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Study of the Variation of Neuroglobinemia and Cytoglobinemia in Homozygous Sickle Cell Subjects

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Abstract: ***Introduction: The data on the variation of neuroglobinemia (CmNgb) and cytoglobinemia (CmCygb) in sickle cell disease (SCD) are unknown. Objective: To determine in the sickle cell subject, the variations of CmNgb and CmCygb, and to study the relationships between these variations and the pathophysiological factors of sickle cell disease. Population and methods: The study involved 30 inter critical sickle cell patients ($HbSS^-$), 20 in crisis ($HbSS^+$) and 36 controls (HbAA). The CmNgb and CmCygb (ng/ml), as well as markers of hypoxia, inflammation, hemolysis and ischemia of the nervous system (NS) were determined. The comparison of variables between different groups, as well as their relationships was analysed ($p < 0.05$). Results: The CmNgb was 8.1 ± 2.3 ng/ml among $HbSS^-$, 6.2 ± 4 ng/ml among $HbSS^+$ versus 5.8 ± 2.2 ng/ml among HbAA ($p = 0.005$). The CmCygb was 1721.8 ± 1971.1 ng/ml among $HbSS^-$, 915.5 ± 835.2 ng/ml among $HbSS^+$ versus 1322.1 ± 986.9 ng/ml among HbAA ($p = 0.081$). Among sickle cell patients, there was a relationship between CmNgb, markers of ischemia of the NS, and hematocrit in the one hand, CmCygb, markers of ischemia of the NS and leukocytes in the other hand. There was no relationship between these two concentrations and markers of hypoxia. Conclusion: The variations in CmNgb and CmCygb observed in SCD suggest a cytoprotective role of these globins.***

Keywords: Neuroglobin – Cytoglobin – Sickle Cell Disease -Pathophysiology

1. Introduction

In humans, actually, four types of globins have been identified **[1, 2, 3]**.These are hemoglobin (Hb), myoglobin (Mb), neuroglobin (Ngb) and cytoglobin (Cygb) **[1-7]**. In general, these globins provide oxygen to the tissues, *via* a prosthetic group of heme containing iron **[1-7]**. As well, the Hb located in red blood cells, distributes oxygen through the circulatory system, **[1, 2, 7-9]**. Mgb is found predominantly in skeletal and smooth striated muscle. It allows oxygen storage *in situ*, while facilitating the deoxygenation of mitochondria and the elimination of excess nitric oxide **[1-4, 6, 10, 11]**. The neuroglobin (Ngb) and cytoglobin (Cygb) have recently been discovered **[1-7]**. They due their names because of preferential expression under physiological conditions, in the nervous tissue for the first **[1-6, 12-20]**, and certain types of neurons and connective tissue cells for the second **[1, 4, 6, 7, 21-23]**.The exact functions of these two proteins, as well as their mechanisms of action are not fully known to date **[1-4, 6]**. However, due to increased concentration during hypoxia-ischemia **[1-7, 23-36]**, oxidative stress **[4, 6, 36-42]** and apoptosis cellular and tissular **[4, 7, 43-46]**, in certain diseases as well Sickle cell disease (SCD), most authors attribute them a cytoprotective role **[4, 6, 12, 13, 46-52]**. SCD is the most common genetic disease in sub-Saharan Africa, particularly in Gabon **[53-67]**. It is due to a mutation of the sixth codon of the first exon of the globin gene (GAG→GTG), the origin of the substitution of glutamic acid by valine. The result is a mutated hemoglobin, characterized by its ability to polymerize in the presence of several factors such as hypoxia, hemolysis, deshydration, inflammation, ischemia and oxidative stress. This ability to polymerize hemoglobin will in turn lead to deoxygenation, which, *in fine* will induce a structural modification of erythrocytes called falciformation **[53-67]**. This falciformation will promote an obstruction of the capillaries then, an endothelial dysfunction, leading to hypoxia in the organs downstream **[58-67]**. This pathological condition will result clinically in the onset of acute or chronic symptoms, particularly

episodes of painful crises, stroke acute chest syndromes (ACS) or pulmonary arterial hypertension **[53, 56-60, 62, 63, 66]**. The direct involvement of hemoglobin (Hb) and myoglobin (Mgb) in the determinism of crisis and complications of SCD *via* determining factors such as hypoxia, hemolysis, inflammation, oxidative stress and ischemia has been widely demonstrated **[8-11, 58, 59, 60, 63, 65, 67]**. Conversely, few data relate to the expression of so-called hexacoordinate globins (Ngb and Cytg) during the sickle cell crisis, even less, their relationship with the main factors involved in this crisis. This work was initiated to determine during the sickle cell crisis, the variations in plasma concentrations of Cytg and Ngb, and to study the relationship between these variations and the pathophysiological factors of SCD.

2. Population and Methods

2.1 Population

This was an observational study, cross-sectional, case-control type, conducted from December to 2017 to April 2019 at Libreville (Gabon). The recruitment of participants was multicenter. The study population was divided into three groups. The first was 20 homozygous sickle cell patients during the crisis ($HbSS^+$). The second included 30 homozygous sickle cell subjects in the basal state ($HbSS^-$) **[55, 58, 62]**. The last group, included 36 healthy people, without no mutation of the gene encoding hemoglobin S (controls, HbAA) **[58]**. This work was carried out according to the recommendations of the Helsinki Ethics Declaration on the use of living beings **[68]**.

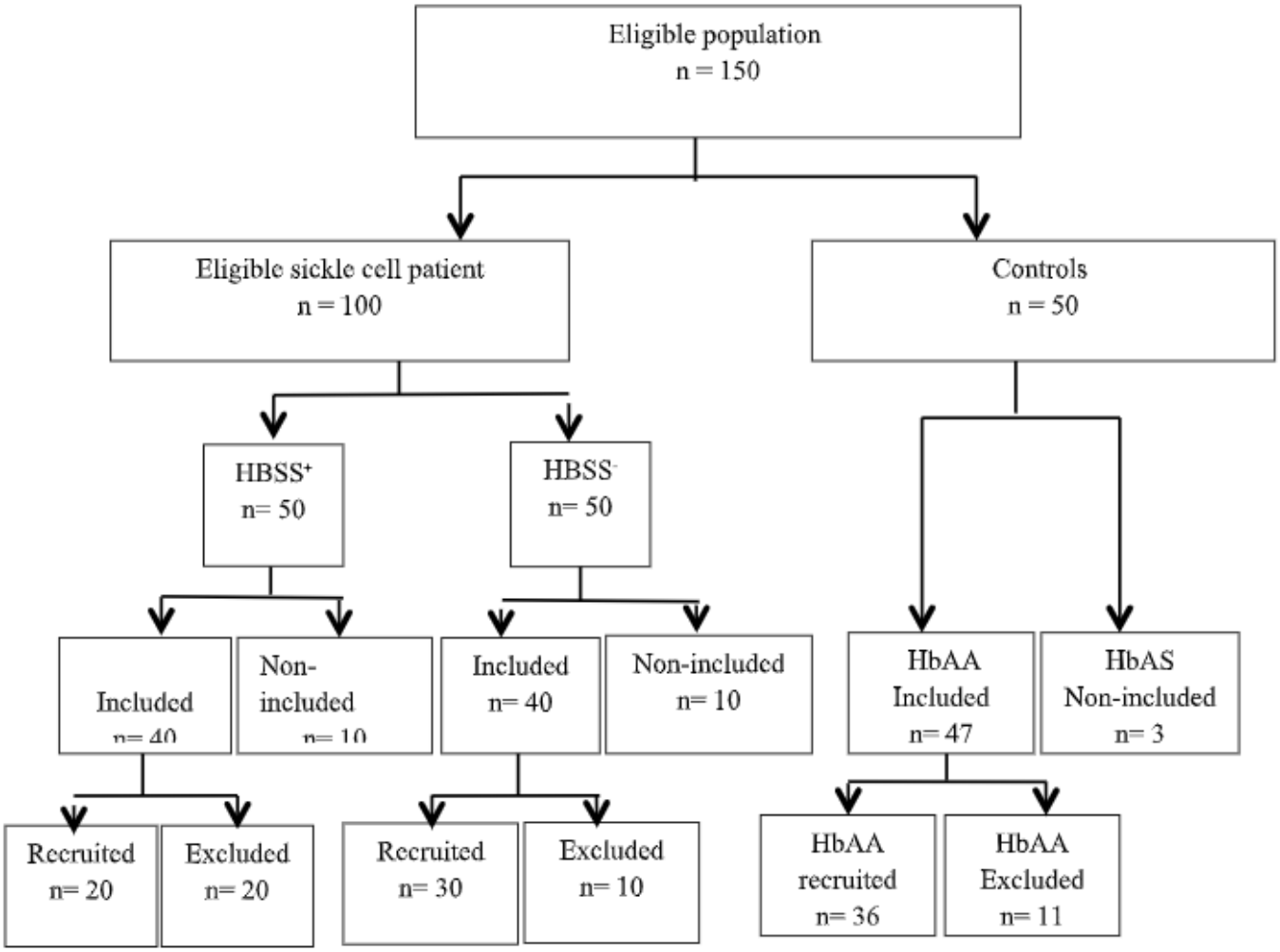
Inclusion criteria
After the ethics committee agrees, and obtains informed consent (parental consent for children), all homozygous sickle cell subjects designated HbSS, regardless of gender, between the ages of 15 and 55, had been selected (history of sickle cell disease, presence of acute or chronic clinically examined manifestations, electrophoresis of hemoglobin on agarose gel with HbSS phenotype) **[53, 58, 59, 62]**. Thereby, the homozygous sickle cell subjects being in crisis or $HbSS^+$ (history of sickle cell disease, presence of acute and/or chronic manifestations, electrophoresis of hemoglobin on agarose gel with HbSS phenotype) **[53, 58, 59, 62, 69]**, or in the basal state, designated $HbSS^-$ (history of sickle cell disease, presence of chronic manifestations on clinical examination, electrophoresis of Hb on agarose gel with HbSS phenotype) **[55, 58, 70, 71]** had been included. Similarly, healthy people, without sickle cell trait (no history of SCD, normal clinical examination, electrophoresis of hemoglobin on agarose gel with HbAA phenotype) had been recruited **[58]**.

