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ORIGINAL ARTICLE

Reduced peripheral blood superoxide dismutase 2 expression in sickle cell disease

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Abstract

Sickle cell disease (SCD), a hereditary form of chronic hemolytic anemia, is characterized by acute vascular occlusion and chronic complications as pulmonary hypertension (PH), a hallmark of higher mortality. This study aimed to determine peripheral blood expression of superoxide dismutase 2 (SOD2), a major mitochondrial antioxidant enzyme in SCD patients on the mRNA level and compared it with SOD2 expression in healthy individuals. It also aimed to detect possible differences in SOD2 expression among patients with/without specific SCD complications and to detect possible correlations with patient laboratory parameters. SOD2 mRNA levels were significantly lower in SCD patients in comparison with controls and correlated with red blood cell count, reticulocyte count, platelet count, C-reactive protein, ferritin, and brain natriuretic peptide values. SCD patients with echocardiographic indications of PH featured significantly reduced SOD2 expression in comparison with patients without such indications. Consequently, SOD2 expression emerges as a potential biomarker of PH in SCD being a link among hemolysis, inflammation, iron overload, oxidative stress, and SCD cardiopathy.

Keywords Sickle cell disease · Superoxide dismutase · SOD2 · MnSOD · Pulmonary hypertension

Introduction

Sickle cell disease (SCD) results from a point mutation on the sixth codon of the beta hemoglobin gene. The substitution of adenine by thymine on DNA causes an amino acid change from glutamate to valine on the beta hemoglobin polypeptide. The subsequent conformational change of hemoglobin renders it prone to polymerization under hypoxic conditions and causes premature extravascular and intravascular red blood cell destruction (hemolysis) and obstruction of the microcirculation. The aforementioned mechanism seems sufficient to explain to a great extent the disease's clinical course and complications. However, despite its monogenic etiology, SCD features high clinical variability, which only partially is explained by variations of the exact beta globin gene genotype. This observation was the stimulus for further research on other pathogenetic mechanisms potentially implied. Intravascular, and to a lesser extent, extravascular hemolysis has emerged as the triggering factor of complex pathophysiologic processes that synergistically determine clinical and laboratory phenotype of patients; chronic inflammation, oxidative stress, nitric oxide depletion, and hypercoagulation were also noted in SCD patients. The extent to which such mechanisms occur in each specific patient depends on both genetic and environmental factors and seems to define the clinical course [1].

Chronic inflammation with acute exacerbations is a hallmark of SCD. Cell-free heme derives from intravascular hemolysis and acts as damage-associated molecular pattern

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(DAMP), leading to innate immune system activation, leukocyte, platelet and endothelium activation, cytokine secretion, and upregulation of adhesion molecules [2]. Even steady state, SCD patients feature increased peripheral blood leukocyte and platelet count and increased acute phase reactants like C-reactive protein (CRP) [3]. The extent of inflammatory process seems to affect prognosis; higher baseline CRP values have been shown to predispose to more vasoocclusive crises that are characterized by further increases of acute phase reactants [4].

Increased oxidative stress has emerged as a key feature of SCD. Intravascular heme and iron release, as well as relapsing ischemia-reperfusion events are major contributors to SCD oxidative damage. Oxidative stress primarily derives from red blood cells (hemoglobin autoxidation, heme and iron release due to intravascular hemolysis) and secondarily affects adjacent cells (white blood cells, platelets, endothelial cells). Other mechanisms, i.e., ischemia-reperfusion cycles and endothelial nitric oxide synthase uncoupling also contribute. Although many oxidative stress markers have been found to be increased in SCD patients, studies on antioxidant enzyme activity are inconclusive; namely, reduced antioxidant enzyme activity has been reported, but it was attributed to oxidative damage of antioxidant enzymes and cofactor depletion. Studies questioning antioxidant enzyme expression are limited in SCD; no enzymic defect contributing to increased oxidative stress is robustly established thus far [1, 5, 6].

Apart from acute ischemic events, recurrent/chronic hemolysis is directly or indirectly responsible for major chronic complications, such as sickle retinopathy, leg ulcers, and pulmonary hypertension (PH) [1]. PH is defined as mean pulmonary arterial pressure ≥ 25 mmHg determined by right heart catheterization (RHC). It can be idiopathic, or it may complicate various cardiovascular, pulmonary, or systematic diseases, i.e., SCD [7]. Its prevalence among SCD patients is 10–30% and its etiology is multifactorial: hyperdynamic circulation due to anemia, nitric oxide depletion due to intravascular hemolysis, chronic thromboembolic disease due to hypercoagulability, and left heart dysfunction mainly due to diastolic dysfunction. Irrespective of etiology, PH is a hallmark of poor prognosis and higher mortality in SCD patients [8]. Because of the invasive diagnostic approach demanded by definition, echocardiography is widely utilized as a substitute noninvasive means of detecting patients with high likelihood of PH. More specifically, a tricuspid regurgitant jet velocity (TRV) ≥ 2.9 m/s has been shown to have a relatively high positive predictive value for RHC-diagnosed PH [9]. Moreover, biochemical markers, namely natriuretic peptides (brain natriuretic peptide—BNP, N-terminal-proBNP—NTproBNP) tend to increase in patients with PH; higher values are predictors of unfavorable prognosis [7].

Superoxide dismutases (SODs) convert the reactive oxygen species (ROS) superoxide to hydrogen peroxide, a less toxic free radical with signaling properties which is further transformed to water by catalase or glutathione peroxidase. SOD2 or MnSOD (manganese superoxide dismutase) is located in mitochondria being responsible for respiratory chain-derived superoxide detoxification. It is encoded by the highly conserved *SOD2* gene, which is located on 6q25 chromosome and tightly regulated by various transcription factors, microRNAs, and DNA methylation [10]. Induction of SOD2, achieved through ischemic preconditioning, has been shown to alleviate ischemia-reperfusion injury in rat cardiomyocytes [11]. Recently, a *SOD2* polymorphism was shown to predispose to sickle vasoocclusive crises and acute splenic sequestration in a pediatric SCD cohort from Brazil [12].

The aims of this study are to determine *SOD2* expression on the mRNA level in SCD patients in comparison with healthy controls and to detect potential correlations between *SOD2* expression and clinical/laboratory parameters of the patients.

Materials and methods

Study population

Twenty-one consecutive Greek SCD patients (16 females and 5 males, age 54.7 ± 9.5 years, 4 homozygous S/S, 17 compound heterozygous beta thalassemia-sickle cell anemia, 5 β^0/S, 12 β^+/S) at steady state (no vasoocclusive, hemolytic, aplastic crises, no blood transfusions during the past 3 months) were studied. Three patients were under hydroxyurea therapy and 8 underwent regular blood apheresis treatment, under a specific protocol previously implemented in our hospital [13]. Patients with ferritin values above 1000 ng/mL were under oral iron chelation therapy. All patients received folate supplementation and acetylsalicylic acid (100 mg/day). History of acute chest syndrome (ACS), femoral head necrosis (FHN), and SCD retinopathy were recorded. *ACS* was defined as the presence of new infiltrates in chest radiography with fever and/or respiratory tract symptoms (cough, sputum production, dyspnea). *FHN* was diagnosed on the basis of clinical and MRI findings. The term *SCD retinopathy* refers to the proliferative retinal involvement. SCD retinopathy was diagnosed on the basis of either neovascularization findings at retinal (fundus) examination or history of laser treatment. All patients had normal left ventricular systolic function (left ventricular ejection fraction > 50%) and no greater than mild valve dysfunction (apart from tricuspid regurgitation). Fourteen Greek healthy volunteers (9 females and 5 males, age 51.2 ± 14.5 years) with normal complete blood count and no history of hemoglobinopathy were tested as well. The study was approved by Laikon General Hospital Ethics Committee in accordance with Helsinki Declaration.

