Relationship between adenosine deaminase polymorphism (c.22G>A) and oxidative stress in sickle cell anemia

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ARTICLE INFO

ABSTRACT

The aim of this study was to identify, in people with sickle cell anemia (SCA), adenosine deaminase (ADA; c.22G>A; rs73598374) polymorphism, and correlating it with oxidative stress markers. We evaluated 95 unrelated and diagnosed Brazilian sickle cell anemia (SCA) patients. All patients received a prophylactic treatment with folic acid of 5 mg/day, while 41 (43.2%) of them were under hydroxycarbamide (HC) treatment (average dose: 22 mg/kg/day). ADA polymorphism was identified by PCR-RFLP. Biochemical parameters were measured using spectrophotometric [catalase, glutathione S-transferase, glutathione peroxidase, glutathione reductase activities] and chromatographic methods [fetal hemoglobin (HbF), glutathione (GSH) and malondialdehyde (MDA) levels]. Among the 95 SCA patients, we identified 80 (84.2%) wild homozygous for ADA (22GG), 15 (15.8%) heterozygous (22GA) and none mutant homozygous (22AA), leading to an allelic frequency of 0.92 for the ancestral allele (22G) and 0.08 for the mutant one (22A). Unexpectedly, we did not observe any influence of ADA polymorphism on oxidative stress markers, as well as interaction effects with HC usage. However, we confirmed a well-described protective effect of HC treatment on decreasing MDA levels ($p = 0.03$). Thus, we concluded that ADA (22G>A) polymorphism does not play significant role in the disruption of sickle erythrocyte redox metabolism.

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1. Introduction

Sickle cell anemia (SCA) is a devastating genetic hemolytic disorder associated with a high morbidity and mortality (Stuart and Nagel, 2004; Rees et al., 2010; Piel et al., 2013). The underlying abnormality is a single nucleotide substitution (c.20A>T; rs334) in the gene that encodes the β-globin chain (Steinberg and Sebastiani, 2012). The mutated globin chain will form the abnormal hemoglobin S (HbS), due to a substitution of valine for glutamic acid at position six of the protein (Bunn, 1997). This substitution creates a hydrophobic patch in the HbS tetramer that results in a propensity to polymerize in its deoxygenated state, forming long polymers that distort the shape of the red blood cells (RBCs) (Hebbel, 1991). However, HbS polymerization is reversible; fibers "melt" as oxygen is taken up by the HbS and the normal discoid shape returns (Stuart and Nagel, 2004). The higher energy expenditure due

to the increased metabolic turnover upon polymerization and depolymerization results in higher reactive oxygen species (ROS) production in sickle erythrocytes (Banerjee and Kuypers, 2004; Akohoue et al., 2007).

Despite our precise knowledge of the molecular defect that is associated with HbS in RBCs (Ingram, 1957; Christoph et al., 2005; Madigan and Malik, 2006) and recent progress in understanding the molecular events that control polymerization of HbS and sickling of erythrocytes (Ferrone, 2004; de Montalembert, 2008), the specific factors and signaling pathways that are involved in this process are unclear. In this way, Zhang et al. (Zhang et al., 2011) demonstrated that adenosine can enhance 2,3-bisphosphoglycerate (2,3-BPG) production via A_{2B} receptor activation, suggesting that elevated adenosine had an unrecognized role in normal RBCs to promote oxygen (O_2) release and prevent acute ischemic tissue injury. However, in sickle erythrocytes, the beneficial role of excessive adenosine-mediated 2,3-BPG induction becomes detrimental by promoting deoxygenation, HbS polymerization and subsequent sickling.

Adenosine displays a complex metabolism in which it is generated intracellularly and extracellularly by degradation of adenine nucleotides

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(Zhang and Xia, 2012). In both media, the enzyme adenosine deaminase, encoded by *ADA* gene (20q13.11), catalyzes the hydrolytic deamination of adenosine or 2′-deoxyadenosine to inosine or 2′-deoxyinosine and ammonia, contributing to the regulation of adenosine levels (Hirschhorn et al., 1994; Fredholm et al., 2005). Furthermore, a common functional variant of the *ADA* gene has been described as a guanine to adenine transition (c.22G>A; rs73598374) which leads to the substitution of asparagine for aspartic acid at the eightieth codon of the gene (Hirschhorn et al., 1994). It has been found that this functional polymorphism leads to a decrease in ADA activity in erythrocytes and lymphocytes (Battistuzzi et al., 1981).