Non-inclusion criteria
In the three months prior to this study, people who had suffered from other pathology than hemoglobinosis S, possible to induce over-expression of neuroglobin or cytoglobin had not been selected **[35, 49]**. In addition, the participants reporting a transfusion less than 3 month old, and sickle cell disease associated with other systemic pathologies had not been selected **[71]**. Otherwise, taking any therapy that may induce neuroglobin synthesis **[34]**, or the presence of retinal, auditory and/or cerebral other lesions than those of an ischemic nature, data of fundus examination and transcranial doppler ultrasound inexploitables were non-inclusion criteria (ophthalmological examination, otorhinoaryngological examination, audiometry, neurological examination, transcranial doppler, brain imaging).

2.2 Methods

Recruitment of the population
The recruitment of sickle cell patients in crisis ($HbSS^+$) took place consecutively in the hospital ward of the hematology department of the University Hospital Centrer of Libreville, Ondo Alain Foundation (FOA) and Jean Bi-Ondo Bidzo and Bethesda Polyclinics. They received a clinical examination (general examination with pain assessment **[72]**, hematological, neurological, ophthalmological and otolaryngological), and a blood sample (neuroglobin, cytoglobin, hypoxia inductible factor-1alpha, C-reactive protein, routine checkup), within 72 hours following their admission. Otherwise, during their hospitalization, functional explorations were carried out (transcranial doppler ultra sound, audiometry), and fundus examination. The patients in the basal state ($HbSS^-$) **[73]** had been selected in the consultation room of the aforementioned structures. They benefited from assessments similar to those of sickle cell patients in crisis (clinical examination, background, functional explorations, and biological assessment). The recruitment of controls (HbAA) was done in voluntary mode, among the general population. The parents of patients and their children, health care workers and medical school students were the controls. They also received a complete clinical examination, followed by functional explorations, fundus examination and a biological assessment superimposed on patients. Any participant, presenting the criteria for inclusion, as well as acute or chronic neurological manifestations of SCD whose ischemic origin was suspected (hematological examination, neurological, ophthalmological and otolaryngological examination, transcranial doppler ultra sound, fundus examination, audiometry) **[53, 58, 67, 74, 75]**, systematically benefited from brain imaging.

During the study period, 150 people with eligibility criteria were identified, among them, 50 sickles cell disease in crisis ($HbSS^+$), 50 in basal state ($HBSS^-$) period and 50 controls (HbAA) **(Figure 1)**. Of the 50 sickle cell subjects in crisis recorded ($HbSS^+$), 10 had been not included, 40 had been included, wich 20 definitely recruited, and 20 removed for refusal of blood test, inexploitable data on TDU, and unavailability. Among the 50 basal sickle cell patients ($HbSS^-$) collected at the beginning of the investigation, 10 were eliminated for refusal to take a sample and unavailability, 30 had been permanently selected. Regarding the control population (HbAA), out of the 50 selected at the start, 47 were included, and 3 were not included because of the presence of the HbAS phenotype in hemoglobin electrophoresis. Of the 47 controls included, 36 were permanently selected, and 11 were recused for unavailability reasons. At the end of the investigation, the study population consisted of 86 participants, which 20 subjects $HbSS^+$, 30 $HbSS^-$ and 36 HbAA.



Flux diagram describing the study population selection

The main variables epidemiological studied were the age of the participants (in years) and the gender. About clinical, annual numbers of VOC, VOA **[62, 69, 73]**, basal hemoglobin rate (g/l) and the notion of intermittent headaches (IH) had been identified. The presence of a crisis or not during the recruitment had allowed to subdivide the group of sickle cell subjects into two groups (HbSS $^{-}$ et HbSS^{+}) **[52, 55, 58, 59, 70, 71]**. The analog visual scale (AVS, in a decimal unit) was used to assess the intensity of the pain **[72]**, and the degree Celsius (°C) to quantify the temperature change. The clinical phenotype was categorized into two distinct groups (hyper viscous or HV, and Hemolytic or HML) **[76]**, as well as the state of conscience (normal or altered according to Glasgow coma scale) **[34]**. The other clinical variables were respiratory rate (resp/min), the pulsed oxygen saturation (SpO_2 in %), and the clinical signs of hemolysis (cutaneous pallor, jaundice, splenomegaly) **[76, 77]**. The biological data analyzed were the electrophoretic phenotype of hemoglobin (HbSS et HbAA), the plasma concentrations of neuroglobin (ng/ml), cytoglobin (ng/ml), HIF-1alpha (pg/ml), C-reactive protein (mg/dl) **[73]**, as well as the rate of hemoglobin (g/dl), white blood cells (/mm^3) and the hematocrit (%). On the level functional, the data collected was audiometric (presence of transmission deafness or not, degree of hearing loss in decibel or DHL in dB) **[78]**, transcranial doppler ultra sound (calculates the average velocity at the right average cerebral artery or TAMMV, in cm /second) **[74, 75]**), and the fundus examination (retinal ischemia present or not **[79])**. For the purposes of the investigation, certain clinical, biological and functional variables obtained from controls were considered a baseline.

Diagnostic methods and classification of the phenotype of SCD

Clinical investigation

The clinical evaluation noted in history of SCD. The physical examination looked for chronic or acute manifestations of the disease (clinical signs of hemolysis **[76, 77]**, VOC, VOA) **[53, 56, 57, 58, 62, 69, 73]**.

Electrophoresis of hemoglobin

The electrophoresis was performed manually on an electric generator (BEKMAN®, BEKMAN COULTER™, USA), using a kit (Hydragel hemoglobine k20® Sebia™, France). The technic used in this investigation was electrophoresis on agarose gel **[54, 57, 69]**. The dosing procedure took place according to the manual dosing protocol proposed by the laboratory Sebia™ **[80]**. The identification of the different hemoglobins (normal hemoglobins or HbAA, abnormal or HbSS, HbAS) was done mainly qualitatively. Electrophoresis of hemoglobin classified the population as homozygous sickle cell patient (HbSS) and control subject (HbAA).

Classification of clinical phenotype

Clinical phenotypes were defined according to Dubert and *al*, as well as Carpentiérie-Pipolo and *al* [74, 76]. Patients with repeated clinical signs of hemolysis in their medical records (Muco-cutaneous pallor, jaundice, splenomegaly), with a rate of hemoglobin less than 7g/dl, of hematocrit less than 20 %, and who would have benefited from several transfusions, corresponded to the hemolytic phenotype (HML) **[53, 57, 58, 60, 76]**. Moreover, those with repeated VOC, with hemoglobin levels ≥ 8g /dl and hematocrit greater than 30% were classified as hyper viscous phenotype (HV) **[53, 57, 58, 60, 76].**

Methods for Studying Markers of inflammation

Clinical markers

Inflammation was clinically identified by pain and fever **[65, 73, 71, 81]**.The pain intensity was assessed using the analog visual scale (AVS) **[81, 82]**. The AVS was normal for a value equal to 0 /10 **[81, 82]**. The temperature measurement was carried out by an electronic thermometer (Temp'10™ Splengler®, France). Temperature was considered normal, when it was between 36.2 and 37.7°C, beyond these values, it was fever **[71, 73, 81]**.

Blood leukocyte level

The blood leukocyte level was wanted in the blood count formula (BCF). During the present study, the BCF was carried out on automat (BC 3000°® Minbraiy™, China), according to the reference method wich is the principle by impedance **[65, 66, 69, 71]**. A white blood cell level greater than 10000/mm^3 defined leukocytosis, and was considered as a biological inflammatory criteria **[60, 65, 66, 67, 82]**.

Dosage of C reactive protein (CRP)

The dosage of C reactive (CRP) was carried out by automatic immuno turbinometric method, on Cobas C111™ (Roche®, Roche diagnostics®, France). One kit (Rohe Cobas CRP. kit™, Roche diagnostics®, France) had been necessary for this dosage. The procedure performed was that described from an earlier study **[82]**. A CRP level lower than 5mg/dl was considered as normal value **[73, 82]**.

Method of Studying Hemolysis Markers

Hemolysis was highlighted on the clinical plan by the presence of pallor muco-cutaneous, jaundice, and splenomegaly **[58, 59, 60, 65, 76, 77]**. On the biological plan, it was evaluated from the hemoglobin (Hb) and hematocrit (Ht) level obtained at the blood count formula (CBF). Hemolysis was defined for Hb and Ht levels lower than those considered usual in each patient **[58, 60, 70]**.

Dosage of Cytoglobin (Cygb)

It was realized manually by ELISA (Enzyme linked immunosorbent assay) technic, using an Elabscience® Kit (Human® Cygb ELISA kit™, N° E-EL-H2471). The procedure was carried out according to the protocol manual dosing proposed by the manufactured **[83]**. For the different incubation phases, it had been used Incucell® MMGROWNY™ device. The optical density was measured on a Biorad PR 3100 ™ reader. The concentration of cygb was obtained by comparison with a standard range treated at the same time as the samples. Plasma cytoglobin (CmCygb) was expressed in ng/ml **[83]**.

