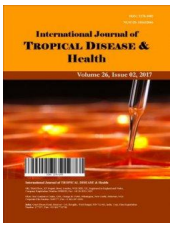

International Journal of TROPICAL DISEASE & Health

37(3): 1-10, 2019; Article no.IJTDH.50379
ISSN: 2278–1005, NLM ID: 101632866

Abnormalities of Hemoglobin and Glucose-6-Phosphate-Dehydrogenase Deficiency in Children with Uncomplicated Malaria and Living in Banfora and Saponé, Two Different Malaria Setting of Burkina Faso

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Authors' contributions

This work was carried out in collaboration among all authors. Authors ESB, ECB, ABT, YT and SBS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AO, IS, IN and AD managed the analyses of the study and the drafting of the manuscript, Authors SSS, JBY, SAC, DK, AZO and LM ensure the participant follow up, collected field data and managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJTDH/2019/v37i330168
Editor(s):
(1) Dr. Muhammad Chutiyami, Lecturer, Shehu Sule College of Nursing and Midwifery Damaturu, Nigeria.
Reviewers:
(1) Itodo, Sunday Ewaoche, Niger Delta University, Nigeria.
(2) Oshim, Ifeanyi Onyema, Nnamdi Azikiwe University, Nigeria.
(3) Ahmed Tabbabi, Jichi Medical University, Japan.
Complete Peer review History: http://www.sdiarticle3.com/review-history/50379

Original Research Article

Received 14 May 2019
Accepted 21 July 2019
Published 26 July 2019

**Corresponding author: Email: eddy.cnrfp@gmail.com, eddy.cnrfp@fasonet.bf;*

ABSTRACT

Aims: The aim of this study is to assess the prevalence of hemoglobin abnormalities and G6PD deficiency and their respective influence on anemia occurring in less than five years old children with clinical *P. falciparum* malaria living in Burkina Faso.

Study Design: The study was a cross-sectional survey with descriptive focus conducted from December 2010 to January 2013 in Saponé health district and from May to October 2011 in Banfora health district. Clinical and laboratory data were collected. Blood smears on slides for malaria diagnosis by microscopy, hemoglobin level and filter paper for the detection of human genetic factors were performed.

Methodology: A total of 386 subjects from Saponé (131) and Banfora (255) were enrolled. DNA collected from each sample was extracted using chelex-100 method and the human genetic resistance factors background was assessed by RFLP-PCR. Abnormal hemoglobin patients were classified as NonAA while AA was defined the normal hemoglobin.

Results: In this study, 70.98% (274/386) were classified normal hemoglobin (AA) while 29.02% (112/386) of subjects were carrying at least one abnormal (NonAA) allele: 24.35%AC, 3.63% AS, 0.78%CC and 0.26%SC. G6PD deficiency was 9.59% (37/386) among which, 4.92% for male and 4.66% in female. However, this gender difference was not statistically significant (p=1.00). 319/367 (86.92%) of the patients were anemic (59.4% with moderate anemia and 20.98% with mild anemia). The prevalence of anemia in G6PD deficient subjects was 83.33% (of which 58.33% were moderate anemia and 22.22% mild anemia). The difference between types of hemoglobin (p=0.64) in the occurrence of anemia (AA 87.64% and Non AA 85.18%) was not statistically significant.

Conclusion: This study showed that the prevalence of these genetic factors was relatively low among children with clinical *falciparum* malaria with high parasite density. In addition, these factors appear to have no effect on anemia.

Keywords: Prevalence; hemoglobin; G6PD; children; malaria; Burkina Faso.

1. INTRODUCTION

The incidence rate of malaria is estimated to have decreased by 21% between 2010 and 2015. The global tally of malaria in 2015 was 212 million new cases and 429,000 deaths [1]. Sub-Saharan Africa still accounts for a disproportionate share of the global burden of malaria with 90% of cases and 92% of deaths due to malaria [1]. Children under five years and pregnant women represent the most affected targets [2]. Some genetic disorders are known to affect malaria development and the prevalence of disease such sickle cell disease (SCD), thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and other red blood cell (RBC) genetic anemia [3].

