Original article

Impact of genetic polymorphisms in key enzymes of homocysteine metabolism on the pathophysiology of sickle cell anemia

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ABSTRACT

This work aimed at studying a possible influence of *methylenetetrahydrofolate reductase* (*MTHFR*; c. 677C > T) and *cystathionine β-synthase* (*CBS*; 844ins68) polymorphisms on overall oxidative status of sickle cell anemia (SCA) patients and on routine markers, correlating them with hydroxycarbamide (HC) treatment. We evaluated 95 unrelated and diagnosed 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). *MTHFR* and *CBS* polymorphisms were identified by Polymerase Chain Reaction. Biochemical parameters were measured using spectrophotometric and chromatographic methods. Routine markers were developed by specialized laboratory. We did not find any effect of 677T and "I" allele combination on the biomarkers evaluated. On the other hand, *MTHFR* 677T mutation was related to a depletion of antioxidant capacity, according to the decreased catalase activity and a reduction about 30% of glutathione levels. Moreover, the presence of the insertion was related to about 23% less biomolecule oxidation levels and lower monocytes count, but about 14% higher lactate dehydrogenase activity. These findings may contribute to highlight that the *MTHFR* and *CBS* polymorphisms involvement in SCA pathophysiology is likely to be far more complex than it was explored to date.

1. Introduction

Sickle hemoglobin (HbS) is caused by single point mutation in *beta-globin* gene (*HBB*; c.20A > T; rs334), and its homozygosity is termed sickle cell anemia (SCA) which is a chronic and progressively debilitating medical condition common worldwide [1,2]. SCA is an hemolytic anemia characterized by a complex pathophysiology that is affected by a number of modifying factors, including co-inheritance of polymorphisms associated to clinical aspects [3,4] and to treatment response [5], as well as a chronic and systemic oxidative stress [6], culminating in an antioxidant deficiency [7,8].

Glutathione (GSH) is a tripeptide, γ-L-glutamyl-L-cysteinylglycine, present at high concentrations (about 2 mM) in healthy erythrocytes as the most abundant non-protein thiol that defends against oxidative

stress [9]. However, GSH concentration is significantly reduced in SCA patients [7,10], with some studies noting a 50% decrease in sickle erythrocytes compared with healthy ones [11], even though its de novo synthesis and recycling rate have been shown to be higher as compared to healthy erythrocytes [7]. GSH is synthesized from cysteine, glutamic acid, and glycine in a two ATP-requiring enzymatic steps, being the availability of the first amino acid a rate limiting of GSH synthesis [9,12]. Under physiological conditions, cysteine is derived from the diet, protein breakdown and transsulfuration pathway that allows the utilization of methionine for GSH synthesis [9,13].

Transsulfuration pathway plays a significant role in the complex folate-dependent homocysteine (Hcy) metabolism along to the re-methylation pathway [14]. Methylenetetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS) are key enzymes that

play a role in the balance of Hcy concentrations by converting it to methionine or cystathionine, respectively [15–17]. Genetic defects occur in either genes encoding for MTHFR and CBS enzymes, leading to an alteration of Hcy levels that may be involved in the cysteine availability. Among the polymorphisms described for these enzymes, the most studied ones are single point mutation in *MTHFR* gene (c. 677C > T; rs1801133) [18] and a 68-bp insertion in the exon 8 coding region of *CBS* (844ins68) [19].

The *MTHFR* 677T allele is associated with reduced enzymatic activity, decreased folate concentrations in serum and red blood cells, increased plasma Hcy levels [18,20], and it is a risk factor for vascular complications [21], including in SCA patients [22–24]. Otherwise, there is no consensus about the 68-bp insertion effect on CBS activity and on Hcy levels [19,25,26]. In spite of that, Alves-Jacob [23] showed that the presence of the 844ins68 mutation in the *CBS* gene is a risk factor for vaso-occlusive episodes in SCA patients. Furthermore, recent studies have demonstrated that defects in either transsulfuration or remethylation pathways are related to increased generation of reactive oxygen species (ROS) [27–29] that could exacerbate the oxidative condition in SCA patients.

Some studies have been carried out to determine the association of the *MTHFR* (677C > T) or *CBS* (844ins68) polymorphisms with higher risk to develop vascular complications in SCA patients. However, there is no report regarding the functional impacts of these polymorphisms on GSH concentration and consequently on oxidative stress and routine markers in SCA patients. For this reason, this work aimed at studying a possible influence of the mentioned polymorphisms on overall oxidative status of SCA patients and on routine markers, correlating them with hydroxycarbamide (HC) treatment.

2. Subjects and methods

2.1. Subjects

We evaluated 95 unrelated Brazilian SCA patients regardless gender (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. 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 following exclusion criteria. 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 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.

The Blood Center of Rio de Janeiro developed the analysis of all patient routine marker, such as complete blood count, hemolytic, hepatic and renal markers. These data were obtained by reviewing the medical records from the Blood Center database, where the information given in questionnaires were also confirmed for each patient, under supervision of clinicians responsible for the patients. Finally, we characterized the baseline outcomes from the patients, regarding demographic data, specific medication use, beta-globin haplotype profile, and the routine markers (Table 1).

