

N-acetylcysteine reduces oxidative stress in sickle cell patients

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Abstract Oxidative stress is of importance in the pathophysiology of sickle cell disease (SCD). In this open label randomized pilot study the effects of oral *N*-acetylcysteine (NAC) on phosphatidylserine (PS) expression as marker of cellular oxidative damage (primary end point), and markers of hemolysis, coagulation and endothelial activation and

NAC tolerability (secondary end points) were studied. Eleven consecutive patients (ten homozygous [HbSS] sickle cell patients, one HbS β^0 -thalassemia patient) were randomly assigned to treatment with either 1,200 or 2,400 mg NAC daily during 6 weeks. The data indicate an increment in whole blood glutathione levels and a decrease in erythrocyte

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outer membrane phosphatidylserine exposure, plasma levels of advanced glycation end-products (AGEs) and cell-free hemoglobin after 6 weeks of NAC treatment in both dose groups. One patient did not tolerate the 2,400 mg dose and continued with the 1,200 mg dose. During the study period, none of the patients experienced painful crises or other significant SCD or NAC related complications. These data indicate that *N*-acetylcysteine treatment of sickle cell patients may reduce SCD related oxidative stress.

Keywords Cell-free hemoglobin · *N*-acetylcysteine · Oxidative stress · Phosphatidylserine · Sickle cell disease

Introduction

Oxidative stress plays a role of major importance in the development of organ damage in sickle cell disease (SCD) [1–4]. Oxidative stress in SCD results from factors such as the unstable auto-oxidative sickle hemoglobin (HbS) [5, 6], chronic intravascular hemolysis [7–9], recurrent ischemia reperfusion injury [4], and low grade inflammation [10]. Increased levels of reactive oxygen species (ROS) lead to further acceleration of hemolysis [8], hypercoagulability [11, 12], decreased nitric oxide (NO) bio-availability [13], and endothelial damage [14].

Given the fact that oxidative stress is a likely major contributing factor in the development of both acute and chronic complications in SCD, the potential of anti-oxidants as therapeutics for SCD should be explored. A major intracellular anti-oxidant is the reduced form of the amino-thiol glutathione (GSH) [15, 16]. Due to increased consumption by excessive levels of ROS, sickle cell patients have decreased levels of plasma and erythrocyte total glutathione and the ratio of GSH to its oxidized form glutathione disulfide (GSSG) is reduced [17, 18]. *N*-acetylcysteine (NAC), the rate limiting substrate for GSH generation, is an important antioxidant with pleiotropic effects on inflammation and vasomotor function [19]. NAC readily enters cells and within the cytoplasm it is converted to L-cysteine, which is a precursor to GSH [20]. Treatment of sickle cell patients with NAC has been demonstrated to have an inhibitory effect on the formation of dense red cells [21]. Augmenting the antioxidant capacity in sickle red blood cells by NAC may reduce oxidative red cell membrane damage and reduce its many down-stream pathophysiological effects, such as hemolysis, endothelial damage, and activation, activation of the coagulation cascade, and the decrease of NO bio-availability [13, 22, 23]. In this randomized open label pilot study, the effects of oral NAC treatment on markers of oxidative stress and hemolysis in sickle cell patients were investigated.

Patients and methods

Study population

Consecutive adult (age ≥ 18 years) homozygous sickle cell anemia (HBSS) or HbS β^0 -thalassemia outpatients (high performance liquid chromatography (HPLC) confirmed), at the Academic Medical Center (AMC), Amsterdam, The Netherlands, were eligible for the study. Exclusion criteria were painful crisis and blood transfusion in the preceding 4 weeks and 4 months respectively, pregnancy, or the desire to get pregnant in the following 3 months, calculated glomerular filtration rate of < 90 ml/min (Cockcroft and Gault formula), known gastric/duodenal ulcers, active infections, auto-immune inflammatory diseases and use of hydroxyurea, vitamin K antagonists or other oral anticoagulants and contraindications for NAC use. Ten race and age-matched healthy volunteers were included as controls for base-line values. All participants received verbal and written explanation of the objectives and procedure of the study and subsequently provided written informed consent. The study was approved by the AMC Medical Ethical Commission and experiments were performed in accordance with the Declaration of Helsinki. The study was registered in the Dutch Trial Registry (www.trialregister.nl; trial ID number NTR1013).

