Gene xxx (xxxx) 146958

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Moderate exercise reveals the influence of *ACTN3* R577X and *ACE* I/D polymorphisms on physical performance in non-athlete active subjects

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ARTICLE INFO

Edited by: De-Jia Li

ABSTRACT

Genome variations contribute to the vast majority of interindividual differences and may decisively influence sports capability. This study was conceived as a means of finding out when exactly polymorphisms start being physically discriminative. The polymorphisms we studied were two of the best characterized ones: *ACE* I/D and *ACTN3* R577X. These germline variants were determined in a cohort of 200 healthy volunteers from the university environment who underwent a series of physical evaluations that included a Cooper test, a 20-meter sprint test and a vertical jump test. Initially, no statistical association was found because the genetic effect was masked by those subjects with sedentary lifestyles. But when only physically active volunteers were considered, the *ACE* and *ACTN3* genotypes were found to have an impact on heart rate after the Cooper test (p-value = 0.033 and 0.032 respectively) and *ACTN3* was found to correlate with the total distance covered in the same test (p-value = 0.051). This can therefore be considered a paradigmatic example in which the environment might hide the genetic effect, with genotypic differences arising only upon training.

1. Introduction

Innate predisposition to physical activity is uneven in the general population. In the field of sports medicine, genetic research has sought profiles capable of predicting better athletic performance, preventing undesired injuries or decreasing recovery time after injury (Lim et al., 2021; Alfred et al., 2011). Although to date several variants have been identified, two genes are probably the best characterized examples: the angiotensin converting enzyme (*ACE*) and the α-actin-3 gene (*ACTN3*) (Ahmetov et al., 2013; Alfred et al. 2011; Chiu et al., 2012). *ACE* is involved in the renin angiotensin aldosterone system and is active in regulating blood volume, blood pressure and electrolyte balance (McCauley et al., 2010). The structural variant rs1799752 consists of an insertion-deletion (I-D) polymorphism which has been associated with improved athletic performance. Allele I carriers of *ACE* show better performance in endurance tests (McCauley et al. 2010; Rodriguez-Romo et al., 2010). On the other hand, ACTN3 belongs to the muscle cytoskeleton and predominates in the sarcomere Z-line (Niemi & Majamaa, 2005). *ACTN3* expressed only in glycolytic fast twitch fibers makes it possible to generate higher contractile capacity, something especially important in sports involving the generation of great speed and muscle power. The genetic variant rs1815739 comprises the substitution of an arginine (R) with a stop codon (X) at ACTN3 amino acid 577, and can create a non-functional version of ACTN3 (MacArthur et al., 2008; Seto et al., 2013).

Regarding the *ACE* I/D polymorphism, different studies have shown a predominance of the D allele in athletes who engage in strength and speed sports (Pereira et al., 2013; Bustamante-Ara et al., 2010; Delmonico et al., 2007). On the other hand, and with regard to the *ACTN3* R577X polymorphism, other researchers have reported the predominance of the RR or RX genotype in elite athletes with high strength and speed performance (Bell et al. 2012; Chiu et al., 2011; Erskine et al., 2014). In the field of gymnastics genetics, Morucci et al. (2014) observed significant decreases in the frequency of the *ACE* II polymor-

Abbreviations: ACE, angiotensin converting enzyme; *ACTN3*, α-actin-3 gene; BMI, body mass index; HWE, Hardy-Weinberg Equilibrium; MAF, Minor allele frequency; HR, Heart rate

[1] In memoriam.

https://doi.org/10.1016/j.gene.2022.146958
Received 18 March 2022; Received in revised form 13 September 2022; Accepted 4 October 2022
0378-1119/

phism with respect to ID and DD. This suggested gymnasts could be disposed towards power conditions, without similar findings in the RR and RX genotypes of *ACTN3*. Although these polymorphisms have been linked with sports performance in athletes, their impact on the general population remains elusive (Bray et al., 2009). We therefore wondered if these genetic variants exert an effect *per se*, or if their physiological benefit is obtained only by subjects undergoing training programs.