2.2. Biological samples

Blood samples (about 8 mL) were collected through venipuncture in ethylenediaminetetraacetic acid (EDTA) tubes. Four milliliters (mL) of

Table 1
Characterization of study group.

Markers	SAMPLE GROUP (*N* = 95)	REFERENCE VALUES
Age [yr: $\overline{X}$ (95% CI)	24.3 (21.5–27.2)	–
Gender [*N* (%)]		–
Female	53 (55.8)	
Male	42 (44.2)	
Medication use[a] [*N* (%)]		–
HC$^+$ (average dose: 22 mg/ kg/day)	41 (43.2)	
HC$^-$	54 (56.8)	
Beta globin haplotype profile [*N* (%)]		–
Bantu/Bantu	42 (44.2)	
Bantu/Benin	19 (20.0)	
Bantu/Cameroon	1 (1.1)	
Bantu/Atypical	8 (8.4)	
Benin/Benin	6 (6.3)	
Benin/Cameroon	1 (1.1)	
Benin/Atypical	2 (2.1)	
Atypical/Atypical	16 (16.8)	
Routine markers [$\overline{X}$ (95% CI)]		
Erythrocytes (k/μL)	2.49 (2.39–2.60)	4.5–6.0
HbF levels (%)	9.6 (8.1–11.1)	0.0–1.0
Hemoglobin (g/dL)	8.3 (8.0–8.5)	11.5–17.0
Hematocrit (%)	23.4 (22.6–24.2)	35.0–43.0
MCV (FL)	93.0 (90.4–95.5)	77.0–100.0
MCH (PG)	33.0 (32.0–34.0)	25.0–34.0
MCHC (G/DL)	35.0 (34.3–35.6)	31.0–37.0
Leucocytes (k/μL)	9.6 (9.0–10.1)	5.0–13.0
Neutrophils (k/μL)	4.4 (4.0–4.8)	1.5–10.0
Lymphocytes (k/μL)	3.8 (3.5–4.0)	1.0–6.0
Monocytes (k/μL)	0.66 (0.60–0.72)	0.08–1.3
Eosinophils (k/μL)	0.43 (0.32–0.53)	0.0–0.60
Basophils (k/μL)	0.15 (0.13–0.16)	0.0–0.20
Platelets (k/μL)	434.4 (406.77–462.1)	140.0–400.0
Reticulocytes (%)	9.9 (9.03–10.7)	0.80–2.0
Reticulocytes (k/μL)	240.6 (222.2–259.1)	60.0–150.0
LDH (U/L)	1142.8 (891.2–1394.5)	< 480.0
AST (U/L)	53.7 (49.5–57.9)	< 35.0
ALT (U/L)	25.3 (21.2–29.4)	< 41.0
TB (mg/dL)	3.3 (2.9–3.7)	< 1.0
DB (mg/dL)	0.60 (0.54–0.66)	< 0.20
IB (mg/dL)	2.7 (2.3–3.1)	< 0.80
ALP (U/L)	145.0 (125.1–164.9)	53.0–141.0
GGT (U/L)	61.0 (44.9–77.0)	8.0–73.0
Creatinine (mg/dL)	0.73 (0.67–0.79)	0.60–1.30

Hb F: fetal hemoglobin; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean cell hemoglobin concentration; LDH: lactate dehydrogenase; AST: aspartate transaminase; ALT: alanine transaminase; TB: total bilirubin; DB: direct bilirubin; IB: indirect bilirubin; AF: alkaline phosphatase; GGT: gamma-glutamyl transferase

[a] All the patients studied received a prophylactic treatment with folic acid of 5 mg/day since the SCA diagnosis

whole blood were used for cytological, electrophoretic and chromatographic hemoglobin identification tests, as well as for DNA extraction from leucocytes for further molecular analysis. The other 4 mL were firstly used to prepare hemolysate for CAT 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 GST, GPx and GR activities and GSH 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. Hb phenotype identification was performed using electrophoresis on cellulose acetate pH 8.6 [30], and agar electrophoresis at pH 6.2 [31]. 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 [32]. *HBB*S* homozygous genotype was confirmed by molecular analysis by PCR-RFLP using *Dde*I as restriction endonuclease (New England BioLabs, Ipswich, MA, USA) [33]. Beta globin haplotypes were determined through the PCR-RFLP analysis of the following polymorphic restriction sites: γG (Hind III), γA (Hind III), ψβ (Hinc II), 3′ψβ (Hinc II) and 5′β (Hinf I), as previously described [34]. *MTHFR* (c.677C > T) polymorphism was assessed through PCR-RFLP using *FastDigest Hinf*I (Thermo Fisher Scientific Inc., Waltham, MA, USA) as restriction endonuclease, according to Frosst et al. [18]. Briefly, the PCR product (198-bp) remained uncleaved for ancestral allele, whereas mutant one produced 175- and 23-bp fragments. *CBS* (844ins68) mutation was detected using PCR as previously described by Tsai et al. [25]. After PCR, we obtained a 184-bp fragment for ancestral allele and a 252-bp for mutant one.