Laboratory screening

Complete blood count, reticulocyte count, and immature reticulocyte fraction (IRF) were determined using the Sysmex XE5000 analyzer. Hemoglobin fraction quantification was performed through high-performance liquid chromatography (HPLC) in a variant II hemoglobin testing system (Bio-rad). Creatinine, lactate dehydrogenase (LDH), total and indirect bilirubin, ferritin, and folate values were analyzed in a Cobas 6000 analyzer (Roche). High-sensitivity CRP (hs-CRP) values were determined using Siemens Advia 1800 assay. BNP values were obtained from patients' peripheral blood samples using the Architect BNP chemiluminescent microparticle immunoassay (Abott).

Real-time RT-PCR

Total RNA was extracted from peripheral blood using the RNeasy Mini Kit after selective lysis of erythrocytes. One microgram of RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). *SOD2* mRNA was quantified using hydrolysis probes technology, and *GAPDH* was used as a reference gene. Primers and TaqMan probes were predesigned and validated by Applied Biosystems. RT-qPCR was performed on a CFX-96 real-time PCR (Bio-Rad). Measurements were performed in duplicate using the iTaq™ Universal Probes Supermix (Bio-Rad).

Relative gene expression values were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak method) [14]. The data are presented as fold change in gene expression normalized to *GAPDH* gene and relative to control. Control group comprised healthy individuals or specific patient subgroups, i.e., patients without a specific complication.

Echocardiography

Patients underwent echocardiographic evaluation with a GE Vivid 7 ultrasound system to assess left ventricular systolic function, valvular abnormalities, and TRV as a measure of pulmonary artery systolic pressure. Two different TRV threshold values (2.5 and 2.9 m/s) were used for comparisons. The first was applied in accordance with 2014 American Thoracic Society practice guideline on PH of SCD [15] and the second in accordance with the most recent guidelines on pulmonary hypertension [7].

Statistical analysis

Quantitative data are presented as mean ± SD. Normality of distribution was determined with Shapiro-Wilk test [16]. Quantitative data not deviating from normal distribution were compared with Student *t* test. Nonparametric Mann-Whitney *U* test was applied to quantitative data not normally distributed. Qualitative data were compared with Fisher Exact test. Correlation analysis with Spearman rho coefficient calculation was applied between relative *SOD2* expression and age/laboratory parameters of the every subject to detect possible correlations. Statistical analysis was performed using the IBM SPSS v25 software package. A less than 0.05 *p* level was considered statistically significant.

Results

Mean age and sex distribution did not differ significantly between patients and controls (independent samples Student *t* test, $p = 0.41$, Fisher exact test, $p = 0.47$, respectively). Clinical, laboratory, and echocardiography data of patients are shown in Tables 1, 2, and 3, respectively.

SCD patients exhibited significantly lower SOD2 mRNA expression in comparison with healthy controls. In fact, SOD2 expression of SCD patients was 73% of the expression observed in controls, $p = 0.008$ (Mann-Whitney *U* test). SOD2 expression did not differ significantly between male and female patients. No correlation was observed between patient's age and SOD2 mRNA levels (correlation coefficient − 0.047, $p = 0.854$, Spearman rho). We subsequently determined the relative SOD2 expression between SCD patients with/without history of specific disease complications (Mann-Whitney *U* test). Although patients with history of FHN showed lower SOD2 expression compared with patients without this complication, the difference was not statistically significant ($p = 0.052$). History of ACS and SCD retinopathy was not related to significantly different SOD2 mRNA levels.

Moreover, we determined the relative SOD2 expression between SCD patients with echocardiographic (increased TRV) or biochemical (increased BNP) markers of PH and patients with no such indications; the second group was considered as the reference group. Relative gene expression in SCD patients presenting TRV > 2.5 m/s was 0.68 compared with patients with TRV ≤ 2.5 m/s, $p = 0.017$ (Mann-Whitney *U* test). Relative gene expression in SCD patients presenting TRV ≥ 2.9 m/s was 0.48 compared with patients with TRV <

Table 1 Demographic-clinical characteristics of patients

Clinical parameter	
Age (years)	54.7 ± 9.5
Sex (male/female)	5/16
Hydroxyurea treatment	3
Blood apheresis treatment	8
ACS	9
FHN	10
Sickle retinopathy	8

ACS acute chest syndrome, *FHN* femoral head necrosis

Table 2 Laboratory parameters of patients. *BNP* brain natriuretic peptide, *hs-CRP* high-sensitivity C-reactive protein, *LDH* lactate dehydrogenase, *RBC* red blood cell count, *SOD2* superoxide dismutase 2

Laboratory parameter	
RBC (10^{12} cells/L)	3.8 ± 1.1
Hematocrit (proportion of 1)	0.27 ± 0.05
Hemoglobin (g/L)	88.8 ± 16.6
White blood cell count (10^9 cells/L)	7.9 ± 2.2
Platelet count (10^9 cells/L)	346.7 ± 194.3
Hemoglobin S (proportion of 1)	0.75 ± 0.08
Hemoglobin F (proportion of 1)	0.10 ± 0.08
Absolute reticulocyte count (10^9cells/L)	232.5 ± 93.2
Reticulocyte count (proportion of 1)	0.070 ± 0.042
Immature reticulocyte fraction (proportion of 1)	0.29 ± 0.08
Creatinine (μmol/L)	62.8 ± 15.0
Total bilirubin (μmol/L)	49.6 ± 44.5
Indirect bilirubin (μmol/L)	33.4 ± 37.1
LDH (U/L)	407.6 ± 165.2
hs-CRP (mg/L)	5.1 ± 2.5
Ferritin (μg/L)	552 ± 741
Folate (nmol/L)	42.8 ± 10.9
BNP (ng/L)	71.5 ± 36.0

2.9 m/s, $p = 0.029$ (Mann-Whitney *U* test). Regarding BNP, as no cutoff values have been introduced in SCD (as opposed to NTproBNP), median value was determined (74.5 ng/L) and patients were categorized as high BNP (values above the median) or low BNP (values below the median). Relative SOD2 expression in patients with high BNP was 0.26 compared with those with low BNP, $p = 0.0001$ (Mann-Whitney *U* test). Subsequently, relative gene expression of patients with TRV > 2.5 m/s and BNP > 74.5 ng/L compared with those with TRV < 2.5 m/s and BNP < 74.5 ng/L was determined as 0.28 $p = 0.001$ (Mann-Whitney *U* test).