2. Materials and methods

2.1. Volunteer selection

Volunteers were recruited from the University of Malaga environment. The exclusion criteria used were: being below the age of majority, being over 40 years old, presenting a cardiopulmonary pathology that discouraged the performance of moderate physical activity, having a body mass index (BMI) above 40, and having a relative participating in the same study. The participants completed a detailed questionnaire covering anthropometric variables, drinking and smoking habits and their weekly physical activity, including any sports or physical exercise performed continuously for at least 30 min. The design of this study complied with the provisions of the Helsinki Agreement (World Medical Association, 64th General Assembly, Brazil 2013), with (Spanish) Law 14/2007 on Biomedical Research regarding the anonymous processing of data and the duty to guarantee confidentiality and implement security measures, and with current legislation on the protection of personal data. The study was approved by the Ethics Committee of the Andalusian Regional Government.

2.2. Physical performance tests

The individuals who agreed to participate in the study all gave their informed consent. Anthropometric variables were measured just before phenotyping, and a habits questionnaire was filled in. The tests started with a Cooper test to determine endurance capacity (Cooper, 1969). The participants were asked to run at a constant rate along a running track for 12 min. Heart rate (HR) was assessed with the HR Polar® RS 800CX monitor immediately at the end of the test and at 3 and 5 min after the test. Maximum HR was calculated in beats per minute subtracting the age in years to 220 as previously indicated (Deborah Riebe, Jonathan K. Ehrman, Gary Liguori, 2018). The total distance covered and the perceived exertion levels (from 1 to 10) were also recorded at the end. Next, to assess speed, a 20-meter sprint test was conducted using a Chronojump® photocells system. Participants did two runs, recovering totally in the interval, and the lower time was chosen. Finally, to assess explosive strength, flight time in vertical jumps without counter movements was recorded (Asmussen & Bonde-Petersen, 1974, Bosco C, 1994). In this test, the participants started from an upright position with their hands at their waists to avoid the influence of trunk extension on lower limb performance. The higher flight time of two attempts was recorded using a Chronojump® jump mat system (Pueo et al., 2020).

2.3. DNA extraction

After phenotyping, the volunteers provided a mouthwash with 12 ml of 0.95 % sterile saline. This was cryopreserved at 4 °C until processed. Samples were centrifuged for 3 min at maximum speed and the supernatant was discarded. The pellet was suspended in 250 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 2 % SDS, 10 mM EDTA and 1 μg/ml proteinase-K) and incubated at 50° C for 15 min. 30 μl of precipitation buffer (3 M sodium acetate) was then added and the sample was placed in ice for 5 min before being centrifuged again for 5 min at maximum speed. The supernatant was mixed with an equal volume of isopropanol and centrifuged again for 5 min. DNA pellets were washed with 250 μl of cold 70 % ethanol. The precipitate was resuspended in 50 μl of distilled water.

2.4. ACE I/D genotyping

PCR was performed in a final volume of 25 μl with SYBR® Green Master Mix 2X reaction mixture (BioRad), 20 ng of each DNA sample and 0.5 pmol of the previously described primers (Forward: 5′ - TGGAGACCACTCCCATCCTTTCT and Reverse: 5′ – GATGTGGCCATCACATTCGTCAGAT) (Fiuza-Luces et al 2011). The PCR was performed in an Applied Biosystems 2700 Thermal Cycler as per the following program: initial denaturation at 95° C for 5 min followed by 30 amplification cycles of: 95°C 30 *sec*, 58°C 30 *sec* and 72°C 1 min, and a final extension at 72 °C for 3 min. Amplicons were run in 1.5 % agarose gels with ethidium bromide. The 477 bp band represented the insertion allele and the 190 bp band represented the deletion allele.

2.5. ACTN3 R577X genotyping

A PCR was performed in a final volume of 25 μl with SYBR® Green Master Mix 2X reaction mixture (BioRad), 20 ng of each DNA sample and 0.5 pmol of each primer (Forward: 5′- CGCCCTTCAACAACTGGCTGGA and reverse: 5′- GGGTGATGTAGGGATTGGTGGAG) (Aranalde, L.C.R., et al 2016; Ortiz et al, 2021). The program followed was: initial denaturation at 95° C for 5 min, 30x amplification cycles of: 95°C 30 s, 60°C 30 s and 72°C 30 s, and a final extension at 72 °C for 3 min. We then added 12 μl of the PCR product to 2.5 μl of 10X Cutsmart buffer and 0.3 μl *Dde*I, 12.5 μl H_2O) and incubated it for one hour at 37° C. With a 3 % agarose gel electrophoresis, R allele was observed as a 489 bp band while the Stop (X) allele was observed as 392 bp + 97 bp bands.