Dosage of Neuroglobin (Ngb)

The plasma dosage of Ngb was determined manually by ELISA (Enzyme linked immunosorbent assay), and using the Human NGB ELISA kit™ (N° E-EL-H1768). The procedure was performed according to a protocol established by previous studies **[30, 34, 35]**. The average plasma Ngb concentration (CmNgb) was expressed in ng/ml.

Method to Detecting Nervous System Ischemia

Cerebral ischemia

The research of cerebral ischemia began by an interrogation (notion of neurological VOA, i.e, intermittent headaches or IH, stroke), accompanied by complete neurological examination, then transcranial doppler ultrasound (TDU) (SONOACER7® SONNY™, Germany). The ultrasound procedure was carried out according to the recommendations of learned societies **[74, 75, 84-87]**. Once the temporal window in front of the tragus of right ear of the participant was identified, the probe (micro convex, high frequency) was positioned to visualize cerebral peduncles. From this window, the right middle cerebral artery was visualized (MCA), followed the recording and the calculation of different measurements (systolic velocity or SV, diastolic or DV, average or AV, average of maxima velocities or TAMMV) of this artery. A TAMMV lower than 200 cm/s was considered normal **[74, 75, 84-87]**.

Ischemia of Audtitory Nerve

The auditory nerve ischemia was investigated using pure tone audiometry (Interacoustics® AD629™, France). It is preceded by examination of ear canal (Smartled 5500® Spengler ™, France). The audiometric assessment was done according to a protocol established in earlier study **[78]**. The presence of conductive deafness in sickle cell subject defined ischemia of auditory nerve **[78, 88]**. In addition, the audiometry of patients revealing a hearing frequency value exceeding 40 decibels (dB), were considered to be abnormal **[60, 65, 67]**. Thereby, the audiometric data made it possible to classify the sickle cell population into two subgroups, according to the presence or not of auditory ischemia.

Retinal ischemia

The biomicroscope assisted eye examination (BM 900® Haag-Streit Diagnostics 900™, Germany) was bilateral. It consisted in the realization of fundus examination after maximum dilation of the pupil with tropicamide (Mydriaticum® Thea pharma™, France) and phenylephrine (Neosynephrine® Europhta ™, France). A drop of anesthetic eye drops (Oxybuprocaine, commesine®) was instilled, followed by the installation of a glass with 3 mirrors of Goldman. Between the glass and the cornea, a 974P carbomaire gel (lacryvisc® Alcon™, France) was applied. The examination began by the posterior pole, then the middle and periphery retinal over 360 degrees. The lesions were recorded according to the nature and location. They were classified into 5 stades according to the Goldberg classification **[89]**. Patients with sickle cell retinopathy were offered fluorescein angiography and then laser treatment of the lesions. Any patient presenting the criteria for inclusion,

as well as acute or chronic neurological manifestations of SCD whose ischemic origin was suspected (hematological examination, neurological, ophthalmological and otolaryngological examination, transcranial doppler ultrasound, fundus examination, audiometry) **[53, 58, 67, 74, 75]**, systematically benefited from brain imaging. Then, they were designed HbSS SN^+.

Method of Studying Hypoxia

Clinical signs of hypoxia

The clinical examination evaluated the respiratory rate as well as the pulsed oxygen saturation (SpO_2).These measurements were made using a portable pulse oximeter (OXY3® Beijing Safe Heart Technology™, China) **[90]**.The SpO_2 measurement procedure involved placing the sensor on the thumb of the participant's right hand, lying down for at least 15 minutes. The hand carrying the sensor is placed on the examination bed. SpO_2 greater than 98 % in ambient air was considered normal.

Determination of hypoxia inducible factor -1α (HIF-1α)

It was carried out by the ELISA (Enzyme linked immunosorbent assay) technic. For this, a kit (Human HIF-1α ELISA kit™, Elabscience® N ° E-EL-H1277) was necessary. The dosing procedure was carried out according to the manual dosing protocol proposed by Elabscience® **[83]**. HIF-1α concentrations were expressed in pg/ml.

Statistical Methods

The choice of sample was convenient, consisting of 86 people, including 20 $HbSS^+$, 30 $HbSS^-$ and 36 HbAA. The data from this survey were collected on a survey sheet, then reported in an Excel file from Microsoft office® 2013.The statistical analysis was done using Epi info™ 7.2.0.1 software from CDC and SPSS® Statistic 21 from IBM®. It used calculations of proportions, means and standard deviations. The Chi-2 test was used to compare the proportions. That of Spearman made it possible to study the correlations between quantitative variables. The relationships between qualitative and quantitative variables were studied by the Mann-Whitney and Wilcoxon test. The difference was statistically significant when the p was less than 0.05 ($p < 0.05$).

3. Results

Descriptive study

Epidemiological parameters of the study population

The average age of the general population was 26.9±9.7 years. It was 30.2±8.3 years in HbAA's, 28±8.2years in $HbSS^-$, and 19.8±10.9years in $HbSS^+$. Men accounted for 45.3% of the total population, with a male/female ratio of 0.8. Taking into account the two subgroups of the sickle cell population ($HbSS^-$ and $HbSS^+$), men represented 40% (n=12/30) of the $HbSS^-$ population and 55% (n=11/20) that of $HbSS^+$, i.e male to female ratio of 0.6 in $HbSS^-$, and 1.2 in $HbSS^+$.

Clinical and biological characteristics of sickle cell patients

Table I summarizes the clinical characteristics of sickle cell patients. Pain was noted in 45% of $HbSS^+$. Hyperthermia was found in 16.6% of $HbSS^-$ and 40% $HbSS^+$. Concerning neurological localization VOA's, the history of retinal ischemia was found in 16.6% of $HbSS^-$ subjects and 5% of $HbSS^+$ subjects. Lesions evoking a notion of auditory ischemia were noted in 15% (n=3/20). One $HbSS^+$ patient i.e 3.3 % have had an ischemic stroke. The notion of intermittent headache (IC) was noted in 17 $HbSS^-$ subjects (56.7%) and 6 $HbSS^+$ (30%). SpO_2 lower than 98% were identified in 70 % of subjects $HbSS^-$, and $HbSS^+$ respectively. Respiratory frequencies exceeding 15 cycles/ min were objectified in 95% of $HbSS^+$ subjects. Clinical signs of hemolysis were observed in 45% of $HbSS^+$, and 10% of $HbSS^-$. The HV phenotype was found in 22% of sickle cell an all types, and that of HML in 76% of cases. The Glasgow score was 15/15 in 100% of the participants.

4. Analytical Study

Comparisons of the biological parameters of basal sickle cell subjects ($HbSS^-$), and in crisis ($HbSS^+$), with those of controls (HbAA).

Regarding the Hb level, its average value (TmHb) was 13.8±1.9g/dl in HbAA's, 8.2±2.1g/dl in $HbSS^-$, and 7.8±4g/dl in $HbSS^+$ (p=0.0005). The average white blood cell count (TmGb) in HbAA's was 5.3±1.6mm^3, compared to 7.5±4mm^3 in $HbSS^-$, and 10.2±8.4mm^3 in $HbSS^+$ (p=0.008) **(Figure 2)**.The average plasma concentration of CRP (CmCRP) was 3.1±5.9 mg/dl in HbAA, 10.2±15mg/dl in $HbSS^-$, and in $HbSS^+$ of 32.7±48, 9mg/dl (p=0.0004) **(Figure 3)**.In HbAA's, the average neuroglobin concentration (CmNgb) was 5.8±2.2 ng/ml, 8.2±2.3ng/ml in $HbSS^-$, and 6.2±4 ng/ml in $HbSS^+$ (p=0.005) **(Figure 4)**.The average plasma concentration of cytoglobin (CmCygb) was 1322.1 ±986.9 ng/ml in HbAA, 1721.8 ± 1971.1 ng/ml in $HbSS^-$, and 915.5±835.2 ng/ml in $HbSS^+$ (p=0.081) **(Figure 5)**. In HbAA's, the average plasma concentration of HIF-1α (CmHIF-1α) was 2.6±2.3ng/ml, 3±2.5 ng/ml in $HbSS^-$, and 4.2±2.7ng/ml in $HbSS^+$ (p=0.035) **(Figure 6)**.

Comparison of the average of the maximum blood velocities of the right middle cerebral artery (TAMMV), of the degree of hearing loss (DHL), of the temperature, of the pulsed oxygen saturation (SpO_2) of sickle cell anemia subjects ($HbSS^+$), and to basal state ($HbSS^-$) with that of controls (HbAA)

The results from the comparison of the average of TAMMV (cm/s), DHL (dB), temperature (°C) and SpO_2 (%) of HbAA, to those of $HbSS^-$ and $HbSS^+$ are summarized in **Table II**. The TAMMV was 21 ±4.4cm/s for HbAA's, compared to 24.5±6.9cm/s for $HbSS^-$ and 23.9±4cm/s for $HbSS^+$ (p=0.0004).The DHL was 35±8.4dB for HbAA's, 38.5±20.3dB for $HbSS^-$, and 28.6±6.9dB for $HbSS^+$ (p=0.267). The average body temperature was 37°C in HbAA, 37.3±0.8°C in $HbSS^-$, and 37.7±1.1°C in $HbSS^+$(p=0.005).The average of SpO_2 was 100% in HbAA, 90.4±4.6% in $HbSS^-$, and 91.1±4.7% in $HbSS^+$ (p=0.0001).