Table 4 and Chart 1 depict the DeltaCt values of patients with/without history of specific disease complications, with/without echocardiographic markers of pulmonary hypertension and with/without BNP values above the median value of 74.5 ng/L. DeltaCt values indicate the difference in the degree of expression between *SOD2* and *GAPDH* (reference gene).

Furthermore, we performed correlation analysis between $2^{-\Delta\Delta Ct}$ values for SOD2 mRNA levels (normalized to GAPDH gene and relative to controls) and the patients' laboratory values, in order to elucidate possible relations between the enzyme's expression and markers of anemia, hemolysis, inflammation, hemostasis, renal function, folate levels, iron overload, and myocardial stress. Results are shown on Table 5.

Table3 Echocardiography parameters of patients

Echocardiography parameters	
TRV (m/s)	2.7 ± 0.3
SPAP (kPa)	5.1 ± 0.9
TRV ≥ 2.9 m/s	$n = 6$
TRV > 2.5 m/s	$n = 13$

TRV tricuspid valve regurgitant jet velocity, *SPAP* pulmonary artery systolic pressure

Discussion

SCD, the first genetic disease defined at molecular level, is characterized by high pathophysiological complexity and clinical heterogeneity that is not adequately interpreted. Some patients tend to exhibit a benign course with few acute crises and chronic complications, while others suffer a debilitating course and have their lifespan substantially shortened. Moreover, its complications causing substantial morbidity and mortality cannot be accurately predicted due to lack of well-established biomarkers. Chronic inflammation, nitric oxide depletion, and increased oxidative stress are fundamental characteristics of SCD and have been linked with disease severity.

To the best of our knowledge, this is the first study indicating reduced expression of an antioxidant enzyme in SCD. The difference found cannot be attributed to sex or age differences; the above parameters did not differ significantly between patients and controls; no significant differences regarding SOD2 expression were noted between male and female patients; and age did not correlate significantly with SOD2 expression among patients. We compared relative SOD2 expression between patients with history of specific disease complications (ACS, FHN, proliferative retinopathy) and patients without them in order to determine whether SOD2 reduction correlates with clinical severity. Patients with history of FHN featured decreased SOD2 expression, but the difference was not statistically significant. FHN is a major cause of debilitation in SCD patients and its etiology is multifactorial [17]. Although coagulation disorders are thought to be a major predisposing mechanism, antioxidant enzyme genetic defects have emerged as risk factors [18]. On the contrary, no differences were obtained with reference to ACS and sickle retinopathy. However, as some of the above complications (i.e., ACS) are characterized by acute occurrence and gene expression is a dynamic phenomenon continuously adapted to various stimuli, the likelihood of SOD2 expression changes during ongoing complications cannot be ruled out and might be subject to future studies. Other recognized complications of SCD, i.e., splenic sequestration, stroke, leg ulcers, and priapism were rare in our study population and statistically relevant comparisons were not possible.

Table 4 DeltaCt (Ct_{SOD2}-Ct_{GAPDH}) as a measure of SOD2 expression in patients with/without specific complications/elevated TRV/elevated BNP. Ct represents the time needed in order that fluorescence produced by PCR products reaches a specific threshold. Higher Ct values may indicate reduced gene expression, consequently higher DeltaCt values indicate reduced relative SOD2 expression

Clinical/echocardiography/laboratory parameter	SOD2 expression DeltaCt (Ct_{SOD2}-Ct_{GAPDH}) values	
	Patients with complication	Patients without complication
ACS	0.82 ± 1.50	0.81 ± 1.07
FHN	1.11 ± 1.32	0.34 ± 1.40
Sickle retinopathy	1.02 ± 1.14	0.76 ± 1.36
TRV > 2.5 m/s	1.23 ± 1.23	− 0.01 ± 0.78
TRV ≥ 2.9 m/s	1.59 ± 0.37	0.51 ± 1.34
BNP > 74.5 ng/L	1.77 ± 0.68	− 0.23 ± 0.75

ACS acute chest syndrome, *BNP* brain natriuretic peptide, *FHN* femoral head necrosis, *PCR* polymerase chain reaction, *SOD2* superoxide dismutase 2, *TRV* tricuspid valve regurgitant jet velocity

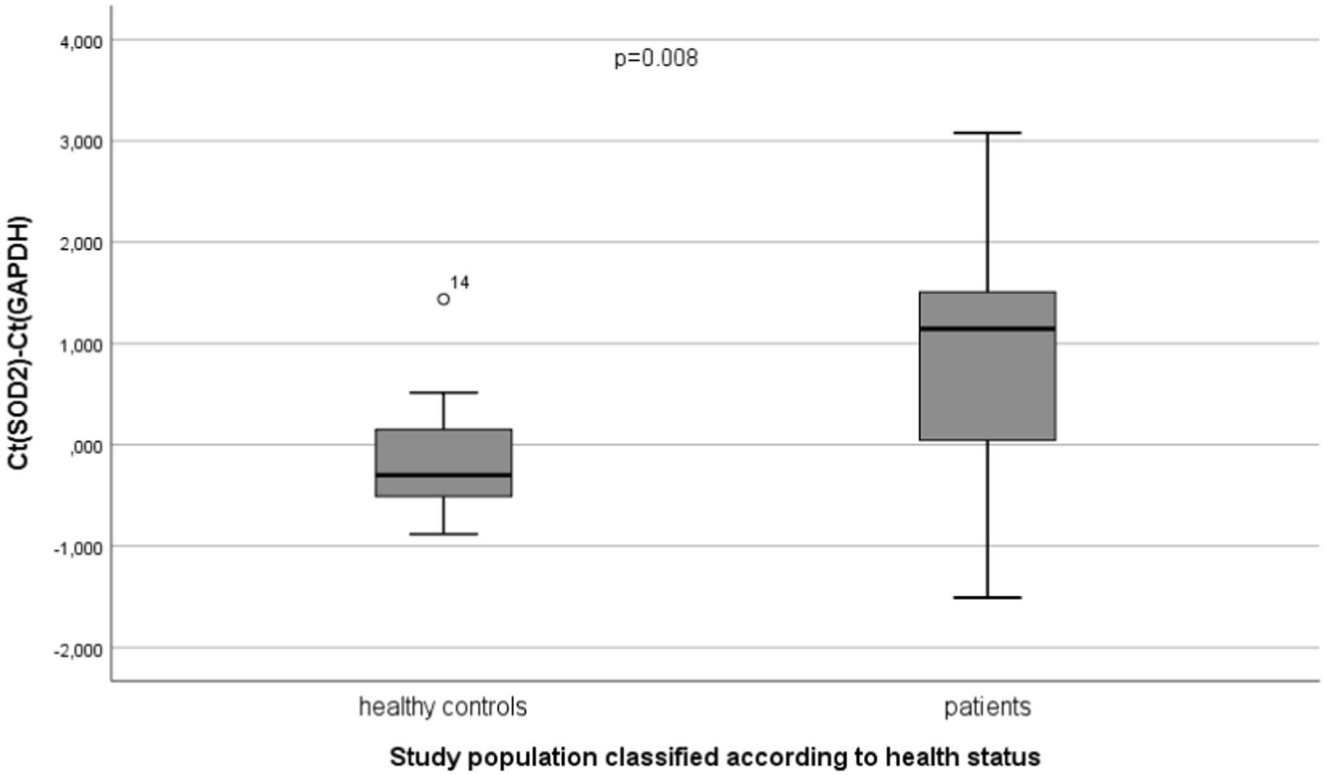
As far as laboratory parameters are concerned, reduced SOD2 expression tended to coincide with worsening of most parameters, namely lower red blood cell count, hematocrit, hemoglobin, lower hemoglobin F fraction, higher white blood cell and platelet counts, higher reticulocyte count, absolute reticulocyte count and IRF, higher LDH, total and indirect bilirubin values, higher hs-CRP, ferritin, and BNP (Table 5). However, only correlation with red blood cell count, reticulocyte count, absolute reticulocyte count, IRF, platelet count, hs-CRP, ferritin and BNP was statistically significant at the 0.05 level (Table 5).