End point

The primary end point of the study was reduction of erythrocyte phosphatidylserine (PS) expression as a direct indicator of erythrocyte membrane (oxidative) damage. Changes in markers of hemolysis (hemoglobin, reticulocytes, lactate dehydrogenase (LDH), and bilirubin) hypercoagulability, endothelial activation, and inflammation, and tolerability of oral NAC were secondary end points.

Study protocol

After baseline measurements and randomization to either 1,200 or 2,400 mg of NAC per day, patients started taking NAC (acetylcysteine 600 mg tablets dissolved in water; Pharmachemie B.V. Haarlem, The Netherlands) orally twice daily during 6 weeks followed by another 6 weeks of follow-up after NAC cessation. Both during NAC treatment (visits 0–3) and in the post-treatment period (visits 4–6) patients were seen two-weekly for follow-up visits during which questionnaires pertaining to side effects were completed, weight, blood pressure, and pulse were measured and a blood sample was drawn via venipuncture. Patients kept a daily pain score diary (visual analogue scale pain score). Patient compliance was monitored by history taking and pill counts. If possible, NAC treatment would not be

discontinued in case of an (hospital admission due to) acute vaso-occlusive pain crisis or other clinical event.

Blood samples

Standard blood counts were performed in EDTA anti-coagulated blood (Cell-Dyn 4000; Abbott, Illinois, USA). LDH and total and direct bilirubin levels were measured in heparinized plasma with spectrophotometry (P800 Modular; Roche, Basel, Switzerland). Citrate, serum, and EDTA samples were centrifuged immediately for 15 min at 3,000 rpm (4°C). Aliquots were stored at –80°C until further analysis. L-arginine (immediate precursor of NO), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) levels were measured in EDTA plasma using reversed-phase HPLC as described elsewhere [24, 25]. NT-proBNP levels were measured in EDTA plasma with electrochemiluminescence immunoassay (Roche Diagnostics). Ultra-sensitive C-reactive protein (US-CRP) was measured with ELISA according to the manufacturer's instructions (Biokit, Barcelona, Spain). Prothrombin fragment 1+2 (F1+2) and thrombin–antithrombin (TAT) complexes were determined using sandwich enzyme-linked immunosorbent assay (ELISA; Enzygnost, Dade Behring Marburg GmbH, Germany). Plasma levels of von Willebrand Factor antigen (vWF-ag) were determined in citrate plasma with in an in-house ELISA. Soluble vascular adhesion molecule-1 (sVCAM-1) levels were determined in serum (R&D Systems; Minneapolis, MN, USA).

Glutathione levels

Concentrations of GSH and GSSG were determined according to Tietze et al. and Sacchetta et al., respectively [26, 27]. After measurement in EDTA full blood, the glutathione values were adjusted for erythrocyte numbers and mean corpuscular volume (MCV). GSH is the difference between total glutathione and GSSG concentrations.

Phosphatidylserine exposure

For measurement of PS expression, first 5 µl blood (EDTA) was washed in a HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 2.1 mM MgCl₂, pH 7.4), centrifuged at 3,650 rpm for 90 s and after adding 5 µl annexin V antibodies (Annexin V-FITC; IQP-120F, IQ products) incubated for 10–15 min at 4°C in the dark. After the incubation period, the erythrocytes were washed, resuspended in 500 µl HEPES buffer, and erythrocyte PS expression was measured by flow cytometry using FACSCalibur (BD Biosciences, CA, USA). Assuming that especially mature old sickle erythrocytes have higher levels of external PS expression leading to their removal, we also analyzed

the percentage of sickle erythrocytes having external PS expression more than one log greater than the PS negative erythrocytes.