About 5% of the worldwide population are healthy carriers of a sickle cell or thalassemic gene; with this Figure reaching 25% in some regions [4] and more than 300,000 children with severe hemoglobinopathy are born every year [5]. Of all the hemoglobinopathies, the S-form or sickle-cell remains the most widespread. It mainly affects African and is currently present on several continents because of the population migration. In Burkina Faso, the prevalence of the sickle cell trait varies from 8 to 10% [6,7]. Several authors have shown that heterozygous hemoglobinopathies (AS, AC) rarely have malaria [7]. These hemoglobinopathies also appear to confer protection against severe anemias [8,9]. Glucose-6-Phosphate Dehydrogenase (G6PD) which is an enzyme present in the cytoplasm of all cells in the body is involved in the first step of the metabolic pathway of pentose phosphates, thus producing NADPH [10]. The G6PD deficit affects more than 400 million people worldwide [11]. G6PD deficit represents the most frequent erythrocytic enzymopathy [12,13]. The global distribution of this enzymatic deficiency is particular and the highest frequencies are observed in hyper-endemic malaria setting [14]. In Burkina Faso, the prevalence of G6PD deficiency is estimated between 8 to 9% [6]. Previous studies (in vitro or in vivo) were carried out to characterize on molecular, biochemical and cellular basis the mechanism that could underlie the protection of the G6PD deficient subject against malaria [15,16]. Then, both hemoglobin abnormality and G6PD deficiency seem to confer protection against malaria and prevent anemia [17].

The aim of this study is to assess the distribution of beta-globin abnormalities and G6PD deficiency and their influence on the prevalence

of anemia in children with *Plasmodium falciparum* malaria and living in two different malaria-endemic areas in Burkina Faso. This will provide data on the prevalence of these two abnormalities in a population with uncomplicated malaria in Burkina Faso.

2. METHODOLOGY

2.1 Study Sites

The study was conducted in two areas covering the health district of Banfora and Saponé. Banfora health district in the Comoé province is located in southwestern part of Burkina Faso, at about 450 km from Ouagadougou, the capital city of Burkina Faso, where malaria is endemic. Malaria transmission in that area is permanent with seasonal peaks during the rainy season from May to November. The health district of Saponé is located 50 Km south-west of Ouagadougou. In this area malaria transmission even seasonal is short compared to the one of Banfora health district (June to October). According to the Ministry Health, in Burkina Faso malaria incidence was 364‰ in 2010 and 413‰ in 2013 [18,19].

2.2 Study Population, Design and Period

A total of 386 children aged between 6 to 59 months were recruited. It was cross-sectional surveys with descriptive focus conducted from December 2010 to January 2013 in Saponé health district and from May to October 2011 in Banfora health district. The study was part of a clinical trial study, assessing the efficacy of two Artemisinin combination therapies (ACT). The inclusion criteria were as followed : 1) fever (axillary temperature ≥ 37.5ºC) and/or a history of fever within the past 48 hours; 2) asexual *P. falciparum* mono-infection identified microscopically on blood smears with parasite density between 2000 and 200000 parasites/µl of blood; 3) no history of anti-malarial drug administration in the last two weeks; 4) no history of serious adverse effects to the study drugs (mefloquine, quinine, artesunate, chloroquine and sulphadoxine-pyrimethamine); 6) no evidence of a concomitant febrile illness; 7) no sign/symptoms of severe malaria as defined by WHO.

2.3 Samples Collection

For each subject included in this study, physical examination, capillary blood samples on slides, venous blood samples (1mL) and filter papers were collected. Slides were used for the diagnosis of malaria parasites, venous blood samples for hemoglobin concentration and the filter papers for the analysis of human genetic factors.