Taken the above observations into consideration, we hypothesized that the co-inheritance of SCA and *ADA* (22G>A) polymorphism would enhance adenosine levels due to *ADA* (22G>A) mutation, and through adenosine-mediated 2,3-BPG induction mechanism, exacerbate ROS generation inside the sickle erythrocytes, culminating in the worsening of the SCA patient oxidative condition. Thus, the aim of this study was to identify, in people with SCA, *ADA* (c. 22G>A; rs73598374) polymorphism, and correlating it with oxidative stress and antioxidant capacity markers.

2. Methods

2.1. Subjects

We evaluated 95 unrelated Brazilian SCA patients (53 women and 42 men; mean age: 24.4 years old; range: 10–59 years old). All subjects were regularly in clinical follow-up in the Blood Center of Rio de Janeiro (RJ, Brazil) and had access to the same medication protocol established and regulated by the Brazilian Ministry of Health for the entire national territory. All the patients studied received a prophylactic treatment with folic acid of 5 mg/day since the SCA diagnosis, while 41 (43.2%) of them were under hydroxycarbamide (HC) treatment (average dose: 22 mg/kg/day). The Data Safety Monitoring Board (DSMB) according to Brazilian Regulations approved the study.

After they gave their informed consent, all patients have answered a questionnaire in order to screen them according to the exclusion criteria listed as follows. Patients were asked specifically whether they were taking any nutritional supplements, nonsteroidal anti-inflammatory drugs (NSAID), opioids, or iron chelating agents, e.g. Further exclusion criteria were pregnant women, smokers or drinkers and patients who had had a stroke, pain and/or hemolytic crisis, or received blood transfusion within the 120 days prior to the study start. The medications used were accessed and those ones taking medication known to affect the analyzed parameters (such as acetylsalicylic acid, antibiotics or vitamins) within 24 h of sample collection were excluded. We confirmed the information given in questionnaires by reviewing each patient's medical records from the Blood Center database under supervision of clinicians responsible for the patients.

2.2. Biological samples

Blood samples (about 8 mL) were collected through venipuncture in ethylenediamine tetra acetic acid (EDTA) tubes. Four milliliters (mL) of whole blood were used for cytological, electrophoretic and chromatographic hemoglobin identification tests, as well as for DNA extraction from leukocytes for further molecular analysis. The other 4 mL were firstly used to prepare hemolysate for catalase activity analysis - whole blood diluted in ultrapure water (1:50, v/v). Then the blood samples were centrifuged in a refrigerated centrifuge at 800 g for 15 min at 4 °C in order to obtain plasma for lipid peroxidation assay while buffy coat were carefully removed. The erythrocytes obtained were washed three times with cold phosphate-buffered saline (PBS - 136 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4). The supernatant and buffy coat were carefully removed after each wash. Finally, the erythrocytes were diluted in a 3.5 μM 2-mercaptoethanol 10 μM NADP 2.7 mM

EDTA hemolyzing solution (1:20, v/v) for glutathione *S*-transferase, glutathione peroxidase and glutathione reductase activities and glutathione content measurement. All hemolysates and plasma samples were immediately frozen at −80 °C until the analysis.

2.3. Hemoglobin identification tests

Cell morphology microscopic analysis was performed on the stained blood using May-Grünwald-Giemsa at photonic microscopy using $40\times$ objective (Bonini-Domingos, 2006). Hb phenotype identification was performed using electrophoresis on cellulose acetate pH 8.6 (Marengo-Rowe, 1965), and agar electrophoresis at pH 6.2 (Vella, 1968). The Hb fraction quantification was obtained using high performance liquid chromatography (HPLC) by the automated VARIANT™ equipment (Bio-Rad Laboratories, Hercules, CA, USA), according to manufacturer's manual.

2.4. Molecular analysis

DNA was extracted from peripheral blood leucocytes and isolated by phenol-chloroform methodology, according to Sambrook et al. (Sambrook et al., 1989). *HBB*S* homozygous genotype was confirmed by molecular analysis by PCR-RFLP using *Dde*I as restriction endonuclease (New England BioLabs, Ipswich, MA, USA) (Saiki et al., 1985). *ADA* (c.22G>A) polymorphism was assessed through PCR-RFLP using *FastDigest Taq*I (Thermo Fisher Scientific Inc., Waltham, MA, USA), according to Safranow et al. (Safranow et al., 2007).

