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Pyruvate Kinase and Fcγ Receptor Gene Copy Numbers Associated With Malaria Phenotypes

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Genetic factors are associated with susceptibility to many infectious diseases and may be determinants of clinical progression. Gene copy number variation (CNV) has been shown to be associated with phenotypes of numerous diseases, including malaria. We quantified gene copy numbers of the pyruvate kinase, liver, and red blood cell (*PKLR*) gene as well as of the Fcγ receptor 2A and Fcγ receptor 2C (*FCGR2A*, *FCGR2C*) and Fcγ receptor 3 (*FCGR3*) genes using real-time quantitative polymerase chain reaction (RT-qPCR) assays in Gabonese children with severe (n = 184) or and mild (n = 189) malaria and in healthy Gabonese and white individuals (n = 76 each). The means of *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* copy numbers were significantly higher among children with severe malaria compared to those with mild malaria ($P < .002$), indicating that a surplus of copies of those genes is significantly associated with malaria severity. Copy numbers of the *FCGR2A* and *FCGR2C* genes were significantly lower ($P = .005$) in Gabonese individuals compared with white individuals. In conclusion, CNV of the *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* genes is associated with malaria severity, and our results provide evidence for a role of CNV in host responses to malaria.

Keywords. malaria; *PKLR*; *FCGR2A*; *FCGR2C*; *FCGR3*.

Clinical phenotypes of malaria are heterogeneous, including asymptomatic infection, fever, mild and severe malaria with anemia, and cerebral malaria [1]. The course of infection can vary significantly between individuals, with many determinants of this variability remaining to be identified [2]. Many human genetic factors have been proposed to provide relative protection from malaria, and genome-wide interpopulation variation has been associated with resistance or susceptibility to malaria [3]. Approximately one-quarter of the total variability in malaria incidence is accounted for by genetic factors [4]. Any contribution of copy number variation (CNV) to the clinical phenotype of infectious disease is of interest [5]. In particular, a pathogenic potential of CNV has been claimed since associations of DNA duplication with Charcot-Marie-Tooth disease and with hereditary neuropathy with liability to pressure palsies were recognized [6, 7]. In infectious diseases, a lower *CCL3L1* (C-C motif chemokine ligand 3-like 1) gene copy number enhances susceptibility to human immunodeficiency virus (HIV)/AIDS [8], and *CCL3L1* CNV was shown to be associated with susceptibility to tuberculosis and malaria [9, 10].

Associations of human erythrocyte disorders including enzyme deficiencies with resistance to malaria are well established [11]. Splice variants and frame shift mutations cause pyruvate kinase (PK) deficiency if the mutations occur on both chromosomes, and are associated with protection against invasion and maturation of *Plasmodium falciparum* in vitro [12]. In particular, a study has shown that the 1529A mutation in the pyruvate kinase, liver, and red blood cell gene (*PKLR*) protects against infection with *P. falciparum*, the causative agent of the severe form of human malaria [13]. The human *PKLR* gene is located on chromosome 1q21 (OMIM 609712) and encodes the enzyme through use of different transcription units with 2 promoters, which are mutually incompatible [14]. The tissue-specific isoenzyme L-PK (liver PK) is expressed in the liver, small intestine, and renal cortex, whereas the R-PK (red blood cell PK) is expressed exclusively in erythrocytes [15]. The *PKLR* gene has been shown to occur in variable copy numbers in the human genome [16]. However, the role of *PKLR* CNV in malaria is yet unclear.

Human low-affinity immunoglobulin gamma Fc region receptors II and III (FcγII and FcγIII) are glycoproteins binding the Fc fragment of IgGs and interacting with polyvalent immune complexes expressed on different effector cells. FcγII and FcγIII are encoded by *FCGR2* (Fcγ receptor 2) and *FCGR3* genes, respectively, which are located on chromosome 1q23-24 [5, 17]. Two copies of the *FCGR3* gene (*FCGR3A* and *FCGR3B*) and 3 copies of the *FCGR2* gene (*FCGR2A*, *FCGR2B*, and *FCGR2C*) exist [5]. Fcγ receptors have broad biological activities, including clearing of antigen–antibody immune complexes and enhancement of phagocytosis and antigen presentation [18]. These activities confer an important role to Fcγ receptors in immune regulation and responses in infectious diseases. *FCGR2A* gene polymorphisms were shown to be associated

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with an increased risk of recurrent respiratory tract infections in Turkish children [19] and with disease severity in patients with meningitis caused by *Neisseria meningitidis* [20]. Recent findings reveal an association of *FCGR2* and *FCGR3* CNV with several autoimmune conditions [21–23].

