Genetic Cofactors Modulators in Congolese Sickle Cell Anemia patients

Etude des cofacteurs génétiques modulateurs de la drépanocytose en milieu congolais

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Résumé

L'hémoglobine fœtale (HbF) et l'alpha-thalassémie sont les deux facteurs génétiques reconnus aujourd'hui comme modulateurs de la drépanocytose.
Notre objectif était de déterminer les fréquences des associations HbF-drépanocytose et alpha-thalassémie-drépanocytose et leur influence sur l'expression clinque de la drépanocytose en milieu congolais.
Il s'agit d'une étude transversale qui a porté sur 256 patients drépanocytaires homozygotes. Les patients étaient repartis en trois sous-phénotypes cliniques : le phénotype clinique bénin (PCB), le phénotype clinique modéré (PCM) et le phénotype clinique sévère (PCS). Les analyses suivantes ont été réalisées : l'hémogramme, le dosage de l'HbF, de la LDH et de la CRP. Le diagnostic de la drépanocytose a été confirmé par la mise en évidence de la mutation E6V. Le diagonstic de l'alpha-thalassémie a été posé par la technique de Multiplex ligation dependent Probe Amplification (MLPA).
L'âge des patients a varié de 5 à 52 ans. L' HbF a varié de 0,5 à 33% avec une moyenne de 6,4%. La persistance héréditaire de l'HbF a été rencontrée chez 49,41% des patients avec une moyenne et écart type de 16,5±11%. En fonction des sous-phénotypes cliniques, 95,9% des patients avec PCB étaient porteurs de l'HbF. L'HbF a été associée avec une réduction statistiquement significative des GB, réticulocytes, plaquettes, LDH et CRP. Sur 106 patients explorés par la technique de MLPA, 25,50% étaient porteurs d'une délétion homozygote $\alpha^{3.7}\alpha^{3.7}$, 23,60% avaient la délétion hétérozygote $\alpha^{3.7}$ et 11,30% étaient porteurs d'une implication α. Le généotype normal $\alpha\alpha/\alpha\alpha$ a représenté 39,60%. La délétion homozygote $\alpha^{3.7}$ a été associée au taux de la microcytose le plus élevé.
Les associations HbF-drépanocytose et alpha-thalassémie-drépanocytose sont fréquentes dans notre milieu. Ces associations s'accompagnent d'une modulation du phénotype drépanocytaire

Mots clé : cofacteur génétique, modulateur, drépanoctose, RD Congo

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Summary

***Background*:** Despite the high incidence of the sickle cell anemia, data on association between HbF and SCA, and between alpha thalassemia and SCA are unknown in the Democratic Republic of Congo. This study aimed at determining the prevalence of these associations among sickle cell patients, and their influence on the clinical and hematological profile.
***Methods*:** A cross-sectional study was conducted among 256 sickle cell patients classified as asymptomatic clinical phenotype (ACP), moderate clinical phenotype (MCP) and severe clinical phenotype (SCP).
***Results*:** In this cohort, HbF was found in 49.4% of patients. Hb F rate was 95.9, 30.9 and 2.9% of patients with ACP, MCP and SCP, respectively. WBCs, Platelets, reticulocytes, CRP, LDH levels, sickle cell crises and complications increased significantly from ACP to the SCP ($p<0.05$). In this cohort, 39.6% of patients had normal genotype ($\alpha\alpha/\alpha\alpha$), 49.1% had a deletion alpha-thalassemia and 11.3% had $\alpha^{3.7}$ triplication. Among them, 23.6% patients with alpha-thalassemia had $\alpha^{3.7}$ heterozygous deletion ($-\alpha/\alpha\alpha$ or $\alpha\alpha/-\alpha$), and 25.5% had $\alpha^{3.7}$ homozygous deletion ($-\alpha/-\alpha$). Among patients with $\alpha^{3.7}$ homozygous deletion, type 3, type 4 and type 6 were found in 7.5, 2.8 and 15.1% of patients, respectively. The $\alpha^{3.7}$ triplication and the normal genotype ($\alpha\alpha/\alpha\alpha$) were found in 11.3 and 39.6% of patients, respectively. The Hb level was significantly higher in patients with homozygous and heterozygous $\alpha^{3.7}$ deletion. The WBCs and Platelets count was significantly higher in patients with $\alpha^{3.7}$ triplication. Hb F was not found in patients with $\alpha^{3.7}$ triplication.
***Conclusions*:** The present study shows that these associations are common in sickle cell Congolese patient. This work might also be a decisive trigger to conduct new studies in our midst.