Red blood cell count, together with hematocrit and hemoglobin, is a parameter indicative of the degree of anemia. In SCD, it is of less clinical utilization and less well-studied compared with hematocrit/hemoglobin. However, a recent large retrospective study connected red blood cell count with left ventricular end-diastolic dimensions, left atrial dimensions, and cardiac index in SCD patients. Of note, despite correlation with other hematological parameters (i.e., hematocrit, hemoglobin), red blood cell count, and HbF%, were the only independent predictors of the above parameters [19].

Reticulocyte and absolute reticulocyte counts are major hemolysis markers (higher values indicate increased hemolysis rate) and also indicate adequate bone marrow counterbalance of premature red blood cell destruction. Although they do not discriminate between intravascular and extravascular hemolysis, higher values have been shown to predispose to stroke and chronic kidney disease and to predict higher mortality [20, 21]. IRF, also called Reticulocyte Maturation Index (RMI), represents the immature reticulocyte population, and as a marker of hemolysis, it is more sensitive than reticulocyte count. Higher reticulocyte and IRF values imply higher hemolysis rate and in our study significantly coincided with lower SOD2 expression.

CRP, the most widely studied marker of inflammation, produced in the liver has been shown to be increased in SCD patients [3]. Krishnan et al. [4] showed that higher CRP values predispose SCD patients to more frequent vasoocclusive crises and are related with lower hemoglobin, higher platelet, and LDH values. Interestingly, Bhagat et al. [22] found a positive correlation between CRP and oxidative stress in SCD. The negative correlation between SOD2 expression and hs-CRP values was an unexpected finding of our study, as inflammatory process tends to upregulate SOD2 expression to compensate for increased oxidative stress; Nf-kβ, the main mediator of inflammation on the transcriptional level, is a positive regulator of *SOD2* gene expression [10]. It can be speculated that inflammation and SOD2 downregulation share a partially common etiology in SCD patients that abrogates inflammation-mediated SOD2 upregulation. In fact, it is possible that SOD2 downregulation acts as an inflammation enhancer, as mitochondrial ROS are indispensable for innate immunity activation and inflammasome induction [23], and reduced SOD2 might lead to higher mitochondrial ROS load.

A strong negative correlation between SOD2 expression and ferritin was also obtained in our study. Ferritin, a highly conserved protein, is located both in the intracellular and extracellular compartments and acts as an iron storage protein. As iron is highly pro-oxidant when uncoupled, intracellular ferritin partially protects cells from iron's harmful consequences. Extracellular ferritin has less well-understood functions but is a clinically useful index of body iron stores. Nevertheless, it is upregulated under inflammatory stimuli together with other acute phase reactants or in neoplasia [24]. Conversely, iron overload is an independent enhancer of inflammation in SCD [25]. Patients with SCD tend to suffer from iron overload due to excessive blood transfusions, while iron absorption status seems to vary among patients. It seems possible that higher ferritin values reflect both inflammation and iron overload in SCD [24, 26]. A recent meta-analysis revealed a survival benefit for SCD patients undergoing chelation therapy [27]. Our results strongly support the rationale of iron removal in SCD patients, either via iron chelation therapy or phlebotomies in selected patients, and provide a mechanistic explanation for iron chelation beneficial action,