Cell-free hemoglobin

Plasma levels of cell-free hemoglobin were determined in citrate plasma with a spectrophotometer (Shimadzu UV-2401 PC) according to the methods of Kahn et al. [28] which adjusts for hyperbilirubinemia and lipemia.

Advanced glycation end products

Two different advanced glycation end products (AGEs) were measured (i.e., pentosidine and N^ε-(carboxy-methyl)lysine (CML)) at baseline and after the 6 weeks of NAC treatment. Pentosidine and CML were measured in EDTA plasma using single-column HPLC with fluorescence detection and ultra performance liquid chromatography–tandem mass spectrometry respectively, as previously described [29, 30].

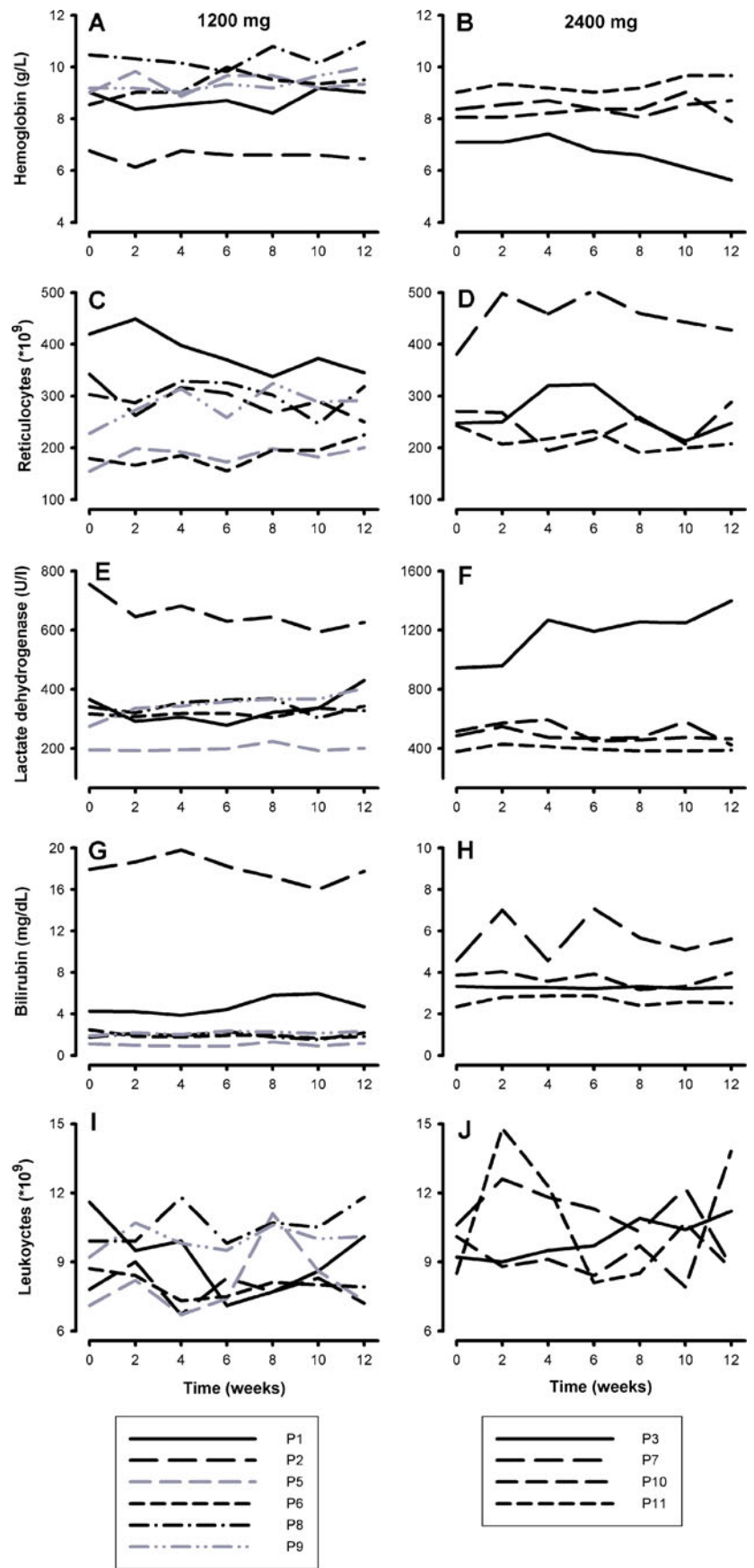
Statistical analysis

Friedman test for repeated measures of non-parametric data was used for comparisons between different time points within a treatment groups. The Wilcoxon signed rank test was used for comparisons between two related samples within a treatment group. The Mann–Whitney *U* test was used for comparisons between two groups. Continuous data are presented as medians with corresponding inter quartile ranges (IQR), unless stated otherwise. *P*<0.05 was considered statistically significant (SPSS 16.0, Chicago, IL, USA).

Results

Eleven patients (10 HbSS and 1 HbS-β⁰-thalassemia; median age 23 years (range 20–47), 6 male, 5 female) who met eligibility criteria, were included in the study. One patient (P4) discontinued using NAC after 3 weeks and withdrew from the study. Two patients who used <80% of prescribed dosing are shown in