Key words: Sickle cell anemia; fetal hemoglobin; alpha-thalassemia; clinical phenotype; Kinshasa; the Democratic Republic of Congo; Africa

Introduction

Sickle cell disease (SCD) is characterized by its extreme phenotypic heterogeneity (1, 2). The natural history of the disease showed the existence of genetic factors modulators (2). Indeed, high levels of fetal hemoglobin (HbF) ($\alpha_2\gamma_2$) decreased the polymerization (3). In adults, the expression of HbF is a quantitative variable associated with the inactivation of fetal γ-globin gene. This period corresponds to the progressive extinction of γ-globin genes and the activation of δ- and β-globin (4). However, some adults have a high HbF levels, called hereditary persistence of HbF (HPFH). Associated with hemoglobin-pathies such as sickle cell anemia, HPFH is a modulating factor of the severity of the disease (5-10). The mechanism is the formation of hybrid polymers ($\alpha\beta^s\gamma$) which interrupt the polymerization of HbS (5). Clinically, HbF reduces the frequency and severity of sickle cell crises. Biologically, the F cells have better survival (11). The expression of HbF is variable among sickle cell patients and has a hereditary transmission (5, 12).

The second genetic factor modulator of SCD is alpha-thalassemia. The modulator and protective effect of alpha-thalassemia is due to the reduction of the production of the synthesis of one or two channels-α. This mechanism causes a decrease in the erythrocyte concentration of Hb and reduces the risk of polymerization of HbS (13).

Alpha-thalassemia and sickle cell anemia are very common in malaria-endemic areas.

In Central Africa, an estimated frequency from 24% to 45% of alpha-thalassemia was associated in patients suffering from sickle cell anemia (SCA) (14-17). Silent alpha thalassemia (-α/αα) and minor alpha-thalassemia minor (-α/-α) are the most encountered forms in this region. Clinically, these deletion forms are often asymptomatic, however they may be associated with microcythemia, a hypochromic anemia and hyper reticulocytosis (18).

The $\alpha^{3.7}$ kb deletion is a silent form very common in sub-Saharan Africa (19–22). This deletion takes away the majority of α_1 gene. Heterozygous carriers of sickle cell disease and the holders of silent forms and minor alpha-thalassemia seem to be protected against severe malaria (23). The Democratic Republic of Congo is located in malaria-holoendemic areas and the incidence of the disease was estimated at 39,700 newborns (CI: 32,600 (14%)–48,800) in 2010 (24). Despite the high prevalence and incidence of the disease, data on association between HbF and SCA, and between alpha thalassemia and SCA are unknown. This study aimed at determining the prevalence and these association among Congolese suffering from SCA, and their influence on the clinical and hematological profile in our midst.

Patients and Methods

Study design and participants

This report was a cross-sectional study conducted in the Sickle Cell Center of Yolo at Kinshasa, the Democratic Republic of Congo. This health facility provides most of the non-private beds for sickle cell patients in the DRC. Patients were consecutively selected in the outpatient clinic of Sickle Cell Centre of Yolo. All patients were free of pain and had not been hospitalized or transfused for at least 100 days before the study. Patients under hydroxyurea therapy or chronic transfusion program were excluded. For this study, 256 sickle cell patients in steady state were recruited. The mean age was 18.6±10.7 years, in this cohort. The study population was categorized in three phenotypic subgroups according to the clinical severity of the disease: (i) asymptomatic clinical phenotype (ACP); (ii) the moderate clinical phenotype (MCP); (iii) severe clinical phenotype (PCS). This classification is based on local practice for assessing the severity of

the disease. Clinical evaluation criteria include: the number of vaso-occlusive crises (VOC) per year, the number of transfusions per year, and chronic complications as femoral head necrosis, malleolar ulcer and cholelithiasis. The patient was classified in the subgroup of ACP when he had a total score ≤3, MCP for a score between 4 and 6, and SCP for a score >7 (25).