2.6. Database creation and variable construction

The participants were phenotypically characterized blind to their genotypes. HR was assessed on the basis of a maximum HR, estimated as previously reported (Fox et al., 1971). Cardiac recovery variables were calculated as percentages of the maximum at 3 and 5 min after the end of the Cooper test. Vertical jump power was calculated with a modification of the Lewis formula (Mathews & Fox, 1976).

2.7. Statistical analysis

Allelic frequencies were calculated from the participants' genotypes. Genotypic distribution in the population was evaluated for Hardy-Weinberg equilibrium (HWE) using the University of Munich web tool (https://ihg.gsf.de/ihg/snps.html). Statistical analysis was performed with IBM's SPSS version 24.0 (SPSS Inc., Chicago, IL, USA). A chi-square test was used to study the distribution of categorical variables. Both U-Mann Whitney and T-tests were applied to study continuous dependent variables. To analyze the joint effect of the polymorphisms studied and environmental factors on each of the measured physical variables, multiple linear regression was used. Values of $p < 0.05$ were considered significant.

3. Results

This study was carried out in a non-consanguineous population of 209 volunteers with an average age of 22 ± 4 years (standard deviation). 49 % of the sample were women. The sample showed enrichment in non-smokers (90 %) and the weekly frequency of physical activity showed bimodal distribution, since 27 % of the sample reported no regular exercise per week while 23 % reported it at least three times a week. BMI was slightly higher among men: 24.2 ± 3.6 *vs* 22.3 ± 3.3 (average, standard deviation). The genotyping results are shown in

Table 1. The allele frequencies were in accordance with what we expected for a Caucasian population, and the genotypic distribution fitted the Hardy-Weinberg equilibrium.

In a first approach, two-sided correlation assays were performed taking vertical jump power, speed, the distance run in the Cooper test, perceived exertion, HR, and recovery as output variables. The results are shown in Table 2. Weekly exercise was significantly associated with every physical variable (Fig. 1), suggesting that any genetic effect would need to be adjusted taking this variable into account. Similarly, BMI was strongly associated with different outputs, such as vertical jump power. This, however, was due to the BMI components, since longer femur lengths increase flight time and the higher the weight the higher the power. Age also negatively correlated with different strength and endurance variables. We could not observe any effect associated with tobacco consumption, probably due to a lack of power.

We then looked at how the two genotypes contributed to the output variables measured. Univariate non-parametric statistics did not show any statistically significant differences when the entire series was analyzed (not shown), so we wondered to what extent the sedentary subjects might be introducing noise to the analysis. We therefore split the series into two subsets, one including those with sedentary lifestyles and the other made up of physically active volunteers (defined as individuals who reported that they engaged in physical activity at least three times per week) (See supplementary Table 1).

Splitting the data in this way clearly revealed the differences in athletic performance between the sedentary and active subgroups. Regarding the distance run in the Cooper test, the sedentary group averages ranged from 1.8 to 1.9 Km, in contrast to the 2.1–2.4 Km seen in the active group. Likewise, HRs in the sedentary group reached 80–87 % of their maximum as opposed to the 66–79 % observed in the active group. Univariate analysis highlighted some statistically significant differences that could be observed only in the physically active group, such as the one between *ACE* I/D and HR after the Cooper test: those with the II genotype showed a HR of 79 %, whereas those with the DD genotype showed a HR of 64 % (T-test, $p = 0.011$). In the sedentary group, maximum HR was a uniform 85 %, regardless of genotype.