In malaria, *FCGR2A* and *FCGR3B* gene polymorphisms were associated with increased susceptibility to cerebral falciparum malaria [24, 25], and *PKLR* single-nucleotide polymorphisms (SNPs) and haplotypes have been shown to be associated with malaria in a Thai population [26]. So far, no associations of CNV in the human *PKLR*, *FCGR2*, and *FCGR3* genes with clinical progression of malaria have been identified. Here, we studied the association of CNV in *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* genes with malaria severity in Gabonese patients with either severe or mild malaria.

MATERIALS AND METHODS

Study Subjects and Sampling

A total of 373 children with either severe (n = 184) or mild malaria (n = 189) presenting at the Albert Schweitzer Hospital, Lambaréné, Gabon, and the Centre Hospitalier de Libreville, Gabon were recruited [27, 28]. Severe malaria was defined as *P. falciparum* hyperparasitemia (>250 000 parasites/μL) with severe anemia (hemoglobin <50 g/L), a Blantyre coma score ≤2, hypoglycemia (glucose ≤2.2 mM), and hyperlactatemia (lactate >5 mM). Mild malaria was defined as parasitemia of 1000–50 000/μL on admission, no schizontemia, circulating leukocytes containing malarial pigment <50/μL, not homozygous for hemoglobin (Hb) S, Hb >80 g/L, platelets >50/nL, leukocytes <12/nL, lactate <3 mM, and blood glucose >50 mg/dL [29]. Exclusion criteria were signs of severe malaria or other acute infections and prior hospital admission for any reason, to exclude possible previous severe malaria as well as intake of antimalarial drugs during the preceding week. Chronic diseases and malnutrition were also exclusion criteria. Mild and severe cases were matched for gender, age and similar residence. In addition, 76 Gabonese and 76 white individuals were included and blood was taken from 35 healthy German adults for measurement of PK activities.

The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital and the ethics committee of the Medical Faculty, University of Tübingen. Informed written consent was obtained from the parents/guardians of all children and from adult participants.

Estimation of Gene Copy Number Variation

The estimation of *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* CNV was performed using duplex real-time quantitative PCR (RT-qPCR) assays. The albumin gene *ALB* was used as reference gene for a single copy gene. The *FCGR3A* and *FCGR3B* genes share approximately 98% sequence identity and the *FCGR2A* gene shares the last exon (exon 7) with *FCGR2C* [5, 30]. Therefore, the primers and probes for *FCGR2* can quantify both *FCGR2A* and *FCGR2C*, whereas the primers and probes for *FCGR3* can quantify both *FCGR3A* and *FCGR3B*. The TaqMan primers and probes used for CNV estimation of those genes are given in Supplementary Table 1.

The duplex amplification reaction mixture was performed in a volume of 25 μL. Every reaction volume contained 12.5 μL of the 2x QuantiTect Multiplex PCR NoRox Master Mix (Qiagen, Hilden, Germany), 0.2 μM of the TaqMan probes of the reference (*ALB*) and the target genes *PKLR*, *FCGR2A*, *FCGR2C*, *FCGR3A*, and *FCGR3B*; 0.4 μM of forward and reverse primers for the reference and target genes; 100 ng of DNA in 3 μL of RNase-free water (Qiagen, Hilden, Germany). All reactions were run in duplicate. In addition, a no-template control sample and a calibrator were incorporated into each run. Thermal cycling conditions were as follows: initial PCR activation step of 15 minutes at 95°C, followed by 35 cycles of 2-step cycling (denaturation 60 seconds at 94°C, annealing/extension 15 seconds at 60°C). The RT-qPCR was performed using the Rotor Gene 3000 and data evaluation was performed using the Rotor-Gene 6.1.81 software version (Corbett Robotics, Sydney, Australia).