Comparison of fundus examination (presence of retinal ischemia or no), transcranial doppler ultrasound (average of the maximum blood velocities of the right middle cerebral artery i.e TAMMV lower or higher of 200 cm/s) and audiometric data (presence of transmission deafness or no, and degrees of hearing loss or DHL, in dB) between sickle cell patients in crisis ($HbSS^+$) and basal state ($HbSS^-$)

Four (4) $HbSS^-$ subjects presented retinal ischemia (13.3%), against one $HbSS^+$ subject (p=0.024). In $HbSS^-$, 10% of subjects (n = 3/30) had a transmission deafness. However, no deafness was diagnosed in $HbSS^+$ (p=0.051).On TDU, TAMMV of less than 200 cm/s were observed in 100% of sickle cell patients in crisis, and in basal state (p=0.018). Hearing loss degrees of less than 200dB were noted in 100% of $HbSS^+$ and $HbSS^-$ subjects (p=0.0002) **(Table III)**.

Relationship between CmNgb and clinical markers of ischemia of the central nervous system (CNS), hypoxia, hemolysis, inflammation, as well as clinical phenotypes in sickle cell anemia subject in basal state and in crisis

In sickle cell subjects who had no history of neurological localization VOA, the CmNgb was 7.6±2 ng/ml in $HbSS^-$, and 4±2.7ng/ml in $HbSS^+$(p=0.042). In sickle cell patients with intermittent headaches (IH), the CmNgb was 8.5±2.3ng/ml in $HbSS^-$, and 3±3.3 ng/ml in $HbSS^+$ (p=0.002).In $HbSS^-$, the CmNgb of the subjects having a stroke was 11.87±5.3ng/ml, compared to 4.3±2.47ng/ml in those who did not (p=0.025) **(Table IV)**. Conversely, no relationship existed between CmNgb and the other markers as well as the clinical phenotypes.

Relationship between CmCygb, clinical markers of ischemia of the central nervous system (CNS), hypoxia, hemolysis, inflammation, as well as the clinical phenotype in sickle cell anemia subject in basal state and in crisis

The CmCygb of $HbSS^+$ subjects with a history of IH was 511.7±458.1pg/ml compared to 1651±1430.1pg/ml in $HbSS^-$ subjects who had the same history (p=0.03).In $HbSS^-$, the CmNgb of the subject having a stroke was 2811±1285pg/ml, against 1320±2200pg/ml in those who did not (p=0.047). However, no relationship existed between CmCygb, the other markers and the clinical phenotypes **(TableV)**.

Correlation between CmNgb and biological markers of inflammation, hypoxia, and hemolysis in sickle cell patients in basal state and in crisis

The results from the multivariate analysis by the Spearman test (rho, p <0.05), taking into account the CmNgb, the average white blood cell count (TmGb), hemoglobin (TmHb), hematocrit (TmHt) and average plasma concentration of CRP (CmCRP) in $HbSS^+$ and $HbSS^-$ were summarized in **Table VI**. There was a correlation between, CmNgb and TmHt in sickle cell subjects in crisis (r=0.44; p=0.05).

Correlation between CmCygb and the biological markers of hypoxia, inflammation and hemolysis, in sickle cell patients in basal state and in crisis

Data from multivariate analysis by the Spearman test (rho, p <0.05), taking into account CmCygb, TmGb, TmHb, TmHt, and CmCRP in $HbSS^+$ and $HbSS^-$ have been summarized in **Table VII**. In $HbSS^-$, there was a correlation between, CmCygb and TmGb (r=-0.37; p=0.049).

Relationship between, CmNgb and the presence of ischemic lesions of the nervous system (CNS and SNP) in sickle cell subjects

The results derived from the comparison between, the CmNgb of the sickle cell patients having presented ischemic lesions ($HbSS\ SN^+$), to that of the controls (HbAA) are represented in **figure 7**. In the HbAA, the CmNgb was 5.79±2.22ng/ml, compared to 7.1±3.15ng/ml in $HbSS\ SN^+$ (p=0.0201).

Relationship between CmCygb and the presence of ischemic lesions of the nervous system (CNS and SNP) in sickle cell patients

The results from the comparison of the CmCygb of the $HbSS\ SN^+$ subjects to that of the HbAA subjects are shown in **figure 8**. The CmCygb of the $HbSS\ SN^+$ was 2732.7±3470.9ng/ml, against 1322.1±3.15 ng/ml in HbAA (p=0.2567).

Table I: Clinical caracteristics of sickle cell subjects

Parameters	**$HbSS^-$**	**$HbSS^+$**
	n (%)	n (%)
Symptoms		
Pain	4 (13.3)	9 (45)
Rate respiratory (Cylce/min)		
< 15	0 (0)	1 (5)
≥ 15	30 (100)	19 (95)
SpO_2 (%)		
< 98	21 (70)	14 (70)
≥ 98	9 (30)	6 (30)
Temperature (°C)		
< 37.5	25 (83.3)	12 (60)
≥ 37.5	5 (16.6)	8 (40)
VOC*pains		
Member	1(3.3)	5 (25)
Vertebral	1(3.3)	7 (35)
Others	0(0)	4 (20)
VOA**		
Retinal ischemia	5 (16.6)	1 (5)
Auditive ischemia	0(0)	3 (15)
Pain according to AVS***(Decimal)		
< 3/10	29 (96.6)	4 (20)
≥ 3/10	1 (3.3)	16 (80)
Clinical signs of hemolysis		
Pallor jaundice splenomegaly	3 (10)	9 (45)
Clinical phenotype		
HV ****	8 (26.7)	4 (20)
HML*****	22 (73.3)	16 (80)
Glasgow coma scale (/15)		
< 15	0 (0)	0 (0)
≥ 15	30(100)	20 (100)

*vaso-occlusive crisis;**vaso-occlusive-accident ;***analog visual scale; ****hyper viscous phenotype ; *****hémolytic phenotype

