Association between Oxidative Stress, Genetic Factors, and Clinical Severity in Children with Sickle Cell Anemia

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Objectives To investigate the associations between several sickle cell disease genetic modifiers (beta-globin haplotypes, alpha-thalassemia, and glucose-6-phosphate dehydrogenase deficiency) and the level of oxidative stress and to evaluate the association between oxidative stress and the rates of vaso-occlusive events.

Study design Steady-state oxidative and nitrosative stress markers, biological variables, genetic modulators, and vaso-occlusive crisis events requiring emergency admissions were measured during a 2-year period in 62 children with sickle cell anemia (58 SS and 4 Sβ^0). Twelve ethnic-matched children without sickle cell anemia also participated as healthy controls (AA) for oxidative and nitrosative stress level measurement.

Results Oxidative and nitrosative stress were greater in patients with sickle cell anemia compared with control patients, but the rate of vaso-occlusive crisis events in sickle cell anemia was not associated with the level of oxidative stress. The presence of alpha-thalassemia, but not glucose-6-phosphate dehydrogenase deficiency or beta-globin haplotype, modulated the level of oxidative stress in children with sickle cell anemia.

Conclusion Mild hemolysis in children with alpha-thalassemia may limit oxidative stress and could explain the protective role of alpha-thalassemia in hemolysis-related sickle cell complications. *(J Pediatr 2017;■■:■■-■■).*

Sickle cell anemia (SCA) is a severe monogenic hemoglobinopathy characterized by the synthesis of an abnormal hemoglobin (sickle hemoglobin [HbS]).[1] When deoxygenated, HbS polymerizes and causes the sickling of red blood cells (RBCs). Sickle RBCs are more fragile and rigid than normal RBCs, leading to chronic hemolytic anemia and frequent vaso-occlusive crises, respectively. The clinical expression of SCA is heterogeneous and influenced by several genetic factors, such as the concomitant presence of alpha-thalassemia,[2-9] β^S-haplotypes,[10,11] fetal hemoglobin levels,[11-14] and glucose-6-phosphate dehydrogenase (G6PD) deficiency.[3,15]

The high rate of HbS auto-oxidation, repetition of ischemic–reperfusion injuries, and chronic hemolysis with release of heme and free iron into the plasma are major sources of reactive oxygen species (ROS) production.[16,17] Indeed, recent studies have demonstrated enhanced oxidative stress in children with sickle cell disease compared with healthy individuals.[18] It is hypothesized that chronic oxidative stress could play an important role in the pathophysiology of SCA.[19-21] However, the exact clinical impact of oxidative stress remains unclear as well as its relationships with the major genetic modifiers of SCA (ie, alpha-thalassemia, β^S-haplotypes, and G6PD deficiency). The main goal of this study was to investigate the possible associations between several known genetic modulators of clinical status in SCA, oxidative stress markers, and clinical severity in a pediatric cohort regularly followed at the academic medical center of Lyon.

AOPP	Plasma advanced oxidation protein products
Ben/Ben	Benin/Benin
BILI	Total bilirubin
CAR	Central Africa Republic
FRAP	Plasma ferric–reducing antioxidant power
G6PD	Glucose-6-phosphate dehydrogenase
HbS	Sickle hemoglobin
HI	Hemolytic index
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
NO	Nitric oxide
NOx	Sum of nitrite and nitrate (NOx = NO2 + NO3)
RBC	Red blood cell
RET	Reticulocytes
ROS	Reactive oxygen species
SCA	Sickle cell anemia
SOD	Superoxide dismutase

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Methods

Children with SCA (58 SS and 4 Sβ^0) aged from 5 to 18 years (median: 10.5 years) and regularly followed for at least 2 years (median follow-up: 7.4 years) at the Institute of Pediatric Hematology and Oncology (Lyon, France) were included between January 2013 and November 2016. All subjects were in steady-state conditions, ie, no blood transfusion in the previous 3 months and the absence of any acute episodes (infection, vaso-occlusive crisis, acute chest syndrome, stroke, or priapism) for at least 1 month.[22] Twelve ethnic-matched children without sickle cell disease also participated as healthy controls (AA) for the determination of oxidative and nitrosative stress levels. All children and their parents were informed about the purpose and procedures of the study, which was approved by the "Hospices Civils de Lyon — CPP Est" (ethics committee number: L14-127). To diagnose SCA, 3 complementary biochemical tests were performed according to expert recommendations[23] and genetically confirmed thereafter.