2.4 Malaria Diagnosis by Microscopy

After making the thick and thin blood smears, the slides were air-dried. The thick and thin blood films were stained with Giemsa 6% for 35 min. The parasites were counted against 200 leukocytes and then extrapolated to parasites per microliter of blood. At least one hundred power film fields were examined before assigning a negative malaria diagnosis. The number of parasites per microliter of blood was calculated using the last full blood count of the patient or the theoretical value of 8000 leucocytes/µl. The Parasite Density (PD) was estimated using the following formula:

$$PD = N \times 8000/X$$

With N = number of parasites counted and X = number of counted leucocytes or the value of the full blood count.

Two expert microscopists who read each blood slide were blinded from each other's reading. All discordant readings were re-read by a third microscopist who was blinded from the previous results.

2.5 Hemoglobin Concentration

Hemoglobin levels were determined using an ABX Pentra 60 hematology analyzer (HORIBA ABX SAS, France) according to the CNRFP SOP. Daily internal quality controls were followed as quality measures [20]. Analysis of samples was performed within 8 hours of blood draw.

2.6 Blood Spots Samples and DNA Extraction

Blood from finger prick spotted onto Whatman filter Papers (Whatman 3 mm, GE Healthcare, Pittsburg, USA), was labeled with patients' study numbers, air-dried, and individually placed into plastic bag marked and containing a desiccant to protect against humidity. The bags were stored at room temperature until DNA extraction. Parasite DNA was extracted using Chelex methods [21]. Briefly, three pieces of filter paper was soaked overnight in a solution of 10%

saponin in PBS and was subsequently washed in PBS. Thus, 50 µl of 20% Chelex® 100 solution (Bio-Rad Laboratories) were added to 1.5 ml microcentrifuge tube containing 3 fragments of filter paper sample. Then, 100 µl of sterile water were added and the microcentrifuge tube placed onto a heating block at 95-100ºC for 10 minutes of incubation. During the incubation phase, the tube was gently whirled and returned to the heat block every two minutes. The samples were centrifuged twice and the final supernatant about 150 µl was conserved in a new labeled tube and stored at -20ºC until it was used for the amplification reaction.

2.7 Human Genetic Factors Genotyping

2.7.1 Hemoglobin genotyping

The hemoglobin in the β-chain of the globin gene at codon six was determined by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The PCR conditions were as follows: one (1) cycle of 5 min at 96ºC, 30 cycles of 96ºC for 30 secs, 60ºC for 1 min, 72ºC for 30 secs and 72ºC for 7 min. DNA samples were amplified. The expected fragment length was 358 bases pairs (bp). The fragment obtained was digested for three hours at 37ºC with MnlI for discrimination between HbAA (173 bp, 109 bp, and 60 bp), HbSS/HbCC and HbSC (173 bp, 109 bp, and 76 bp), HbAS/HbAC (173 bp, 109 bp, 76 bp and 60 bp). A second digestion with DdeI allowed for further discrimination for ambiguous results between HbSS (331 bp), HbCC (201 bp and 130 bp), HbSC (130 bp, 201 bp and 331 bp), HbAS (130 bp, 201 bp and 331 bp) and HbAC (201 bp and 130 bp). PCR products were analyzed by electrophoresis in a 1.5% agarose gel.

2.7.2 G6PD genotyping

DNA was amplified and analyzed for the presence or absence of one of the common G6PD mutations G→A 202 using Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. PCR amplification was done using primers forward and reverse (Table 1). Details of the PCR-RFLP process are described elsewhere [22].

2.8 Statistical Analysis

Double entry of data in Excel 2010 was performed and analyzed using R version 3.5.1 (2018-07-02). The statistical tests done based on Pearson chi-square for the comparison of proportions and frequencies or the Fisher test for the comparison of proportions when the theoretical number is less than 5; the Student test for comparison of means. P values were reported, with differences considered significant at $p<.05$.