gray in the figures. One patient on the 2,400 mg NAC dose had gastro-intestinal complaints that disappeared after switching to 1,200 mg on the second day of treatment which she continued using (P6). No other patient reported adverse events. Levels of hemoglobin, LDH, and bilirubin and reticulocyte and leukocyte counts did not change significantly (Fig. 1). Sickle cell patients had significantly lower whole blood total glutathione and GSH levels as compared to healthy controls (Fig. 2a). Total glutathione levels increased during treatment period in patients of both 1,200 mg (from 136 (100–198) to 169 (121–221) µmol/l) and 2,400 mg (from 150 (136–165) to 163 (142–

Fig. 1 Markers of hemolysis during the 12 weeks of study period. Panels **a** and **b** hemoglobin, panels **c** and **d** reticulocytes, panels **e** and **f** lactate dehydrogenase, panels **g** and **h** bilirubin, panels **i** and **j** leukocytes. Panels on the *left*: 1,200 mg and panels on the *right*, 2,400 mg. Patients are numbered in the order of inclusion. Two patients with compliance of <80% are shown in *gray*. Patient number 4 (P4) discontinued using NAC and withdrew from the study



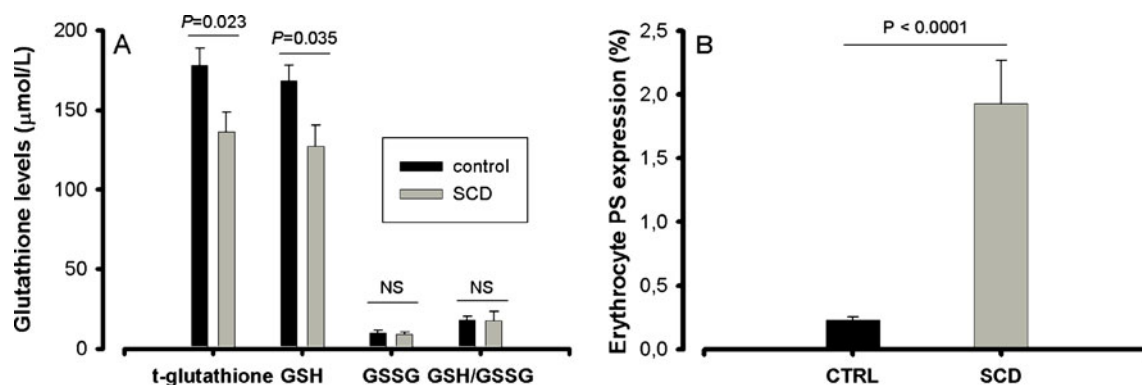


Fig. 2 Glutathione levels and erythrocyte phosphatidylserine (PS) expression in sickle cell patients (SCD) as compared to healthy controls (CTRL). **a** Levels of total glutathione (t-glutathione) and the reduced glutathione (GSH) are significantly lower in sickle cell patients (gray bars) than in race-matched healthy controls (black bars).