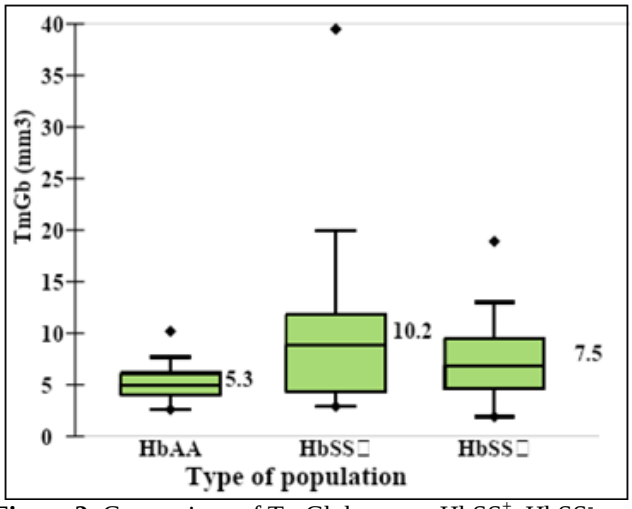


Figure 2: Comparison of TmGb between HbSS^{+}, HbSS^{-} and HbAA

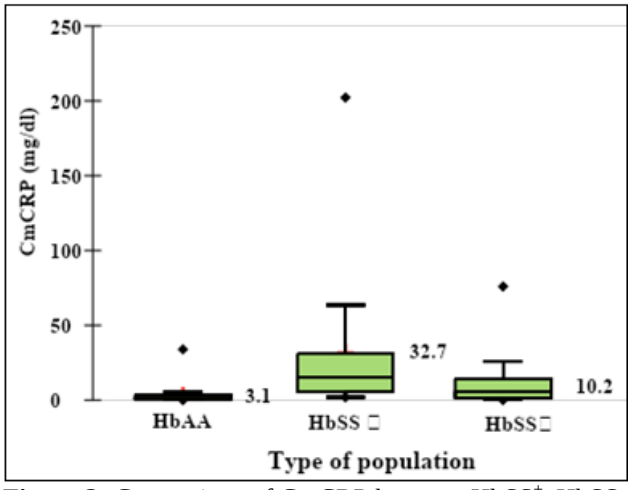


Figure 3: Comparison of CmCRP between HbSS^{+}, HbSS^{-} and HbAA

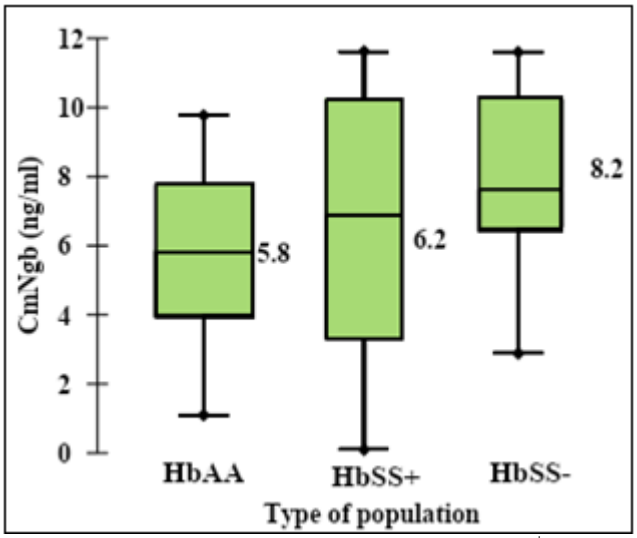


Figure 4: Comparison of CmNgb between HbSS^{+}, HbSS^{-} and HbAA

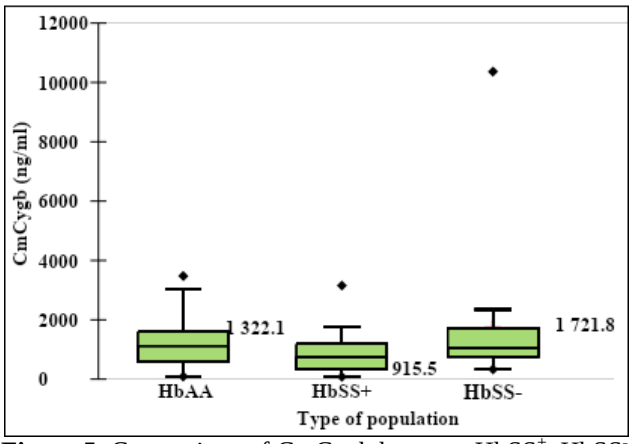


Figure 5: Comparison of CmCygb between HbSS^{+}, HbSS^{-} and HbAA

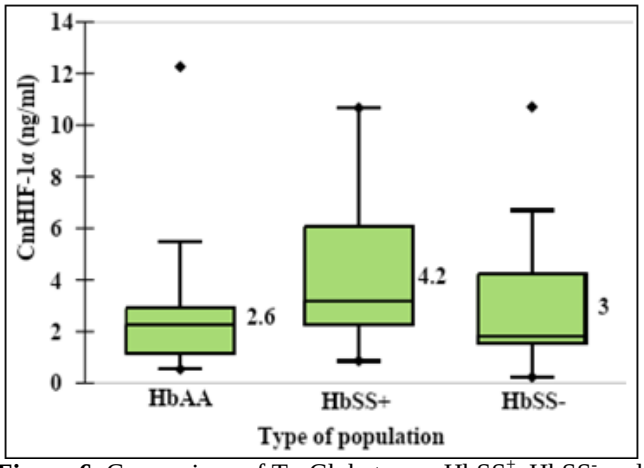


Figure 6: Comparison of TmGb between HbSS^{+}, HbSS^{-} and HbAA

Table II: Comparison of the average of the maximum blood velocities of the right middle cerebral artery (TAMMV), of the degree of hearing loss (DHL), of the temperature, of the pulsed oxygen saturation (SpO_2) of sickle cell anemia subjects (HbSS^{+}), and to basal state (HbSS^{-}) with that of controls (HbAA)

	HbAA	**HbSS^{-}**	**HbSS^{+}**	p
Parameters				
TAMMV (cm/s)	21 ± 4.4	24.5 ± 6.9	23.9 ± 4	**0.0004**
DHL (dB)	35 ± 8.4	38.5 ± 20.3	28.6 ± 6.9	0.267
Temperature (°C)	37 ± 0	37.3 ± 0.8	37.7 ± 1.1	**0.005**
SPO_2 (%)	100 ±0	90.4 ± 4.6	91.1 ± 4.7	**0.0001**

Table III: Comparison of fundus examination (presence of retinal ischemia or no), transcranial doppler ultrasound (average of the maximum blood velocities of the right middle cerebral artery i.e TAMMV lower or higher of 200 cm/s) and audiometric data (presence of transmission deafness or no, and degrees of hearing loss or DHL, in dB) between sickle cell patients in crisis (HbSS^{+}) and basal (HbSS^{-})

Parameters	**HbSS^{-}**	**HbSS^{+}**	**p**
	n (%)	**n (%)**	
Retinal ischemia			
Yes	**4 (13.3)**	**1 (5)**	
No	26 (86.6)	19 (95)	0.024
Transmission deafness			
Yes	**3 (10)**	**0 (0)**	
No	27 (90)	20 (100)	0.051

DHL (dB)			
≤ 40	20 (66, 7)	14 (70)	
> 40	10 (33.3)	7 (30)	0.0002
TAMMV (cm/s)			
200 <	30(100)	20 (100)	
≥ 200	0(0)	0 (0)	0, 018

Table IV: Relationship between CmNgb (± SD) and clinical markers of ischemia of the CNS, hypoxia, hemolysis, inflammation, as well as clinical phenotypes in sickle cell anemia in basal state and in crisis

Parameters	CmNgb (ng/ml)		p
	$HbSS^-$	$HbSS^+$	
Marker of hypoxia			
Respiratory rate (cycle/min)			
< 15	9.8±2.6	10.9 ± 0	0.095
≥ 15	7.5 ± 3.2	7.1 ± 3.4	0.6966
SpO_2 (%)			
< 98	7.6 ± 2, 9	7.2 ± 3, 5	0.8137
≥ 98	7.3 ± 3.7	7.5 ± 3.5	1
Markers of inflammation			
Temperature (°C)			
< 37.5	7.7 ± 2.8	7.8 ± 3	0.7952
≥ 37.5	6.8 ± 4.8	6.5 ± 3.9	1
AVS*			
< 3	8.1 ± 2, 4	7.32 ± 4.1	0.7202
≥ 3	6.7 ± 3.9	7.3 ± 3.3	0.5686
Marker of hemolysis			
Pallor jaundice splenomegaly			
Yes	1.1 ± 1.5	6 ± 3.9	0.0522
No	1 ± 1.2	7.3 ± 2.5	0.0612
Markers of ischemia			
History of VOA			
Yes	8.5 ± 2.3	7 ± 4.2	0.757
No	7.6 ± 2	4 ± 2.7	**0.042**
History of IH			
Yes	8.5 ± 2, 3	3 ± 3.3	**0.002**
No	7.6 ± 2.2	7.6 ± 3.5	0.627
Clinical Phenotype			
Hyper viscous	8.2 ± 2.6	7.9 ± 3.9	0.8383
Hemolytic	7.3 ± 3.4	7.2 ± 3.5	0.9528

*Analog visual scale

Table V: Relationship between CmCygb, clinical markers of ischemia of the central nervous system (CNS), hypoxia, hemolysis, inflammation, as well as the clinical phenotype in sickle cell anemia subject in basal state and in crisis

Parameters	**CmCygb (ng/ml)**		p
	$HBSS^-$	**$HBSS^+$**	
Markers of hypoxia			
Respiratory rate (cycle/min)			
< 15		820 ± 0	
≥ 15	1727.1 ± 1995.4	912.2 ± 752.5	0.0710
SpO_2 (%)			
< 98	1886.9 ± 2293.1	771.9 ± 489.2	0.0549
≥ 98	1353.9 ± 1031.9	1224.1 ± 1116.4	0.7237
Markers of inflammation			
Temperature (°C)			
< 37.5	1788.6 ± 2161.3	1196.1 ± 783.5	0.6732
≥ 37.5	1419.51 ± 836.56	474.8 ± 368.7	0.0570
AVS**			
< 3	1528.4 ± 1214.3	1690.7 ± 1089.9	0.5307
≥ 3	1986.8 ± 2743.6	711.8 ± 487.9	0.1248
Markers of hemolysis			
Pallor jaundice splenomegaly			
Yes	761.7 ± 887.1	946.5 ± 1039.9	0.9263
No	534.8 ± 634.1	612.4 ± 456.8	0.714
Markers of ischemia			
History VOA			
Yes	1970.7 ± 2326.2	956.5 ± 807.5	0.1330
No	1158.5 ± 621.5	711.8 ± 275.2	0.1649
History of IH			
Yes	1651.04 ±1430.1	511.7 ± 458.1	**0.030**
No	1310.1± 1100	1250.2 ± 821.3	0.071
Clinical phenotype			
Hyper viscous	1640.9 ± 871.8	1020.9 ± 735.3	0.2207
Hemolytic	1758.4 ± 2289.4	899.3 ± 776.2	0.2872

Table VI: Correlation between CmNgb and biological markers of inflammation, hypoxia, and hemolysis in sickle cell patients in basal state and in crisis

	CmNgb (ng/ml)	
Parameters	**$HbSS^-$**	**$HbSS^+$**
Markers of hypoxia		
CmHIF1-alpha (ng/ml)		
Rho	0.14	0.357
p	0.44	0.122
n	30	20
Markers of inflammation		
TmGb (mm^3)		
rho	0.006	-0.057
p	0.97	0.81
n	30	20
CmCRP (mg/dl)		
rho	-0.056	-0.395
p	0.768	0.094
n	30	20
Markers of hemolysis		
TmHb (g/dl)		
rho	-0.09	0.42
p	0.606	0.06
n	30	20
TmHt (%)		
rho	-0.25	**0.44**
p	0.19	**0.05**
n	30	20

Table VII: Correlation between CmCygb and the biological markers of hypoxia, inflammation and hemolysis, in sickle cell patients in basal state and in crisis

Parameters	**CmCygb** (ng/ml)	
	$HbSS^-$	**$HbSS^+$**
Markers of hypoxia		
CmHIF-1alpha (ng/ml)		
rho	0.054	0.005
p	0.78	0.98
n	30	20
Markers of inflammation		
TmGb (mm^3)		
rho	**-0.37**	-0.12
p	**0.049**	0.6
n	**30**	20
CmCRP (mg/dl)		
rho	0.14	0.09
p	0.45	0.716
n	30	20
Markers of hemolysis		

TmHb (g/dl)		
rho	0.21	0.128
p	0.25	0.60
n	30	20
TmHt (%)		
rho	-0.009	0.23
p	0.96	0.31
n	30	20