First, we determined the prevalence of hemoglobin and G6PD type in the study area. After we compared *P. falciparum* parasite density between normal subjects and those with abnormality, then we analyzed the link between human genetic factors and anemia.

3. RESULTS

3.1 Socio-demographic Characteristics

A total of 386 subjects were enrolled, of whom 190 (49.22%) were male and 196 (50.78%) female. There is no predominance by gender ($p = 0.72$). The sex ratio M/F is 0.97. The majority of the population was 12-23 months old and 24-35 months old (28.50% and 27.20% respectively). On the other hand, children aged 6-11 months were the least represented, with 12.43%. The mean age was 28.87 ± 1.44 months.

3.2 Prevalence of Human Genetic Factors

The Table 2 compares within each group the genotypic frequency observed with that expected from the calculated allelic frequencies. The study population follows the Hardy-Weinberg equilibrium.

The prevalence of the normal hemoglobin type was higher than abnormal type ($p<0.001$). Overall, 274 (70.98%) children did not have abnormal hemoglobin type and 94 (24.35%) were HbAC carriers. Hemoglobin types were similar among sites (Table 3).

The estimated prevalence of G6PD deficiency frequency (Table 4) in our population was 9.59% (37/386), with a statistically significant difference between G6PD Deficient and G6PD Normal ($P<0.001$).

3.3 Human Genetic Factors and *P. falciparum* Parasite

There was no significant difference in the *P. falciparum* parasite means densities between hemoglobin and G6PD types ($p=0.94$ and $p=0.87$

respectively). However, the results showed (Table 5) significant difference in the means of gametocytes density Hemoglobin genotypes carriers (p<0.001).

3.4 Human Genetic Factors and Anemia

Anemia types were classified following that of the WHO definition. The different ranges are: Normal >11 g/dl; Middle 10-10.9 g/dl; Moderate 7-9.9 g/dl; Severe <7 g/dl. The mean values of hemoglobin rate permit us to have all subjects' groups (when the population was subdivided by human genetic factors) with a moderate anemia (Fig.1).

The prevalence of anemia was 87.64% (227/259) and 85.18% (92/108) in subjects with normal and abnormal hemoglobin, respectively. For subjects with G6PD deficiency, a total of 83.33% (30/36) were anemic or 58.33% (21/36) had moderate anemia and 22.22% (8/36) middle anemia (Table.6). After analysis of anemia type, there were no differences (p>0.05) between normal subjects and those with abnormality (G6PD deficiency and abnormal hemoglobin).

4. DISCUSSION

Children aged 6 -11 months are the least represented in our study population. Born from mothers living in high transmission areas, the study population has a certain level of clinical and parasitological immunity for a period of 3 to 6 months after birth. A study in older children confirmed the protective effect of passive transfer of antibodies [23].

Table 1. Primers sequences for hemoglobin type and G6PD amplification

Genes	Primer name	Allelic-specific primers
Hemoglobin type	Forward	AGGAGCAGGGAGGGCAGGA
	Reverse	TCCAAGGGTAGACCACCAGC
G6PD type	Forward	GTGGCTGTTCCGGGATGGCCTTCG
	Reverse	CTTGAAGAAGGGCTCACTCTGTTTG

Table 2. Genotypic frequencies of hemoglobin type

Genotype		Theoretical number	Theoretical frequency (%)	Observed number	Observed frequency (%)	p	IC (95%)
AA		278	71,65	274	70,98	0,90	[66,13-75,41]
Non AA		110	28,35	112	29,02	0,90	[24,59-33,87]
	AC	85	21,90	94	24,35	0,47	[20,22-29,01]
Non AA	AS	15	3,86	14	3,63	1,00	[2,08- 6,15]
	CC	7	0,43	3	1,80	0,34	[0,20-2,45]
	SC	2	0,51	1	0,26	1,00	[0,01-1,66]
All		388	100,00	386	100,00	0,47	[98,77-100,00]