The oxidized disulfide form of glutathione (GSSG) and GSH/GSSG ratios were comparable between patients and controls. **b** Sickle erythrocytes (SCD, gray bar) have a significantly higher outer membrane PS expression as compared to erythrocytes of race matched healthy controls (CTRL, black bar)

178) µmol/l) dose groups (Fig. 3, panels a and b), with the differences being statistically significant when analyzing the two dose groups combined (from 150 (113–168) to 167 (142–179) µmol/l, $P<0.05$).

Red blood cell PS expression was significantly higher in sickle cell patients as compared to healthy controls (Fig. 2b) and decreased after 6 weeks of NAC treatment in patients of both 1,200 mg (from 1.64 (1.35–2.89)% to 1.46 (0.76–2.60)%) and 2,400 mg (from 2.44 (1.12–3.53)% to 1.42 (1.00–2.97)%) NAC groups (Fig. 3, panels c and d) reaching statistical significance when analyzing both dose groups together (from 1.64 (1.32–3.37)% to 1.43 (1.00–2.6)%), $P=0.036$). Also, the percentage of sickle erythrocytes with very high external PS expression (more than one log greater than the PS negative erythrocytes) decreased during the treatment and returned towards baseline values after cessation of NAC treatment (Fig. 3, panels e and f). The decrease was statistically significant in the 2,400 mg group ($P=0.039$) and when analyzing both dose groups together ($P=0.030$).

Plasma levels of cell-free hemoglobin decreased after NAC treatment and returned towards baseline levels after cessation of the treatment in patients of both 1,200 (from 5.0 (1.4–16.7) to 2.7 (2.1–8.5) µmol/l) and 2,400 mg (from 6.5 (4.5–10.4) µmol/l to 6.5 (3.4–7.8) µmol/l) groups, though the differences were not statistically significant (Fig. 3, panels g and h). Both pentosidine and CML were significantly higher in sickle cell patients at baseline as compared to healthy controls, and CML decreased significantly after 6 weeks of treatment when analyzing the two dose groups together (Fig. 4). None of the other measured parameters changed during the treatment period (Table 1).

During the treatment period, none of the study patients was admitted with SCD related complications. The daily pain score did not change during treatment (data not shown).