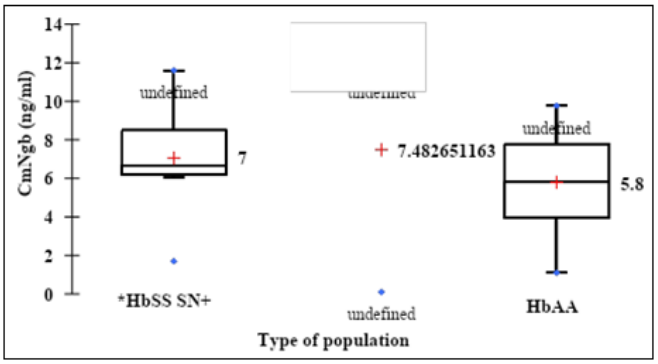


Figure 7: Relationship between, CmNgb and the presence of ischemic lesions of the nervous system (CNS and SNP) in sickle cell subjects
HbSS SN**: HbSS subjects with nervous system ischemia

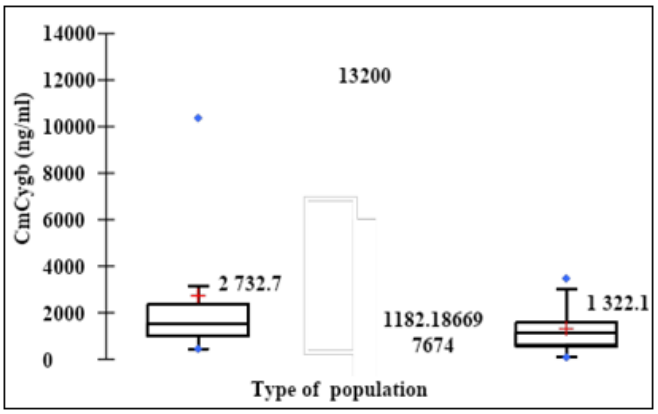


Figure 8: Relationship between, CmNgb and the presence of ischemic lesions of the nervous system (CNS and SNP) in sickle cell subjects
HbSS SN^{+}*: HbSS subjects with nervous system ischemia

5. Discussion

Study Limits

Neuroglobin and cytoglobin are recently discovered globins **[1-4]**.Most authors give them a cytoprotection role due to overexpression during pathologies associating hypoxia, ischemia, oxidative stress, inflammation and apoptosis, as in sickle cell anemia **[1-7, 23-36]**. Sickle cell anemia is an autosomal recessive disease, with a chronic course, and most of the curative treatments are under study **[54-67]**. This work was initiated because there are few data on the expression of these two new proteins during the sickle cell crisis, and even less their relationships with the main factors behind the triggering of this crisis. To do this, we determined the levels of expression of cytoglobin and neuroglobin, and study the probable relationships of these two molecules with the pathophysiological factors of this disease, in 20 patients with homozygous sickle cell patients in crisis ($HbSS^{+}$), and 30 to basal state ($HbSS^{-}$).This study meet several difficulties, including unavailability, refusal to participate, and especially blood samples. All of these constraints had a considerable impact on the sample size. However, despite these limits, it emerges that the average plasma concentration of neuroglobin (CmNgb) was higher in sickle cell anemia, all types combined (6.2±4ng/ml in $HbSS^{+}$ *versus* 8.1±2.3 ng/ml in $HbSS^{-}$), compared to control subjects (5.8±2.2ng/ml) (p=0.005). At the same time, the average plasma concentration of cytoglobin (CmCygb) was higher in $HbSS^{-}$ (1721.8±1971.1ng/ml), compared to that in $HbSS^{+}$ subjects (915.5±835.2ng/ml) and controls (HbAA) (1322.1±986.9 ng/ml) (p=0.081). In sickle cell anemia, on the one hand, there was a relationship between CmNgb, markers of ischemia of the NS and hematocrit, and on the other hand, between CmCygb, markers of ischemia of the NS and leukocytosis. However, probably due to the size of the sample, no relationship existed between these two concentrations and the other markers.

6. Analytical Study

Comparison of the average white blood cell count (TmGb) of sickle cell subjects with that of controls

During the present study, the TmGb of sickle cell an all types, was significantly higher than that of controls (HbAA). This result is similar to that of Thiam and *al* **[55]**. Indeed, this difference in the level of white blood cells count is related to leukocytosis which is almost physiological in SCD according to Doupa and *al*, as well as several other authors **[53, 56, 58, 59, 65, 66, 89]**.This leukocytosis is explained by the inflammatory processes which play a major role in the pathogenesis and pathophysiology of this condition **[63, 65, 67]**. Furthermore, during our study, the TmGb observed in $HbSS^{+}$ was 1.4-fold higher that of $HbSS^{-}$. These data are similar to those found by certain authors **[65, 89, 91]**. Indeed, Dahmani and *al* found a higher white blood cell count in sickle cell patients in crisis compared to those in the basal state **[65, 89, 91]**. Thus, the difference in TmGb that we observed between the two groups could be explained by the fact of the crisis. In fact, the level of white blood cells in sickle cell patients in crisis would be a biological marker of inflammation on the one hand, but also a prognostic marker on the other hand. It would thus make it possible to monitor HbSS patients being on hydroxycarbamide **[65]**. Thus, our data confirm the existence of leukocytosis during sickle cell anemia. The latter would translate an acute inflammatory process in $HbSS^{+}$, subacute and chronic in $HbSS^{-}$. At the same time, our results confirm on the one hand TmGb as a biological marker of inflammation and as a prognostic marker of sickle cell anemia by differentiating the stable state from the crisis on the other hand.

Comparison of the average plasma CRP concentration (CmCRP) of sickle cell subjects with that of controls

At the end of our work, the CmCRP of $HbSS^{+}$ subjects were 15.8-fold higher than that of HbAA, and 4-fold higher than that of $HbSS^{-}$.This observation is similar to that of Gueye and *al* **[92]**. In fact, during their study, the authors noted that sickle cell patients in crisis had significantly higher CRP concentrations than those of sickle cell patients in the basal state, as well as those of controls **[92]**.Therefore, the increase in CRP that we observed in sickle cell subjects

compared to controls demonstrates that during sickle cell anemia, there is an inflammatory process which is almost constant in the basal state ($HbSS^{-}$), and acute during a crisis ($HbSS^{+}$) **[65, 66, 69, 71, 73.81]**. It also confirms CRP as a biological marker of inflammation, but also a prognostic marker during this pathology **[65, 71, 73, 92, 81]**.

Comparison of CmNgb of sickle cell subjects with that of controls

It appears from our work that, the CmNgb of $HbSS^{+}$ was significantly higher than that of the controls ($p = 0.005$). This variation in concentration between these two populations is related to the fact that there are phenomena of ischemia, hypoxia, inflammation and oxidative stress at the systemic level during sickle cell anemia **[53, 56, 58, 59, 61, 63]**. In the literature, these phenomena are described as being capable of causing lesions in the nervous system **[93, 94]**. We believe that these lesions are in turn responsible for the production of neuroglobin. In fact, neuroglobin has been shown to be overexpressed in conditions involving ischemia, hypoxia, oxidative stress and apoptosis **[1-3, 4, 6, 18, 19, 25, 26]**. Ovono and *al*, had recently observed an increase in plasma neuroglobin in subjects with primary glaucoma (4.7±4.6ng/ml) compared to controls (0.9±1.1ng/ml). According to them, glaucoma being a condition whose pathogenesis associates the phenomena of ischemia, hypoxia, oxidative stress and apoptosis, the results observed suggested a role of bio-prognostic marker of this protein **[34]**. Furthermore, according to Xie and *al*, neuroglobin is highly induced during neurological conditions involving inflammatory processes, oxidative stress and ischemia **[4]**. In addition, when comparing the two sickle cell subgroups ($HbSS^{-}$ and $HbSS^{+}$) of the study population, the CmNgb of $HbSS^{-}$ was 1.3-fold significantly higher than that of $HbSS^{+}$.This result suggests that there is an overexpression of neuroglobin in $HbSS^{-}$ compared to $HbSS^{+}$.Therefore, the difference in concentration observed could be explained by the fact that we found approximately 13.3% of $HbSS^{-}$ with retinal ischemia lesions, against 5% in $HbSS^{+}$ ($p=0.024$). However, it has been proven by several authors, that neuroglobin would be strongly induced during lesions of the nervous system (retina, brain, auditory nerve) **[17, 18, 19, 25, 26, 34, 35]**. Thus, on the basis of our results, we can say that during sickle cell anemia, there is an induction of Ngb probably related to the pathophysiological phenomena associated with this disease. This Ngb induction would be more important during the crisis, and especially in the presence of lesions (ischemia, hypoxia, apoptosis, and oxidative stress) of the NS. As a result, our data suggest not only a role as a biomarker, but also a neuroprotective of this protein during sickle cell anemia.