2.5. Biochemical measurements

As an oxidation marker, we measured colored adducts produced by the reaction with thiobarbituric acid in the plasma samples [35], according to Silva et al. [36]. The products were detected by high performance liquid chromatography coupled to UV/Vis detector (UV/Vis-HPLC) [37,38]. The HPLC system consisted of ESA584 pump and an ESA526 UV/Vis detector set in 532 nm. The 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.

As antioxidant markers, catalase (CAT) [39], glutathione *S*-transferase (GST) [40], glutathione peroxidase (GPx) [41], and glutathione reductase (GR) [42], activities were determined spectrophotometrically, while GSH concentration was determined HPLC coupled to a coulometric electrochemical detection (ECD-HPLC) [43]. We filtered the hemolysate aliquot through Millex syringe filter units (0.22 μm) and we directly injected into the HPLC system. The system consisted of ESA584 pump, a model 5021 conditioning cell that was maintained at a potential of $+900$ mV and a dual cell model 5011 analytically with potential set at 250 mV (first cell) and 650 mV (second cell) (ESA Coulochem III, Bedford, MA, USA). The column was an ACE 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-1.

Chromatogram monitoring and peak identification and quantification were performed using the same software used in the oxidation marker 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 logarithmically transformed ($\log_{10}$) 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 (GLM), because these approaches generalize to unbalanced designs and designs with more factors, including crossed and nested, and combinations of categorical and continuous variables [44,45]. Thus, allowing checking HC usage outcome, individual mutation effects, as well as any interaction effects. That way we have established the *MTHFR* and *CBS* genotypic distributions and HC usage as predictors with adjustment for gender, and age at investigation 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 sizes [44].

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 [44,45].

3. Results

Among the 95 SCA patients evaluated, we found 66 (69.5%) wild homozygous for *MTHFR* (677CC), 27 (28.4%) heterozygous (677CT) and two (2.1%) mutant homozygous (677TT), leading to an allelic frequency of 0.84 for the ancestral allele (677C) and 0.16 for the mutant one (677T). The allelic frequency obtained for *CBS* (844ins68) mutation was very similar to the *MTHFR* one: 0.85 for the ancestral allele (A) and 0.15 for the insertion (I). Moreover, we identified the following genotyping distribution for *CBS* gene −69 (72.6%) ancestral homozygous (AA), 23 (24.2%) heterozygous (AI) and three (3.2%) insertion homozygous (II). In addition, the co-heritance prevalence of both mutation in at least one chromosome (T_/I_) was 8.4%.

In order to address the proportion of generalized variance of SCA pathophysiology that is accounted by *MTHFR* 677T polymorphism and *CBS* 844ins68 insertion, we evaluated 31 biochemical parameters (25 clinical follow-up parameters and 6 oxidative stress markers), in people with SCA treated or not with HC. Unexpectedly, according to the statistical analysis adopted, we did not find any protective effect of HC treatment on oxidation marker and antioxidants evaluated. On the other hand, people with SCA under HC use presented almost twice the HbF values than those patients not treated (Fig. 1a), leading to improvement of MCV (Fig. 1b) and MCH (Fig. 1c), since HbF levels correlated positively with these two erythrocyte indices (Fig. 1d).

Still with regard to HC use, we found other well-documented pharmacological effects such as average reduction about 20% of leukocyte count (Fig. 2), as well as an improvement of hemolytic markers (Fig. 3). We did not observe any other influence of HC treatment on the remaining parameters evaluated.