2.5. Biochemical measurements

Lipid peroxidation levels were assessed in the plasma samples by the product formed from malondialdehyde (MDA) and thiobarbituric acid (TBA), according to Silva et al. (Silva et al., 2015). The product was detected by high performance liquid chromatography coupled to UV/Vis detector (UV/Vis-HPLC) (de Almeida et al., 2003; de Almeida et al., 2004). The HPLC system consisted of ESA584 pump and an ESA526 UV/Vis detector set in 532 nm. The MDA–TBA derivative extracts were separated by a Shimadzu (Kyoto, Japan) C18 column (150×4.6 mm, 5 μm) that was fitted with a guard column cartridge of the same composition. The mobile phase (potassium phosphate 0.05 M, pH 7.0, with 40% methanol) was pumped at an isocratic flow of 1 mL min^{-1}. Chromatogram monitoring and peak identification and quantification were performed using the EZ Chrom Elite software (Agilent Technologies, Santa Clara, CA, USA). The calculations were based on a calibration curve previously constructed and prepared according to same procedure used for the samples from authentic standards. The results were expressed in ng/mL.

Catalase (CAT) (Beutler, 1975), glutathione *S*-transferase (GST) (Keen et al., 1976), glutathione peroxidase (GPx) (Sies et al., 1979), and glutathione reductase (GR) (Beutler, 1969), activities were determined spectrophotometrically, while GSH concentration was determined HPLC coupled to a coulometric electrochemical detection (ECD-HPLC) (Rodriguez-Ariza et al., 1994). A hemolysate aliquot was filtered through Millex syringe filter units (0.22 μm) and directly injected into the HPLC system, consisted of ESA584 pump and an electrochemical coulometric detector (ESA Coulochem III, Bedford, MA, USA) with potential set at 650 mV. The column was an ESA C18 (250×4.6 mm, 5 μm) also fitted with a guard column cartridge of the same composition. The mobile phase consisted of sodium phosphate 0.05 M, octanesulfonic acid 0.025 M, pH 2.5, with acetonitrile (2%; v/v) was pumped at an isocratic flow of 0.9 mL/min.

Chromatogram monitoring and peak identification and quantification were performed using the same software used in the lipid peroxidation assay. The calculations were based on a calibration curve previously constructed by injecting authentic GSH standards into the

HPLC system. The antioxidant enzymes were expressed in U/mL, while GSH levels were in μM.

2.6. Statistical analysis

Statistical analysis were performed in groups with at least three individuals using the Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA), while the graphics were done using GraphPad Prisma version 5.01 for Windows (GraphPad Software, La Jolla, CA, USA).

We assessed data normality by using Normal Probability Plots of Residuals. Thus, some data were either transformed into $\log_{10}$ or identified as outliers and removed from comparison analysis. We also tested data homoscedasticity by Levene's test, assuming a significance level of 0.05. For comparison analysis, we used General Linear Models (GLMs), because these approaches generalize to unbalanced designs and designs with more factors, including crossed and nested, and combinations of categorical and continuous variables (Quinn and Keough, 2002; McDonald, 2014). That way we have established the ADA genotypic distribution and HC usage as predictors, age and gender as covariates, and the biochemical parameters evaluated as dependent variables. If appropriate, we applied the Tukey–Kramer post hoc test, a simple and reliable multiple comparison for unequal sample size (Quinn and Keough, 2002).

Wilk's λ test was performed as a multivariate counterpart of a univariate R^2, that is, it indicates the proportion of generalized variance in the dependent variables that is accounted for by the predictors. In order to assess association degree between biochemical parameters and HbF levels, we used Pearson's correlation. Data were expressed as mean ± 95% confidence intervals of their biological values and we considered $p < 0.05$ as statistically significant (Quinn and Keough, 2002; McDonald, 2014).

3. Results

Among the 95 SCA patients evaluated, we identified 80 (84.2%) wild homozygous for ADA (22GG), 15 (15.8%) heterozygous (22GA) and none mutant homozygous (22AA), leading to an allelic frequency of 0.92 for the ancestral allele (22G) and 0.08 for the mutant one (22A). Due to the lack of mutant homozygous, and as HC is an important modulator of SCA oxidative status, we have compared the effects of ADA 22A polymorphism between wild homozygous (GG) and heterozygous (GA), the influence of HC using between patients treated (HC$^+$)] or not treated with HC (HC$^-$), as well as their interaction (ADA 22A*HC usage).