Comparison of the CmCygb of sickle cell subjects with that of controls

Although this difference is not statistically significant, the comparison of CmCygb between the different groups reveals that the CmCygb of basal sickle cell subjects ($HbSS^{-}$) was 1.3-fold higher than the control value. In our opinion, this result is related to certain factors which induce the synthesis of cytoglobin **[3, 4, 6, 7, 37, 39]** while being involved in the pathophysiology of SCD **[53, 56, 58, 59, 61]**. Indeed, ischemia, hypoxia, inflammation and oxidative stress are known to be factors that cause systemic overexpression of cytoglobin **[3, 4, 6, 7, 33, 37, 38]**.However, according to several studies, these factors are constantly found in SCD **[53, 56, 58, 59, 61, 63]**. Another explanation of our result would be the fact that our population be constituted of a proportion of people with lesions of the NS (83.3% of subjects $HbSS^{-}$ filled the criteria of ischemia of the NS, and 35% in $HbSS^{+}$). In this case, these lesions would be more ischemic and asymptomatic in nature (auditory nerve, optic nerve, retina, brain), especially since the neurological evaluation was normal in almost all sickle cell patients (abnormal neurological examination and CT scan in one patient). In fact, experiments carried out both *in vitro* and *in vivo* have shown that Cygb is induced on the rise by hypoxia and especially ischemia of neuronal cells **[4]**. Shu-feng and *al*, for example, had studied the potential functions of cytoglobin in neonatal rats during brain damage caused by ischemia and hypoxia. Data from this study demonstrated that increased cytoglobin reduced lesions due to hypoxia and cerebral ischemia.These data highlighted, according to them, the neuroprotective role of this protein during brain damage caused by hypoxia and ischemia **[28]**. Thus, the overexpression of Cygb observed in $HbSS^{-}$ confirms the presence of ischemic phenomena in this population, and suggests Cygb as a biomarker of ischemic lesions of the NS in sickle cell anemia in inter critical period. However, although remarkable, the variation in CmCytgb between HbAA and $HbSS^{-}$ found during our work was not statistically significant ($p=0.087$). We believe that this result supports the notion that the production of cytoglobin under physiological conditions remains controversial. Indeed according to Xie and *al* **[4]**, this globin is produced at low concentrations under conditions of physiological hypoxia according to some authors, and is overexpressed under the same conditions for others. The reasons for this variability would notably be the type of population studied, the models and especially the technic for detecting this globin. For example, the detection technic used during our work was identical to that of the study by Tayyar and *al* **[95]** (plasma assay of Cygb by Elisa). However, the cytoglobinemia found in the controls by these authors is far below ours (2.84 ng/ml *versus* 1322.1ng/ml). Apart from the reasons mentioned above by Xie and *al*, we believe that the ubiquitous distribution of this protein within the body would also be another explanation **[3, 4, 5, 7]**. At the same time, the CmCygb found in $HbSS^{+}$ during the present survey was 1.8-fold significantly lower than that of $HbSS^{-}$. These data are probably related to our sample. They can be explained in particular by the fact that 13.3% of the population size of $HbSS^{-}$ subjects had presented ischemia of the NS, against 5% in $HbSS^{-}$ ($p=0.024$). In fact, several studies have shown that cytoglobin is higher in disorders of the NS associated with ischemic and hypoxic lesions **[3, 6, 28]**. Therefore, our analysis confirms the neuroprotective role of cytoglobin during attacks of ischemia-hypoxic types of the NS in general, on the one hand. On the other hand, the result we have obtained suggests that during sickle cell anemia, Cygb would also play its cytoprotective role **[30]**. Another explanation of our result would also be the fact in the existence in $HbSS^{+}$ of a factor inhibiting the synthesis of cytoglobin, such as dysthyroidism. A study by Oliveira and *al* had effectively demonstrated that the secretion of thyroid hormones decreased the production of cytoglobin in

thyroidectomized rats receiving injections of thyroid hormones, compared to the control **[16]**. In addition, cytoglobin is thought to play a major role in maintaining the tone and rheology of the vessel *via* nitric oxide dioxigenase (NOD) **[38, 39]**.Thus, the down regulation of cytoglobin would prevent the endothelial dysfunction of high blood pressure mediated by the angiotensin system. In our opinion, the fact that the CmCygb of $HbSS^+$ is lower than that of $HbSS^-$ suggests a NOD deficiency associated with a decline in cytoglobin activity in sickle cell patients in crisis. This deficit is thought to contribute to endothelial dysfunction, oxidative stress, apoptosis, major arterial remodeling and vasomotor disorders seen in this population **[7, 37, 38, 39]**. Finally, a final reason for the variation would be due to the duration and intensity of the hypoxia. In $HbSS^+$ subjects oxygen therapy and hyper hydration are generally prescribed **[56, 58, 62, 96]**. However, the production of Cygb decreases with the improvement of tissue oxygenation and endothelial dysfunction **[3, 4, 7, 21, 33, 37, 38, 39]**.

Comparison of CmHIF-1alpha of sickle cell subjects with that of controls

From our work, it emerges that the CmHIF-1α of sickle cell subjects in crisis ($HbSS^+$) was 1.6-fold higher that of controls (HbAA). HIF-1α is described in the literature as a biomarker of hypoxia par excellence in human pathologies **[97, 98, 99]**. It would thus regulate the expression of more than 100 target genes involved in a wide range of physiological functions, including the response to hypoxia. Consequently, our result shows that the overexpression of HIF-1α testifies to the hypoxic phenomena evoked during sickle cell anemia **[53, 56, 58, 59]**. Still in the same idea, our data corroborate those of the study by kato and *al*, as well as Damahourie and *al*. Indeed, these authors had described in their various works, the role of hypoxia in the pathophysiological mechanism of sickle cell anemia **[60, 65]**.To conclude, we believe that the difference in concentration of HIF-1alpha observed between sickle cell subjects in crisis and controls during our work confirms that hypoxia is indeed present in the pathophysiology of sickle cell anemia. Moreover, this makes us suggest HIF-1alpha as a biological marker of hypoxia during the sickle cell crisis. The plasma HIF-1alpha level of $HbSS^+$ was 1.4-fold higher that of $HbSS^-$.Since the level of expression of HIF-1 alpha is a prognostic marker for hypoxia in general **[97, 98, 99]**, this result confirms an intensification of hypoxic phenomena during the sickle cell crisis, compared to the basal state. At the same time, he suggested that CmHIF-1alpha be a prognostic marker for hypoxia during sickle cell anemia.

Comparison of the average of the maximum blood velocities (TAMMV) of the right middle cerebral artery, the degree of hearing loss (DHL), the temperature, the pulsed oxygen saturation (SpO_2) of the controls (HbAA), with those of sickle cell subjects in basal state ($HbSS^-$) and in crisis ($HbSS^+$)

The average value of the TAMMV of the $HbSS^+$ subjects and that of the controls was very much below 200 cm / s (value considered to be normal during our work). However, in sickle cell anemia, all types combined, it was significantly higher (24.5±6.91cm/s in $HbSS^-$, and 23.91±4.02cm/s in $HbSS^+$) compared to that of controls. (20.96 ± 4.41cm/s) (p=0.004). Despite the controversies over the use of transcranial doppler in adult sickle cell anemia, these data join those of Sampaio and *al*, Adegoke and *al*, Brewin and *al*, as well as Edjlali and *al* **[84-87]**. In fact, all these authors find higher average blood velocities of the cerebral arteries in sickle cell subjects compared to controls.According to some of them, these results suggest an approximately 220-fold higher risk of stroke in sickle cell anemia compared to the general population **[84-87]**. In addition, in sickle cell children where the problem of accessibility of the temporal window is minor (less than 1%), Herinirina and *al* **[100]**, as well as other authors also find velocities abnormally increased blood levels **[74, 75]**. Thus, despite the fact that the TAMMV of the $HbSS^+$ subjects in our survey are within physiological limits (<200 cm/s) our data suggest reconsidering the values for TAMMV and certain other common parameters of doppler transcranial that are assumed to be references in these adult patients.

The average degrees of values of controls (35dB) and sickle cell an all types (38.46 dB in $HbSS^-$ and 28.57 dB in $HbSS^+$) were normal, and did not vary significantly between the different groups (p = 0.267).The absence of a hemoglobin S phenotype in our sample could be a reason. Indeed, central auditory ischemia is mainly found in sickle cell subjects with the HbSC phenotype. It appears to be in the form of sudden and silent ischemia responsible for functional hearing impairment **[58, 62, 68]**. Therefore, the low frequency of patients with conductive hearing loss in our working population (approximately 10% of $HbSS^-$ subjects) would be an explanation. Another reason would be the method of detecting auditory nerve ischemia. During our study, a DHL value greater than 40dB was chosen as abnormal **[60, 65, 67]**. However, some studies use as a reference value for DHL less than 20 dB. This, according to these authors, improves the sensitivity of the results **[78]**.Still, the heterogeneity of the study population (subgroups by age and sex, duration of the disease) could also influence our result.The average value of body temperature was higher in sickle cell an all types (37.28°C in $HbSS^-$ and 37.68°C in $HbSS^+$) compared to controls (37°C) (p = 0.005).Body temperature being a clinical marker of inflammation, the difference in value noted between controls and sickle cell patients therefore testifies to the existence of an inflammatory syndrome during sickle cell anemia **[55-59, 65]**. This inflammation would evolve chronically during the inter-critical period, and acutely during the crisis. Therefore, this situation could explain the difference observed between $HbSS^+$ and $HbSS^-$ (60% of $HbSS^+$ had a body temperature above 37.5°C, while 40% of $HbSS^-$ were febrile). Thus, our survey confirms the data from the literature. In fact, according to these studies, during sickle cell anemia, there is an inflammatory process which, perhaps constant depending on whether one is in the basal state ($HbSS^-$), or accentuated in the event of a crisis ($HbSS^+$) **[65, 66, 67]**. Thus, our survey confirms the data from the literature. In fact, according to these studies, during sickle cell anemia, there is an inflammatory process which, perhaps constant depending on whether one is in the basal state ($HbSS^-$), or accentuated in the event of a crisis ($HbSS^+$) **[65, 66, 67]**. Compared to controls, the average SpO_2 of sickle cell subjects of any type was significantly lower than the control value (in HbAA 100%, in $HbSS^-$ 90.4%, and 91.1% in $HbSS^+$) (p=0.0001).The value of SpO_2 is recognized as a clinical

marker of high sensitivity hypoxia **[101]**. Therefore, our result confirms the presence of hypoxia during sickle cell anemia **[59, 60, 67, 68]**, and therefore suggests SpO_2 as a clinical marker of hypoxia during this pathology. According to the same data, hypoxia was found in 70% of subjects in crisis, but also in the basal state. This supports the idea that hypoxia is constant during sickle cell anemia **[59, 60, 67, 68]**. This chronic hypoxia could also explain, at least in part, the polypnea that we have observed in this population. Thus, this compensatory polypnea, associated with oxygen therapy which is often recommended in the event of a crisis **[96]**, could explain the fact that the SpO_2 of $HbSS^+$ is lower than that of HbSS.