Laboratory analysis

Five ml of venous blood sample was drawn from each study participant into an EDTA tube, used to determine hematologic parameters. Hematologic parameters were performed using an automate Sysmex XS – 1000 i (Lincolnshire, USA). For LDH assay, 1 ml of blood the samples were collected in dry tubes. The serum LDH assay was performed with a spectrophotometer at 340 ηm with Thermo GENESYS 10S Bio apparatus (USA). The kit was provided by Cypress diagnostics (Landrop-Belgium). The reference values at 30 °C were 160-320 U/L. CRP was measured with the immune-turbidimetric method using an automated CYANSTart apparatus (Cypress diagnostics, Belgium). The assay kit was provided by CRP-Vital diagnostics. All analysis was performed at Institut National de Recherche Biomédicale (INRB) at Kinshasa, the DRC.

DNA extraction

Genomic DNA was extracted locally at the INRB using the standard "salting out" method as previously described (26). DNA samples were later normalized to 50 ηg/µl with a Dropsence® robot (Trinean, Belgium) at the Center for Human Genetics, KU Leuven, Belgium.

Diagnosis of sickle cell anemia and determination of Hb F

Sickle cell screening was performed using an automated agarose gel electrophoresis technique with the Hydrasis II apparatus (SEBIA, France). Sickle cell anemia (SCA) was diagnosed in presence of production of mostly Hb S with no Hb A. The concentrations were measured by an integrated densitometer.

Confirmation of the diagnosis of SCA

SCA diagnosis was confirmed by PCR-RFLP at Laboratory for Center for Human Genetics of

Katholieke Universiteit te Leuven in Belgium. The 440 bp PCR fragments on HBB was amplified by standard PCR using following primers: F-TGTGGAGCCACACCCTAGG GTTG and R-CATCAGGAGTGGACAGA TCC. The PCR program comprised an initiation denaturation for 5 min at 95°C, 32 amplification cycles including a short denaturation at 95°C for 30 min, annealing at 58°C for 30 min and extension at 72°C for 30 sec. A final extension at 72°C for 5 min terminated the program. The PCR control product was performed electrophoresis on QIAxcel 3190 apparatus (Qiagen). This fragment was later restricted using DdeI (Roche) restriction enzyme following standard protocol. Normally, this enzyme cuts the PCR product (CTGAG/) on the SCA mutation spot (*E6V*) located 201 bp far from the 5'-end of the fragment and on a second site 167 nucleotides downstream the mutation site. The normal allele produces 3 fragments with 201 bp, 167 bp and 72 bd size respectively. When the mutation is present on the allele, the restriction site on the mutation spot is removed (CTGAT>CTGTG). Thus, the enzyme makes only one cut at 167 bp downstream and this allele gives 1 fragment with 368 bp and a second with 72 bp. The digestion product was controlled on 2 % agarose gel. Normal individuals show 3 bands (72 bp, 167 bp and 201 bp), heterozygotes have 4 bands (72 bp,

167 bp, 201 bp and 368 bp) whereas homozygotes have 2 bands (72 bp and 368 bp).

The diagnosis of thalassemia

The diagnosis of alpha-thalassemia was performed by the technique of multiplex ligation dependent probe amplification (MLPA) with the T1 Thermocyler 3144 apparatus (Biometra) in the Center for Human Genetics, University Hospital, KU Leuven, Belgium. The SALSA MLPA kit P140 probemix HBA provided by MRC-Holland was used for MLPA assay.

The heterozygous deletion $\alpha^{3.7}$ kb was characterized by the presence of single copy (ratio = 0.5) of the 08498-L08422, 04633-L23748, 18096-L22520, 18880-L24428, 08494-L08417, 14855-L23604, 18093-L22517 probes (27). The homozygous deletion HBA type 6 was characterized by zero copy (ratio = 0) of the above mentioned probes (27). HBA deletion type 4 was characterized by zero copy of the probes 08498-L08422, 04633-L23748, 18880-L24428 and associated with a copy in less (ratio = 0.5) of the probes 18096-L22520, 08494-L08417, 14855-L23604, 18093-L22517. HBA deletion type 3 was characterized by a copy in less of the probes 18096-L22520, 18880-L24428, 08494-L08417, 14855-L23604, 18093-L22517 and zero copy of the probes 08498-L08422, 04633-L23748 (28). The triplication $\alpha^{3.7}$ type A was characterized by an extra copy (+1 copy) of the probes 08498-L08422, 04633-L23748, 18096-L22520, 18880-L24428, 08494-L08417, 14855-L23604, 18093-L22517 (28).