Regarding the influences of the mutations investigated, we adopted a dominant genetic model. For *MTHFR* (677C > T), we compared wild homozygous (CC) against the rare homozygous variant combined with the heterozygous (T_), while for *CBS* (844ins68) the comparison was between ancestral homozygous (AA) against the association of heterozygous and insertion homozygous (I_). Furthermore, we performed for each gene individually interaction with HC treatment. In this regard, we did find none gene-HC treatment interaction, thus, confirming that
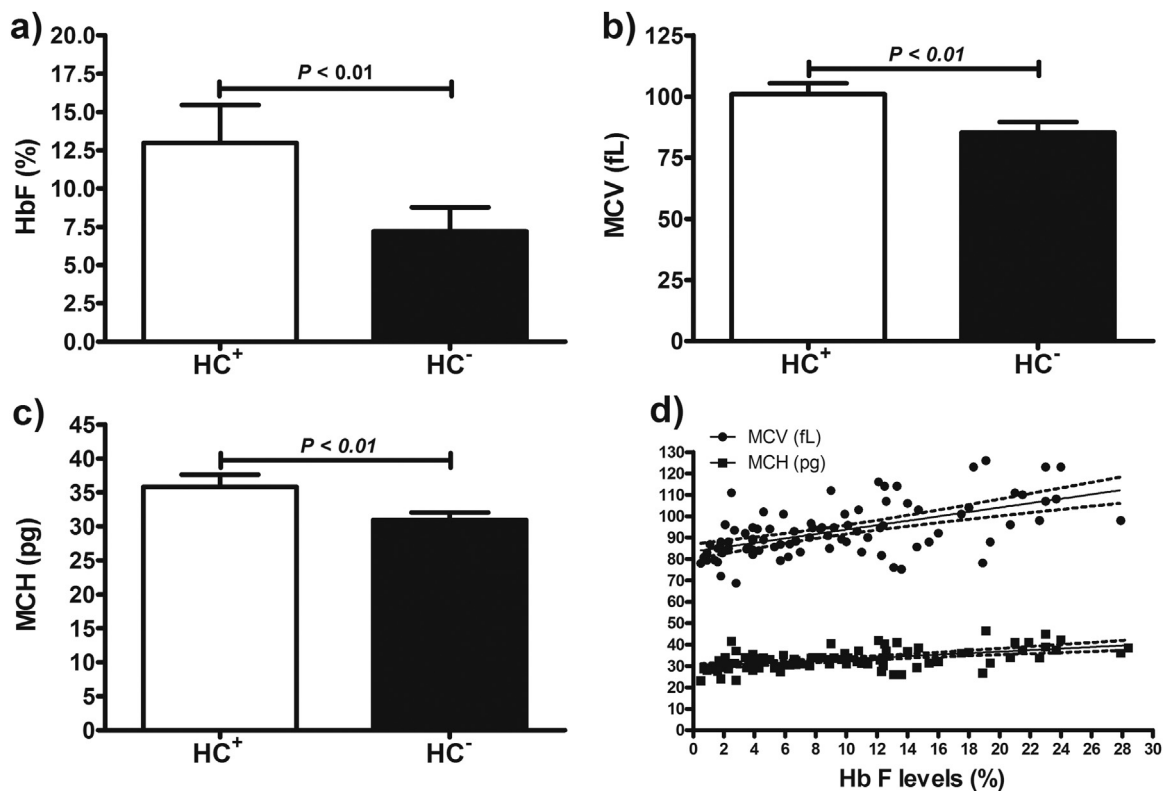
Fig. 1. HC beneficial effects on erythrocyte markers. A) Higher HbF amount in the subgroup of people with SCA under HC use compared to those ones not treated. Improvement of MCV (B) and MCH (C) indices due to HC treatment. D) HbF levels associated positively with MCV ($r = 0.58$; $P < 0.01$) and MCH ($r = 0.54$; $P < 0.01$). Hb F: fetal hemoglobin; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; HC$^+$: subgroup of patients treated with hydroxycarbamide; HC$^-$: subgroup of patients not treated with hydroxycarbamide. Statistical comparisons were performed by General Linear Models (GLM). Association degree was performed by Pearson's correlation test. Data were expressed as mean ± 95% confidence intervals.