PKLR, *FCGR2A*, *FCGR2C*, and *FCGR3* copy numbers were quantified using the comparative cycle threshold ($C_{T)}$ method (ΔΔCt) [31], which requires a calibrator. The construction of our plasmid DNA calibrator for quantification of copy number variants was as follows: RT-qPCR product of *ALB* and target genes *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* cloned into the pCRII-TOPO 4 Kb vector according to the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany). Plasmid DNA isolation and purification were performed using the Qiagen DNA isolation kit (Qiagen, Hilden, Germany). Plasmids containing single inserts of *ALB*, *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* fragments were identified through DNA sequencing (BigDye Terminator version 1.1 Cycle Sequencing Kit on an automated sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, California). Analysis of the insert sequences were done with the BioEdit sequence Alignment Editor Software (North Carolina State University, Raleigh). The sequences were blasted using the online nucleotide blast service of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/).

After spectrophotometric assessment of plasmid DNA concentrations, the plasmid copies were calculated according to the QuantiTect Multiplex PCR NoRox kit (Qiagen, Hilden, Germany) using the formula: (Xg/μL DNA/ [plasmid length in base pairs × 660]) × 6.022 × 10^{23} = Y molecules/μL. A 1:1 plasmid construct mixture including equal *ALB* and *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* copies was diluted in 1:10 series. The 1:10^4 dilution was chosen as the calibrator, as it was similarly amplified in comparison with co-amplified genomic samples. To validate the plasmid calibrator mix, 3 plasmid construct mixes were made with different ratios (1:1, 1:2 and 1:3),

keeping the amount of the *ALB* gene constant, while amounts of the *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* genes were varied. The 4-dilution series $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$ was measured.

Pyruvate Kinase Enzyme Activity

Pyruvate kinase enzyme activities were measured in whole blood from 35 volunteers by a commercial biochemistry laboratory (Labor Prof. Seelig and Kollegen, Karlsruhe).

Statistical Analyses

For 2-group comparisons, a *t* test or Mann–Whitney *U* test was used to compare mean or median where appropriate. The variance of analysis was considered for the correlation analysis. All statistical data analyses were conducted using the software JMP 5.0.1 (http://www.jmp.com/en_us/software/jmp.html). The level of significance was set at a *P* value of <.05. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated using Stata 9.2 software (http://www.stata.com/).

RESULTS

Validation of the Comparative C_T Method

To use the comparative C_T method, a validation experiment must indicate that both target and reference genes have equal efficiencies of amplification. If the log of the initial DNA quantity is plotted against the ΔC_T value, the slope of the graph should be in between −0.1 and 0.1 [31]. Therefore, we examined this correlation in different individual genomic DNA samples by creating validation curves of comparative C_T method for the *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* genes (Supplementary Figure 1*A*, 1*C*, and 1*E*). The curve slope is −0.035, 0.006, and 0.01 for the *PKLR*, *FCGR2A-FCGR2C*, and *FCGR3* quantification, respectively. These results indicate that the amplification efficiency of the reference gene *ALB* and the target genes *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* are well comparable. Validation of the plasmid construct mix was conducted using a genomic DNA sample. When the ratios (reference/target 1:3, 1:2, and 1:1) were plotted against the gene copy numbers in a genomic DNA sample, a high correlation ($R^2 > 0.99$) was observed, where the numbers of *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* copies was relatively decreased according to the given ratios (1:3, 1:2, and 1:1), respectively (Supplementary Figure 1*B*, 1*D*, and 1*F*).

PKLR CNV and Malaria Severity

PKLR CNV was determined and compared in a total of 373 malaria patients with either severe (n = 184) or mild (n = 189) malaria. The results showed that the number of *PKLR* copies in severe malaria cases was significantly higher compared to that observed in mild malaria cases ($P < .0001$) (Figure 1A). Looking at rounded CNV, the comparison clearly indicates that the severe cases had significantly higher *PKLR* gene copies (mean, 2.6 ± 0.5; median, 3) compared with mild cases (mean, 2.4 ± 0.5; median, 2) ($P < .0001$). When unrounded *PKLR* CNV was compared, the *PKLR* copies in the severe cases were also significantly higher (mean, 2.6 ± 0.4; median, 2.65) in comparison to those in mild cases (mean, 2.5 ± 0.3; median, 2.45) ($P = .002$). Stratification of patients according to *PKLR* CNV in groups with 2, 3, or 4 copies showed a difference in distribution of gene copy numbers (Figure 1B). Severe malaria patients significantly had 3 or 4 copies than severe malaria patients with 2 copies only. Having >2 copies of *PKLR* conveys a 2.4-fold higher risk of severe malaria (OR, 2.4 [95% CI, 1.5–3.7]; $P = .0001$).