Ethical considerations

All major participants provided written consent for study participation. Since some participants were minors, they provided oral assent and their legal guardians provided written consent for study participation. The aim and the procedures of the study were explained to the participants. The participants were informed that they could withdraw anytime without further obligation. Anonymity of the participants was guaranteed and no personal details were recorded This consent procedure and the study were reviewed and approved by the National Ethical Committee of the Public Health School of the University of Kinshasa, Kinshasa, the DRC (ESP/CE/027B/2011), in compliance with the principles of the Helsinki Declaration II.

Data management and analysis

Results were manually entered into a microcomputer and analyzed using the Excel Version 2002 (CDC) and they were exported on SPSS 17.0 for further analysis. Data are represented as means ± SD when the distribution was normal and median with range when the distribution was not normal. Frequency of various clinical and laboratory findings are expressed as percentages. ANOVA test were used to compare differences among categorical variables. Correlations of variables were calculated with the Pearson test. Statistical significance level was set at p=0.05.

Results

Prevalence of fetal hemoglobin in the study population

The HbF ranged from 0 to 33.3% with a median of 6.4%. In this cohort, HbF was found in 127 (49.4%) patients. In this group of patients with HbF, the mean rate of HbF was 16.5 ± 11.0% (figure 1).

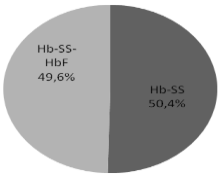

Figure 1: Prevalence of fetal hemoglobin in the study population

The frequencies of HbF in clinical sub-phenotypes in the study population.

Fetal hemoglobin was found in 95.9% of patients with ACP, 30.9% of patients with MCP and 2.9% of patients with SCP. Overview is shown in Table 1.

Table 1. Frequencies of HbF by clinical phenotypes

Phenotypes	Age (years)	Frequency of HbF		Total (%)
		M	F	
ACP (n=73)	19.4±11.8	19 (26.0%)	51 (69.9%)	95.9
MCP (n=149)	17.9±9.8	18 (12.1%)	28 (18.8%)	30.9
SCP (n=34)	20.3±12.1	1 (2.9%)	0 (0%)	2.9

ACP: asymptomatic clinical phenotype; MCP: moderate clinical phenotype; SCP: severe clinical phenotype

Influence of HbF on hematological and biochemical parameters

The concentrations of Hb and HbF decreased from the ACP to the SCP and the difference was statistically significant. White blood count (WBCs), Platelets count, reticulocytes, CRP and LDH levels increased from ACP to the SCP and the difference was statistically significant. Other details are presented in table 2.

Table 2. The influence of HbF on haematological and biochemical parameters according to the clinical phenotype

variables	ACP (n=73)	MCP (n=149)	SCP (n=34)	*p*
HbF (%)	16.7±8.4	2.7±1.4	0.2±1.1	<0.001
Hb (g/dl)	9.2±1.4	7.3±0.9	6.5±0.7	<0.001
WBC ($x10^3$/µl)	7.94±1.62	12.67±3.70	14.81±4.82	<0.001
Reticulocytes (%)	7.56±5.03	1654±11.01	15.47±7.27	<0.001
Platelets ($x10^3$/µl)	250.35±94.97	272.44±145.58	360.41±246.85	0.002
CRP (mg/l)	63±24	129±31	207.8±24.3	<0.001
LDH (U/L)	433±157	787±208	1214±148	<0.001

Correlations between Hb and WBCs, reticulocytes and platelets

A significant negative correlation was observed between HbF and WBCs respectively, in patients with the ACP and MCP. Overview is shown in table 3.

Table 3. Correlations between Hb and WBCs, reticulocytes and platelets

Variables	ACP (r_1)	MCP (r_2)	SCP (r_3)
WBCs	-0.454*	-0.234*	-0.006
Platelets	-0.221	-0.051	0.114
Reticulocytes	-0.211	-0.009	0.025

*: significant correlation ($p < 0.001$) ACP: asymptomatic clinical phenotype; MCP: moderate clinical phenotype; SCP: severe clinical phenotype

Influence of HbF on acute and chronic sickle cell complications

The frequency of acute complications and chronic complications increases with the decrease in HbF. Details are presented in table 4.