Unexpectedly, the presence of ADA 22A allele did not influence any of the oxidative stress parameters assessed, as well as we did not observe any interaction effects (Fig. 1). We only observed a well-documented HC effect on lipid peroxidation levels. Those patients treated with HC showed lower MDA levels [514.8 ng/mL (95% CI: 452.1–577.5)] than the patients untreated [378.8 ng/mL (95% CI: 309.9–447.7)], regardless the presence of mutation ($p = 0.03$). Furthermore, Wilk's λ statistic agreed with the univariate analysis, showing that a
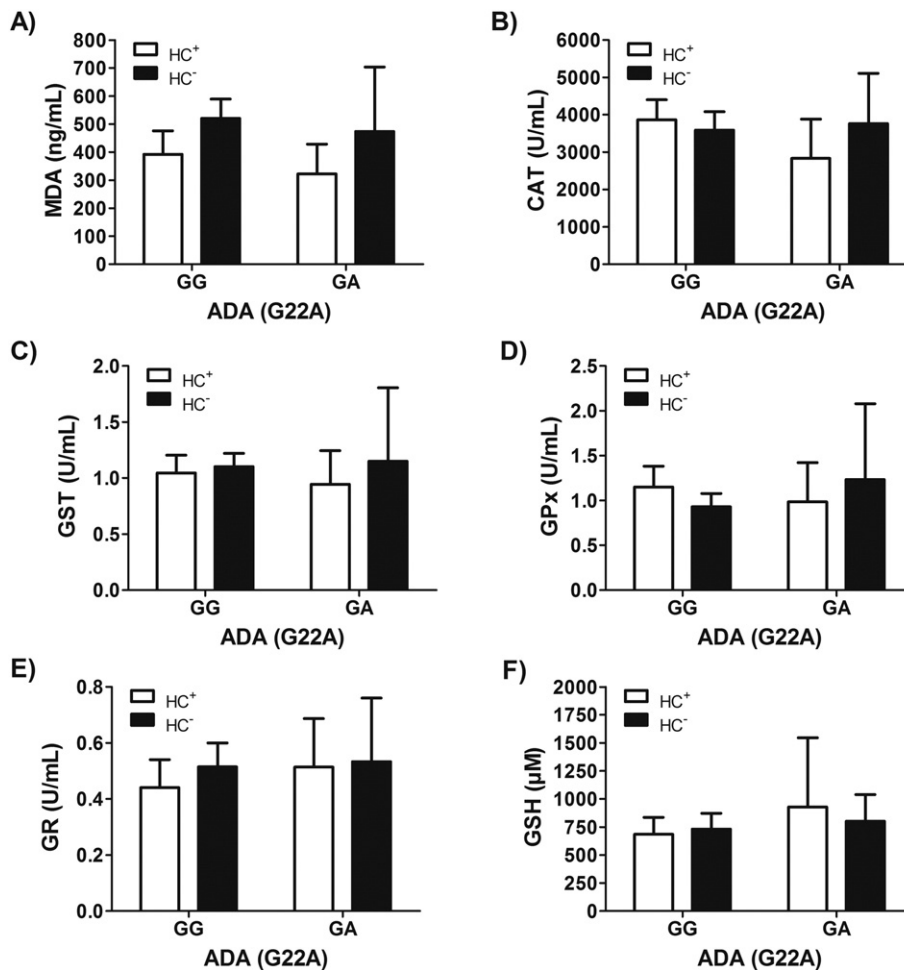

Fig. 1. None influence of ADA (22G>A) polymorphism and its interaction with HC treatment on oxidative stress markers. (A) Lipid peroxidation measured through malondialdehyde levels. Enzymatic activities of (B) catalase; (C) glutathione S-transferase; (D) glutathione peroxidase; (E) glutathione reductase. (F) Glutathione concentration. GG (HC$^+$): ancestral homozygous under HC use ($N = 31$); GA (HC$^+$): heterozygous patients under HC use ($N = 7$); GG (HC$^-$): ancestral homozygous not treated with HU ($N = 43$); GA (HC$^-$): heterozygous patients untreated with HU ($N = 6$). Comparison analysis were performed by using General Linear Models ($P > 0.05$).

Table 1
Effects of *ADA* 22A polymorphism, HC usage and their interaction on the biochemical parameters measured.

Source	df	Wilk's λ	F	P values
ADA 22 A polymorphism	7, 46	0.90	0.72	0.64
HC usage	7, 46	0.74	2.30	0.04
ADA 22 A*HC usage	7, 46	0.87	0.97	0.46

ADA: adenosine deaminase gene; HC: hydroxycarbamide; df: degree of freedom; *F*: ratio of two mean square values.

significant proportion of the variance in the combination of response variables (MDA levels, activities of CAT, GST, GPx and GR, and GSH content) is accounted for HC usage effects (Table 1).

