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Evaluation of gene polymorphisms in exercise-induced oxidative stress and damage

ARTHUR KENJI AKIMOTO[1], ANA LUISA MIRANDA-VILELA[1], PENHA CRISTINA ZAIDAN ALVES[1], LUIZ CARLOS DA SILVA PEREIRA[2], GRACIANA SOUZA LORDELO[1], CASSIA DE OLIVEIRA HIRAGI[1], IZABEL CRISTINA RODRIGUES DA SILVA[1], CESAR KOPPE GRISOLIA[1] & MARIA DE NAZARÉ KLAUTAU-GUIMARÃES[1]

[1]*Departamento de Genética e Morfologia, Laboratório de Genética, Instituto de Ciências Biológicas, Universidade de Brasília, Brasília/DF, Brazil, and* [2]*Laboratório Sabin de Análises Clínicas, Brasília/DF, Brazil*

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Abstract

Many potentially significant genetic variants related to oxidative stress have been identified and performance in endurance sports is a multi-factorial phenotype. Thus, it was decided to investigate the influences of the haptoglobin (Hp), MnSOD (Val9Ala), CAT (21A/T), GPX1 (Pro198Leu), ACE, glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) genes' polymorphisms on the oxidative stress and damage suffered by human athletes (runners). Blood samples taken immediately after a race were submitted to genotyping, comet and TBARS assays, biochemical analyses of creatine kinase (CK), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). MnSOD significantly influenced results of CK and a possible association between Hp1F-1S and Hp1S-2 genotypes with superior TBARS values was found. Higher or lower TBARS and CK values or DNA damage also depended on the interaction between Hp and ACE or GST genotypes, indicating that MnSOD and Hp polymorphisms can be determining factors in performance, at least for runners.

Keywords: *Exercise-induced damage, biochemical markers of tissue injuries (AST, ALT, CK), TBARS assay, comet assay, antioxidant enzyme polymorphisms.*

Introduction

Reactive oxygen species (ROS) are continuously generated by aerobic organisms, mainly as a result of normal oxidative metabolism in the mitochondria [1,2]. Under normal circumstances, ROS are neutralized by an elaborate antioxidant defense system consisting of enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) and numerous non-enzymatic antioxidants [1,3,4]. Because exercise increases oxygen consumption, it can cause an imbalance between ROS and antioxidants, which is referred to as oxidative stress [5–7]. Acute intensive exercise is a well accepted model to induce oxidative stress [6,8], leading to an increase in plasma lipid peroxidation [9] plus oxidative damages to DNA, muscles and other tissues [2,4,7,9,10].

Studies have identified elevations in blood oxidative stress markers after acute exercise [5,9,11,12], such as malondialdehyde (MDA), a by-product of lipid peroxidation that can be measured by TBARS assay [11,12]. At the same time, the increase in cytosolic proteins such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) in the circulation after exercise reflects cellular injury and can be used as an exercise-induced damage marker [13–18]. Because strenuous exercise has been shown to induce DNA damage in peripheral leukocytes, comet assay could be used to evaluate this damage [19,20]. Although this is not the only way to measure oxidative DNA damage, it is one of the most sensitive and accurate [21].

Correspondence: Ana Luisa Miranda-Vilela, Departamento de Genética e Morfologia, Instituto de Ciências Biológicas, Universidade de Brasília, Brazil. Tel: +55 61 3307-2161. Fax: +55 61 3273-4942. Email: mirandavilela@unb.br; analuisamv@uol.com.br

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Many potentially significant genetic variants related to oxidative stress have already been identified. Several single nucleotide polymorphisms (SNPs) have been reported to result in changes in the levels or the activities of antioxidant enzymes, which can lead to reduction in protection against oxidative stress [22]. They include the Val9Ala in the mitochondrial targeting sequence of the MnSOD gene (NCBI, refSNP ID: rs1799725), 21A/T in the promoter region of the CAT gene (NCBI, refSNP ID: rs7943316) and Pro-198Leu of the GPX1 gene (NCBI, refSNP ID: rs1050450), among others [23–27]. In addition, it has been demonstrated that the ability of the serum glycoprotein haptoglobin (Hp) to bind free haemoglobin (Hb) in the plasma and block Hb-induced oxidative damage is phenotype-dependent [28]. Hp polymorphism has been associated with the prevalence and clinical evolution of many inflammatory diseases, including infections, atherosclerosis and autoimmune disorders [29]; such associations can be explained by functional differences among the phenotypes [28,29].

