Ferroportin Q248H mutation was not found to be protective against malaria and anemia in children under 5 years living in South Kivu/Democratic Republic of Congo, an endemic area of *Plasmodium* infection

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HIGHLIGHTS

- Frequency of ferroportin Q248H is high in African population.
- Ferroportin has been described to protect red blood cells against oxidative stress and malaria infection.
- Ferroportin allele frequency was similar in anemic and non-anemic children.
- No difference according to ferroportin allele frequency was observed among children in endemic area of *Plasmodium* infection.
- Ferroportin Q248H mutation, G6PD deficiency and sickle cell anemia did not affect susceptibility to malaria in endemic area.

ABSTRACT

**Background:** Ferroportin (FPN) is known as an iron exporter and its effect on RBC iron could therefore hamper the growth of malaria parasites, since parasites are in need of iron. The aim of this study was to examine the prevalence of FPN Q248H in South Kivu/DRC and to evaluate its role in *Plasmodium* infected children and to explore its relationship with anemia.

**Materials and methods:** We conducted a cross-sectional study in the health zone of Miti Murhesa in South Kivu/DRC. 1088 children aged under five years were included. The FPN Q248H mutation was analyzed by PCR (*N* = 1071). Allele frequency was calculated based on Hardy-Weinberg equation. *Plasmodium* infection was assessed by LAMP malaria assay (*N* = 1057). Statistical analysis was done using Medcalc® software. *P*-values < 0.05 were considered significant.

**Results:** We found 11.4% FPN Q248H mutation. T allele frequency was estimated to be 0.0588 ± 0.0052. No significant differences for frequencies of anemia and malaria were observed between FPN Q248H mutation and FPN wild type. However, *Plasmodium* infected carriers of the FPN Q248H mutation had lower hemoglobin values than wild type children.

**Conclusion:** Even though FPN Q248H mutation is associated with lower hemoglobin values in *Plasmodium* infected children, it was not found to be protective against malaria and anemia in children under 5 years living in malaria endemic area of South Kivu/Democratic Republic of Congo.

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1. Introduction

Ferroportin (FPN), a highly conserved transmembrane protein encoded by SLC40A1 gene [1, 2, 3] has been reported to be expressed and to play a critical role in several different tissues involved in mammalian iron homeostasis, including duodenal enterocytes (iron uptake and export into circulation), hepatocytes (storage), syncytiotrophoblast (transfer to embryos) and reticuloendothelial macrophages (iron recycling from senescent red blood cells) [2]. FPN is the only known mammalian iron exporter and is essential for transport of iron from cells to blood. Zhang et al. have shown that FPN is an important cornerstone of systemic iron homeostasis that enables erythroid cells to return significant amount of iron directly into the blood [4]. The high abundance of FPN in red blood cells (RBCs) [5] and its effects on RBCs iron could therefore hamper the growth of malaria parasites, since malaria parasites require iron. It has been described that Plasmodium parasites may be able to access both intra-erythrocytic and serum iron for maturation. Increased labile iron was observed during parasite maturation which was consistent with increased iron demands of the parasite during trophozoite and schizont stage [6]. FPN has indeed been described to protect RBC against oxidative stress and malaria infection [5]. The SLC40A1 gene mutation c.744G>T; p (Gln248His), further referred to as Q248H is localized in exon 6. It has been identified at relatively high frequencies in populations of African ancestry e.g. in African-Americans [7] scoring allele frequencies between 6-26% [8, 9, 10, 11, 12, 13, 14, 15, 16, 17]. In South Kivu/DRC, FPN Q248H mutation has been found to have a prevalence between 12% and 14 % [10, 11]. The mutation results in a partial insensitivity of FPN to hepcidin [18, 19] leading to a continuous and increased iron export and eventually decreased intracellular iron levels. As a consequence, FPN Q248H mutation might confer an advantage against malaria [5] since the parasite will be more deprived from iron. On the one hand, FPN Q248H mutation might be expected to be associated with anemia due to its iron-expelling effect, on the other hand due to its protective effect against malaria, FPN Q248H mutation might show protection from malaria-associated anemia. Indeed, as demonstrated in a small group of Zambian children hospitalized for uncomplicated malaria, the FPN Q248H patients experienced less fulminant malaria with reduced parasitemia, additionally the mutated patients showed lower hemoglobin values than wild type patients [5]. Malaria remains a large public health problem. In 2018, an estimated 228 million cases of malaria occurred worldwide. Most of malaria cases (93%) were in the African region and the DRC is one of the most affected countries. Malaria causes 405,000 deaths each year and children aged under 5 years are the most vulnerable group, they accounted for 67% of all malaria deaths worldwide [20]. The protective effect of FPN Q248H in endemic setting is not clearly demonstrated. In the high endemic area of Zambia, Zhang et al. described a reduced episode of fulminant malaria with reduced parasitemia in FPN Q248H children [5], while Manake et al. in the low endemic area of Botswana, did not find any protective effect of FPN Q248H against malaria [21]. In other African countries no effect on the prevalence of malaria was found in FPN Q248H carriers compared to wild type children [15]. The effect of FPN Q248H mutation on anemia and malaria in children living in South Kivu/DRC where malaria is endemic and anemia is prevalent, is yet unexplored. The aim of this study was to examine the prevalence of FPN Q248H in South Kivu/DRC and to evaluate its role in Plasmodium malaria infection in a pediatric population and to explore its relationship with anemia.