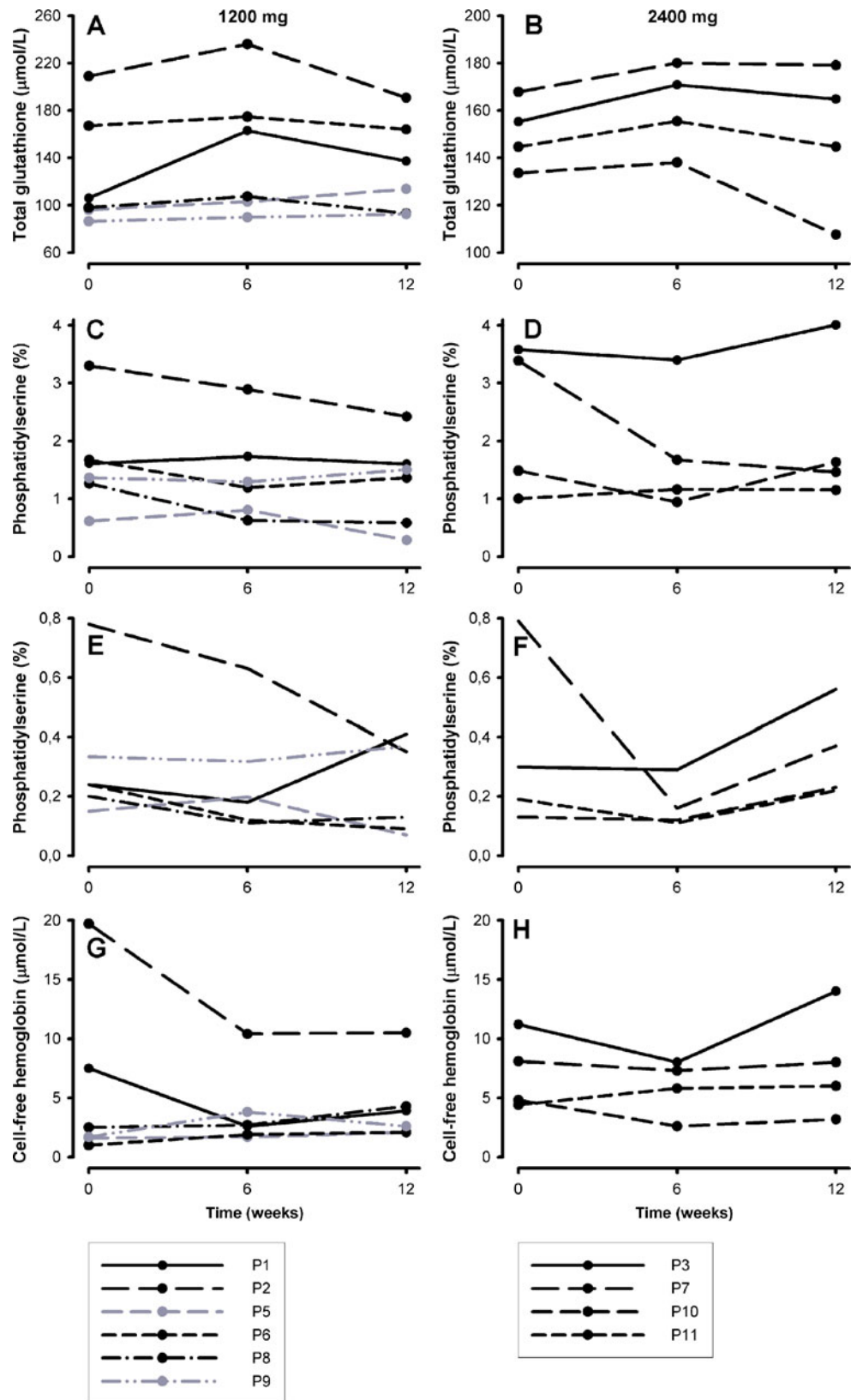
Discussion

In this pilot study, 6 weeks of NAC treatment seemed to reduce oxidative stress in SCD, as reflected by reduced red cell membrane PS expression. Also, plasma levels of AGEs and cell-free hemoglobin seemed to decrease during NAC treatment. None of the other primary end points changed during the treatment period.

Through enzymatic reactions PS is normally restricted to the inner monolayer of the cell membrane [31]. Increased intracellular generation of ROS leads to (per-)oxidative damage to the erythrocyte inner membrane and proteins responsible for maintaining normal PS asymmetry, resulting in abnormal PS externalization [32]. NAC treatment seemed to result in a decrease of surface membrane PS expression with both 1,200 and 2,400 mg dose groups. With a reduction in red cell membrane damage, hemolysis would be expected to be reduced. While the standard markers of hemolysis did not change, there was a decrease in plasma levels of cell-free hemoglobin, though the differences were not statistically significant [28]. Downstream events of pathophysiological PS exposure such as thrombin generation and increased expression of sVCAM-1 did not change [33, 34]. Given large variability of such parameters, we cannot rule out that a potential ameliorating effect of NAC supplementation on endothelial and coagulation activation has been missed due to the small number of included patients.