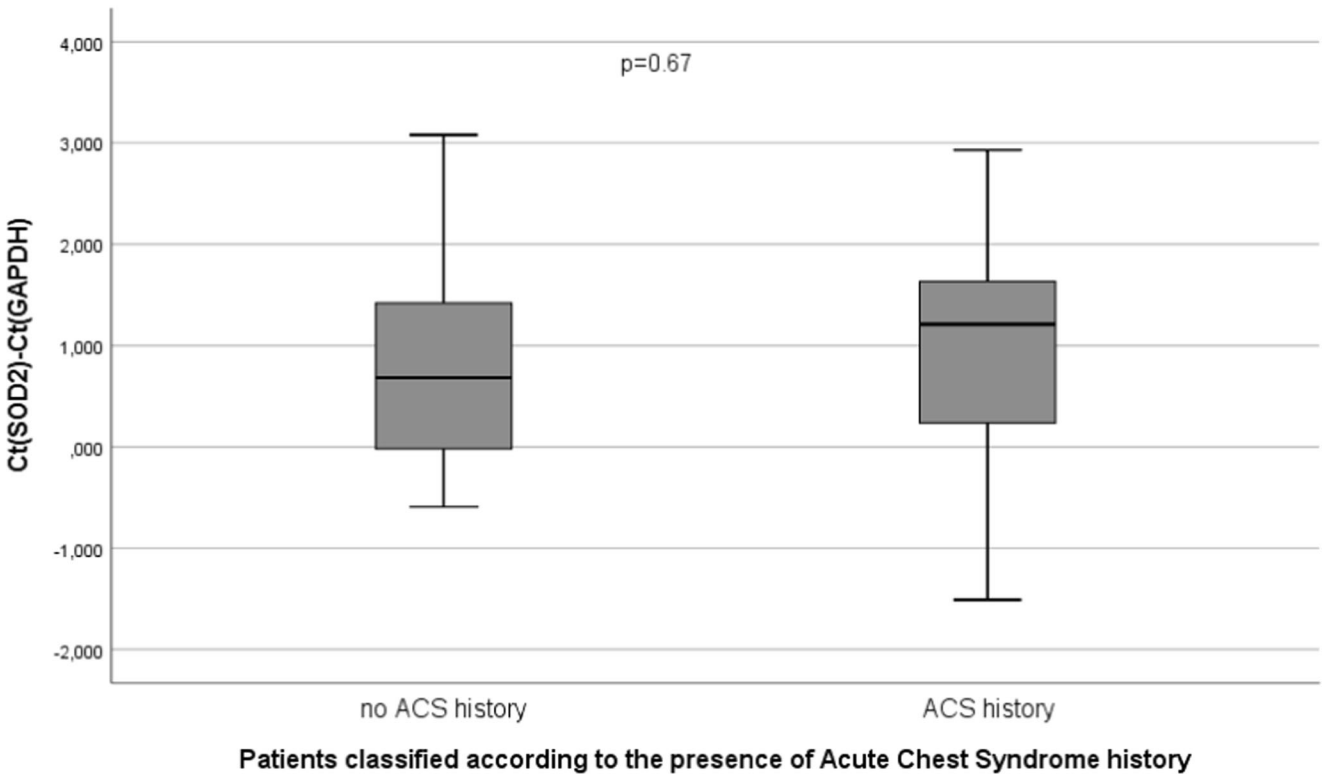
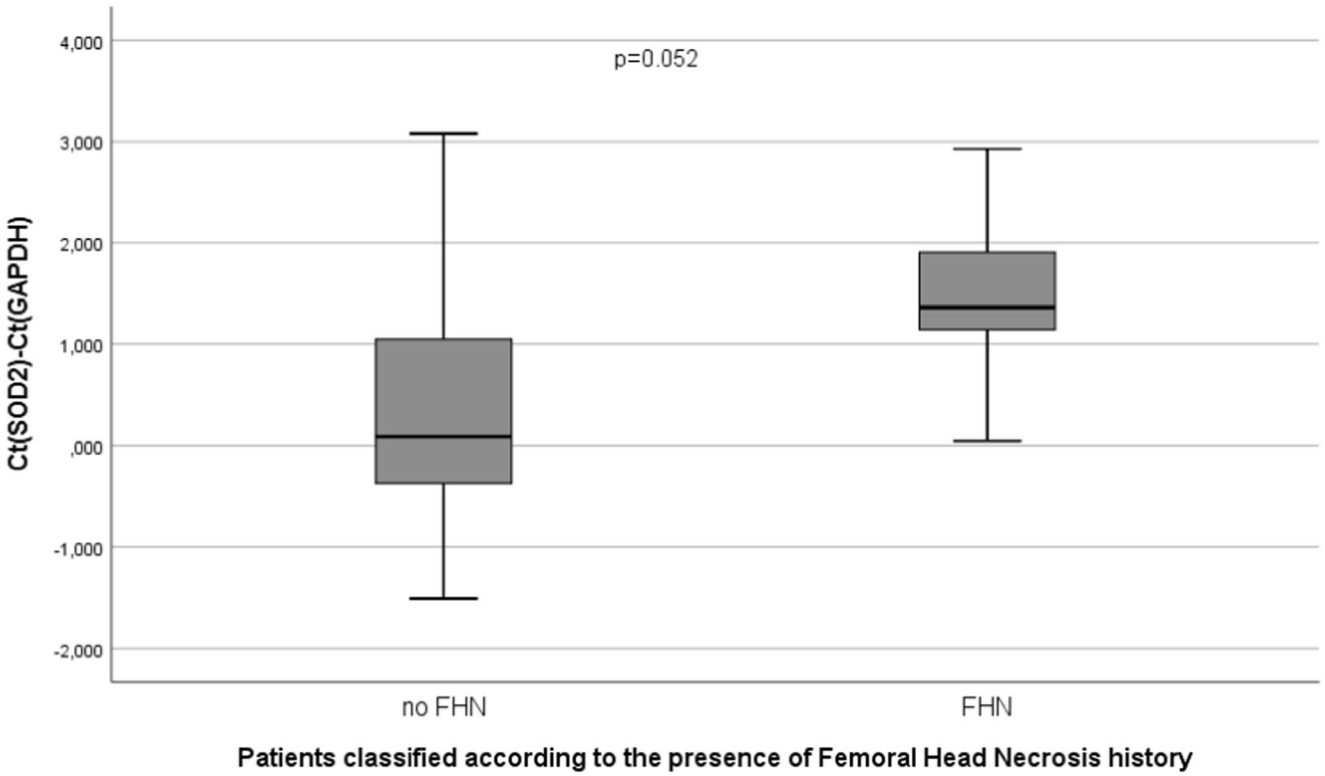
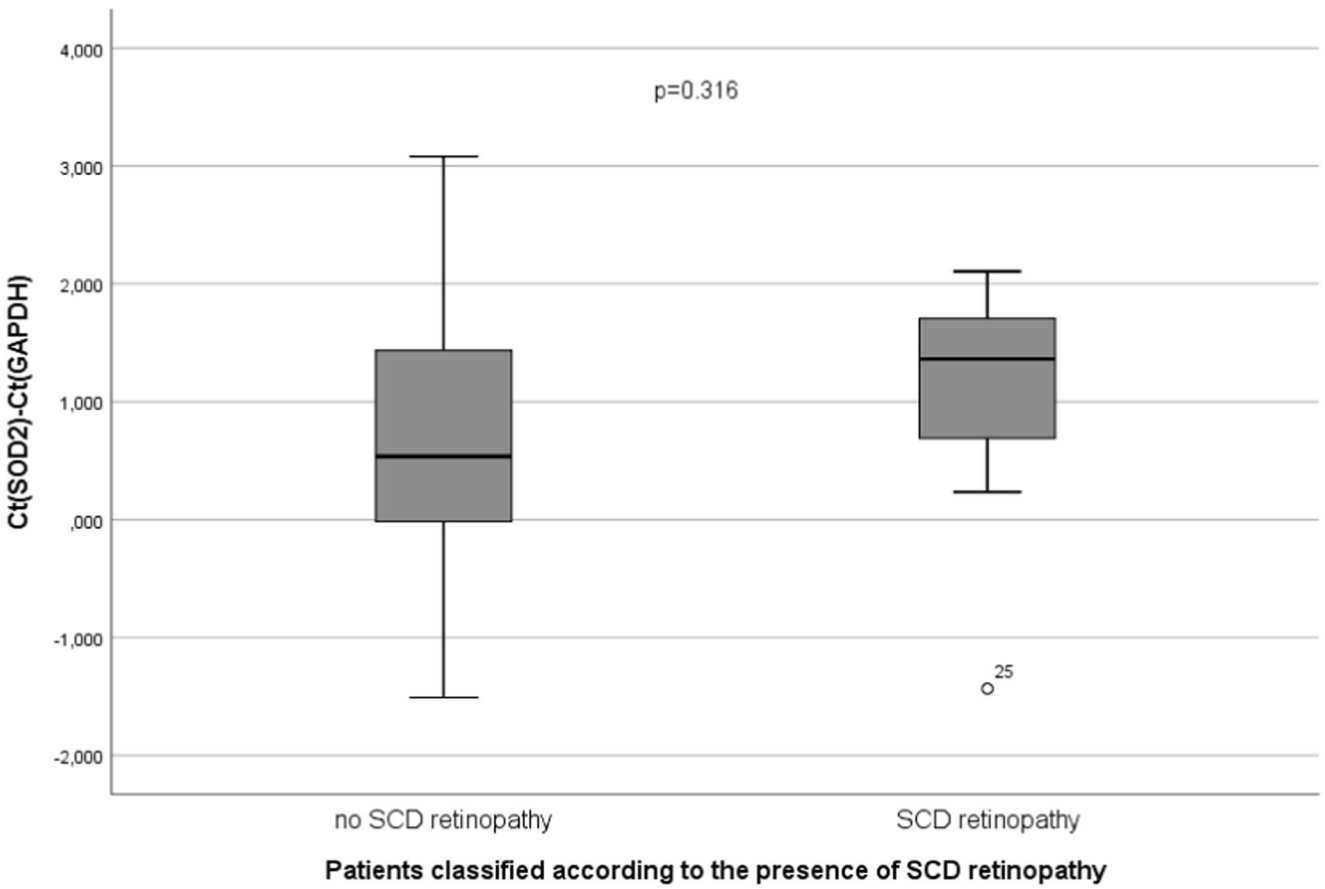