Table 4. Assessment of sickle cell complications based on clinical phenotypes

Variables	ACP (n=73)	MCP (n=149)	SCP (n=34)	*P value*
HbF (%)	16.7±8.4	2.7±1.4	0.2±0.1	<0.001
Pain crisis /year	0.2±0.0	3±1	6±0.1	<0.001
Transfusion/year	0.1±0.0	2±1	4±1	0.001
cholelithiasis (%)	8.2	6.0	17.7	0.001
femoral head necrosis (%)	2.7	28.9	79.4	<0.001
Leg ulcer (%)	0	3.4	35.3	-

Prevalence of alpha-thalassemia

Of a total of 106 sickle cell patients investigated with the technique of MLPA, 42 (39.6%) patients had normal genotype (αα/αα), 52 (49.1%) had an alpha-thalassemia deletion and 12 (11.3%) were carriers of $\alpha^{3.7}$ triplication (figure 2).

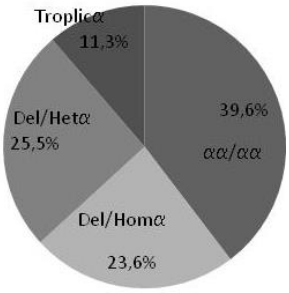

Figure 2: Frequencies of alpha-thalassemia deletion in the study population

Patients with alpha-thalassemia deletion were divided as follows: 25 (23.6%) patients had silent thalassemia or $\alpha^{3.7}$ heterozygous deletion (-α/αα or αα/-α), 27 (25.5%) patients were carriers of minor thalassemia or $\alpha^{3.7}$ homozygous deletion (-α/-α). This deletion included 3 types: The type 3 was found in 8 (7.5%) patients, the type 4 in 3 (2.8%) patients and the type 6 found in 16 (15.1%) patients. The $\alpha^{3.7}$ triplication was found in 11.3% of patients and the normal genotype (αα/αα) in 39.6% of patients. Others details are shown in table 5.

Table 5. Frequencies of alpha-thalassemia polymorphism

Alpha-thalassemia polymorphism	Genotypes		Frequency
$\alpha^{3.7}$ heterozygous deletion	$\alpha^{3.7}$ heterozygous	-α/αα or αα/-α	25 (23.6%)
$\alpha^{3.7}$ homozygous deletion	Type 3	-α/-α	8 (7.5%)
	Type 4		3 (2.8%)
	Type 6		16 (15,1%)
$\alpha^{3.7}$ triplication	type A	αα/αα	12 (11.3%)

The alpha-thalassemia polymorphism and hematological changes

In patients with homozygous and heterozygous $\alpha^{3.7}$ deletions, the Hb level was significantly higher than in the patients with triplication.

The WBCs and Platelets counts was significantly higher in patients with $\alpha^{3.7}$ triplication compared to others polymerphisms.

Hb F was not found in patients with $\alpha^{3.7}$ triplication.

Patients with $\alpha^{3.7}$ homozygous deletion had a low MCV compared to others polymorphisms. Overview is shown in table 6.

Table 6. The alpha-thalassemia polymorphism and hematological changes

Variables	$\alpha^{3.7}$ heterozygous deletion (n = 25)	$\alpha^{3.7}$ homozygous deletion (n = 27)	αα/αα (n = 42)	$\alpha^{3.7}$ triplication (n =12)	p
Age (years)	20.1±13.6	23.8±9.5	22.5±16.1	23.8±13,3	0.123
Hb (g/dl)	9.0±1.2	8.6±1.2	8.6±1.4	7.2±0.4	0.001
Htc (%)	26.5±3.5	26.4±2.7	26.5±3.9	23.3±2.2	0.028
HbF (%)	13.6±7.6	9.7±7.8	9.1±8.1	1,4±0,01	0.000
HbA_2 (%)	1.7±0.7	2.0±0.6	2.0±0.5	1.9±0.5	0.089
WBCs ($x10^3$/µl)	10.3±4.0	8.3±2.6	9.0±3.1	11.1±6.3	0.080
Reticulocytes (%)	12.8±7.9	12.2±6.8	9.9±6.3	14.1±6.3	0,921
MCV (*f*l)	81±11	73±8	78±9	80±10	0.020

Discussion

Fetal hemoglobin and alpha-thalassemia are two genetic factors recognized for their modulation of the expression of SCD (29). Central Africa is one of the regions with the highest sickle cell population. However, data on the modulators of the disease are rare (16, 30, 31). The objective of this study was to determine the factors in a cohort of patients with sickle cell disease living in the DRC.

