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Coinheritance of polymorphic alleles of *PIEZO1*, *G6PD* and *HBB* enhances protection against malaria

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ABSTRACT

Malaria has exerted potent selective pressure on the human genome over millennia and has been a significant force in shaping human evolution. We have determined in 424 individuals living in malaria-hyperendemic areas in Ghana and in the Democratic Republic of Congo, the genotypes at the loci *PIEZO1*, *G6PD*, *HBB*, and *PKLR*. By qPCR we have also estimated *P. falciparum* parasitemia in all these subjects. We found that 41% of individuals tested had one protective variant, 20.5% two variants, 6.4% three variants, and 0.7% four variants. We have confirmed that *Pz_E756del*, *G6pd A-*, and *HbS* are associated with lower parasite density. The highest allele frequency was that of *Pz_E756del*, approaching 0.2, and we found that it is in linkage disequilibrium with *Pz_E750Q*. While overall malaria prevalence did not differ significantly among the groups, non-pregnant individuals with multiple protective alleles had lower rates of high-density parasitaemia, suggesting an additive effect of these variants against severe malaria infection, while pregnancy showed different allele protection profile. The high frequency of individuals carrying two or more protective polymorphisms might have implications for malaria transmission and parasite reservoir maintenance. Thus, the significance of additive or possibly synergistic effects of multiple protective genes co-existing in the same person deserves further investigation.

1. Introduction

Malaria, caused by parasites of the genus *Plasmodium*, is a vector-borne disease believed to have caused half of the deaths of all humans who have ever lived [1,2]. In 1949 J B S Haldane suggested that the high frequency of β-thalassaemia in Mediterranean regions might be explained by heterozygotes being more resistant to malaria: this was the first formulation of the so-called "malaria hypothesis" [3]. Since then, a large body of evidence has confirmed this hypothesis, and it has also led to the identification of several other polymorphic genes expressed in red cells that confer some degree of protection against malaria. These polymorphisms appear to have been subjected to positive selection over hundreds of generations: to the extent that malaria is regarded as having played a significant role in shaping human evolution [2,4,5].

Abbreviations: (DRC), Democratic Republic of Congo; (dNTPs), deoxynucleotide triphosphates; (DBS), dried blood spot; (DSS), dried serum spot; (*G6PD*), glucose-6-phosphate dehydrogenase; (Hb), haemoglobin; (*HbB*), Hb β-chain gene; (IPTp), intermittent prophylactic treatment; (LOD), limit of detection; (MM), microscopic malaria; (NI), non-infected; (OR), odds ratio; (PG), pregnancy group; (*PKLR*), pyruvate kinase L/R gene; (RFLP), restriction fragment length polymorphism; (*HbAS*), sickle cell trait; (SM), submicroscopic malaria; (SP), sulfadoxine–pyrimethamine..

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Hereditary disorders in haemoglobin (Hb) structure or rate of production are some of the genetic alterations that have the greatest known protective action against malaria. Such haemoglobinopathies include the structurally distinct forms of Hb (*HbS* or *HbC*) and thalassaemias, in which there is a decreased production of either the α- or the β-chains of Hb [6]. *HbS*, caused by the substitution of glutamic acid for valine (Glu6Val) in the Hb β-chain gene (*HBB*) [7] results in the formation of sickle-shaped erythrocytes upon deoxygenation [8]: HbA/S heterozygotes, who are haematologically asymptomatic, have protection against severe malaria [9–11] due to a direct effect on parasite growth and survival [12–14], boosting both adaptive [15] and acquired immune response [16,17]. A collateral damage of positive selection of heterozygotes is that the frequency of the HBB^{E6V} allele becomes high in the population, resulting in the birth of homozygous *HbS* individuals (*HbSS*), who suffer from sickle cell disease with severe clinical manifestations, resulting in drastically reduced life expectancy [18]. This is a classic example of a balanced polymorphism, whereby heterozygotes are at an advantage in malaria endemic regions, at the expense of an increased prevalence of a severe congenital condition. *HbC*, in turn, produced by a glutamate-to-lysine mutation in the same codon as *HbS* (Glu6Lys) [19] is mainly distributed in West Africa [20] and is associated with protection against severe malaria [21–23], mainly in homozygotes [22], through impaired export of the PfEMP1 protein [24,25], a virulence factor essential for parasite reproduction and immune evasion.

The red cell enzymopathy most widespread in the world is the X-linked glucose-6-phosphate dehydrogenase (G6PD) deficiency, a condition affecting more than 400 million people [26]. The enzyme encoded by *G6PD* is the rate-limiting step of the pentose phosphate pathway, catalysing the production of NADPH, central to the erythrocyte's antioxidant defence [27]. Currently, more than 200 mutations in the *G6PD* gene are known, most compromising enzyme stability and/or decreasing its catalytic activity [28]. Numerous studies have shown that females heterozygous for G6PD-deficiency have a selective advantage against severe malaria [29–32], a notion further supported by the fact that independently arisen polymorphic variants of *G6PD* have high prevalence in malaria-endemic regions [33]: an example of convergent evolution. *G6pd A-* variant, the most prevalent in African populations [34,35], is caused by two mutations in tandem: the Asn126Asp mutation, which alone causes the *G6pd A* variant but does not cause enzyme deficiency, plus the Val68Met mutation: the two mutations together produce the G6PD deficient phenotype [35]. The main mechanism of action responsible for its protective effect lies in the enhanced susceptibility to oxidative stress of infected G6PD-deficient erythrocytes, resulting in increased early phagocytosis [36,37].