Moreover, performance in endurance sports is a multi-factorial phenotype, influenced by several factors, including physique and biomechanical, physiological, metabolic, behavioural, psychological and social characteristics. Hence, it is unlikely that the genetic component of these traits will be explained by DNA sequence variation at only a few genes. It is more likely that several gene loci, each with a small but significant contribution, will be responsible for this genetic component [30]. A limited number of candidate genes have been tested in this context and the gene locus encoding angiotensin I-converting enzyme (ACE, EC 3.4.15.1) is the most widely studied [30,31]. The ACE gene is located on the long arm of chromosome 17 (17q23). It is characterized by a polymorphism resulting from the presence or absence of a 287 base pair (bp) fragment of an Alu repeated sequence within intron 16 of the ACE gene, the corresponding designation of I (for Insertion) or D (for Deletion) of the two resulting alleles [32,33].

Metabolic characteristics are one of the factors that influence endurance performance [30] and the isoenzymes of the glutathione S-transferase (GST) family (EC 2.5.1.18) play a vital role in phase 2 of biotransformation of many substances, including products of oxidative stress [34]. The glutathione S-transferases M1 (GSTM1) and T1 (GSTT1) genes code for the cytosolic enzymes GST-μ (mu) and GST-θ (theta), respectively. These enzymes catalyse reactions involved in the conjugation reactions between reduced glutathione (GSH) and a variety of electrophilic compounds [34,35]. It is thought that most GST substrates are xenobiotics or products of oxidative stress [34]. Thus, gene polymorphisms in that family can influence exercise-induced oxidative stress and endurance performance. The genes glutathione S-transferase M1 (GSTM1, chromosome 1p13.3) and glutathione S-transferase T1 (GSTT1, chromosome 22q11.2) may be deleted (null alleles/null genotypes) and these polymorphisms have been shown to be associated with susceptibility to cancer [34,35]. However, these loci have never been studied in the context of endurance performance or exercise-induced oxidative stress.

Hence, the aim of this study was to investigate the influences of the Hp, MnSOD (Val9Ala), CAT (21A/T), GPX1 (Pro198Leu), ACE, GSTM1 and GSTT1 genes' polymorphisms on the oxidative stress and damage suffered by runners.

Materials and methods

Study design and participants

The trial was conducted from August 2007–April 2008. Volunteers of both sexes (82 men and 53 women) and different age groups (15–58) were recruited in high schools, colleges, universities, clubs and companies in Brasília (Federal District, Brazil). The selection criterion (inclusion/exclusion criterion) used for the runners was that they had at least a 4000 m run performance, which means that only trained sportsmen were included. The race took place outdoors on flat tracks and the athletes could choose the distance that they would cover (4-21 Km), according to the type, intensity and length of weekly training. The volunteers were grouped for distance chosen, so that the same route was covered within the same time for each group of athletes, guaranteeing the same intensity (time needed to finish the race). The volunteers were informed about the purpose of the study and all of them received a random number generated by a computer.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee for Health Sciences Faculty Research of the University of Brasília and by the National Commission for Ethics in Research (CONEP). Written informed consent was obtained from all subjects.

Procedures and measurements

Blood samples were drawn with EDTA immediately after the race. Blood samples were used to verify DNA damage by comet assay (single-cell gel electrophoresis or SCGE) and genes' polymorphisms, while serum samples were used for AST, ALT, CK and TBARS assays.

Comet assay

The comet assay (alkali method) was carried out according to Singh et al. [36] with few modifications. Microscope slides were dipped briefly into 1.5% hot

(60°C) normal melting agarose (NMA) prepared in phosphate-buffered saline (PBS). The slides were dried overnight at room temperature and then stored at 4°C until use. Subsequently, freshly collected EDTA peripheral blood from each sample (20 μL) was suspended in 120 μL of 0.5% low melting point agarose in PBS (LMA) (GIBCO BRL, Grand Island, NY, USA) at 37°C and pipetted onto six microscope slides pre-coated with a layer of normal melting point agarose. This mixture was allowed to set at 4°C for 10 min Then the slides were immersed in a freshly prepared cold (4°C) lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, NaOH to pH 10.0–10.5, 1% lauroyl sarcosine; 1% Triton X-100 and 10% dimethyl sulphoxide were added directly before use) at 4°C for 1 h After lysis the slides were placed in an electrophoretic tank in 300 mM NaOH and 1 mM EDTA, pH $>$ 13.0 for 30 min Subsequently, electrophoresis was carried out at 25 V, 300 mA for 40 min Soon after neutralization (3 $\times$ 5 minin 0.4 M Tris, pH 7.5 at 4°C), the slides were stained with ethidium-bromide (EB) at 20 μg/mL, fixed by 100% ethanol for 5 minand analysed with a Zeiss Axioskop 2 fluorescence microscope (filter 510–560 nm, barrier filter 590 nm) with total increase of 400×. All slides were duplicated. One hundred comets on each slide were scored visually as belonging to one of the five classes proposed by Collins et al. [37] and the DNA damage (DD) was calculated according to Jalonszynski et al. [38].