Our study showed that the association between HbF-SCA and alpha-thalassemia-SCA are common in Congolese environment. In this study, HbF-SCA and alpha-thalassemia-SCA represented 49.4% and 49.1% of patients, respectively. The influence of HbF on hematological and chemistry parameters was recognized by previous studies (2). The modulating effect of alpha-thalassemia sickle cell anemia is due to the reduction of the erythrocyte concentration of Hb and reduction of MCV (13).

Our study showed that 49.4% of our study population expressed HbF with a median of 6.4%. Tshilolo *et al*. reported similar values (7.2%) of Hb-F in patients with SCA in a recent study in the DRC (31). However, our results were lower than those observed by Ngo *et al*. in Arab-Indian haplotypes (32). This difference is related to the change of HbF between different haplotypes (33, 34). Bantu haplotype predominates in the DRC and is associated with the most severe form of the disease because of low HbF (35).

Our study showed that patients with the ACP were associated with high levels of HbF compared to the other two phenotypes. In addition, these patients had low morbidity markers as the WBCs, the reticulocyte and platelet count. The influence of HbF on improving haematological parameters is related to its ability to inhibit the polymerization of HbS. Hemolysis and hyper inflammation state are reduced (5, 11).

Our study showed that the decrease in the rate of HbF is accompanied by increased frequencies of CVO, hemolysis and chronic complications from ACP to SCP. The increase in HbF was correlated with the reduction in markers of the severity of the disease. In this cohort, a significant negative correlation between the HbF and the number of WBCs was found. This is inconsistent with related previous studies (36, 37).

In this report, 49.1% of our study population was carrying an alpha-thalassemia syndrome. This high frequency of SCD with alpha-thalassemia has been reported by previous studies (18, 31, 38).

In our midst, the alpha-thalassemia syndrome included two main forms. Silent alpha thalassemia (heterozygous deletion $\alpha^{3.7}$) and minor alpha thalassemia (homozygous deletion $\alpha^{3.7}$). Each form includes a quarter of the patients. Our findings reported in this study

were found to be similar to those described in sub-Saharan Africa (18, 31).

One patient out of 4 had homozygous deletion $\alpha^{3.7}$. This statistics was lower than those found by Hassan *et al*. in Oman (38), but higher than those found by Rumaney *et al*. in Cameroon (18). This difference could be due to diagnostic techniques and different haplotypes predominant in these countries. In this cohort, the most form found was type 6. This deletion was also predominant in previous studies in Africa (39, 40).

The influence of alpha-thalassemia on hematological changes in sickle cell patients is controversial. However, it is known that alpha-thalassemia reduces the risk of polymerization of HbS. This situation implies a decrease of the erythrocyte concentration of Hb. Hematologic changes related to the decrease in WBCs count, reticulocytes and increased HbA_2 are still contradictory. El-Hazmi and al reported that the hematological changes influenced by the alpha-thalassemia related to the decrease in the MCV and the MCHC (41). Decreasing VGM improves rheology, while the decrease of the MHC reduces the risk of polymerization of HbS (13).

Our study showed that patients with homozygous and heterozygous $\alpha^{3.7}$ deletion 3.7 had an Hb concentration, the WBCs count, reticulocyte rate and the rate of HbA_2 similar to those in patients with normal thalassemia genotype ($\alpha\alpha/\alpha\alpha$). Our observation confirms the controversy of the action of alpha-thalassemia on hematological parameters.

Conclusion

The present study is the first attempt to describe the prevalence of the association between SCA and HbF, and SCA and alpha-thalassemia in the sickle cell population in the DRC. These associations are common in sickle cell Congolese patient. In this cohort, $\alpha^{3.7}$ triplication was associated with an increase in biomarkers of the severity of the disease. This work might also be a decisive trigger to conduct new genetic studies in the sickle cell patients living in the DRC.

Competing interests

The authors have declared that no competing interests exist.

Authors' contributions

TMM, PLT, and JMMM conceived, designed, deployed and directed the case–control study at the Sickle cell center of Yolo and at the Faculty of Medicine of University of Kinshasa. TMM carried out patient recruitment and follow-up, sample collection, storage and transport. TMM, AL, JMMM, PLT, AL wrote the manuscript, TMM, PLT, JMM, AL, VR, DKK analyzed data, MNA, GM, KD, VR brought some precious corrections.. All authors read and approved the final manuscript.

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