Chart 1 DeltaCt (Ct_{SOD2}-Ct_{GAPDH}) as a measure of SOD2 expression in peripheral blood leukocytes of study subjects. Higher values represent lower relative SOD2 expression. ACS acute chest syndrome, FHN femoral head necrosis, SOD2 superoxide dismutase 2, SCD sickle cell disease, TRV tricuspid regurgitant jet velocity





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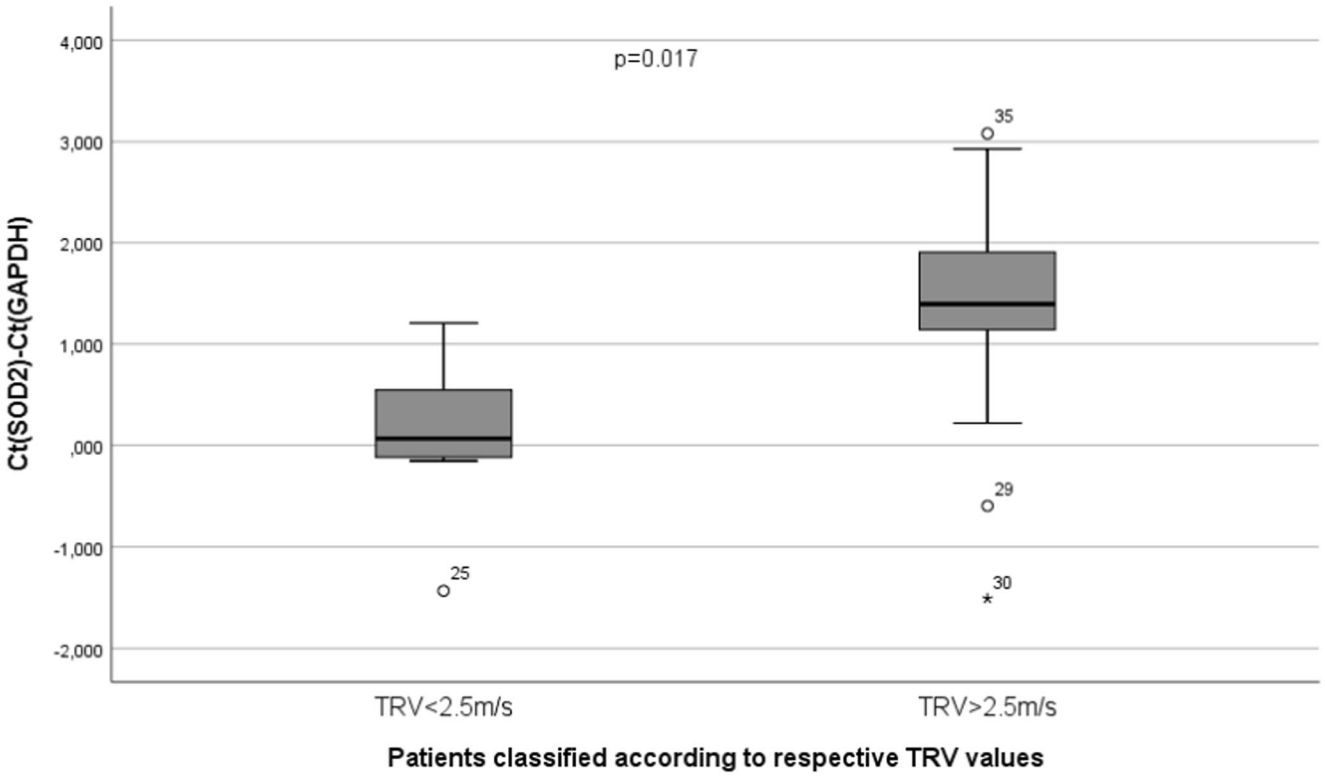