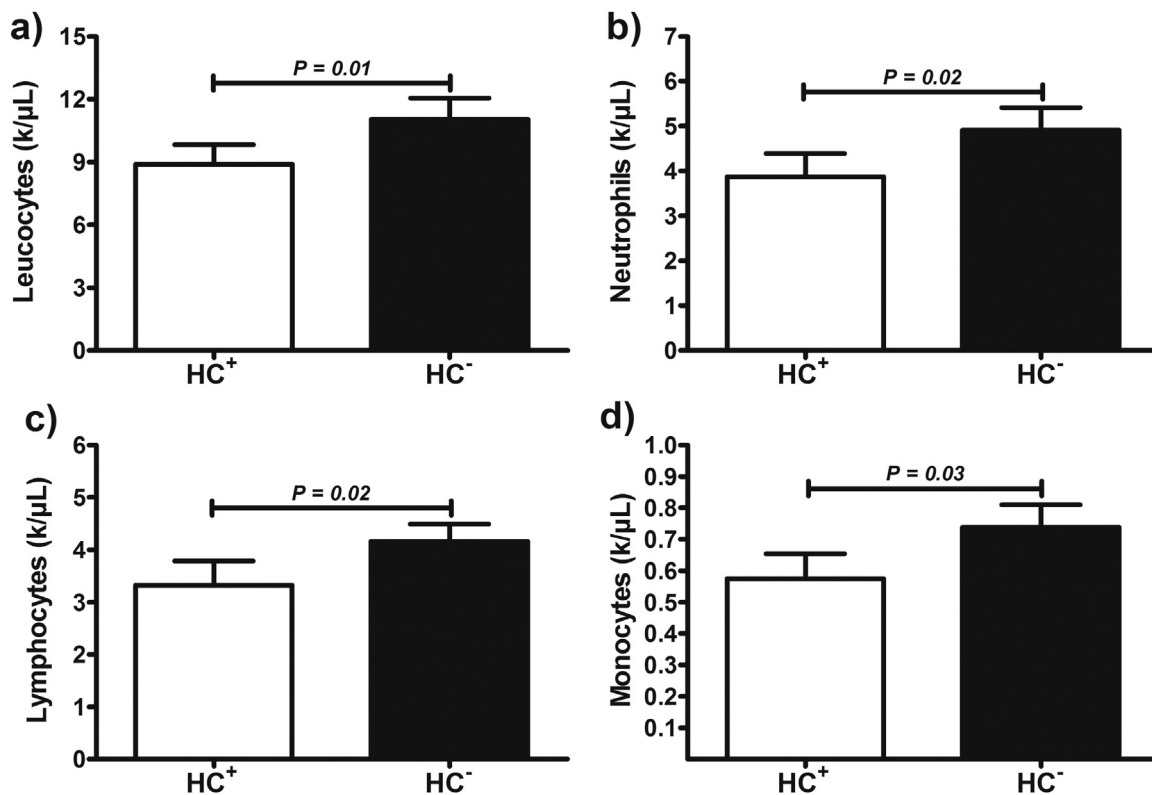
Fig. 2. Antiinflammatory effects of HC treatment. HC use by SCA patients promoted reduction of total leukocyte number (A), and neutrophil (B) lymphocyte (C) and monocyte (D) counts. HC$^+$: subgroup of patients treated with hydroxycarbamide; HC$^-$: subgroup of patients not treated with hydroxycarbamide. Statistical comparisons were performed by General Linear Models (GLM). Data were expressed as mean ± 95% confidence intervals.
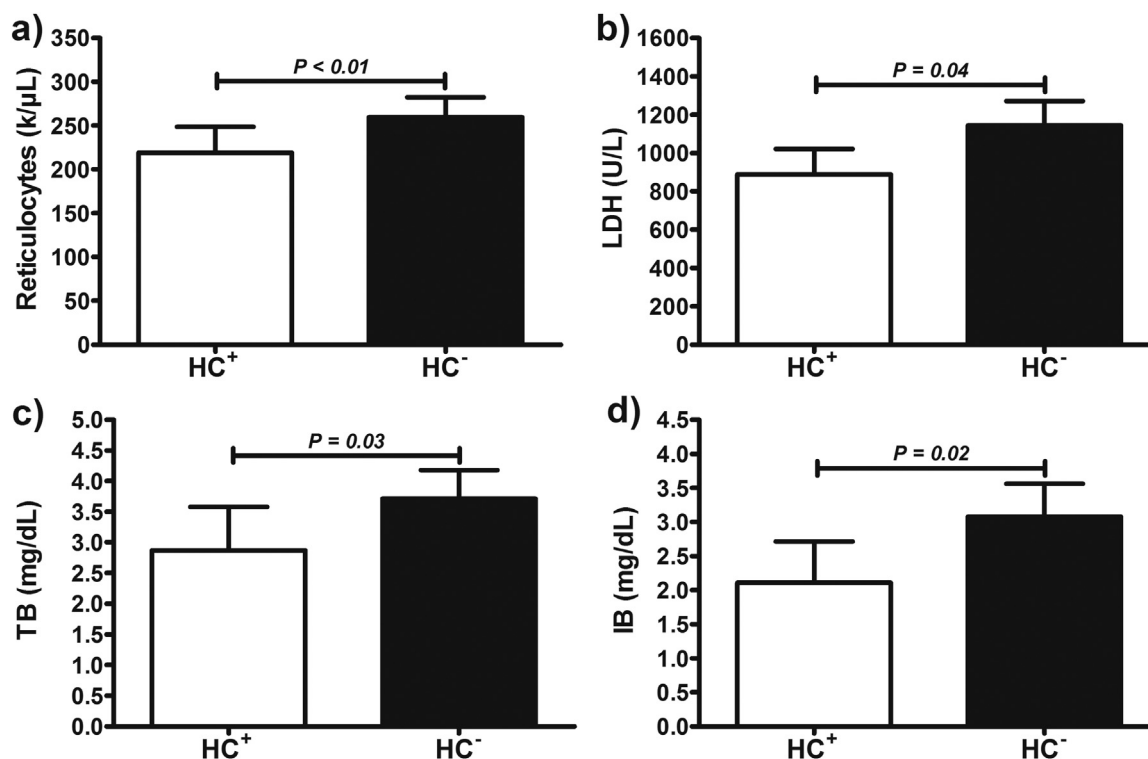
Fig. 3. Improvement of hemolytic markers mediated by HC treatment. People with SCA under HC use presented a reduction of reticulocyte count (A), LDH activity (B), and TB (C) and IB levels when compared to those patients not treated. HC$^+$: subgroup of patients treated with hydroxycarbamide; HC$^-$: subgroup of patients not treated with hydroxycarbamide; LDH: lactate dehydrogenase; TB: total bilirubin; IB: indirect bilirubin. Statistical comparisons were performed by General Linear Models (GLM). Data were expressed as mean ± 95% confidence intervals.