2. Material and methods

2.1. Study location, study design and sample size

This cross-sectional study was conducted in the health zone of Mitu Murhesa in South Kivu province of Democratic Republic of Congo (DRC). Data collection has been described elsewhere [22, 23]. The sample size for this study was based on the proportion in a single cross-sectional survey, and the estimate of proportion was based on the prevalence of malaria in South Kivu, which was estimated to be 10% [24].

1088 children aged between 6 and 59 months, in good general health, were recruited. Details on inclusion criteria were described elsewhere [22].

2.2. Blood collection and laboratory tests

Blood samples were collected by venipuncture in an EDTA-tube and immediately stored in cooler boxes containing ice packs and transported to the reference laboratory of the Hospital of Bukavu. Hemoglobin (HB) was measured on the spot by finger prick using a Hemocue® analyzer (Hemocue® 301 + Angelholm, Sweden) and adjusted for altitude as proposed by Sullivan et al. [25]. Anemia was defined as Hb < 11 g/dl using WHO criteria [26]. For the detection of the FPN Q248H mutation, genomic DNA was isolated from whole blood using the NucleoMag Blood 200 µl kit (Macherey Nagel) on a Hamilton STARlet. A part of exon 6 of FPN was amplified using forward primer 5'-AGG AGA GAT CAT GTT CAG TT-3' and reverse primer 5'-CTA GGT TGT AAA GCT GGT CTT A-3'. Primers were used at final concentration of 300 nM in 20 µl total volume, using SsoFast EvaGreen supermix (Bio-Rad) and 2 µl of genomic DNA. PCR was performed on a CFX96 cycler (Bio-Rad), using the following protocol: 98 °C for 2 min, followed by 45 cycles of 98 °C for 5 s and 62 °C for 20 s. A melting curve was determined by heating the samples from 65 °C to 82 °C with 0.02 °C/s. Clustering of the samples was done using the Precision MelT Analysis software (Bio-Rad). 1071 children had results available for ferroportin with 17 missing data due to invalid results related to technical issues.

Plasmodium infection was assessed by loop-mediated isothermal amplification (LAMP) assay as described elsewhere [22]. 1057 children had results for LAMP malaria assay with 31 missing data (11 were due to invalid results due to technical issues and 20 due to lack of sample).

In a subgroup of children, Hb electrophoresis (n = 500) was performed for HbS detection and G6PD activity (n = 361) was measured using the fluorescent spot test (Path, formerly Program for Appropriate Technology in Health, Seattle, Washington, USA). For the latter a qualitative result is generated: normal, intermediate or deficient. Intermediate G6PD activity was considered as normal.

The prevalence of FPN Q248H and allele frequencies were calculated. Allele frequency was calculated based on Hardy-Weinberg equation. According to the number of children included, and the percentages scoring positive for malaria in the FPN wild type group, we calculated the power of the study envisaging a 50% reduction (with α = 0.05).

2.3. Statistical analysis

For statistical analysis, MedCalc® software, version 9.4.2.0 was used. Categorical variables are expressed as proportions or frequencies and continuous variables are expressed as mean ± SD. Pearson’s chi-square test was used to compare the proportions of FPN Q248H allele between children. The mean Hb concentration was compared using the Student’s t-test. Because carriers of sickle cell disease (sickle cell trait) and G6PD deficiency are associated with anemia and/or decreased malaria susceptibility in endemic areas, we evaluated the effect of sickle cell trait, G6PD deficiency and FPN Q248H mutation on malaria associated anemia in children. Frequencies were compared using the Pearson’s chi-square test. A p-value < 0.05 was considered significant.

