphism to distinguish elite athletes from non-athletic population. Sixty professional soccer players (age 22.5 ± 2.2) and sixty healthy volunteers (age 21.2 ± 2.3) were enrolled. Samples of venous blood was used to prepare genomic DNA. The polymorphic sites were scanned using PCR-RFLP protocols with different enzyme. We used a multivariate logistic regression analysis to demonstrate an association between the five PEPs and elite phenotype. We found statistical significance in NRF2 (AG/GG genotype) polymorphism/soccer players association ($p < 0.05$) as well as a stronger association in ACE polymorphism ($p = 0.02$). Particularly, we noticed that the ACE ID genotype and even more the II genotype are associated with soccer player phenotype. Although the other PEPs had no statistical significance, we proved that some of these may work indirectly, amplifying the effect of another polymorphism; for example, seems that PPAR α could acts on NRF2 (GG) enhancing the effect of the latter, notwithstanding it had not shown a statistical significance. In conclusion, to establish if a polymorphism can influence the performance, it is necessary to understand how they act and interact, directly and indirectly, on each other.

Key Words: Polymerase chain reaction-restriction fragment length polymorphism, performance-enhancing polymorphisms, performance

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The abilities of athletes to stand out in particular physical qualities are determined, among other things, by adaptation to stress of circulatory, respiratory, muscular and other biological systems. The effectiveness of these mechanisms can certainly be improved with training. Biologically, it joins changes in tissues and cells, in turn depending on local variations of gene expression. The presence of specific gene variants decides the prowess of some physical traits, such as speed, muscle strength, greater possibility of injury and even emotional control.^{1,2} Invaluable contributions were provided by both the Human Genome Project,³ that sequenced 20.000 to 25.000 genes, and subsequently by the International HapMap Project, that identified variations in the human

genome and genes related to common diseases such as asthma, cancer, diabetes and cardiovascular diseases.⁴ Brey et al.⁵ have instead developed a genetic polymorphisms map that may be associated with predispositions of better fitness and sports results. 239 fitness genes were identified: 7 in chromosomes X, 18 in mitochondrial DNA and 214 in autosomes. Since several years, researchers are focusing their interest on research of the “right” genetic profile that can contribute to athletic performance and to determine the mechanisms that can guide a person to a specific athletic field.⁶⁻⁸ Contributions of genotype and phenotype to achieve elite performance is still not known. Adaptations to a resistance effort are strongly supported by the

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Table 1 Information on genotyping methods for each polymorphism

GENE	PRIMERS	TEMPERATURE ANNEALING	RESTRICTION ENZYME
PPAR α intron 7G/C	F 5'-3': TCACTCCTTAAATATGGTGG	59°C	TAQ I
	R 5'-3': AAGTAGGGACAGACAGGACCAGTA		
PPAR γ C1-Gly482Ser	F 5'-3': TAAAGATGTCTCCTCTGATT	50°C	HPA II
	R 5'-3': GGAGACACATTGAACAATGAATAGGATTG		
ACE	F 5'-3': GCCCTGCAGGTGTCTGCAGCATGT	66°C	---
	R 5'-3': GGATGGCTCTCCCCGCCTTGTCTC		
CKMM	F 5'-3': GGGATGCTCAGACTCACAGA	50°C	NCO I
	R 5'-3': AACTTGAATTTAGCCCAACG		
NRF2 AG/GG	F 5'-3': AGTTTAGTGTCTCCCAGTGT	50°C	RSA I
	R 5'-3': CTTAGTTTTCTTGTATCCGT		

mitochondrial functions: it is determined by genes of nuclear and mitochondrial DNA that encode enzymes of energy metabolism and are associated with aerobic physical fitness and insulin sensitivity, a factor that could play a key role in the pathophysiology of type 2 diabetes. For example, the genes coding for the peroxisome proliferator-activated receptor (PPAR) - delta and gamma coactivator 1 alpha, which will be discussed in details below, are very important for proper functions of mitochondria. PPAR δ , in particular, regulates the expression of genes involved in lipid and carbohydrate metabolism and one of its functional polymorphism has been associated with a predisposition for an endurance performance.⁹