In order to better understand the non-participation of *ADA* 22A polymorphism in the expression of oxidative stress markers, further investigation was carried out according to HbF amount of SCA patients, because HbF is capable of changing the intracellular concentrations of HbS that dictate the pace and extent of polymerization(Damanhouri et al., 2015). Thus, we analyzed the association degree between HbF levels and biochemical markers studied in SCA patients heterozygous for *ADA* polymorphism, since they showed high HbF levels variation (mean value: 8.6%; range: 0.5–23%). However, we found no correlation between HbF amount and each variable measured (Fig. 2).

4. Discussion

Even with improved knowledge of the human genome, development of new genomic tools and identification of single nucleotide polymorphisms (SNPs) associated with subphenotypes of sickle cell disease (SCD) by genome-wide association studies (GWAS) (Fertrin and Costa, 2010), there is still a major challenge to combine all these variables and establish potential predictors of the SCD severity (Lettre, 2012). In addition, the understanding of SCA pathophysiology has gradually increased (Rees and Gibson, 2012), and among the new evidences, oxidative stress has been increasingly related to both cause and consequence of inflammation, hemolysis, vasculopathy, vaso-occlusion, infection, injury by ischemia/reperfusion, e.g. (Rees et al., 2010). However, studies associating genetic and biochemical markers are scarce, thus this study, to our knowledge, yields a unique opportunity in which both genetic factor (*ADA* polymorphism) and oxidative stress markers were simultaneously measured and correlated with HbF levels and HC use in persons with SCA.

The frequency of mutated allele in *ADA* gene (22A) is estimated at 0.06 in Western populations, lower among individuals of African descents and higher in Southeast Asian populations (Hopkinson et al., 1969; Weissmann et al., 1982; Hirschhorn et al., 1994). Previous studies from Brazilian population showed different allele frequencies. For instance, Dutra et al. (Dutra et al., 2010) obtained a 0.11 allele (22A) frequency for individuals from Rio Grande do Sul State. While, Nunes et
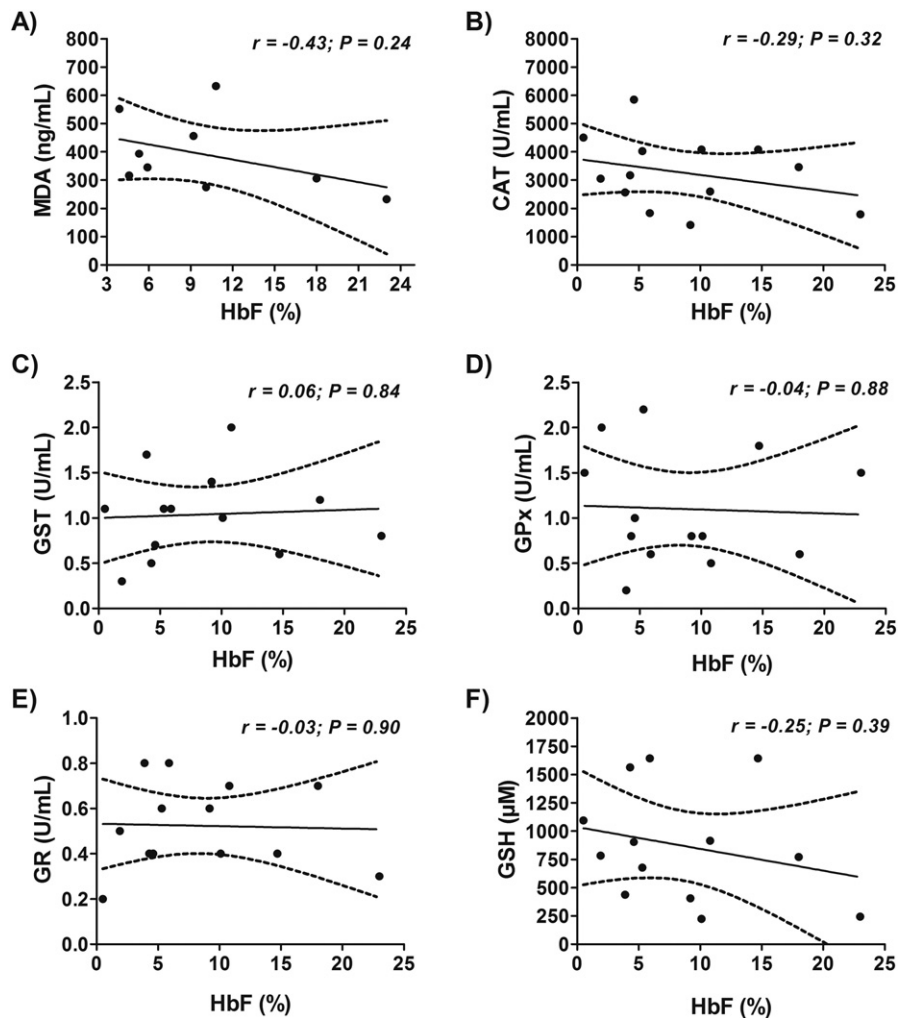