We next examined the correlation of *PKLR* CNV with PK enzyme activities in 35 volunteers and observed that the rounded number of *PKLR* copies was not significantly correlated with enzyme activity ($R^2 = 0.06$; $P > .05$) (Figure 1C). Similarly, the unrounded *PKLR* copy number also was not correlated with enzyme activities in the volunteers ($R^2 = 0.04$; $P > .05$).

FCGR2A and *FCGR2C* CNV and Malaria Severity

FCGR2A and *FCGR2C* CNV was quantified in patients with mild (n = 125) or severe (n = 102) malaria. The comparison revealed that severe cases had significantly higher copies compared with those of patients with mild malaria (mean, 5.3 ± 0.9 vs 4.3 ± 0.8 copies; $P < .0001$) (Figure 2A). Rounding of copy numbers did not influence statistical significance (median, 4 vs 5; $P < .0001$). With regard to the malaria phenotype, we observed a difference in distribution among patients with respect to *FCGR2A* and *FCGR2C* copy numbers (Figure 2B). We grouped patients according to *FCGR2A* and *FCGR2C* CNV and observed that individuals with <5 copies of each gene had a reduced risk to develop severe malaria (OR, 0.16 [95% CI, .08–.3]; $P < .0001$). In contrast, individuals bearing >5 *FCGR2A* and *FCGR2C* gene copies had a 20-fold higher risk to develop severe malaria (OR, 20 [95% CI, 7–81]; $P < .0001$). In addition, *FCGR2A* and *FCGR2C* CNV was measured in each of 76 healthy Gabonese and European individuals, indicating that the gene copy number among Europeans was significantly higher compared to that in Gabonese individuals (rounded median, 6 vs 5 copies; $P = .005$) (Figure 2C).

FCGR3 CNV and Malaria Severity

FCGR3 CNV was quantified in patients with mild (n = 125) or severe (n = 102) malaria. Severe cases had a higher gene copy number compared with mild cases (mean, 2.8 ± 0.7 vs 2.3 ± 0.6; $P < .0001$) (Figure 3A). We had a similar observation when gene copy numbers were rounded (median, 2 vs 3; $P < .0001$). In addition, a difference in distribution among patients with respect to *FCGR3* gene copy numbers was observed (Figure 3B). We grouped patients according to *FCGR3* CNV and observed that the risk of severe malaria was higher with ≥3 *FCGR3* copies (OR, 4 [95% CI, 2–7]; $P < .0001$). In addition, *FCGR3* CNV was quantified in the 76 Gabonese and 76 European individuals. The gene copy number of *FCGR3* in Gabonese individuals did not differ significantly between white and Gabonese individuals