Increased production and tissue accumulation of AGEs due to oxidative stress are associated with disease severity and organ complications in diabetes and inflammatory diseases [35–37]. AGE interaction with intra- and extracellular tissue structures leads to distortion of normal tissue architecture [23], resulting in (micro-)vasculature basement membrane thickening with reduced vascular wall elasticity, reduced filtration rate across the vessel lumen and diminished arteriolar

Fig. 3 Total glutathione levels, erythrocyte phosphatidylserine (PS) expression, and cell-free hemoglobin levels at baseline, after 6 weeks of NAC treatment and 6 weeks after cessation of NAC. Panels **a** and **b** total glutathione, panels **c** and **d** erythrocyte PS expression, panels **e** and **f** erythrocytes with very positive external PS expression (>1 log greater than the PS negative erythrocytes) and panels **g** and **h** cell-free hemoglobin. Panels on the *left*, 1,200 mg NAC; panels on the *right*, 2,400 mg



vasodilatory response all contributing to tissue ischemia [37, 38]. Furthermore, the interaction of AGEs with their receptor (RAGE) enhances the production of pro-inflammatory

cytokines, adhesion molecules, and more oxidants, inducing a pro-inflammatory response and further increasing oxidative stress [35]. Steady state plasma AGEs are increased in both

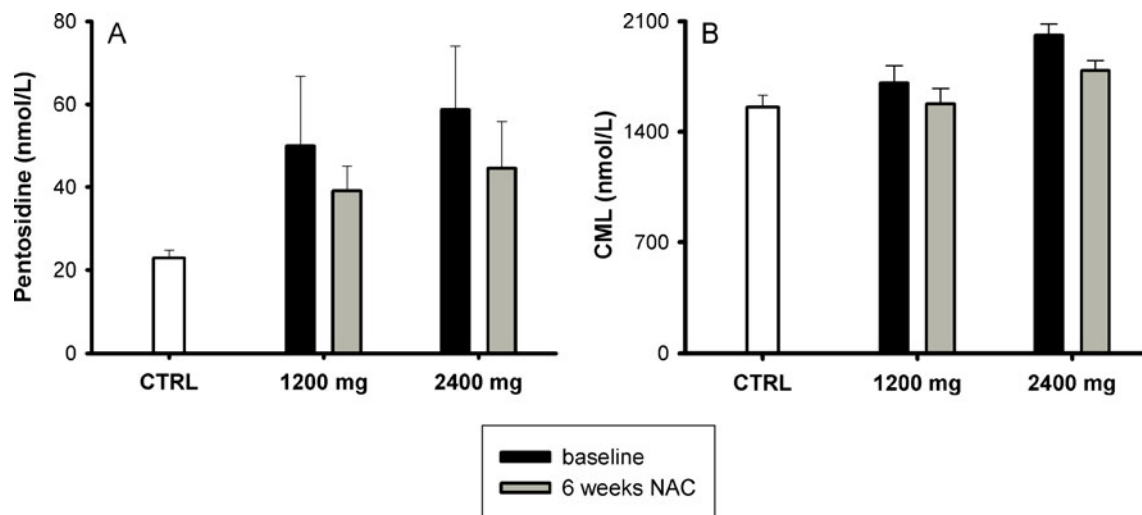


Fig. 4 Plasma levels of AGEs (pentosidine and N^{ϵ} -(carboxy-methyl) lysine (CML)) in controls (CTRL; white bar) and sickle cell patients (SCD) at baseline (black bar) and after 6 weeks (gray bar) *N*-acetylcysteine (NAC) treatment. **a** Baseline pentosidine levels were higher in sickle cell patients than in controls ($P < 0.0001$). Pentosidine decreased

after 6 weeks NAC treatment in both 1,200 and 2,400 mg groups, though the differences were not statistically significant. **b** CML levels at baseline were also higher in sickle cell patients than in controls ($P = 0.019$) and decreased after 6 weeks NAC treatment in both groups. Means \pm SEM

children and adults with SCD, and we have recently demonstrated AGEs to be significantly associated with both the degree of hemolysis and the presence of hemolysis-related organ complications in adult sickle cell patients [18]. Somjee et al. [18] reported a strong inverse correlation between plasma AGEs and GSH levels in sickle erythrocytes. Decreased levels of GSH may play an important role in the increased formation of AGEs since GSH is an essential cofactor for glyoxalase 1, the enzyme that detoxifies the major AGE precursor methylglyoxal to *S*- γ -lactoyl-glutathione [39]. The decrease in AGE levels during NAC treatment could suggest that production and tissue accumulation of AGEs and thus

oxidative tissue damage can be reduced by enhancing GSH production with NAC treatment.