HC influence on patient hematological and hemolytic profile, regardless the presence of the mutations studied.

We also assessed a possible gene-gene interaction effect on HC response and on biomarkers evaluated. In this way, the statistical comparisons were performed through a combined dominant model: ancestral homozygous for both *MTHFR* (677C > T) and *CBS* (844ins68) polymorphisms (CC/AA) against those patients at least heterozygous for both genetic variants studied (T_/I_). Unexpectedly, we did not find any additive, synergic or even antagonistic effect of 677T and "I" alleles on the biomarkers evaluated.

Notwithstanding, we found only individually gene effects. The presence of the 677T allele in at least one chromosome was related to a depletion of antioxidant capacity, according to the decreased CAT activity (Fig. 4a) and a reduction about 30% of GSH levels (Fig. 4b) in the T_ subgroup when compared to the CC counterpart. In spite of this, we did not find any statistical difference in biomolecule oxidation levels, as well as in the other antioxidant evaluated. The co-inheritance of *HBB*S* and *MTHFR* 677T did not influence any of the routine markers assessed.

Remarkably, the co-inheritance of *HBB*S* and *CBS* (844ins68) influenced the biomarkers analyzed in an entirely different and dual way. On the one hand, people with SCA and at least heterozygous for *CBS* (844ins68) showed about 23% less oxidation product levels (Fig. 5a) and lower monocytes count (Fig. 5b) than those patients without the insertion. These effects might be considered beneficial or protective ones. On the other hand, the I_ subgroup presented about 14% higher LDH activity when compared to the AA one (Fig. 5c), which might be interpreted negatively for people with SCA. The other antioxidant and routine markers analyzed did not differ statistically between the *CBS* subgroups.

4. Discussion

Although SCA is one of the first disorders to be clearly defined at

molecular level, genetic understanding of the basis for disease expression variability is still unclear [46]. In this way, despite HbS presence is indispensable for the disease establishment, several other phenomena affected by a multitude of genes other than the one directly involved (*HBB*S*) play an important role [47]. Thus, to our knowledge, this is the first report focusing on the possible influence of the polymorphisms in *MTHFR* and *CBS* genes and their association on oxidative stress markers and routine measurements, and on HC treatment in SCA patients.

The prevalence of *MTHFR* (677C > T) and *CBS* (844ins68) mutations reveals an extensive ethnic and geographic variability [48,49]. For instance, the prevalence of *MTHFR* homozygosity (677TT) may vary between 1% and 30% in the general population [50], while *CBS* homozygosity shows a much lower variance, ranging from absent to 4% [48]. However, the genotypic frequency data obtained for these polymorphisms in our study agrees to previous studies in Brazilian SCA patients [22,23,51].

HC administration is the only currently available disease-modifying therapy for SCA [52–54]. HC is an antineoplastic drug, which its main pharmacological action is to increase HbF levels [52]. It has other potentially beneficial effects including improved nitric oxide (NO) metabolism, reduced red cell-endothelial interaction, decreased erythrocyte density [52], improvement of MCV and MCH [55,56], and reduction of white cell counts [57], lipid peroxidation levels [58,59] and hemolysis [60]. We observed these expected pharmacological effects, regardless the presence of both polymorphisms investigated. Despite patients' response to HC variation be attributed to several single nucleotide polymorphisms (SNPs) in various genes that are linked or not to the β-globin gene cluster [61], we did not observe any HC differential response promoted by the polymorphisms evaluated but both mutations have individually influenced one or other biomarker assessed.

The SNP at position 677 in the *MTHFR* gene had the functional influence on 30% and 65% reduced enzyme activity in heterozygous (CT) and homozygous (TT) variants, respectively [62]. This loss of
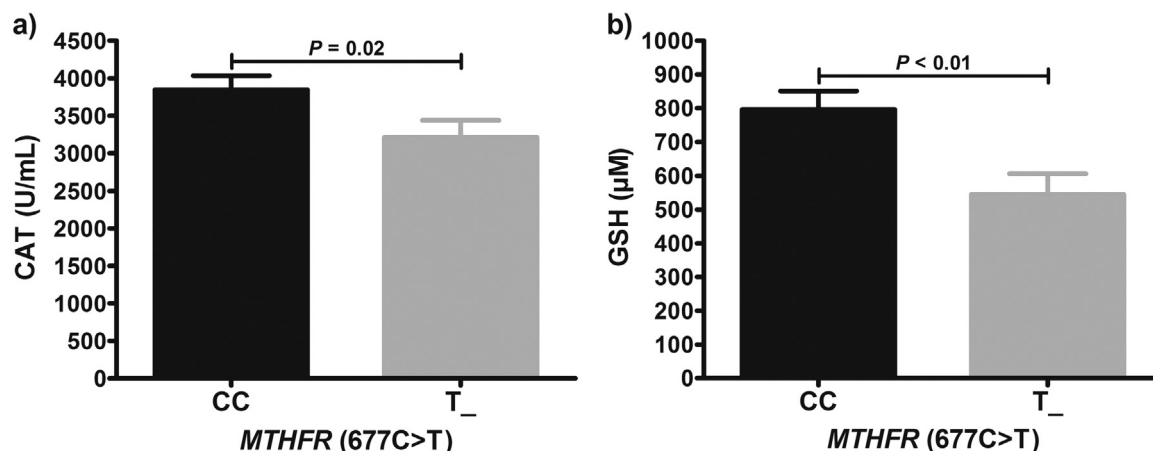
Fig. 4. Influence of *MTHFR* 677T allele on antioxidant capacity. T_ subgroup showed decreased catalase activity (A), as well a reduction of GSH levels (B) when compared to CC subgroup, regardless HC treatment. CC: wild homozygous patients for *MTHFR* (677C > T) polymorphism; T_: patients at least heterozygous for *MTHFR* (677C > T) mutation. CAT: catalase enzyme; GSH: glutathione; MTHFR: Methylenetetrahydrofolate reductase. Statistical comparisons were performed by General Linear Models (GLM). Data were expressed as mean ± 95% confidence intervals.

activity promotes a significantly reduction in the production of 5-Methyl tetrahydrofolate (5-MTHF, the primary form of plasma folate), culminating in lower plasma and erythrocyte folate concentrations [63]. In addition, SCA patients normally present lower serum cobalamin (Vitamin B12) levels, without macrocytosis or hypersegmented neutrophils [64–66]. Deficiency in any one of these micronutrient can cause increased erythroid progenitor cell death through impaired DNA synthesis and its consequences during erythropoiesis [67]. Moreover, according to Moestrup [68], during increased hemolysis (one charac-

teristic pathophysiological event of SCA), erythropoiesis is accelerated by erythropoietin (EPO), making this process extremely sensitive to deficiency of cobalamin and folate. In this way, we expected that the co-inheritance of *HBB*S* and *MTHFR* 677T would worsen clinical outcome of SCA patients. However, *MTHFR* polymorphism did not significantly affect any of routine markers.