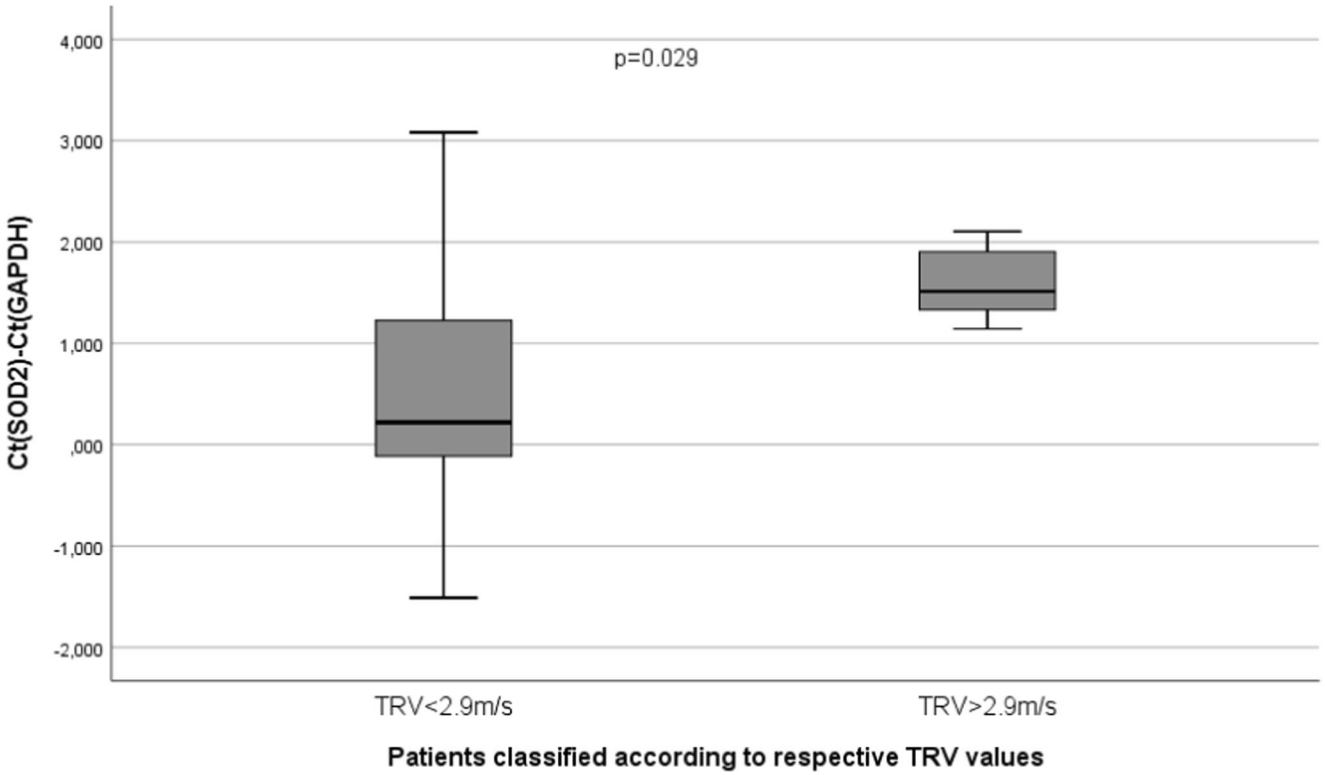

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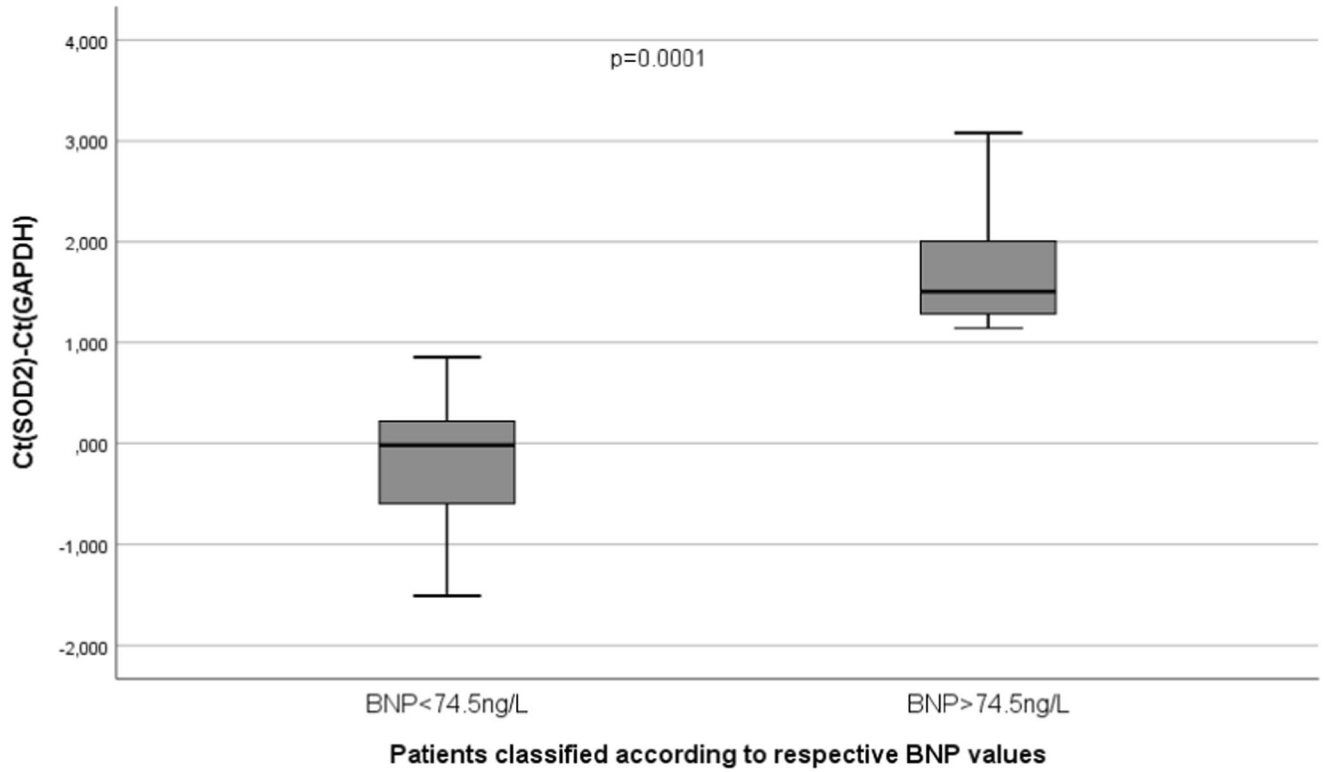
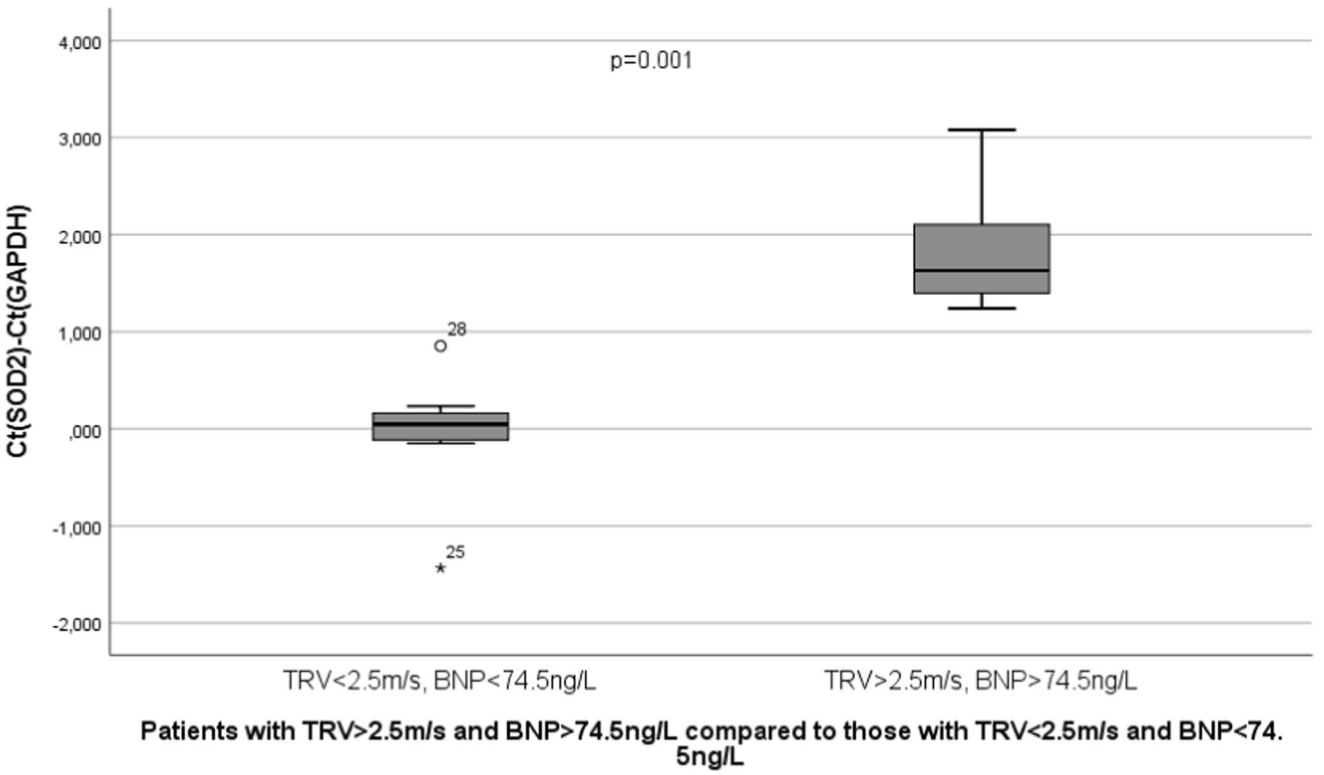
as this strategy might lead to upregulation of SOD2 [1, 13]. Future clinical studies might verify this hypothesis.

Platelet count showed a negative correlation with SOD2 expression. Although platelets do not express hemoglobin





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genes, they are activated in SCD patients and exhibit a proinflammatory, prothrombotic phenotype. They form complexes with sickle red cells and leukocytes that promote vascular obstruction, endothelial dysfunction, and inflammation [1].