We then performed a multivariate regression analysis taking into account the two polymorphisms under study and using a codominant model. No significant results were obtained for *ACTN3* R577X when vertical jump power was analyzed by age, weekly exercise and height, or when run speed was analyzed. Equivalently, the *ACE* I/D results were not statistically significant for the Cooper test distance run or for HR after the run (Table 3). Interestingly, under the same conditions the genotypes did show an association, or at least a trend towards association, under linear regression analysis. This occurred, for example, in the Cooper test among physically active subjects, where the *ACTN3*-R allele showed a higher average in the distance run (2,404 *vs* 2,107 m for *ACTN3* RR and XX, respectively, $p = 0.071$), and higher performance in the vertical jump (802 *vs* 645 W for *ACTN3* RR and XX, respectively, $p = 0.051$) (Table 3, Fig. 2). With regard to *ACE* I/D we found that physically active subjects with the II genotype had higher HRs immedi-

Table 1
Genotyping results. MAF = Minor allele frequency. HWE= Hardy-Weinberg Equilibrium

Locus	Genotype	Observed	MAF	HWE
ACTN3 R577X	RR	42	0.46	0.08
	RX	92		
	XX	28		
ACE I/D	DD	61	0.42	0.44
	ID	80		
	II	34		

Table 2
Spearman's correlation between endurance capacity, speed and explosive strength measurements and non-polymorphic variables.

		Gender	Smoking	Weekly exercise	Age	Height	Weight	BMI
Power	Rho	−0.610	0.040	0.147	−0.034	0.588	0.667	0.474
(Watts)	p-value	< 0.001	0.567	0.036	0.626	< 0.001	< 0.001	< 0.001
Speed	Rho	−0.588	0.131	0.324	−0.424	0.432	0.146	−0.100
(mps)	p-value	< 0.001	0.062	< 0.001	< 0.001	< 0.001	0.038	0.155
Cooper	Rho	−0.622	0.019	0.480	−0.331	0.449	0.180	−0.055
Distance	p-value	< 0.001	0.792	< 0.001	< 0.001	< 0.001	0.011	0.439
Cooper	Rho	−0.052	0.053	−0.220	0.160	0.090	0.206	0.204
Effort	p-value	0.483	0.470	0.003	0.028	0.221	0.005	0.005
Heart Rate	Rho	−0.151	0.015	−0.403	0.221	0.203	0.297	0.280
(To)	p-value	0.040	0.835	< 0.001	0.003	0.006	< 0.001	< 0.001
Recovery	Rho	−0.166	0.025	−0.185	0.142	0.118	0.220	0.233
3 min	p-value	0.034	0.748	0.018	0.070	0.132	0.005	0.003
Recovery	Rho	−0.162	0.040	−0.227	0.182	0.154	0.257	0.231
5 min	p-value	0.037	0.609	0.003	0.019	0.047	0.001	0.003

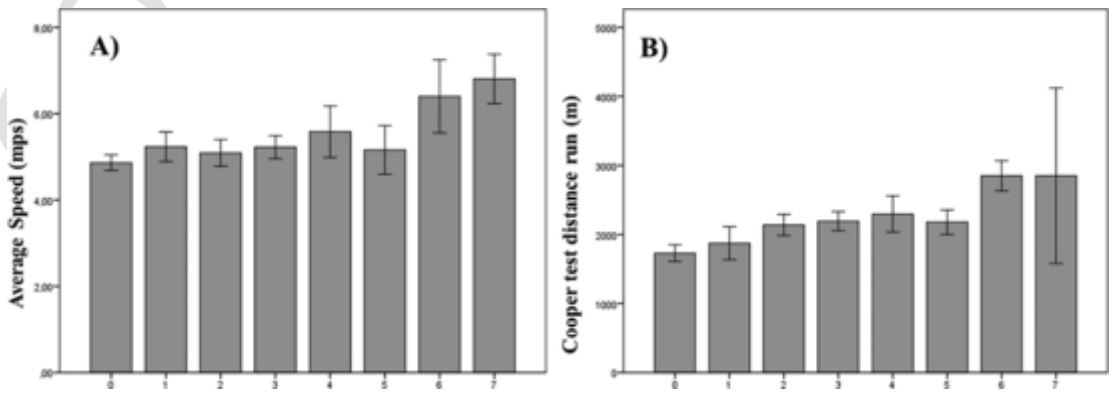

Fig. 1. Effect of weekly exercise on two illustrative variables: the 20 m sprint test (A) and the Cooper test distance run (B) (the bars represent average ± 95 % confident interval).