Further, 5-MTHF is found to be an effective scavenger of superoxide [69]. Thus, long-term depletion of folate/methyl is shown to decrease reduced/oxidized GSH ratio, alter activity of manganese-dependent
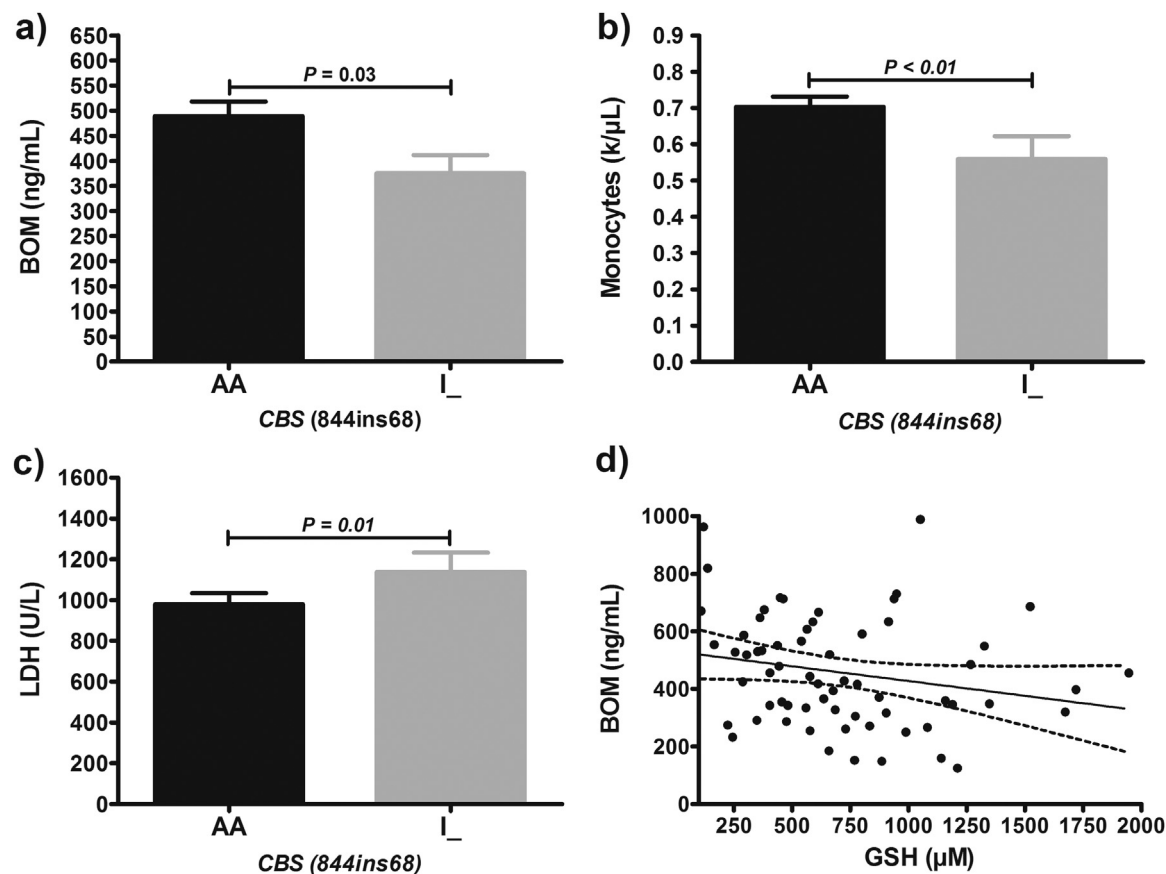

Fig. 5. Dual effects of *CBS* (844ins68) insertion on SCA pathophysiology. A) Patients with the insertion in at least one chromosome showed lower peroxidation levels when compared to those wild homozygous. B) I_ subgroup presented a reduction of monocyte count in relation to the AA subgroup. C) The presence of insertion was related to worse LDH activity in people with SCA. BOM: biomolecule oxidation marker; LDH: lactate dehydrogenase; CBS: cystathionine β-synthase. AA (HU$^+$): ancestral homozygous *CBS* (844ins68) subgroup; I_: association of heterozygous and insertion homozygous subgroup. Statistical comparisons were performed by General Linear Models (GLM). Data were expressed as mean ± 95% confidence intervals.