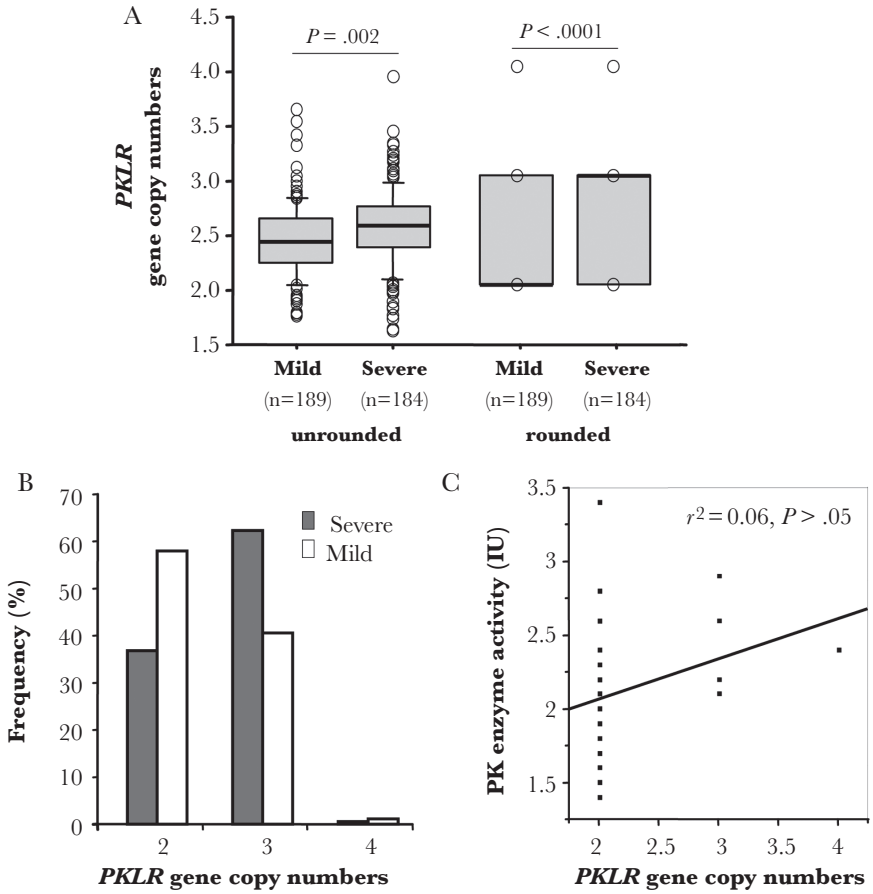

Figure 1. Distribution of *PKLR* gene copy number in malaria patients. *A*, Distribution of *PKLR* copy numbers in patients with severe (n = 184) or mild (n = 189) malaria. *B*, Distribution of *PKLR* copy number variation (CNV) groups (2, 3, and 4 copies) in severe and mild cases. *C*, Correlation of *PKLR* CNV with the pyruvate kinase (PK) enzyme activity units per gram hemoglobin (U/gHb[erythrocytes]) in 35 healthy volunteers. The plots depict 10th, 25th, 50th, 75th, and 90th percentiles and the circles represent outlying values, below and above 10th and 90th percentiles, respectively. *P* values were calculated by using Student *t* test to compare mean between groups.

(mean, 2.4 ± 0.5 vs 2.2 ± 0.4; P = .06). A similar result was observed when gene copy numbers were rounded (median, 2 vs 2) (P > .05; Figure 3C).

DISCUSSION

Genomic imbalances such as copy number variation (CNV) can contribute to the shaping of human disease phenotypes [32]. Many diseases including distinct forms of cancer, lupus glomerulonephritis, and HIV type 1 infection have been found to be associated with CNV [21, 33]. Here, we quantified copy numbers for the *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* genes and show that the CNV of those genes is associated with the risk of severe malaria. We also observed a significant difference of *FCGR2A*, *FCGR2C*, and *FCGR3* CNV between samples of Gabonese and European individuals.

Genomic imbalances in the *PKLR* gene locus are inconsistently reported in the literature. A first CNV map catalogued 5 gains of the *PKLR* gene in Nigerians [34] and a CNV region (CNVR 370.1) has been defined, including the *PKLR* region [35]. The impact of host genetic determinants, including CNV, on malaria has been extensively documented [11]. The present study illustrates that *PKLR* gene dosages are significantly associated with the malaria outcome, and a surplus of gene copies seems to be a susceptibility factor for malaria. Other studies have shown that PK deficiency is associated with protection against malaria [13, 36]. Although the correlation of the *PKLR* CNV with PK enzyme activities was weak only, our results point to the same direction, namely, that less enzyme activity contributes to protection against severe malaria. *PKLR* is located in the CNVR 370.1 [35]. Other genes than *PKLR*—for example, the secretory carrier membrane protein 3 (*SCAMP3*), a potassium channel (*HCN3*), a CDC-like kinase (*CLK2*), or a hypothetical protein, which are all located on the same CNVR—might be responsible for increased susceptibility caused by multiple copies [35].

Although an association of *PKLR* polymorphisms with malaria has been shown in a Thai population [26], information on polymorphisms in African populations is sparse. So far, 192 *PKLR* mutations associated with PK-deficient function have been reported, with few variants only identified in sub-Saharan Africa [12, 13, 37]. The short tandem repeat and SNPs in *PKLR*