Table 3
Regression analysis using either the entire series or those with an active lifestyle. The regression models used a codominant genetic model adjusted by subject age, height, and physical activity, taking *ACTN3*-RR and *ACE*-DD as reference genotypes.

		ACTN3 R577X			*ACE* I/D		
		Entire series	Sedentary	Active	Entire series	Sedentary	Active
Power (Watts)	Beta	0.002	0.149	**−0.181**	−0.031	0.010	−0.09
	p-value	0.973	0.166	**0.071**	0.656	0.920	0.366
Speed (mps)	Beta	0.076	−0.143	−0.059	−0.057	0.060	−0.127
	p-value	0.323	0.160	0.574	0.444	0.536	0.244
Cooper Distance	Beta	0.091	−0.003	**−0.210**	−0.061	0.034	−0.145
	p-value	0.195	0.980	**0.051**	0.353	0.729	0.184
Cooper Effort	Beta	0.055	−0.087	−0.175	−0.053	**−0.212**	0.064
	p-value	0.485	0.459	0.141	0.487	**0.059**	0.589
Heart Rate (To)	Beta	0.056	0.160	**−0.230**	0.095	−0.094	**0.223**
	p-value	0.426	0.137	**0.033**	0.153	0.358	**0.032**
Recovery 3 min	Beta	−0.079	0.168	−0.005	0.018	−0.040	0.077
	p-value	0.358	0.155	0.970	0.825	0.727	0.567
Recovery 5 min	Beta	−0.077	**0.221**	−0.052	0.011	−0.104	0.104
	p-value	0.356	**0.052**	0.689	0.889	0.351	0.420

ately after the run (79 *vs* 64 % for II and DD genotypes, respectively, p = 0.032) (Table 3, Fig. 2D).

4. Discussion

Single nucleotide polymorphisms are the most frequent functional variants of our genome and one of the main factors that identify us in our interaction with the environment, modifying organs and tissue function to varying extents. In the case of sport, and depending on the discipline, genome variations may prove decisive. In this work, we focused on two of the more paradigmatic polymorphisms. *ACE* and *ACTN3* germline variants play a significant role in determining the type of physical activity pursued by elite athletes. Previous studies have observed that elite sportspeople are the result of an almost genetic process of selection, highly biased towards either speed or endurance disciplines (Eynon N, et al 2013; Jacob Y et al 2018). We wanted to learn more about the precise moment in which polymorphisms start being physically discriminative. Could their physiological effects have an impact at lower levels of physical activity? Would their contribution be observable even among subjects with sedentary lifestyles or basic levels of training? Could uncontrolled amateur training improve sports performance? To try to answer these questions, we studied the influence of *ACE* and *ACTN3* polymorphisms on physic responses in a general population which included both sedentary individuals and people with physically active lifestyles.

After analyzing a population of non-athlete subjects with two levels of physical activity defined as 1) sedentary lifestyle and 2) from three to seven sessions of physical activity per week, the results obtained revealed that, among sedentary people, the absence of a functional ACTN3 (allele X), has a favorable codominant effect on higher vertical jumping power. This contradicts most studies carried out in athletes