Biochemical analyses

Serum AST, ALT and CK analyses were run on the automated chemistry analyser ADVIA 1650 (Bayer Diagnostics USA, Minnesota, USA), using the appropriate Advia chemistry r eagents and protocols. The TBARS assay was carried out according to Wasowicz et al. [12] and the fluorescence was measured with a Jasco FP-777 spectrofluorometer (excitation: 525 nm emission: 547 nm).

Haptoglobin (Hp), antioxidant enzymes, Ace, GST M1 and GST T1 genotyping

DNA was isolated from the buffy-coat layer using the Blood genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK). DNA samples underwent amplification in an MJ PTC-100 (MJ. Research Inc., Waltham, MA, USA).

Hp genotypes were determined by allele-specific polymerase chain reaction (PCR) as described by Yano et al. [39], while Mn-SOD, CAT and GPX1 genotypes were determined by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assays performed as described respectively by Mitrunen et al. [40], Ukkola et al. [41] and Zhao et al. [42]. DNA fragments containing I/D polymorphism in intron 16 of the ACE gene were amplified by PCR as previously described by Rigat et al. [43], using DMSO (dimethyl sulphoxide) as recommended by Odawara et al. [44], to avoid mistyping of the DD genotype. The glutathione S-transferase (GST) GSTM1 and GSTT1 fragments were amplified simultaneously as proposed by Chen et al. [45], using β-globin as positive control. The absence of an amplification product combined with the presence of a positive control band (268 bp DNA fragment of β-globin) indicated the null (variant) type for both polymorphisms (Table I).

The PCR and PCR-based RFLP products were resolved in non-denaturing polyacrylamide gels stained with silver nitrate.

Statistical analyses

Allelic and genotypic frequencies were estimated by gene counting and the goodness of fit of the genotype distribution to the Hardy-Weinberg equilibrium (HWE) was assessed by the chi-square (χ^2) test. Values of $p > 0.05$ indicated HWE. Data for genetic diversity were assessed by comparing the observed and expected heterozygosities and FIS (inbreeding coefficient). Probability (p) values for co-dominant markers (Hp, MnSOD, CAT, GPX1 and ACE) were generated using Genepopweb Statistical Program version 3.4 (http://genepop.curtin.edu.au). For dominant markers (GSTM1 and GSTT1), χ^2 test was calculated using a chi-square calculator program. Since the PCR method is not suitable for distinguishing between homozygous (+/+, wild type) and heterozygous (+/-)these two groups were considered together (non-null genotypes) and compared with the variant group (null genotypes).

Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 17.0. Data were expressed as mean $\pm$ SE (standard error of mean) and values of $p < 0.05$ were considered statistically significant. The continuous variables were tested for normal distribution with Shapiro-Wilk. Differences between sexes were evaluated by the Student t-test and the possible correlations between the parameters sex/age groups and age groups/covered distance were analysed through Chi-square correlation test. Possible differences among the groups analysed were investigated through ANOVA or Kruskal-Wallis test (when the data presented heterogeneous variability). For significant ANOVA results, Bonferroni's post-hoc test was chosen to carry out 2-to-2 comparisons (between the genotypes). For significant Kruskal-Wallis results, Mann-Whitney U-test was performed to verify differences between genotypes (2-to-2 comparisons). For ANOVA or Kruskal-Wallis significant results, the ODDS Ratio (OR) with 95% confidence intervals (CI) was calculated to estimate whether genotypes were associated with higher/lower damage or lipid peroxidation. For

Table I. Chromosome location, primer sequences and restriction enzymes used in the genotyping of Haptoglobin (Hp), MnSOD, CAT, GPX1, ACE, GSTM1 and GSTT1 genes' polymorphisms.

Genetic markers	Chromosome location	Primer sequences	Restriction enzymes	References
Hp*1F	16q22.1		—	[39]
F3		5′ CAGGAGTATACACCTTAAATG 3′		
C72		5′ AATTTAAAATTGGCATTTCGCC 3′		
Hp*1S				
C51		5′ GCAATGATGTCACGGATATC 3′		
S2		5′ TTATCCACTGCTTCTCATTG 3′		
Hp*2				
F3		5′ CAGGAGTATACACCTTAAATG 3′		
C42		5′ TTACACTGGTAGCGAACCGA 3′		
MnSOD	6q25.3		*Ngo MIV*	[40]
Sense		5′ ACCAGCAGGCAGCTGGCGCCGG 3′		
Antisense		5′ GCGTTGATGTGAGGTTCCAG 3′		
CAT	11p13		*Hinf I*	[41]
Sense		5′ AATCAGAAGGCAGTCCTCCC 3′		
Antisense		5′ TCGGGGAGCACAGAGTGTAC 3′		
GPX1	3p21.3		*Apa I*	[42]
Sense		5′AGCCCAACTTCATGCTCTTC 3′		
Antisense		5′CAGGTGTTCCTCCCTCGTAG 3′		
ACE	17q23		—	[43]
Sense		5′ CTGCAGACCACTCCCATCCTTTCT 3′		
Antisense		5′ GATGTGGCCATCACATTCGTCAGAT 3′		
GSTM1	1p13.3		—	[45]
GSTM1/6		5′ GCTTACCGTGTTATGGAGGTTC 3′		
GSTM1E7A		5′ TTGGGAAGGCGTCCAAGCGC3′		
GSTM1E7		5′ TTGGGAAGGCTGCCAAGCAG 3′		
GSTT1	22q11.2		—	
Sense		5′ TTGGGAAGGCGTCCAAGCGC3′		
Antisense		5′ TTGGGAAGGCGTCCAAGCGC3′		
β-globin	11p15.5	Positive control	—	
Beta 1		5’ CAACTTCATCCACGTTCACC3’		
Beta 2		5’ GAAGAGCCAAGGACAGTTAC3’		