Table 3. Distribution of subjects by hemoglobin type

Hemoglobin type	Site			All
	Banfora	Saponé	p-value	
HbAA (n) (%)	100 (76.34%)	174 (68.23)	0.12	274 (70.98%)
HbAC (n) (%)	30 (22.90%)	64 (25.10)	0.72	94 (24.35%)
HbAS (n) (%)	1 (0.76%)	13 (5.10%)	0.06	14 (3.63%)
HbCC (n) (%)	0 (0.0)	3 (1.18%)	0.52	3 (0.78%)
HbSC (n) (%)	0 (0.0)	1 (0.39%)	1.00	1 (0.26%)
Total (n) (%)	131 (33.94%)	255 (66.06%)	NS	386 (100.00%)

Table 4. Distribution of subjects by G6PD type

Parameters	G6PD type			All
	G6PD Deficient	G6PD Normal	p-value	
Frequency (n) (%)	37 (9.59%)	349 (90.41%)	<0.001	386 (100.0%)
Male (n) (%)	19 (10.00%)	171 (90.00%)	<0.001	190 (49.22%)
Female (n) (%)	18 (9.18%)	178 (90.82%)	<0.001	196 (50.78%)

Table 5. Distribution of subjects by human genetic factors, gametocyte carriage and *P. falciparum* parasite density

Parameters		Gametocyte carriage (n) (%)	Parasite density (parasites/µl) 95% confidence interval (CI)
Hemoglobin type	HbAA (274)	22 (8.03%)	52167.93 [46745.80-57590.06]
	HbAC (94)	10 (10.64%)	47075.59 [38666.80-55484.37]
	HbAS (14)	3 (21.43%)	50084.64 [19602.83-80566.45]
	HbCC (3)	1 (33.33%)	31527.67 [-33535.00-96590.34]
	HbSC (1)	1 (100.0%)	14081.00 NS
	p-value	0.005	0.94
G6PD type	Deficient (37)	4 (10.81%)	50407.26 [45722.75-55091.76]
	Normal (349)	33 (9.45%)	52346.89 [36934.72-67759.06]
	p-value	1.00	0.87
	All (386)	37 (9.58%)	50593.18 [46129.26-55057.09]

Note: HbAA: homozygous wild type genotype, HbAS: heterozygote sickle cell hemoglobin, HbAC: heterozygote hemoglobin C, HbSC: heterozygote hemoglobin S and C, HbCC: homozygote hemoglobin C

In this study, 29.02% of subjects had hemoglobinopathy. A higher frequency of AC hemoglobin carriers (24.35%) had been obtained in our population compared to that identified in Mali (13%) by Travassos in 2015 [24]. This could be explained by the fact that hemoglobin C has a maximum frequency in Burkina Faso [25]. The prevalence of hemoglobin AS 3.63% is low to findings already reported in Ghana (9.10%) in 2014 [26] and in Burkina Faso (8.07%) in 2015 [27]. No homozygous SS was met in our study but the prevalence of hemoglobin S was low. These low rates may be due to our sampling or to the fact that sickling is a hindrance to parasite development. This prevalence is comparable to that reported by Modiano et al in 2001 in malaria infected population.

The prevalence of G6PD deficient subjects in our population was low. This frequency could be explained not only by sampling but also by the fact that the relatively high parasitemia (>2000 parasites) cause us to lose positive subjects. Malaria Atlas Project data show a prevalence of G6PD deficiency in Burkina Faso which is 9.38% [5.6-15]. Similar studies in other regions give lower or higher frequencies. Carter in 2011 found frequencies of 17.4% in Ghana and 19.7% in Mali [28]. A prevalence of 15.8% was found in Tanzania [29]. In our study, there were no distributions of G6PD deficiency according to the sex. This is in contrast with previous studies where high prevalence's among men was observed. Ouattara and colleagues in 2014 had a male prevalence of 14.3% and 6.0% among women [30].