Alpha-Thalassemia, β^S-Haplotypes, and G6PD Status

Multiplex gap-polymerase chain reaction was used to detect the 5 common alpha-thalassemia deletions (−3.7 kB, −4.2 kB, −20.5 kB, SEA, MED).[24] The β^S-haplotypes were characterized by fluorescence resonance energy transfer or high-resolution melting methods.[25] The 3 main G6PD-deficient variants in Africa, ie, A(−), Betica, and Med, were screened by dedicated high-resolution melting methods[26,27] to assess the G6PD status.

Hematologic Measures and RBC Deformability

Total bilirubin (BILI), lactate dehydrogenase (LDH), and aspartate aminotransferase were measured via the use of standard biochemistry methods. Hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, RBCs, platelets, white blood cells, neutrophils, and reticulocyte (RET) counts were determined with a hematology analyzer (ADVIA; Siemens, Rungis, France). RBC deformability, reported as an elongation index, was determined at 37°C and 30 Pa by ektacytometry (LORRCA MaxSis; RR Mechatronics, Hoorn, The Netherlands) via the recent methodologic standardization guidelines for patients with sickle cell disease.[28,29]

Oxidative and Nitrosative Stress Markers

After centrifugation of the EDTA tube, plasma was collected and stored at −80°C until analysis. Plasma advanced oxidation protein products (AOPPs) were measured according to the semiautomated method developed by Witko-Sarsat et al.[30] Malondialdehyde (MDA) plasma concentrations were determined as previously described[31,32] based on thiobarbituric acid reactions. The superoxide dismutase (SOD) activity was determined via the method described by Oberley and Spitz.[33] Plasma catalase activity was measured by the method of Johansson and Borg.[34] Plasma glutathione peroxidase activity was determined by the modified method of Paglia and Valentine.[35] Plasma ferric-reducing antioxidant power (FRAP) concentrations were measured as previously described[31] based on the ferric-reducing ability of plasma.[36] Nitrotyrosine plasma concentrations, an end product of protein nitration by $ONOO^-$, were measured by enzyme-linked immunosorbent assay.[37] Nitric oxide (NO) metabolites were assessed as the sum of nitrite and nitrate ($NOx = NO_2 + NO_3$) concentrations.[38] After nitrate reduction by nitrate reductase, the fluorimetric quantification of NOx was based on the reaction of nitrite with 2,3-diaminonaphthalene.

Clinical Data

Clinical reports of subjects were reviewed retrospectively by 2 physicians to collect a history of hospitalized vaso-occlusive crisis and acute chest syndrome events since the beginning of their regular follow-up. An acute painful episode was considered as a vaso-occlusive crisis if it lasted for more than 4 hours, the patient felt that the pain was typical for vaso-occlusion, no other etiology could be identified by the physicians, and the patient was admitted to the pediatric emergency department to treat the pain with parenteral opioids.[7] This working definition excluded hand–foot syndrome, acute chest syndrome, and osteomyelitis. Acute chest syndrome was defined as previously described.[7] The annual vaso-occlusive crisis and acute chest syndrome rates during the previous 2 years were calculated for each child.

Statistical Analyses

Results are presented as median with [25th; 75th] percentiles. To evaluate the influence of the different genetic modifiers investigated, each was divided into 3 subgroups: (1) alpha-thalassemia: no alpha-thalassemia ($\alpha\alpha/\alpha\alpha$), heterozygous alpha-thalassemia ($\alpha-/\alpha\alpha$), and homozygous alpha-thalassemia ($\alpha-/\alpha-$); (2) β^S-haplotypes: Benin/Benin (Ben/Ben), Central Africa Republic (CAR/x, where x is any haplotype), and Senegal (Senegal/n, where n is any haplotype except CAR)[39]; and (3) G6PD status: normal (norm), heterozygous deficiency (hetero), and homo- or hemizygous deficiency (homo-hemi). To evaluate the clinical severity, patients were classified into 2 subgroups: (1) vaso-occlusive crisis/acute chest syndrome (+): vaso-occlusive crisis rate ≥2/year or acute chest syndrome rate ≥0.5/year over the last 2 years and (2) vaso-occlusive crisis/acute chest syndrome (−): vaso-occlusive crisis rate <2/year, no acute chest syndrome during the last 2 years.

A multivariate linear regression model adjusted for alpha-thalassemia (presence vs absence) and hydroxyurea treatment was used to evaluate the association between RBC deformability and plasma AOPP. Hemolytic measures (ie, RET, BILI, aspartate aminotransferase, and LDH) were combined into 1 variable (hemolytic index [HI]) by principal component analysis, as previously done.[40,41] Nonparametric correlation was performed to test whether hemolysis (measured by HI) and oxidative stress (measured by plasma AOPP level) were associated. The χ^2 test was used to compare categorized variables between the different groups. The significance level was defined as $P < .05$. Analyses were conducted with SPSS (SPSS Statistics, version 22; IBM Corp, Armonk, New York).

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