this, the Pearson chi-square test was applied and differences were considered significant at $p < 0.05$. To calculate the OR the following parameters were used: for the TBARS assay the median was used, which was >0.027 and <0.027 nmol/mL of MDA for both sexes; for CK they were >170 and <170 UL for males and >145 and <145 for females, according to reference values proposed by Freire et al. [46]. The interactions between two genetic markers in the results of AST, ALT, CK and Comet assays were also analysed through Univariate Analyses of Variance (UNIANOVA).

Results

Results indicated a significant deviation from Hardy-Weinberg equilibrium (HWE) for the Hp (Hp sub-types: $p = 0.0093$), MnSOD ($p < 0.001$), GSTM1 ($p < 0.001$) and GSTT1 ($p < 0.001$) loci. The Hp locus was appropriate for a heterozygote deficit ($p < 0.001$), while the MnSOD locus was for a heterozygote excess ($p < 0.001$). For GSTM1 and GSTT1, results were compatible with heterozygote deficit, due to homozygous (+/+, wild type) and heterozygous (+/-) being considered together within non-null genotypes, since the PCR method is not suitable for distinguishing these genotypes. When the Hp*1 alleles were treated as a single block, the genotypic distributions were in accordance with the EHW ($p = 0.1678$). The genotypic distributions of CAT, GPX1 and ACE loci were in accordance with HWE ($p = 0.2952$ for CAT, $p = 0.4153$ for GPX1 and $p = 1.0000$ for ACE loci). MnSOD locus presented a

Table II. Values of AST, ALT, CK and Comet assays for the total and gender groups.

	n (%)	AST* (U/L)	ALT (U/L)	CK* (U/L)	Comet* (ua)	Tbars (nmol/mL of MDA)
Total	135 (100.0)	29.60 ± 0.75	23.36 ± 0.99	282.64 ± 28.72	244.50 ± 2.45	0.0273 ± 0.001
Men	82 (60.7)	30.99 ± 0.91	24.34 ± 1.22	361.46 ± 42.98	240.28 ± 2.93	0.0272 ± 0.001
Women	53 (39.3)	27.51 ± 1.24	21.89 ± 1.66	162.19 ± 22.97	251.02 ± 4.16	0.0274 ± 0.001
p-value		0.022	0.226	0.000	0.032	0.883

The data correspond to the means and to the standard error (SE). $*p < 0.05$; differences between sexes by the Student t-test. U/L = unit per litre; ua = arbitrary units; n = sample size.

Table III. Influences of distance covered (Km) on the total group for AST, ALT, CK and Comet assay values.

Km	*n* (%)	AST* (U/L)	ALT (U/L)	CK (U/L)	Comet (ua)	Tbars* (nmol/mL of MDA)
4–5	55 (40.7)	27.35 ± 1.07[a]	20.72 ± 1.25	215.58 ± 27.6	242.96 ± 2.98	0.026 ± 0.001[a]
6–7	40 (29.6)	29.38 ± 1.34	24.56 ± 1.68	339.28 ± 73.14	252.03 ± 5.17	0.031 ± 0.001[b]
8–10	32 (23.7)	32.34 ± 1.65	24.75 ± 2.59	313.03 ± 60.43	235.31 ± 5.33	0.026 ± 0.001[a]
16–21	8 (5.9)	34.88 ± 2.60[b]	29.75 ± 4.35	346 ± 89.69	254.12 ± 10.67	0.027 ± 0.003
p-values		0.004	0.096	0.271	0.119	0.004

U/ L= unit per litre; ua=arbitrary units; n=sample size. p-values for ALT and CK were generated by ANOVA, while p-values for AST, Comet and TBARS assays were by the Kruskall-Wallis test. [a] and [b] indicate significant differences between the covered distances detected by the Mann-Whitney U-test in the 2-to-2 comparisons: [a]=significant compared to [b]: p=0.001 in the comparison of 4–5 and 6–7 Km; p=0.009 in the comparison of 6–7 and 8–10 Km.

heterozygosity-observed (Ho) value higher than the heterozygosity-expected (He) value and an FIS value (FIS= −0.5451) compatible with selection in favour of heterozygotes.