Normal hemoglobin carriers (AA) have a higher parasitic density than carriers of abnormal hemoglobin. The mechanisms by which the HbS trait protects against *P. falciparum* are still only partially understood, but the implication of two factors seems to dominate [25]. The first line of defense is the acceleration of the falciformation of the parasitized cells, which facilitates their withdrawal from the circulation. The parasites which have escaped this process then see their growth hampered when the host cell is subjected to hypoxia and adheres to the endothelium of the venules. Several authors have shown that heterozygous sickle cells and AC carriers rarely have malaria attacks [6,7] and low parasitaemias [6]. Among subjects with abnormal hemoglobin, AS subjects have a higher parasitaemia. All subjects with hemoglobin CC and SC had the lower parasitaemia. This is consistent with the in-vitro tests that demonstrate that *Plasmodium* develops poorly in HbCC erythrocytes. Indeed, studies carried out in vitro on oxygenated CC cells have produced the following results [31]: invasion by the parasite is normal; the first growth cycle is normal, but the number of ring forms is substantially reduced after the schizont stage, schizonts are seen to degenerate on the fourth day; in comparison with normal parasitic cells, resistance to osmotic lysis is increased. These cells have trouble breaking and releasing merozoites in a normal manner.

The highest parasitic density is observed in G6PD normal subjects. This difference was not statistically significant (p = 0.87). One might think that the intra-erythrocytic replication of *P. falciparum* is not affected by the existence of a G6PD deficiency. Studies have shown that parasitic density does not differ as a function of G6PD status. Martin's work in 1994 had questioned the hypothesis that G6PD deficiency

Table 6. Anemia type in the population by human genetics factors

Parameters	Hemoglobin type				G6PD type		
	All (367)	AA (259)	NonAA (108)	p-value	Normal (331)	Deficient (36)	p-value
Normal n (%)	48 (13.08%)	32 (12.35%)	16 (14.81%)	0.64	42 (12.69%)	6 (16.67%)	0.68
Mild n (%)	77(20.98%)	61 (23.55%)	16 (14.81%)	0.08	69 (20.84%)	8 (22.22%)	1.00
Moderate n (%)	218 (59.40%)	146 (56.37%)	72 (66.67%)	0.08	197 (59.52%)	21 (58.33%)	1.00
Severe n (%)	24 (6.54%)	20 (7.72%)	4 (3.70%)	0.24	23 (6.95%)	1 (2.78%)	0.54

AA: homozygous wild type genotype, NonAA: Abnormal hemoglobin (HbAC, HbAS, HbCC, HbSC), Deficient: G6PD Deficient and Normal: G6PD normal