Nuclear respiratory factors NRF1 and NRF2 coordinate the expression of nuclear and mitochondrial genes relevant to mitochondrial biogenesis and cellular respiration. The polymorphism carriers in the opening translation sequence ATG of the gene NRF2 prove to have a greater run economy than non-carriers, which could potentially explain inter-individual differences in endurance capacity.¹⁰ PPARGC1 α is an important co-factor that regulates gene expression related to oxidative phosphorylation and ATP production in target tissues through coactivation of nuclear receptors. Studies in mice show that it increases performance during a test carried out at VO₂ peak, showing an increase of oxidative peak capacity or greater oxygen consumption throughout the body.¹¹ Studies have shown that PPARGC1A (Gly482Ser) polymorphism is associated with state athletic strength / power, and in particular, the PPARGC1A Ser / Ser genotype seems more favorable to athletes more powerful than controls. In these athletes there was an increase in the contribution of the anaerobic system to the production of energy needed for exercise. Athletes undergoing a heavyweight training program that had this genotype showed an advantage in developing resistance.¹² The mitochondrial synthesis is stimulated by the pathway PPARGC1A (peroxisome proliferator-

activated receptor coactivator 1 Gamma Alpha)-NRF (Nuclear Respiratory Factor)-TFAM (Mitochondrial Transcription Factor A). In summary, the receptor Peroxisome Proliferator-Activated Receptor- δ (PPAR- δ) leads the promotion of PPARGC1A, which is the first stimulator of mitochondrial biogenesis. Factors NRF1 and NRF2 are intermediate transcription factors that stimulate the synthesis of TFAM, and the latter is the final effector that activates replication of mitochondrial DNA molecules.¹³ Among the factors that may affect this pathway, some genetic variations should be included, which can have effects individually or in combination with physical activity. For example, the functional polymorphism C294T (rs2016520) of the gene encoding PPAR- δ and Gly482Ser polymorphism (rs8192678) of the gene encoding PPARGC1A could play a key role in the activity of protein and/or mRNA. The C allele of the C294T polymorphism of PPAR- δ (site in exon 4) is associated with a highest transcriptional activity of the PPAR- δ promoter, inducing a binding site for the transcription factor Sp-1.¹⁴ The minor allele of PPARGC1A Ser482 is associated with a more modest capacity of endurance during physical activity.¹⁵ The G allele of the variant A/G intron 3 of the subunits β 1 of the NRF2 gene (rs7181866) is associated with endurance and maximum oxygen consumption in response to resistance training.¹⁶

Common genetic variation that separates endurance athletes by sprinter, is probably due to natural selection. For example, the actin-binding protein alpha-actinin-3 (ACTN3) is highly present in the mechanical contraction of the fast muscle fibers; in sarcomere it is the largest component of the Z line and plays a fundamental organizing and regulating function for the muscle contraction. ACTN3 is nearly always expressed among professional athletes of power,¹⁷ while the polymorphism R577X (results in premature stop codon), which is associated with an absolute deficiency of ACTN3, it is prevalent to a greater extent among professional

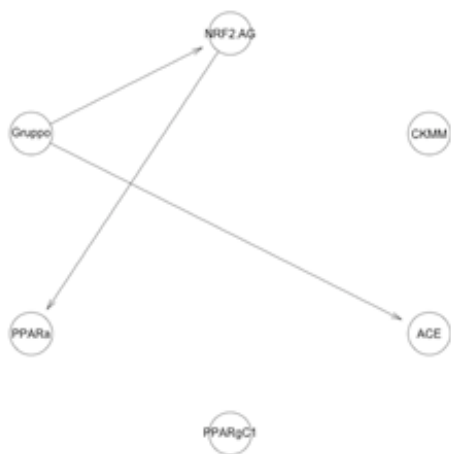


Fig 1. Graphical model to evaluate the relative importance of each predictor to the regressors with regression type graphical models