There was a significant correlation between age groups and the distance covered ($p < 0.001$). Significant differences appeared for AST, CK and Comet assays between men and women (Table II) and for distance covered (p=0.004). For the latter, the Mann-Whitney U-test showed significant differences between 4–5 and 6–7 Km (p=0.001) and between 6–7 and 8–10 Km (p=0.009) (Table III). No significant differences were found among the age groups (Table IV).

For the genetic markers, differences were detected only for MnSOD polymorphism in CK values (p=0.049); the Ala/Ala genotype presented significant differences in respect to the Val/Val (p=0.013) and Val/Ala (p=0.025) genotypes (Table V). Although Haptoglobin sub-types showed a p-value of 0.051 for TBARS assay, the Mann-Whitney U-test detected significant differences between Hp 1F-1S and Hp 1S-1S (p=0.006) and between Hp 1S-1S and Hp 1S-2 (p=0.003) in the 2-to-2 comparison (Table V). No other significant values were found in the other analyses of the genetic markers (Tables V and VI). For TBARS and CK values, respectively, the Haptoglobin (Hp) and MnSOD polymorphism genotype distributions were presented, plus the Odds Ratio (OR) with 95% confidence intervals (CI) in Table VII.

When the interaction between two genetic markers was performed, significant results appeared for CK with Hp/GSTM1 (p=0.005) and Hp/GST (p=0.007), for Comet assay with Hp/ACE (p=0.048) and for Tbars assay with Hp/ACE (p=0.044). For CK, the greatest damage was presented by individuals carrying Hp 1F-1S and GST M1+T1+ genotypes; while the least damage was observed for individuals Hp 1F-1F/GST M1+T1+. For Comet assay, the greatest damage was observed in subjects Hp 1F-2/ACE DD and II, while the least damage was presented by 1F-1F/ACE ID individuals. Concerning TBARS assay, the highest lipid peroxidation was observed for Hp 1F-1S/ACE II and the lowest for Hp 1S-1S/ACE II (Figure 1).

Discussion

A weak production of ROS is necessary for normal contractile activity of skeletal muscles [47] and physical training is a known to induce antioxidant enzymes [9,10]. However, intensive or prolonged exercise, above habitual intensity of effort or training with very elevated frequency, overloads the endogenous antioxidant system's capacity, leading to an increase in plasma lipid peroxidation [9] plus oxidative damage to DNA, muscles and other tissues [2,4,7,9,10]. Most exercise-induced physiological and biochemical changes have already been well studied [3,4,7–10,18,48] and our study did not aim to evaluate such alterations. We investigated the influences of some genes' polymorphisms on the oxidative stress and damage suffered by runners, after a race run outdoors on flat tracks. Only trained sportsmen were included and they could choose the distance that they would cover, according to the type, intensity and length of their weekly training. In this context, the significant correlation between age groups and the distance covered obtained in this study could be expected, because

Table IV. Influences of age groups (year-olds) on the total group for AST, ALT, CK and Comet assay values.

Age groups	*n* (%)	AST (U/L)	ALT (U/L)	CK (U/L)	Comet (ua)	Tbars (nmol/mL of MDA)
15–19 (teenager)	23 (17)	31.48 ± 1.99	21.48 ± 1.27	406.09 ± 121.37	240.87 ± 4.51	0.0249 ± 0.001
20–40 (young adult)	83 (61.5)	29.06 ± 0.95	22.72 ± 1.40	280.98 ± 30.04	243.40 ± 3.17	0.0278 ± 0.001
41–58 (middle age)	29 (21.5)	29.64 ± 1.52	26.79 ± 1.99	186.18 ± 25.48	250.52 ± 5.92	0.0278 ± 0.001
p-values		0. 868	0. 182	0. 619	0. 763	0. 478

U/L=unit per litre; ua=arbitrary units; *n*=sample size. *p*-values for ALT were generated by ANOVA, while *p*-values for AST, CK, Comet and TBARS assays were by the Kruskall-Wallis test.

Table V. Influences of Haptoglobin (Hp), MnSOD, CAT, GPX1 and ACE genes' polymorphisms on the AST, ALT, CK and Comet assay values.