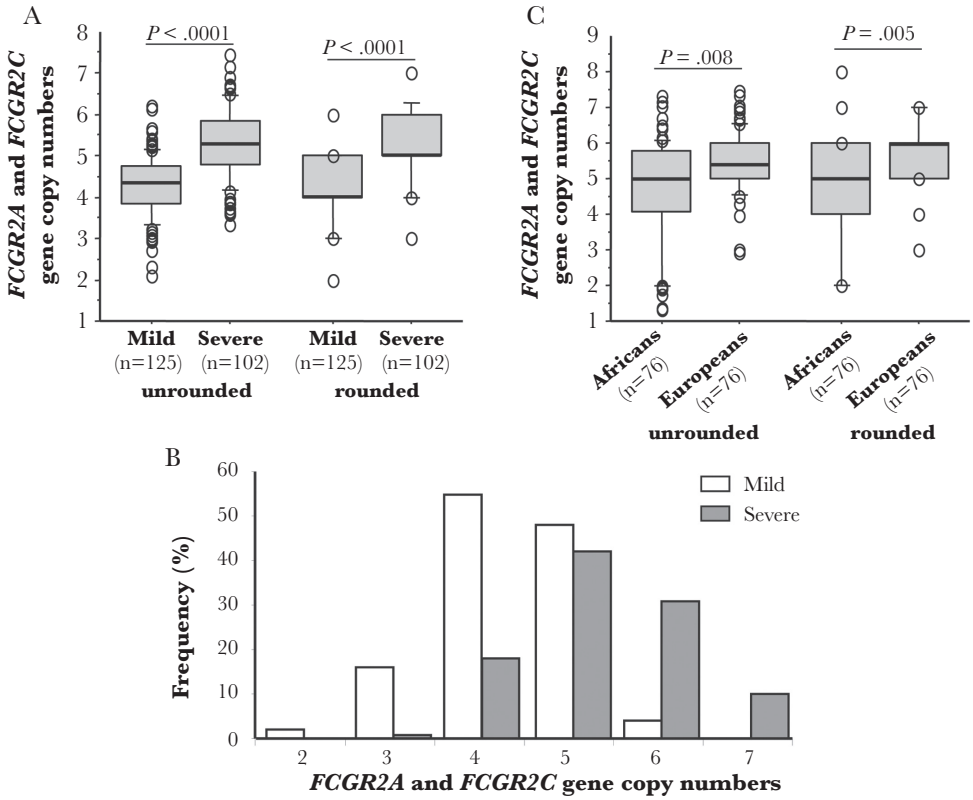

Figure 2. Distribution of *FCGR2A* and *FCGR2C* gene copy numbers in malaria patients. *A*, Distribution of *FCGR2A* and *FCGR2C* copy number variations (CNVs) in the patients presenting with severe (n = 125) or mild (n = 102) malaria. *B*, Distribution of groups (2, 3, 4, 5, 6, and 7 copies) with different *FCGR2A* and *FCGR2C* gene copies in mild and severe malaria patients. *C*, *FCGR2A* and *FCGR2C* CNV distribution in Gabonese (n = 76) and white (n = 76) individuals. The plots depict 10th, 25th, 50th, 75th, and 90th percentiles and the circles represent outlying values, below and above 10th and 90th percentiles, respectively. *P* values were calculated by using Student *t* test to compare means between groups.

gene have been found associated with malaria selective pressure in sub-Saharan African populations [38]. However, the 4 mutations 269T > A, 1456C > T, 1705A > C, and the T10/19 repeat are not associated with malaria [38]. Moreover, a frequent missense mutation (G829A; Glu277Lys) was identified to relate to PK deficiency in sub-Saharan Africa; however, this mutation was not associated with malaria infection and outcome [39]. Although PK-deficient individuals have serious disadvantages such as hemolytic anemia, hemochromatosis, splenomegaly, and others [40], we would, however, not expect to identify deleterious polymorphisms in a small sample size.

Similar to *PKLR* CNV, we also observed that patients with severe malaria had higher *FCGR2A*, *FCGR2C*, and *FCGR3* copy numbers compared to those presenting with mild malaria. This observation is in line with another study showing that having more *FCGR2C* copies is associated with idiopathic thrombocytopenic purpura [41]. The functional variants of *FCGR2A* have been associated with severe malaria, including anemia and cerebral complications [42, 43]. An additional study indicates an association of *FCGR2A* in combination with *FCGR3B* variants with malaria only, without stratifying for distinct phenotypes of malaria [24]. *FCGR3* CNV predisposes to glomerulonephritis, rheumatoid arthritis, and ankylosing spondylitis [21–23]. *FCGR3* CNV influences protein expression and the functionality of FcγIII [44], which may contribute to severe courses of malaria. Higher *FCGR* copy numbers are also associated with inflammatory disorders that result in excessive leukocyte proliferation and an overstimulated immune response [45]. Furthermore, expression and activation of Fcγ receptors may cause release of proinflammatory mediators [46].