endurance athletes.¹⁸ In a recent work on soccer players it was showed a prevalence of ACTN3RR genotype, that was linked with a high production of ACTN3 and for the first time it was correlated with the training load and overall the mtDNA copy number increase was assess as a bioenergetics biomarker.¹⁹ Some authors observed a significantly lowest frequency of the X/X genotype in power athletes compared with the control group.²⁰ The gene Creatine kinase-MM (CK-MM) encoding the cytosolic muscle isoform of CK, responsible for the rapid ATP regeneration during intense muscle contraction.²¹ Reduced expression of this enzyme may be responsible for muscle fatigue most likely due to increased intracellular concentration of inorganic phosphate. Moreover, studies of changes in CK-MM gene sequence showed a significant association between some of its polymorphisms and increased cardiorespiratory endurance, peak performance and lower decline in force generation; particularly, GG genotype and G allele are represented in power-oriented athletes.²² Several beneficial effects on athletic performance in endurance and sprint, were also observed in different genotypes of a single locus of angiotensin converting enzyme (ACE). The ACE gene, despite some controversy, seems to present variations that can be associated with many inherited traits, including physical, psychological and performance parameters,²³ it has two alleles, called "I" and "D". The latter is associated with power-oriented athletes and anaerobic sports: the mechanism that underlies it is probably governed by the differences in strength gain at skeletal muscle level, because D allele was associated with greater increases in quadriceps strength after training, especially DD genotypes presented better performance for example during jump and sprint tests. In contrast, I allele could influence

resistance performance improvements through a better use of substrate and muscle efficiency, with a consequent saving of energy supplies. On the other hand, DD genotypes may benefit athletes in activities that require strength and speed, while II ACE genotype in endurance activities.²⁴

Materials and Methods

Sixty sub-elite male Italian soccer players (age 22.5 ± 2.2) and sixty sedentary male volunteers (age 21.2 ± 2.3) were enrolled in this study. Athletes and controls were all Caucasian, in order to ensure the absence of a probable genetic ethnicity inclination and to overcome any problems of population stratification.

All participants gave their informed consent for genotyping, understood that the results would be anonymous and confidential. By standard clinical procedures venous blood samples were obtained, between 8:00 and 10:00 in the morning, at rest.

The samples were treated with anticoagulant (heparin) and used for genomic DNA extraction (PureLink Genomic DNA, ThermoFisher Scientific).

The polymorphic sites were analyzed by PCR-RFLP protocols with different enzymes (Tab 1).

Results

Using a multivariate logistic regression analysis, we tried to figure out if it is possible to speculate the impact of each polymorphism on the expression of elite soccer

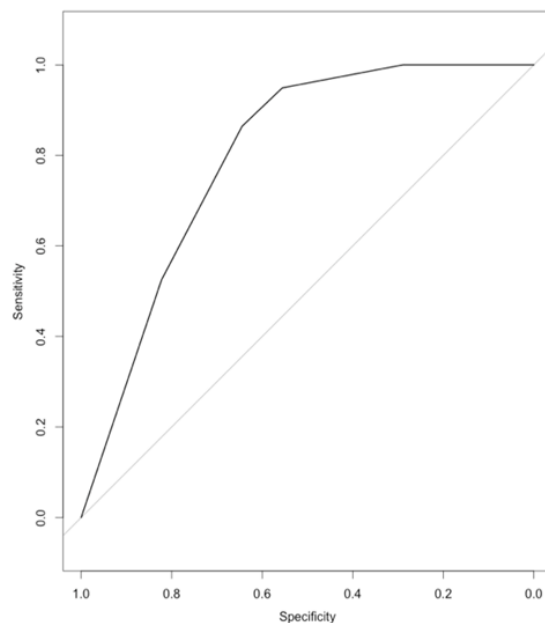


Fig 2. Receiver operating characteristic curve (ROC) summarizing the ability of elite genotype score to classify potential elite athletes from non-athletes (controls). AUC indicates the area under the curve (95% confidence intervals).

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player phenotype. Particularly, we investigated how each polymorphism works directly or through other polymorphism to discern elite athletes from non-athletic population. We found statistical significance in NRF2 (AG/GG genotype) polymorphism/soccer player association ($p=0.03$) as well as in ACE II genotype ($p=0.02$). Particularly, it merges a probability three times higher to become soccer players if you have these two polymorphisms (NRF2 AG/GG and ACE II). Furthermore, despite PPAR α has not a statistical significance, have a positive estimate value (0,5) compare to the others that suggest a possible involving in the induction of the expression of elite phenotype (Tab. 2). Although the others PEPs (PPAR α , PPARGC1, CKMM) had no statistical significance and they didn't show any correlation with the others, it means they probably work independently. Figure 1 summarized all five polymorphisms belong to the performance-enhancing polymorphism family and how it's probably works. Previously, we demonstrated that PPAR α had a significant trend of association between GG polymorphism, especially G allele, and elite soccer player.²⁵ Through this new approach, we realized that the polymorphism investigation could be a marker of the elite phenotype only if it's associated each other. As a matter of fact PPAR α demonstrated a significant statistical association with soccer player group ($p=0.03$) only when is investigated with NRF2 genotype. Definitely PPAR α could acts on NRF2 (GG) enhancing the effects of the latter nonetheless it had not shown a statistical significance (Fig 1). The genotype distribution