Genetic markers	*n* (%)	AST (U/L)	ALT (U/L)	CK (U/L)	Comet (ua)	Tbars (nmol/mL of MDA)
Hp						
1F-1F	9 (6.7)	29.11 ± 2.51	18.78 ± 2.21	250.44 ± 100.08	241.78 ± 9.75	0.029 ± 0.003
1F-1S	9 (6.7)	27.89 ± 3.49	21.56 ± 2.94	462.11 ± 289.95	246.22 ± 8.79	0.031 ± 0.002a
1S-1S	19 (14.1)	28.74 ± 1.3	19.26 ± 1.74	289.00 ± 56.49	246.68 ± 6.07	0.024 ± 0.001b
1F-2	21 (15.6)	27.62 ± 1.68	23.9 ± 2.78	243.76 ± 71.93	251.95 ± 7.75	0.027 ± 0.002
1S-2	38 (28.1)	32.03 ± 1.63	26.14 ± 2.25	276.14 ± 33.67	242.08 ± 4.53	0.029 ± 0.001a
2-2	39 (28.9)	29.29 ± 1.41	23.92 ± 1.76	272.67 ± 43.56	242.00 ± 4.31	0.026 ± 0.001
p-values		0.423	0.132	0.621	0.856	0.051
MnSOD						
Val/Val	25 (18.5)	31.36 ± 1.36	26.72 ± 2.17	293.48 ± 52.42a	239.92 ± 4.59	0.030 ± 0.002
Val/Ala	103 (76.3)	29.20 ± 0.88	22.29 ± 1.1	291.46 ± 35.28a	244.57 ± 2.85	0.027 ± 0.001
Ala/Ala	7 (5.2)	29.14 ± 4.32	26.86 ± 6.33	115.43 ± 21.92b	259.71 ± 13.94	0.025 ± 0.003
p-values		0.199	0.057	0.049	0.409	0.212
CAT						
AA	25 (18.5)	29.04 ± 1.60	24.84 ± 2.29	277.72 ± 45.96	245.20 ± 5.56	0.026 ± 0.001
AT	59 (43.7)	31.18 ± 1.30	24.33 ± 1.82	312.14 ± 42.77	244.64 ± 3.86	0.027 ± 0.001
TT	51 (37.8)	28.12 ± 1.02	21.55 ± 1.12	251.51 ± 53.43	243.98 ± 3.9	0.028 ± 0.001
p-values		0.233	0.529	0.386	0.954	0.872
GPX1						
Pro/Pro	67 (49.6)	29.8 ± 1.07	22.89 ± 1.47	307.88 ± 50.83	239.67 ± 3.37	0.027 ± 0.001
Pro/Leu	53 (39.3)	36.75 ± 6.82	25.49 ± 2.02	658.19 ± 378.63	248.21 ± 3.68	0.028 ± 0.001
Leu/Leu	15 (11.1)	27.2 ± 2.21	22.93 ± 3.19	175.73 ± 25.97	252.93 ± 9.19	0.027 ± 0.002
p-values		0.281	0.629	0.452	0.151	0.945
ACE						
DD	48 (35.6)	30.77 ± 1.48	24.15 ± 2.02	340.83 ± 65.85	248.21 ± 4.42	0.027 ± 0.001
ID	66 (48.90	34.68 ± 5.56	24.46 ± 1.69	567.12 ± 304.84	241.92 ± 3.32	0.028 ± 0.001
II	21 (15.6)	28.19 ± 1.88	21.76 ± 1.92	207.52 ± 26.16	244.10 ± 6.15	0.026 ± 0.002
p-values		0.587	0.881	0.945	0.585	0.446

The data correspond to the means and to the standard error (SE). U/L = unit per litre; ua = arbitrary units; n = sample size. p-values were generated by the Kruskall-Wallis test. The lower-case letters indicate significant differences detected by the Mann-Whitney U-test in the 2-to-2 comparisons between genotypes, being [a] = significant compared to [b]: for MnSOD, p = 0.013 in the comparison between Ala/Ala and Val/Val and p = 0.025 in the comparison between Ala/Ala and Val/Ala; for Hp, p = 0.006 in the comparison between Hp 1F-1S and Hp 1S-1S and p = 0.003 in the comparison between Hp 1S-1S and Hp 1S-2.

aged muscles exhibit reduced antioxidant adaptation to training, when compared to young muscles and strenuous physical exercise can impose oxidative stress on the skeletal muscle and other organs due to increased ROS [4,6,48,49]. In the same way, the significant results for AST also could be expected, since this marker is used to evaluate muscular, myocardial, erythrocyte, hepatic, renal and pancreatic cell injuries [13,14] and significant results appeared between 4–5 and 16–21 Km in the 2-to-2comparisons.

Table VI. Influences of GSTM1 and GSTT1 genes' polymorphisms on the AST, ALT, CK and Comet assay values.