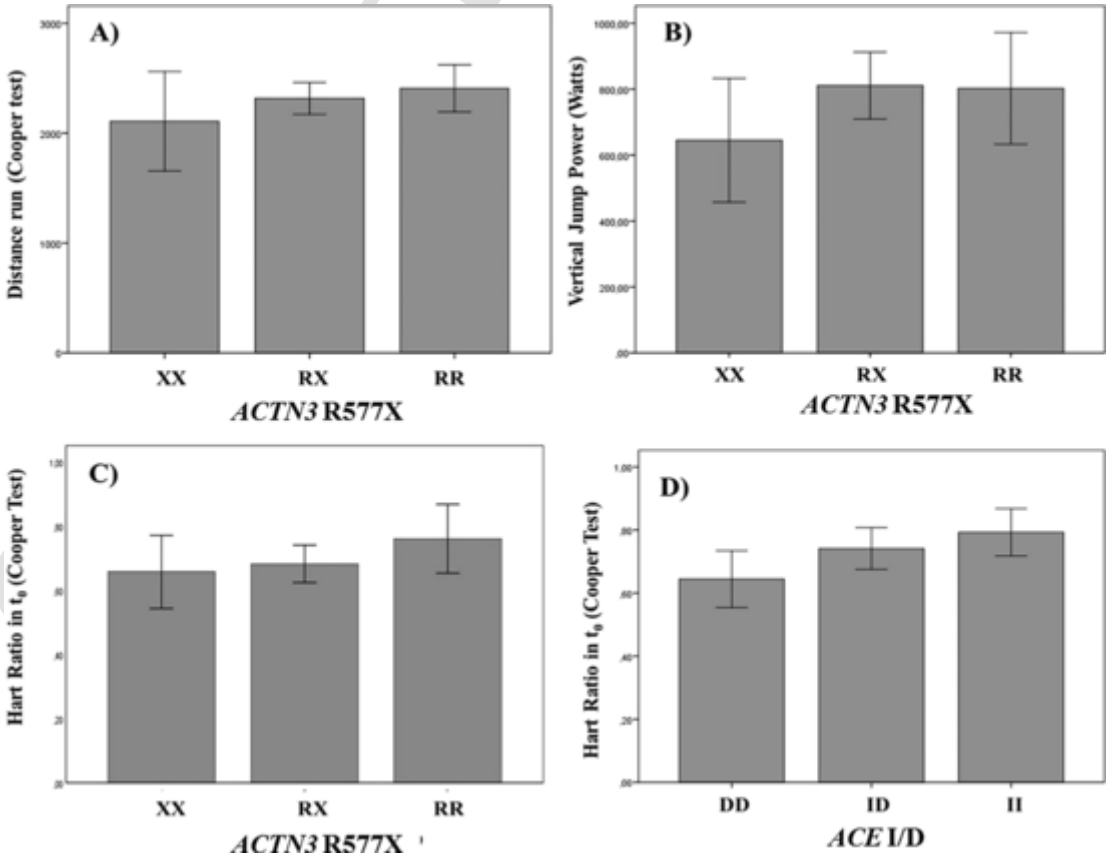

Fig. 2. Graphical representation of different variables measured according to subject genotypes. The bars represent the average and the error bars show the 95% confidence interval. Only those subjects with physically active lifestyles were plotted.

(Jacob, Y.,et al. 2018). ACTN3 XX genotype shows a reduction in this parameter among the active group, perhaps due to muscle damage as already observed in some studies (Zouhal, H., et al 2021). We could speculate that even mild training impairs power performance in subjects without ACTN3. In this particular parameter (vertical jump), there were no appreciable differences with respect to the *ACE* polymorphism. In the Cooper run test, the distance run by sedentary participants tended to be better or greater in genotypes XX and II, suggesting better aerobic adaptation among volunteers without ACTN3 and with less ACE activity. This may corroborate what has been reported about the association with greater aerobic capacity, at least among II subjects. An opposite trend was found in the active group, with higher values in the RR and DD genotypes. HR in sedentary individuals was very homogeneous between genotypes, although the people who improved the most with physical activity and had lower HRs were the XX subjects as opposed to the *ACTN3* RR subjects. Concerning the *ACE* genotype, physically active DD subjects showed a clearly lower HR, the XXDD combination being the one that best correlated to the improvement of heart rate with physical activity. With regard to HR recovery (3 min after finishing), RR subjects in the sedentary group recovered earlier.

The results obtained in this work suggest a clear example of genetic-environment interaction where the genotypic effect is detectable in people with non-professional training.

Uncited reference

Bell et al. (2012).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

Acknowledgements

We thank all the volunteers for their generous contribution to this work and the Molecular Medicine alumni for their technical help during phenotyping and genotyping. The authors would like to dedicate this manuscript to the memory of Jose Ramon Alvero Cruz: colleague, mentor, friend and the driving force behind this research.

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