was in Hardy-Weinberg equilibrium for the whole group (soccer players and control group). The area under the ROC curve is .80 (AUC) that be considered to be "good" at separating elite soccer players from control group. The ROC analysis highlights a significant discriminating accuracy of the model in identifying an elite soccer players (area under the curve [AUC]=0.80; 95% confidence interval [CI]: 0.71-0.88) (Fig 2). Definitely, the results of the statistical analysis for the comparison of elite athletes vs controls indicated that particularly 3 polymorphisms of those analysed were significantly associated with elite phenotype: 2 directly (NRF2 AG/GG and ACE II) and 1 indirectly (PPAR α , through NRF2).

Discussion

Since many years disputes have emerged about the interpretation of association studies involving ACE gene (I/D). Some studies, indeed, related the I allele with power performance instead of endurance performance^{26,27}. The novelty of this study lies in the fact that even in a team sport like football, it is possible to find this type of association. Particularly, we applied a multivariate linear regression to better analyzed which is the impact of each polymorphism alone or together to induce elite phenotype expression. Taken together the results obtained in our previous study²⁵ and in this one, it is clear that the new approach shows power of each polymorphism as performance-enhancing factor. In the attempt to implement experimental designs reducing selection errors and the study of athletes from different sports, comparing them with a control group adequately

Table 2. Estimates and Standard Errors for the Model and the significance of each coefficient in the presence of the others

VARIABLE	ESTIMATES	STD ERROR	Z VALUE	P-VALUE
INTERCEPT	-2,4614	1,5752	-1,56	0,1182
PPARa GC	-0,1094	0,7996	-0,14	0,8912
PPARa GG	0,5458	0,7942	0,69	0,4919
PPARgC1 SG	-0,3521	0,5198	-0,68	0,4981
PPARgC1 SS	-0,6604	0,8459	-0,78	0,4350
ACE ID	0,6882	0,5347	1,29	0,1981
ACE II	3,3034	1,5015	2,20	0,0278 *
CKMM AG	-0,8885	0,5113	-1,74	0,0822 .
CKMM GG	-0,8795	0,7630	-1,15	0,2490
NRF2 AG/AG	3,1164	1,4368	2,17	0,0301 *
NRF2 AG/GG	3,8156	1,7672	2,16	0,0308 *

large, helps to make results achievable and reproducible. In details, during a game of 90 minutes, medium-high level players run for about 8-10 km at an intensity close to the anaerobic threshold (80-90% of maximum heart rate). However, within this endurance context are required numerous flashes of explosive strength, as kicks, jumps, sprints, changes of speed and direction and tackle, in addition to maintaining balance under defensive pressure to control the ball. Since the main component is, however, resistance, this could explain a genotypic profile typical of endurance athletes associated with football players. On the other hand, it should be stressed that athletic performances are multifactorial events: factors such as environment, gene-gene and gene-environment interactions play valuable roles in the complex traits of champions, that can not be reduced to a set of genetic polymorphisms.²⁸ This innovative method begins to be used in many other fields, such as the prevention of injuries and the investigation of muscular morphological changes, such as atrophy or myasthenic syndrome.^{29,30,31}

List of acronyms

ACE - angiotensin converting enzyme
ACTN3 - actin-binding protein alpha-actinin-3
CK-MM - Creatine kinase-MM
NRF - Nuclear Respiratory Factor
PCR-RFLP - Polymerase chain reaction-restriction fragment length polymorphism
PEPs - performance-enhancing polymorphisms
PPAR - peroxisome proliferator-activated receptor
TFAM - Mitochondrial Transcription Factor A
VO₂ - oxygen uptake

Author's contributions

Each author contributed in equal part to the manuscript.

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Conflict of Interest

The authors declare no conflicts of interests derived from the outcomes of this study.

Ethical Publication Statement

Institutional Review Board that approved the protocol for the study: Sport and Exercise Sciences Research Unit, University of Palermo, Italy.

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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