superoxide dismutase (Mn-SOD), CAT and GPx and induce irreparable oxidative DNA damage [70]. Our data corroborated these features, according to the decreased antioxidant capacity in the T_ subgroup. Furthermore, MTHFR reduced activity caused by the polymorphism studied is associated with elevated Hcy levels [71,72], which in turn are responsible for an increased ROS production in several tissues such as bone and vascular endothelium [73,74], and positively correlated with MDA levels [75]. However, this last association was not observed in our study. We believe that the lack of increasing oxidation product levels can be attributed to daily folic acid treatment administered in the SCA patients evaluated. According to Dragani et al. [27], a regimen of folate intake (5 mg daily) is capable to produce a 58% decrease in Hcy levels, improving vascular function and oxidative stress in patients with coronary heart disease. Although SCA patients routinely using folic acid to improve erythropoiesis, this supplementation did not seem to be able of avoid or mitigate antioxidant consumption in SCA patients at least heterozygous for *MTHFR* (677C > T), which can cause other consequences than rise in oxidation biomolecule amount.

For the insertion (844ins68) in the *CBS* gene, it was initially thought to mandate the use of an insertion-associated premature stop codon in the *CBS* mRNA and result in the translation of an inactive truncated enzyme [19]. Subsequently, Tsai et al. [25] showed that the 68-bp insertion generates an alternative splice site that permits the elimination of the entire inserted region, thereby allowing the formation of a normal mRNA transcript and a fully functional CBS enzyme. Moreover, it was speculated that this insertion is associated with somewhat higher CBS enzyme activity [26]. Assuming that the *CBS* (844ins68) mutation causes higher CBS activity, and taking into consideration the fact that the transsulfuration pathway is activated in order to combat oxidative stress and GSH depletion [76], these features justify the lower oxidation marker levels found in SCA patients with the insertion in at least one chromosome.

Other fact that might have contributed to the biomolecule oxidation results obtained is the basic function of CBS, promoting the condensation of Hcy with serine to form cystathionine [14]. Thus, the activation of CBS along with the plausible higher CBS activity promoted by insertion (844ins68) in the *CBS* gene may have decreased the Hcy levels, consequently ROS production and oxidation adducts. This reduction mechanism of Hcy levels hypothesized may also explain our results of monocytes count, since Zhang et al. [77] reported that Hcy is capable of increasing monocyte population in peripheral blood, spleen, and bone marrow in transgenic CBS-deficient mice. Heretofore, one might speculate that *HBB*S* and *CBS* (844ins68) co-inheritance had a positive prognosis, but the deviation to transsulfuration pathway in order to replenish GSH concentration may result in decreased H_2S level that induces vasoconstriction [76], worsening other characteristic pathophysiological event of SCA, vaso-occlusive events (VOE).

VOE are associated with ischemia/reperfusion damage to tissues that lead to pain and acute or chronic injury affecting any organ system, thus being an important cause of end-organ damage [78,79]. Moreover, it is estimated that 10–39% of VOE occurs with hepatic involvement [80]. This observation along to the facts that transsulfuration pathway is particularly active in hepatocytes [13]; and LDH is also an hepatic enzymes [79,81]; should be involved in the worst LDH outcome we found in the I_ subgroup. Thus, we hypothesized that this reflection might be a signal of initial establishment of hepatic dysfunction in the SCA patients from the I_ subgroup. Furthermore, Vitvitsky et al. have addressed a metabolic conundrum by establishing that red blood cells clear sulfide via methemoglobin-catalyzed oxidation of H_2S to thiosulfate and polysulfides, suggesting a detrimental mechanism for SCA patients with the insertion studied, since HbS instability contributes to an accelerated autoxidation and higher methemoglobin levels [82]. In this way, the *HBB*S* and *CBS* (844ins68) co-inheritance may enhance H_2S scavenging, favoring VOE occurrence and its pathophysiological consequences.

Finally, we did not observe any interaction effect of 677T and "I"

alleles on the biochemical parameters evaluated, notwithstanding tight relation between cellular one-carbon and GSH metabolism [83–85]. Anyway, considering that one-carbon metabolism is a metabolic pathway that provides a variety of metabolic intermediates, which react either with pro-oxidants or promote antioxidant defense, along with the crucial physiological importance of a tight regulation of Hcy and H_2S metabolism in persons with SCA, our results leave new perspectives for further studies to address better the above-mentioned hypotheses.

5. Conclusions

A number of limitations of the present study need to be acknowledged, including no functional analysis of MTHFR and CBS enzymes, neither measurements of folate, cobalamin, and even Hcy, and the small sample size in some subgroups evaluated. Nevertheless, this work was an outstanding hypothesis-generating study, suggesting that *MTHFR* (677C > T) and *CBS* (844ins68) polymorphisms involvement in SCA pathophysiology is likely to be far more complex than it was explored to date. Briefly, we provided evidence that: i) the polymorphisms investigated did not affect HC treatment, and apparently, they did not present any interaction effect on SCA pathophysiology; ii) *HBB*S* and *MTHFR* 677T co-inheritance showed a detrimental effect on antioxidant capacity of people with SCA; iii) *HBB*S* and *CBS* (844ins68) co-inheritance had a dual impact on pathophysiological processes in SCA patients.

Conflict of interest

The authors declare no competing financial or other relationship with any people or organization interests.

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