Genetic markers	*n* (%)	AST (U/L)	ALT (U/L)	CK (U/L)	Comet (ua)	Tbars (nmol/mL of MDA)
GSTM1						
−	87 (64.4)	33.97 ± 4.23	24.21 ± 1.43	499.61 ± 231.62	244.45 ± 3.1	0.028 ± 0.001
+	48 (35.6)	29.21 ± 1.39	23.42 ± 1.86	305.87 ± 62.57	244.58 ± 4.03	0.027 ± 0.001
p-values		0.352	0.671	0.998	0.703	0.523
GSTT1						
−	19 (14.1)	28.78 ± 2.06	28.11 ± 3.34	258.42 ± 54.87	246.11 ± 7.27	0.029 ± 0.001
+	116 (85.9)	29.73 ± 0.80	22.62 ± 1.01	286.64 ± 32.37	244.23 ± 2.60	0.027 ± 0.001
p-values		0.621	0.100	0.436	0.791	0.234
GST						
M1-T1−	11 (8.1)	29.80 ± 3.12	30.30 ± 4.78	281.55 ± 80.00	240.18 ± 8.87	0.028 ± 0.002
M1-T1+	76 (56.3)	29.83 ± 0.91	22.40 ± 1.10	267.93 ± 30.37	245.07 ± 3.32	0.028 ± 0.001
M1+T1−	8 (5.9)	27.50 ± 2.67	25.38 ± 4.74	226.63 ± 74.61	254.25 ± 12.3	0.03 ± 0.001
M1+T1+	40 (29.6)	29.55 ± 1.59	23.03 ± 2.05	321.72 ± 73.64	242.65 ± 4.18	0.026 ± 0.001
p-values		0.817	0.316	0.883	0.774	0.380

The data correspond to the means and to the standard error (SE). U/L = unit per litre; ua = arbitrary units; n = sample size. p-values were generated by the Kruskall-Wallis test.

Table VII. The Haptoglobin (Hp) and MnSOD polymorphisms genotype distributions plus the Odds Ratio (OR) with 95% confidence intervals (CI) for TBARS and CK values, respectively.

Genotypes	*n* (%)	OR (95% CI)	*p*-value
Hp			
1F-1F	9 (6.7)	1.375	0.645
1F-1S	9 (6.7)	4.103	0.066
1S-1S	19 (14.1)	0.777	0.597
1F-2	21 (15.6)	0.333	0.040
1S-2	38 (28.1)	2.341	0.029
2-2	39 (28.9)	0.575	0.151
MnSOD			
Val/Val	25 (18.5)	2.111	0.135
Val/Ala	103 (76.3)	0.860	0.721
Ala/Ala	7 (5.2)	0.217	0.053

n = sample size. p-values were generated by the Pearson chi-square test.

Because intensive exercise is a well accepted model to induce oxidative stress [6,8], leading to an increase in plasma lipid peroxidation [9], higher MDA values would be expected with the increase in distance covered, and this did not happen. However, the endurance athletes who participated in this study normally run from 8–10 Km, showing that endurance training is important to avoid increase in lipid peroxidation. In addition, there were significant differences between Hp genotypes and TBARS values. Haemolysis can occur as a result of mechanical trauma in the capillaries of runners' feet [50] and, in this case, extracellular haemoglobin (Hb) becomes highly toxic because of the oxidative capacity of iron-containing heme. This, in turn, participates in the Fenton reaction to produce reactive oxygen species and haptoglobin then prevents Hb-induced oxidative damage [28]. It has been demonstrated that the protective effect of Hp against this oxidative mechanism is also phenotype-dependent [28]. Our results suggest a possible association between the Hp1F-1S and Hp1S-2 genotypes and greater MDA values and between Hp1S-1S and minor lipid peroxidation, although p-value for OR of Hp1F-1S was not seen to have been significant. However, this result could be due to the smaller sample size of the Hp1F-1S genotype.

According to heterozygosity-observed (Ho) and heterozygosity-expected (He) data for the Hp locus, the main factors that contributed to deviation from HWE were the higher frequencies of Hp1F-1F and Hp1S-1S in regard to the expected as well as the lower observed frequency of Hp1F-1S. Given that our research was carried out with athletes, the results suggest that Hp1F-1S is disadvantageous, at least for athletes whose physical exercise can impose oxidative stress due to increased ROS, and mainly for those jointly carrying ACE II or GST M1+T1+ genotypes. This is because the highest or the lowest lipid peroxidation or damage (evaluated by CK and Comet assays) in this study depended on the interaction between Hp and ACE or GST genotypes. Some studies have suggested that ACE II or ID genotypes are associated with better aerobic performance than the ACE DD genotype, while others have not found such association [51]. Although haptoglobin genotypes have never been analysed in the context of human performance, our results suggest that this genetic marker can be one of the determining factors in performance, at least for runners.