The role of FcγRII and FcγRIII on monocytes has been shown in antibody-dependent cellular cytotoxicity (ADCC) leading to the death of malaria parasites [47]. Blockage of FcγRII and FcγRIII abolished ADCC completely. Antibodies binding to FcγRII and FcγRIII and directed against a putative vaccine candidate can trigger ADCC effectively in low concentrations. Fcγ may play a similar role in natural killer (NK) cells and a surplus of receptors on the cell surface may lead to deleterious activation of NK cells, aggravating the symptoms of malaria. NK cells can aggravate autoimmune and infectious diseases [48]. Given the importance of FcγR in antibody-dependent cellular inhibition and its proposed correlation with amount and functionality of FcγR [44, 49], CNV in the *FCGR* cluster may be a well-balanced system to prevent excessive immune

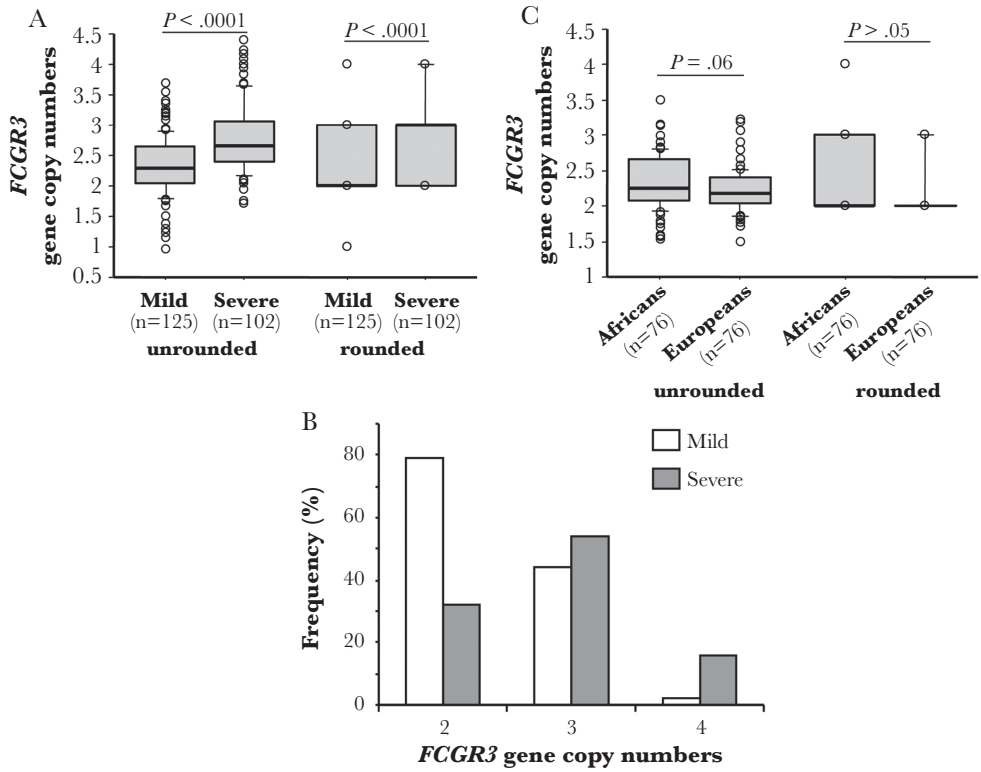

Figure 3. Distribution of *FCGR3* gene copy numbers in malaria patients. *A*, Distribution of *FCGR3* copy number variation (CNV) in patients with severe (n = 125) or mild (n = 102) malaria. *B*, Distribution of groups (2, 3, and 4 copies) with different *FCGR3* copy numbers in mild and severe malaria patients. *C*, *FCGR3* CNV distribution in Gabonese (n = 76) and white (n = 76) individuals. The plots depict 10th, 25th, 50th, 75th, and 90th percentiles and the circles represent outlying values, below and above 10th and 90th percentiles, respectively. *P* values were calculated by using Student *t* test to compare means between groups.

responses by restricting the number of receptors on the cell surface. In addition, the FcRγ-chain has been demonstrated in an animal model to play an important role in predisposition to *Plasmodium berghei* infection through antibody-dependent cell-mediated phagocytosis [50].

In conclusion, we quantified *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* gene copy numbers in Gabonese children with severe and mild malaria. The increased dose of these genes is significantly associated with the risk of severe malaria. The effect of *PKLR*, *FCGR2A*, *FCGR2C*, and *FCGR3* genes on the clinical outcome of malaria may, therefore, be mediated through a gene dose-dependent manner. Additional studies are needed to clarify the role of CNV of these genes in host immune responses against *P. falciparum* infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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