Fig. 2. No association between HbF amount and each oxidative stress parameters evaluated in SCA patients heterozygous for ADA (22G>A) polymorphism. A) Lipid peroxidation measured through malondialdehyde levels. Activities of antioxidant enzymes (B) catalase; (C) glutathione S-transferase; (D) glutathione peroxidase; (E) glutathione reductase. F) Glutathione levels. Dashed line indicates 95% confidence band of best-fit line. All correlation analysis were made by Pearson's test.

al. (Nunes et al., 2011) and Mazzotti et al. (Mazzotti et al., 2012) found a frequency about 0.05 for people from Sao Paulo State. These data are in accordance to studies developed in European populations (Persico et al., 2000; Safranow et al., 2007; Bachmann et al., 2012). This way, we found a frequency lower when compared to those reported for Caucasians and higher than that expected for African descents that might to be only the reflection of ethnic and racial admixture of our population. Moreover, to our knowledge, this is the first report about *ADA* (22G>A) polymorphism in people with SCA from Rio de Janeiro State.

During periods of cellular hypoxia or stress, adenosine is released from cells along with the adenine nucleotides, ATP, ADP, and AMP, which are converted to adenosine by ectonucleotidases (Jacobson and Gao, 2006; Eltzschig and Carmeliet, 2011). This response is even more pronounced in SCA patients due to increased amounts of ATP in the circulation derived from chronic sickle red cell hemolysis and tissue damage from vasoocclusion (Hasko et al., 2008). This observation causes the postulated hypothesis of this study is more plausible, but we did not observe any influence of *ADA* polymorphism on oxidative stress markers evaluated. Firstly, we thought that it could be the result of a known protective effect of HbF levels shown by Dasgupta et al. (Dasgupta et al., 2010) in SCA transgenic mice, and further confirmed in SCA patients by Silva et al. (Silva et al., 2011), in which Hb F protective effect is primarily mediated by decreased intravascular sickling, resulting in a decreased oxidative stress. However, we found no association between HbF levels and each biochemical parameters assessed. It is worth noting that the lack of mutant homozygotes and the small number of heterozygous might be affecting the results. Notwithstanding, even with a small sample size, our results have left perspectives for further studies to better address this hypothesis.

Although, the enzyme encoded by the A22 allele has about 35% less catalytic activity than enzyme encoded by G22 allele, individuals carrying one copy of mutated allele display 15–20% lower activity compared to ancestral homozygotes (Battistuzzi et al., 1981). This way, we demonstrated that enzyme inefficiency caused by mutation is not sufficient to trigger a worsening in oxidative outcome of people with SCA. Nevertheless, Safranow et al. (Safranow et al., 2007) demonstrated an association of *ADA* 22A allele with decreased risk of coronary artery disease. While Mi et al. (Mi et al., 2008) showed that mice lacking ADA developed priapic activity and penile vascular fibrosis. Recently, Cita et al. (Cita et al., 2016) did not observe any relation between the same SNP (*ADA*; rs73598374) and a hemolytic index calculated through lactate dehydrogenase, aspartate aminotransferase, reticulocytes and total bilirubin in 150 SCA patients from Guadeloupe. Thus, further studies with larger sample size and association with clinical manifestations can predict the relative risk generated by co-inheritance of *ADA* (22G>A) polymorphism and SCA in disease progression.

In conclusion, we showed that *ADA* (22G>A) polymorphism does not play significant role in the disruption of sickle erythrocyte redox metabolism. However, taking into consideration the studies summarized above, we suggest that this polymorphism might be involved in the broad spectrum of SCA phenotypic expression.

Conflict of interest disclosure

The authors declare no competing financial or relationship with other people or organizational interests. Furthermore, the authors have materially participated in the research and/or article preparation and approved the final article version.

Acknowledgments

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