Comparison of results obtained at the fundus examination between subjects with sickle cell disease in the basal state ($HbSS^-$) and in crisis ($HbSS^+$)

Sickle cell anemia patients with retinal ischemia were more great in $HbSS^-$ compared to $HbSS^+$ (13.3% *versus* 5%; p=0.024). It was mainly proliferative retinopathy. This result corroborates those of several other studies **[58, 72, 89, 102, 103]**. Indeed, Bilong and *al* **[102]**, Menaa and *al* **[103]**, as well as KA and *al* **[104]** have described sickle cell retinopathy as a chronic complication of this pathology.It would be more frequent in subjects with the HbSC phenotype than HbSS. Thus, it is likely that the small proportion of retinal damage observed during our survey is due to the high prevalence of this complication in SC composite sickle cell anemia. However, we believe that his pathogenesis probably does not depend on the clinical condition of the patient (crisis or inter-critical condition), but rather on age, history of pathology, hemoglobin level and others factors involving a multidisciplinary approach to the disease **[58, 72, 89, 102, 104]**.

Relations between, CmNgb, CmCygb and clinical markers of ischemia of the nervous system (NS) in the sickle cell subjects

A statistically significant relationship has been found between, CmNgb and history of VOA of the nervous system in sickle cell anemia. Indeed, $HbSS^-$ subjects with a history of VOA of the nervous system presented CmNgb significantly higher (8.5±2.3ng/ml) compared to $HbSS^+$ with the same history (7±4.2ng/ml; p=0.045). In the literature, it has been reported that neuroglobin is upregulated in response to lesions of hypoxia-ischemia, and plays a role of biomarker and neuroprotective **[3, 4, 6, 12-16, 19, 20, 29, 34, 35]**. Taking into account the fact that we observed a significantly higher CmNgb in sickle cell subjects compared to controls, we can say that Ngb is overexpressed in sickle cell patients at high risk of ischemic attacks of the NS. Thus, the VOA relation of the NS and neuroglobin suggests CmNgb as a biomarker of ischemia of this system during sickle cell anemia. Despite the fact that the relationship between CmCygb and history of VOA of the NS is not statistically significant, there was however, a large variation in CmCygb between sickle cell subjects with a history of VOA of all types, and those who have never had one.This result also suggests an overexpression of cytoglobin in sickle cell subjects at risk of cerebral ischemia. At the same time, there was a statistically significant relationship between CmNgb, CmCygb, and a history of intermittent headache (IH) and stroke. In fact, the CmNgb and CmCygb of sickle cell patients with a history of IH and stroke were significantly higher than those of patients who did not have them. In the literature, IH's are considered to be warning signs of the risk of having a stroke **[29]**. At the same time, it has been shown that the plasma concentrations of Cygb and Ngb rise during lesions of hypoxia-ischemia of the NS such as stroke **[3, 4, 6, 14, 12, 28, 29, 34, 35]**.Thus, the relationship IH, CmNgb and CmCygb also testifies to the overexpression of these two globins in sickle cell patients exposed to the risk of having a stroke.Furthermore, this result suggests the neuroprotective and diagnostic biomarker role of these two molecules in the event of ischemic attacks of the central NS in the sickle cell subject. Relationships between, CmNgb, CmCygb, and clinical (fever, pain) and biological (TmCRP, TmGb) markers of inflammation. At the end of our study, we found no relationship between, the clinical markers of inflammation, CmNgb, as well as CmCygb. According to the literature, Ngb and Cygb are over-expressed during inflammation of the nervous system **[3, 4, 6, 12]**.Therefore, our results could be explained by the fact that our population is essentially made up of patients who probably do not have inflammatory pathologies of the nervous system apart from the chronic complications of a functional nature (ophthalmic, auditory) of sickle cell anemia. Indeed, of the 45 and 40% of $HbSS^+$ who presented pain and fever respectively, no symptomatic clinical anomalies were objectified during the neurological evaluation (neurological examination, Glasgow score were normal in all patients). Regarding the biological markers of inflammation, there was a negative correlation in HbSS- between, TmGb and CmCygb (r=-0.37 and p=0.049), but not with TmCRP (r=0.14 and p=0.45). This data could be explained by the fact of the existence of clinically asymptomatic dysthyroidism within this group. Indeed, it has been described in the literature that the presence of thyroid hormones inhibits the production of cytoglobin **[4, 16]**.

Relationship between, CmNgb, CmCygb, and clinical (respiratory rate, SpO_2) and biological markers of hypoxia (HIF-1alpha) in sickle cell subjects in basal state and in crisis

No relationships have been found between, CmNgb, CmCygb and clinical markers of hypoxia in sickle cell subjects.However, during the present study, low SpO_2 and CmHIF-1alpha were significantly higher in sickle cell subjects compared to controls. This confirmed, in our opinion, the presence of hypoxia in this population. With respect to Ngb and Cygb, data from studies by Ovono and *al*, as well as shu-fen and *al*, have shown that these two globins are overexpressed during hypoxic nervous system disorders **[28, 30].**This, according to these authors, can be explained by the neuroprotective role of these molecules. Which role would be exercised more during acute than chronic processes. Thus, we think that the absence of acute hypoxia of the nervous system (encephalopathy, neuropathy) observed in the working population is one of the explanations for our result. Similarly, administering oxygen systematically to some sickle cell patients in crisis would be another.

Relationship between, CmNgb, CmCygb, clinical (Cutaneous pallor, jaundice, splenomegaly) and

biological markers of hemolysis (TmHb and TmHt) in sickle cell subject in basal state and in crisis

A positive correlation existed between CmNgb and TmHt in sickle cell patients in crisis (r=0.44; p=0.05).According to contemporary authors, the hematocrit is a main determinant of blood viscosity both in physiological conditions, but even more during sickle cell anemia alone or associated with other pathologies such as diabetes **[105, 106]**.Thus, the elevation of the hematocrit level in the sickle cell subject in crisis exposes to strong rheological disorders with endothelial dysfunction. The hematocrit level would thus be correlated with the risk of the occurrence of ulcers and glomerulopathy in sickle cell phenotypes of the hemolytic type. Conversely, those with a hyper viscous phenotype would tend to develop VOA **[105]**.Unlike the two other human globins (hemoglobin and myoglobin), the expression of which varies depending on hemolytic disorders and many other hemorrhagic processes **[6, 7, 8, 9, 10, 11]**, there are very few data on the expression of neuroglobin and cytoglobin during this type of disease **[1, 4, 7, 12]**. Indeed, cytoglobin, and even more its counterpart neuroglobin would be rather overexpressed during nervous system disorders **[3, 4, 6, 12, 13, 34]**.Therefore, the positive correlation found between CmNgb and the hematocrit level during the sickle cell crisis would reflect abnormal rheological processes in the nervous system. In addition, this result suggests, in our opinion, a cerebrovascular risk in this population.

Relationship between CmNgb, CmCygb and the presence of ischemic lesions of the nervous system (peripheral and central nervous system) in sickle cell patients in basal state and in crisis

Compared to the controls, it was found during the present study, a higher CmNgb, as well as a CmCygb in the sickle cell subjects having presented lesions of ischemia of the nervous system (HbSS SN +) ($p = 0.0201$ and $p = 0.2567$). According to recent studies, the overexpression of these two proteins, both at the tissue and plasma level, would reflect a lesion of the central or peripheral nervous system of a hypoxia-ischemic nature **[3, 4, 6, 12, 13, 14, 16, 17, 19, 20, 30, 34, 45]**. Consequently, our result suggests the role of biomarker diagnostic of these two proteins in sickle cell subjects at high risk of nerve hypoxia-ischemia. In addition, they demonstrate at the same time, the neuroprotective role of neuroglobin and cytoglobin already mentioned by other authors **[28, 29, 31, 3247, 45, 46, 48, 50]**. However, these results ask to be confirmed by a larger sample of patients.

7. Conclusion

Neuroglobin (Ngb) and cytoglobin (Cytg) are two relatively young human globins. The first seems to have neuroprotective properties (anti-ischemic, anti-hypoxic, antioxidant). The second, a little more ubiquitous, would play the same role, not only on neurons, but also on other types of cells in the body. This cyto protection function, which seems to be specific to both, would be mediated by factors involved in the pathophysiology of sickle cell anemia. However, if the implication of myoglobin, and even more of hemoglobin in the pathogenesis of crises has been widely studied, those of Ngb and Cytg remain unknown. The aim of this work was to determine during the sickle cell crisis, the levels of plasma expression of neuroglobin, of cytoglobin, and to study the probable relations of these two molecules with the pathophysiological factors of sickle cell disease. It appears that there are variations in plasma concentrations of neuroglobin and cytoglobin during sickle cell anemia. These variations do not seem to be influenced by the sickle cell crisis.They are labeled with both neuroglobin and cytoglobin, and are more related to ischemic phenomena in the nervous system, probably *via* rheological processes. Thus, the results of the present investigation suggest a role of biomarker but also neuroprotective of these two new globins against ischemia-hypoxia lesions of the nervous system during this pathology. However, given the sample size, these data need to be confirmed by carrying out a larger-scale study.

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