ALT evaluates hepatic lesion [15,16] and total CK is the biochemical marker most used in the literature to evaluate muscular cell injuries, mainly after exhausting exercise accomplished by individuals without illnesses [16–18]. CK release into and clearance from plasma depends on the level of training and type, intensity and duration of exercise performed on the days before the test. Thus, high levels of serum CK in apparently healthy subjects may be correlated with physical training status and they depend on sarcomeric damage: strenuous exercise that damages skeletal muscle cells results in increased total serum CK [18]. In this context, our results corroborate literature where oestrogen has been shown to exert antioxidant properties and to protect skeletal muscle from

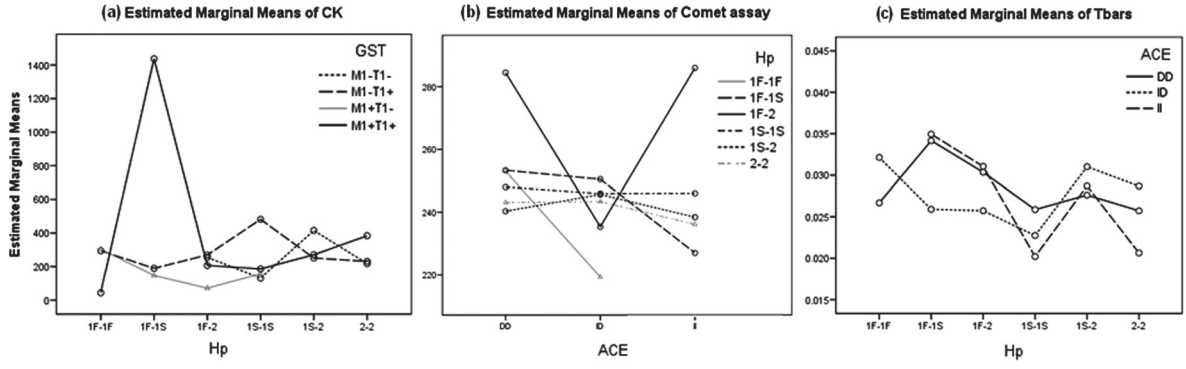

Figure 1. Significant interactions between genetic markers on the values of CK, TBARS and Comet assays: (A) Hp/GST and CK (U/L); (B) Hp/ACE and Comet assay (ua); (C) Hp/ACE and TBARS assay (nmol/mL of MDA). U/L = unit per litre; ua = arbitrary units. p-values were generated by UNIANOVA: (A) p = 0.007; (B) p = 0.048; (C) p = 0.044.

damage [52]. Since for clinical purposes the reference limits for AST, ALT and CK are lower in women than in men [46], those oestrogen properties can be extrapolated at least for AST and ALT results. In addition, athletes have higher CK values than non-athletes [53]. Thus, the upper reference limits for CK, and certainly for AST, could be increased.

Moreover, our results indicate influences of MnSOD genotypes on the values of CK and, for this marker, they suggest a possible association between Ala/Ala genotype and lower damage, despite the smaller sample size. However, previous studies have associated the variant –9Ala allele with decreased MnSOD efficiency against oxidative stress [54] and diseases related to abnormal free radical defense mechanisms, such as exudative age-related macular degeneration, Parkinson's disease and risk of breast, prostate and ovarian cancers [40,54–56]. In this context, our results corroborate the literature, since DNA damage was higher for individuals carrying the Ala/Ala genotype than for other MnSOD genotypes, although significant differences did not appear due to the great standard deviation of this genotype, besides its small sample size. They also suggested that the mechanisms of muscle and DNA damage are independent and could operate in a different way.

On the other hand, the significant deviation from HWE of the MnSOD locus in the present study was compatible with heterozygote excess and this result can be explained by natural selection. Positive selection increases the proportion of rare alleles (with low frequency), while balancing selection increases the proportion of intermediate alleles [57]. At the same time, natural selection can act at the level of genes, if particular genotypes allow for increased fitness in specific environments [58]. Since this work was accomplished with athletes, the heterozygote excess could indicate adaptive advantage instead of balancing selection. Although the MnSOD allele frequencies found in this study (57%Val; 43%Ala) are similar to those found by Mitrunen et al. [40] (56%Val; 44%Ala) in a Finnish Caucasian population and by Bica et al. [59] (59%Val; 41%Ala) in a Brazilian population of Porto Alegre, the genotype frequency distributions were different; none of them presented heterozygote excess. However, both were studies of association with breast cancer and, even though the frequencies above belonged to control groups, they were not formed by athletes.

In conclusion, MnSOD significantly influenced results of CK and a possible association between Hp1F-1S and Hp1S-2 genotypes with higher TBARS values was found. The highest or lowest lipid peroxidation and damage (evaluated by CK and Comet assay) also depended on the interaction between Hp and ACE or GST genotypes, indicating that MnSOD and Hp polymorphisms can be determining factors in performance, at least for runners.

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