Table 5 Correlation analysis between SOD2 expression ($2^{-\Delta\Delta Ct}$ values) and laboratory indices of SCD patients

Laboratory parameter	Correlation coefficient ρ	p
RBC	0.4	*0.034*
Hematocrit	0.329	0.169
Hemoglobin	0.285	0.236
White blood cell count	− 0.254	0.293
Platelet count	− 0.463	*0.046*
Hemoglobin S	− 0.009	0.972
Hemoglobin F	− 0.391	0.098
Absolute reticulocyte count	− 0.552	*0.018*
Reticulocyte count %	− 0.618	*0.006*
Immature reticulocyte fraction	− 0.547	*0.028*
Creatinine	0.067	0.791
Total bilirubin	− 0.37	0.131
Indirect bilirubin	− 0.358	0.144
LDH	− 0.255	0.307
Ferritin	− 0.571	*0.008*
Folate	0.097	0.701
hs-CRP	− 0.505	*0.033*
BNP	− 0.806	*0.005*

BNP brain natriuretic peptide, *CRP* C-reactive protein, *LDH* lactate dehydrogenase, *RBC* red blood cell count, *SCD* sickle cell disease, *SOD2* superoxide dismutase 2

Values in italics represent statistically significant differences (p <0.05)

Platelet count has been correlated with vasoocclusive crises, emergency department admissions, stoke hazard, and pregnancy complications in SCD patients [28–31]. Platelet activation has been implied in the pathophysiology of PH in SCD [32].

We subsequently examined the possible relation between SOD2 expression and PH, a complication related with increased mortality in SCD. Even though RHC is the gold standard for the diagnosis of PH, we performed cardiac ultrasound as a noninvasive substitute to stratify patients according to likelihood of PH. Higher TRV values have been shown to be an independent predictor of higher mortality in SCD [15, 20]. Most studies in SCD patients have defined TRV values of 2.5 m/s as the threshold to classify patients as high or low likelihood for PH, and current SCD PH guidelines recommend the same cutoff point [15, 33]. In our study, we have selected both 2.5 and 2.9 m/s as thresholds. The second was selected in order to increase positive predictive value (at the cost of lower sensitivity) in accordance with the most recent guidelines on PH [7]. Moreover, our study population is older than SCD patients of other studies (age 55 ± 9 years) and older subjects tend to feature higher TRV values [33, 34].

Patients with higher TRV had significantly reduced SOD2 expression in comparison to patients with lower TRV values. The importance of this finding cannot be underestimated. In terms of pathophysiology, it provides a link between increased oxidative stress and SCD PH. Previous studies have connected genetic defects of human antioxidant enzymes with pulmonary hypertension and other complications in SCD [12, 18, 35]; nevertheless, this is the first study directly showing reduced expression of an antioxidant enzyme in SCD. Excessive oxidative stress has been implicated in the pathophysiology of other forms of PH, i.e., idiopathic pulmonary arterial hypertension (iPAH). More specifically, endothelial cells and pulmonary artery smooth muscle cells from iPAH patients feature reduced SOD2 expression, and *SOD2* exon 2 hypermethylation is at least partly responsible for this reduction [36]. Furthermore, despite the initial perception of an organ-specific disorder, iPAH is a systemic syndrome featuring multiorgan metabolic deregulation [36]. Interestingly, Mata et al. [37] showed that peripheral blood leukocytes from iPAH patients are also characterized by decreased SOD2 expression on the mRNA level. Our findings imply that impaired enzyme synthesis leading to excessive oxidative stress is a common pathophysiologic mechanism between SCD PH and iPAH. In SCD, this defect seems to be attributed to hemolysis procedure, which also causes anemia, inflammation, and cardiovascular compromise. The significant correlation between SOD2 expression and red blood cell count, reticulocytes, CRP, and ferritin further supports the above conclusion. Consequently, our finding conceptually connects all major pathophysiologic aspects of SCD, namely hemolysis, inflammation, iron overload, hypercoagulability, increased oxidative stress, and cardiovascular stress-pulmonary hypertension.

On the clinical level, our study findings provide the basis for the establishment of a new biomarker of PH in SCD. A biomarker is a reproducibly and objectively measured characteristic of a biological process [38]. As peripheral blood is an easily approachable and readily available sample source and quantitative real-time RT-PCR availability and clinical application is constantly on the rise, it is tempting to examine the possible role of SOD2 measurement in the diagnosis of PH in SCD patients. SOD2 candidacy as a biomarker is further supported by its close correlation with BNP, a well-established biomarker of increased intraventricular filling pressures. BNP is secreted mainly by left/right ventricular myocardium under volume/pressure overload circumstances, and in heart failure, it closely correlates with left ventricular end-diastolic pressure. In PH, BNP levels predict right ventricular strain [39]. Natriuretic peptide levels have been shown to be increased in SCD patients with PH and diastolic dysfunction [40] and higher levels are related with higher mortality [18]. In this study, BNP was determined instead of NTproBNP. Both peptides come from the cleavage of the same prepeptide called preproBNP, but their metabolism differs. BNP has a shorter half-life and its levels are affected less by chronic kidney disease than the ones of NTproBNP [41]. However, despite being clinically applied if NTproBNP is unavailable, it has not been formally studied in SCD. BNP values may not accurately

represent heart filling pressures if a patient is treated with sacubitril, an inhibitor of the BNP-cleaving enzyme neprilysin. In this study, none of the patients included was under sacubitril treatment; thus, BNP values can be considered an accurate indicator of heart filling pressures.

Without a doubt, the establishment of SOD2 expression as a clinically relevant biomarker requires further research and validation, especially in conjunction with RHC measurements in order to verify PH diagnosis and elucidate the possible relation between RHC-determined PH severity and SOD2 expression. Prognostic information may come from the prospective follow-up of patients with various SOD2 expression levels and the rate of future events (hospitalizations, mortality). Nonetheless, a significant connection with two established predictors of cardiovascular mortality, namely high TRV and BNP, association with anemia (red blood cell count), inflammation (hs-CRP) and iron status (ferritin) and physiologic relevance, may render SOD2 mRNA expression a promising potential biomarker.

Major limitations of this study include the relatively small study population and the absence of RHC data regarding patient hemodynamic parameters. Moreover, the absence of previous studies with BNP (not NTproBNP) in SCD PH (as opposed to other forms of PH) and the inability to accurately convert BNP to NTproBNP values and vice-versa makes comparisons with previously published studies difficult. Future studies might address these limitations and also determine SOD2 expression dynamics under acute complications (i.e., vasoocclusive crises, acute chest syndrome) or after the initiation of specific treatment regiments, i.e., iron chelation or hydroxyurea.

Conclusion

SOD2 expression is paradoxically downregulated on the mRNA level in peripheral blood leukocytes from SCD patients; the degree of downregulation correlates with laboratory indices of anemia, hemolysis, inflammation, iron overload, and cardiovascular stress. Patients with echocardiographic indications of pulmonary hypertension show significantly lower SOD2 expression compared with other patients. Peripheral blood SOD2 expression may represent a promising biomarker of pulmonary hypertension in SCD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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