In contrast to the study by Pace et al. [21] in which glutathione increments were only observed in patients treated with 2,400 mg NAC during 6 weeks, increases in glutathione levels in this study were comparable in both 1,200 and 2,400 mg dose groups. A possible explanation for this discrepancy could be of methodological nature. The comparable glutathione increments with concurrent reductions in erythrocyte PS expression and plasma AGEs in both dose groups may suggest that higher dose than 1,200 mg NAC per day may not be needed. This is an important finding as

Table 1 Markers of endothelial and coagulation activation and NO bioavailability before and after NAC treatment

	1,200 mg ($n=6$)		2,400 mg ($n=4$)	
	Baseline	6 weeks	Baseline	6 weeks
sVCAM-1 (ng/mL)	1,151 (880–1,638)	1,110 (859–1,841)	788 (638–1,578)	905 (778–1,516)
vWF:Ag (%)	181 (152–377)	208 (145–374)	162 (129–217)	162 (130–234)
TAT (μ g/L)	8.2 (4.4–14.1)	5.9 (5.4–12.6)	10.2 (7.8–10.7)	8.4 (6.3–11.2)
F1+2 (pmol/L)	268 (201–341)	269 (174–329)	302 (179–366)	254 (170–360)
Thrombin generation				
Lag time (min)	2.0 (1.8–2.3)	2.0 (1.8–2.0)	1.0 (1.0–1.8)	1.0 (1.0–1.8)
Peak value (nM)	310 (233–375)	343 (282–363)	343 (271–367)	311 (161–358)
ETP (nM*min)	1,136 (844–1,507)	1,225 (1,009–1,315)	1,156 (913–1,287)	1,102 (520–1,313)
Arginine (μ mol/L)	52.6 (45.0–67.9)	57.5 (46.7–73.3)	61.2 (57.6–71.4)	63.5 (60.6–78.0)
ADMA (μ mol/L)	0.62 (0.52–0.70)	0.67 (0.49–0.73)	0.57 (0.51–0.64)	0.61 (0.55–0.64)
NT-proBNP	63.0 (25.5–125)	51.8 (33.9–84.7)	28.3 (24.3–46.9)	33.5 (13.3–64.1)

Numbers are medians with inter quartile ranges. *sVCAM-1* soluble vascular cellular adhesion molecule-1; *vWF-Ag* von Willebrand factor antigen; *TAT* thrombin antithrombin complex; *F1+2* prothrombin fragments 1+2; *ADMA* asymmetric dimethylarginine; *NT-proBNP* N-terminal pro-Brain Natriuretic Peptide. Changes not significant

gastro-intestinal side effects may be dose-limiting (as observed in one patient in the current study).

The obvious limitation of our study is the small number of patients in a pilot study lacking a control (placebo) group as well as the short duration of treatment. Nonetheless, the presented data seem to indicate gradual decrease in red cell surface PS expression, plasma levels of AGEs and cell-free hemoglobin with subsequent increments after NAC cessation. Obviously, these findings need confirmation in larger randomized placebo-controlled clinical trials. One of the 11 patients had HbS- β^0 -thalassemia. The excess of alpha-globin might influence the degree of oxidative stress in this patient. However, both sickle cell and beta-thalassaemia erythrocytes are associated with oxidative stress [40, 41]. Furthermore, disease severity in patients with HbS- β^0 -thalassemia is comparable with those in HbSS [42–44]. *N*-acetylcysteine amide, an amide form of *N*-acetylcysteine has been shown to reduce oxidative stress in beta thalassaemia red blood cells [45]. Exclusion of this patient does not alter the results (data not shown).

In conclusion, *N*-acetylcysteine treatment of sickle cell patients seems to reduce erythrocyte PS expression, plasma AGEs, and cell-free hemoglobin levels. Given the wide availability, safety and low cost of NAC and the findings in this pilot study, we are of the opinion that potential of NAC as a “supportive care therapeutic” in SCD deserves further study.

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