2.4. Ethical consideration

Ethical approval was obtained by the Institutional Ethical committee of the Catholic University of Bukavu, UCB (Ref: UCB/CIE/NC/003/2017).
Table 1. Baseline characteristics of study population.

| Variables              | All, n | FPN Q248H | FPN wild type | p-value |
|------------------------|--------|-----------|---------------|---------|
| Age group (months)     |        |           |               |         |
| 6-23                   | 1071   | 122       | 949           |         |
| 24-59                  | 712    | 82        | 630           | 0.93    |
| Sex                    |        |           |               |         |
| Male                   | 537    | 60        | 477           | 0.89    |
| Female                 | 534    | 62        | 472           |         |
| LAMP malaria           |        |           |               |         |
| Positive               | 355    | 37        | 318           | 0.66    |
| Negative               | 685    | 79        | 606           |         |
| Anemia                 |        |           |               |         |
| Present                | 422    | 50        | 372           | 0.77    |
| Absent                 | 649    | 72        | 577           |         |

3. Results

The ferroportin Q248H mutation was found in 122 children out of 1071, giving a prevalence of 11.4% (11% heterozygous and 0.4% homozygous) with an allele frequency f(T) of 0.0588 ± 0.0052. Q248H status does not differ by age and sex (Table 1).

Table 2 shows the calculated FPN Q248H allele frequencies based on Hardy-Weinberg equation in Plasmodium infected and anemic children. No significant difference in T allele frequency in infected versus uninfected children was observed, nor was a difference found in T allele frequency between anemic versus non-anemic children. However, when we compared the Hb levels within the Plasmodium infected children, we found that FPN Q248H mutation was associated with lower Hb levels than the FPN wild type (p = 0.05) (Table 3).

We evaluated the effect of FPN Q248H mutation, sickle cell trait and G6PD deficiency in Plasmodium infected and uninfected children on the prevalence of anemia (Table 4). In Plasmodium infected children, no significant difference was observed in the prevalence of anemia between wild type and FPN mutated, nor in the group of uninfected. Finally, we did not find any association of G6PD deficiency or sickle cell trait with anemia, neither in the wild type, nor in the mutated group of children.

4. Discussion

We found a prevalence of FPN Q248H mutation of 11.4% (11% and 0.4%, respectively heterozygous and homozygous) in a group of 1071 children in South Kivu/DRC with an allele frequency of 0.0588 ± 0.0052. These scores are similar to results of previous studies performed in African and African-American populations [8, 9, 10, 11, 12, 13, 14, 15, 16, 17].

We did not find a significant difference for the prevalence of malaria between the FPN wild type and FPN Q248H children. Compared to a previous study (Manake et al.) (N = 264), our study included a larger number (N = 1071) of children, however the study of Muriuki et al. included even more subjects (2550 and 11982, respectively for children with mild and severe malaria). The conclusions of the latest study are similar to ours. They also concluded that there was no proof of a protective effect of FPN Q248H mutation against malaria in community-based cohort studies in Uganda, Gambia, Burkina Faso and Kenya [15]. In the low endemic setting of Botswana, Manake et al. demonstrated, in the cohort of 264 (183 uncomplicated Plasmodium falciparum and 81 controls), that the prevalence of FPN Q248H mutation was similar between patients with Plasmodium falciparum malaria and control subjects [21]. In contrast, De-Liang Zhang et al. found parasitemia to be significantly decreased in a cohort of 66 Zambian children under six years of age, carrying FPN Q248H who were hospitalized for uncomplicated P. falciparum malaria. Moreover, they experienced less fulminant malaria [5]. Anemia is a common and serious complication of Plasmodium infection in endemic areas. We described before that asymptomatic Plasmodium infection was significantly associated with anemia in our group of children under five years [22]. We now show that in our study population the FPN Q248H mutation is not associated with the frequency of anemia and it does not protect children against malaria-associated anemia. On the contrary, when comparing the quantitative Hb results, we found that the malaria-infected mutated group had lower Hb values than the infected wild type group of children. Results are conflicting about anemia in children with FPN Q248H mutation. Some authors demonstrated the absence of the protective effect of FPN polymorphism against anemia [5, 9, 15, 27], while others demonstrated the increase in Hb concentration in case of FPN Q248H mutation [13, 14, 15]. Muriuki et al. showed a modest protection of the mutation against anemia in a cohort of children from The Gambia, Burkina Faso, Uganda and Kenya, the Hb concentration was 0.22 g/dl higher in Q248H mutated children compared with WT, p = 0.036 [15]. The lower hemoglobin levels in children with malaria and FPN mutation may be the result of a reduced amount of iron available for hemoglobin synthesis due to hemolysis (malaria) and increased iron export (FPN mutation). However, contrasting that study, in our study FPN Q248H mutation did not protect children against malaria, and we also could not show a protective effect of FPN Q248H against malaria associated anemia in children under 5 years living in South Kivu/DRC. Our results are similar to the results obtained in the study performed by De-Liang Zhang et al. [5]. Due to the relationship of malaria and anemia with sickle cell trait and G6PD deficiency we evaluated the effect of sickle cell trait, G6PD deficiency on malaria associated anemia in a sub-group of children but we did not find any association.