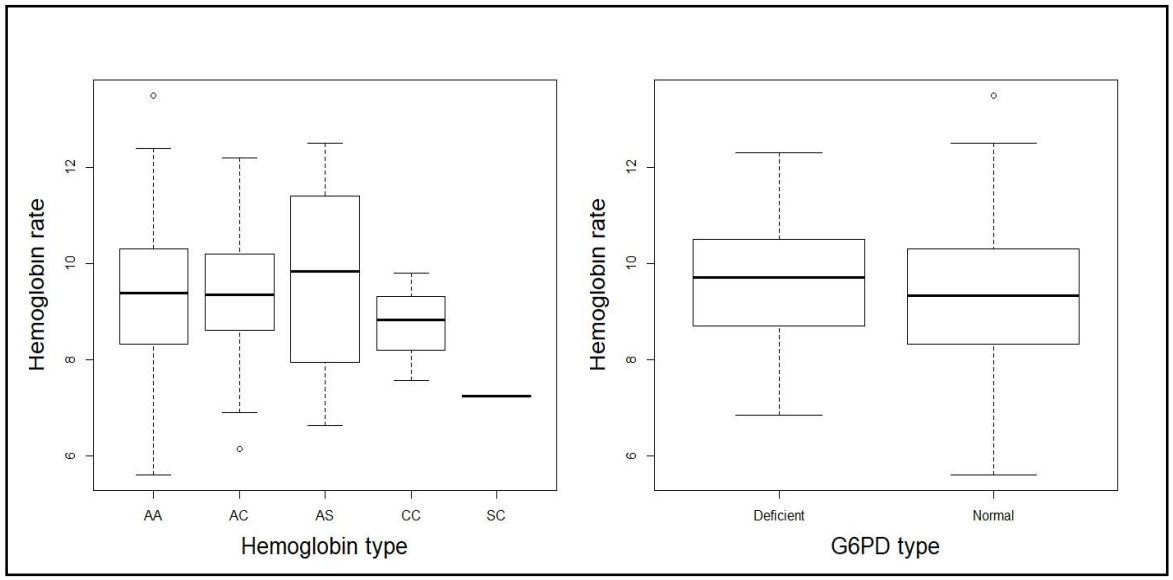

Fig. 1. Mean hemoglobin rate by human genetic factors

Note: HbAA: homozygous wild type genotype, HbAS: heterozygote sickle cell hemoglobin, HbAC: heterozygote hemoglobin C, HbSC: heterozygote hemoglobin S and C, HbCC: homozygote hemoglobin C

would greatly impede the development of the parasite [32]. Indeed, *in vitro*, it has shown that the parasite develops well in G6PD deficient erythrocytes in the absence of oxidative stress.

The gametocyte index obtained in our study was lower (9.58%) than that obtained in Uganda by Bwayo (22.0%) in 2014 [10] in children aged from six months to nine years and in Burkina Faso by Bougouma (30.5%) in 2012 [6] with children under five years. This may be due to the several efforts made since 2005 including provision of artemisinin-based combinations treatments (ACTs), distribution of long-lasting insecticidal nets (LLINs) and scale-up of seasonal malaria chemoprevention with amodiaquine-sulfadoxine-pyrimethamine (AQ-SP) in children aged between 6 to 59 months [33].

A moderate anemia has been observed in all hemoglobin type group. However, Diop in Senegal [34] with subjects aged 3 to 53 years had AS subjects with normal hemoglobin and SC subjects with mild anemia. The majority of G6PD deficient subjects were anemic. This may suggest that the G6PD deficiency is an anemic factor. It should be noted that G6PD plays an important role in the maturation of erythroids [35]. In 2008, in a study by Capelli with G6PD deficient subjects showed that, in the absence of hemolytic seizures and triggering factors, G6PD deficiency was not related to anemia or hemoglobin [36]. After analyzing of these human genetic factors effect, malaria infection has probably a bigger role on the malaria level in our study (we have an average of repeated infestation with *Plasmodium* of 2-3 episodes/year of malaria per child less than 5 years of age). Also**,** malnutrition and iron deficiency that affects these children from lower socioeconomic classes may be the cause of the different types of anemia encountered in this study. However, other nutritional deficiencies (folic acid, vitamin B12 and vitamin A), acute or chronic inflammation, and parasitic infections can also cause anemia.

5. CONCLUSION

In our study the prevalence of abnormal hemoglobin and G6PD is relatively low, probably because of study population specified by a high parasite density. In addition, G6PD deficiency does not appear to influence parasitaemia or to be associated with the occurrence of anemia. The abnormality of hemoglobin, although influencing parasitaemia, does not seem to have any effect.

CONSENT

All authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this paper.

ETHICAL APPROVAL

The study received approval from the Ethics Committee for Health of Burkina Faso before its implementation (DELIBERATION Nº2011-9-59). It was conducted in accordance with good clinical and laboratory practice. In addition, written informed consent was obtained from the parents or guardians of all participating children before enrolment. Confidentiality of information was ensured by assigning identification numbers to subjects.

ACKNOWLEDGEMENTS

We thank the population and local authorities of the health district of Saponé and Banfora for their patience and commitment. We are grateful to the staff of the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) / Institut National de Sante Publique (INSP).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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