Table 2. FPN Q248H allele frequencies were calculated based on Hardy-Weinberg equation in Plasmodium infected and anemic children in South Kivu,

| Variables                | G allele | T allele | Allele Frequency f(T) ± SD | p-value |
|--------------------------|----------|----------|---------------------------|---------|
| Plasmodium infected, n = 710 | 673      | 37       | 0.0521 ± 0.0086           | 0.49    |
| Plasmodium uninfected, n = 1370 | 1287    | 83       | 0.0606 ± 0.0066           |         |
| Overall, n = 2080         | 1960     | 120      | 0.0577 ± 0.0053           |         |
| Anemia present, n = 844   | 792      | 52       | 0.0661 ± 0.0085           | 0.73    |
| No anemia, n = 1298       | 1224     | 74       | 0.0570 ± 0.0066           |         |
| Overall, n = 2142         | 2016     | 126      | 0.0588 ± 0.0052           |         |

* Pearson’s chi square test was applied to compare the FPN Q248H allele among children. Hardy Weinberg equation was used to calculate allele frequencies.

Table 3. Mean Hb concentration according to FPN Q248H mutation.

| Variables             | FPN Q248H mean Hb±SD (N) | FPN wild type mean Hb±SD (N) | p-value |
|-----------------------|--------------------------|-------------------------------|---------|
| Overall               | 11.0 ± 1.7 (122)         | 11.1 ± 1.6 (949)             | 0.51    |
| Sex                   |                          |                               |         |
| Female                | 11.1 ± 1.6 (62)          | 11.2 ± 1.6 (472)             | 0.64    |
| Male                  | 10.8 ± 1.8 (60)          | 11.0 ± 1.6 (477)             | 0.36    |
| Age                   |                          |                               |         |
| 6-23                  | 10.5 ± 1.4 (40)          | 10.8 ± 1.3 (319)             | 0.17    |
| 24-59                 | 11.2 ± 1.8 (82)          | 11.2 ± 1.7 (630)             | 1.00    |
| LAMP malaria          |                          |                               |         |
| Positive              | 9.6 ± 1.8 (37)           | 10.2 ± 1.8 (318)             | 0.05    |
| Negative              | 11.5 ± 1.2 (79)          | 11.5 ± 1.3 (606)             | 1.00    |
Table 4. FPN Q248H mutation, sickle cell trait, G6PD deficiency and their association with anemia in Plasmodium infected and uninfected children.

| Variables                  | Plasmodium infected | p-value | Plasmodium uninfected |
|----------------------------|---------------------|---------|-----------------------|
|                            | All, n (%)          | Anemia, n (%) |                   |
| Ferroportin                | 355 (100)           | 215 (100)  | 685 (100)             |
| FPN wild type              | 318 (89.6)          | 188 (87.4) | 606 (88.5)            |
| FPN Q248H                  | 37 (10.4)           | 27 (12.6)  | 79 (11.5)             |
| G6PD activity              | 195 (100)           | 182 (100)  | 165 (100)             |
| G6PD deficiency            | 50 (25.6)           | 47 (25.8)  | 52 (31.5)             |
| G6PD normal                | 145 (74.4)          | 135 (74.2) | 113 (68.5)            |
| Hb electrophoresis         | 228 (100)           | 207 (100)  | 259 (100)             |
| Hb AS                      | 15 (5.7)            | 13 (6.3)   | 15 (5.8)              |
| Hb AA                      | 215 (94.3)          | 194 (93.7) | 244 (94.2)            |

5. Conclusion

The present study does not establish a protective effect or any other relationship between FPN Q248H and malaria in children under 5 years of age. Although the frequency of anemia was not correlated with the FPN Q248H mutation, in Plasmodium infected children lower hemoglobin levels were found in the presence of the mutation.

Declarations

Author contribution statement

Yvette Lufungulo Bahati: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Joris Delanghe, Jan Philipp: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ghislain Bismwa Balaluka: Analyzed and interpreted the data; Wrote the paper.

Karl Vandepeole, Antoine Sadiki Kishabongo: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Justin Gikomola Cirhuza: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

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