The gene *PIEZO1* encodes a non-selective mechanosensitive cation channel [38] playing an essential role in erythrocyte volume homeostasis, the disruption of which results in erythrocyte lysis and anaemia [39]. Some variants of *PIEZO1* are known to cause congenital xerocytosis (ORPHA:3202) [40], a rare autosomal dominant disorder eliciting haemolytic anaemia [41]. Since 2018, new *PIEZO1* variants have been found to confer protection against malaria [42]: specifically, the *Pz_E756del* variant, produced by in-frame single codon deletion, protects against severe human malaria [43] by a mechanism still unknown; in a mouse model of cerebral malaria, this gene impairs *P. berghei* growth, diminishing blood-brain barrier dysfunction and mortality [42]. It has been hypothesized that in humans a gain of function of the Piezo1 ion channel [42,44,45] may impair the export of virulence factors, such as the membrane protein PfEMP-1 [43]; or an iron overload, as observed in *Pz_E756del* heterozygotes [46].

Other molecular variants regarded as protective against malaria include those of the pyruvate kinase gene (*PKLR*) (ORPHA:766) [47–49]. Pyruvate kinase (PK) is a key glycolytic enzyme that generates ATP, essential for the maintenance of red blood cell structure and function [50]. Deficiency of this enzyme caused by mutations [51] causes inherited haemolytic anaemia, a common and heterogenous disorder requiring regular transfusions [47]. Evidence that PK deficiency protects against malaria has been found in animal models of *P. chabaudi* infection [52] and in *P falciparum* cultures in PK-deficient erythrocytes [48,49]. Protection against malaria in human populations is not yet demonstrated. Nevertheless, the prevalence of this disorder in African populations [51] together with evidence of resistance in non-human models makes some genetic variants of *PKLR* candidates for a protective effect against human malaria.

This study has been prompted by the idea that certain alleles at different genetic loci may have additive or synergistic effects in malaria protection. Previous studies have demonstrated that the protective effects of classical malaria-resistance polymorphisms, such as HbAS and α-thalassaemia, can be modulated by epistatic interactions between co-inherited variants, sometimes diminishing [53] or alternatively enhancing [54] protection against severe malaria. While these earlier studies primarily focused on interactions between specific pairs of well-known variants, our investigation aimed to broaden this perspective by simultaneously analysing the combined effect of a larger set of polymorphisms -including recently described loci such as PIEZO1- within the same population framework and in relation to *P. falciparum* parasitaemia levels. To test this hypothesis, we have conducted our study in two different African populations, both living in highly endemic malaria areas. The choice of the loci analysed was based on three main criteria: (i) existing evidence of protection against falciparum malaria, particularly in relation to parasitaemia control and mild clinical phenotypes; (ii) potential functional mechanisms involving single amino acid substitutions directly affecting erythrocyte physiology or parasite viability; and (iii) genetic simplicity (biallelic or limited variant sites), allowing reliable integration into models assessing additive or synergistic effects. In this respect, we focused primarily on the PIEZO1, G6PD, HBB, and PKLR loci, which not only fulfil these criteria but also represent a combination of classical and newly proposed mechanisms of malaria protection.

2. Material and methods

2.1. Clinical samples

The human samples used in this study were collected in two regions of high malaria transmission in Ghana and the Democratic Republic of Congo (DRC). Samples from Ghana were collected at Our Lady of Grace Hospital in Breman-Asikuma, a rural town located in the central region of Ghana categorised as having high year-round malaria transmission and where 100% of reported malaria infections are caused by *P. falciparum* [55,56]. Samples were collected in May 2017 during the rainy season when malaria parasite transmission peaks. A total of 200 volunteer donors were recruited. Due to the shortage of sample volume in five of the participants, 200 blood and 195 plasma samples were collected in Ghana. Since preservation of blood or plasma in dry format allows for transport and storage of samples at room temperature and has been shown to be a suitable sample type for subsequent DNA or protein extraction [57–59], samples were preserved in dried blood spot (DBS) or dried serum spot (DSS) format. From each individual, 2 mL of peripheral blood was collected by venepuncture and 600 μL were preserved as dried blood, which was arranged as 12 x 50 μL DBS on Whatman FTA classic filter paper cards (GE Healthcare). Plasma was obtained by centrifugating the remaining blood, so DSS were generated in an identical manner. All cards were air dried for 12 hours and stored at room temperature individually in desiccant sealed bags.

A total of 224 blood samples were collected in DRC using the same procedure as for the Ghana samples. These samples were collected in March 2019 from five hospitals or dispensaries in Mbuji-Mayi (Dipumba General Hospital, Institut Superieur de Technique Medical, Kansele General Hospital, Centre Hospitalier Notre-Dame and Centre Hospitalier Kitenge), a city located in south-central DRC where malaria transmission is high with 100% of cases caused by *P. falciparum* [56].

Donor selection in Ghana and DRC followed the same criteria. Any

patient attending the hospital was considered eligible, regardless of age, sex, or reason for consultation. However, the patient questionnaire allowed to exclude as donors those individuals who had taken antimalarial drugs as treatment for malaria fever in the month prior to the visit to avoid an indirect effect on the parasite load at the time of sampling. Only in obstetric check-ups appointments, donors were accepted with intermittent prophylactic treatment (IPTp) sulfadoxine–pyrimethamine (SP) as adopted by health policies. In this context it should be noted that both in Ghana and in the DRC malaria prophylaxis for pregnant women remains nationwide policy, and Breman-Asikuma and Mbujimayi were not an exception at the sampling time. As per the policy, pregnant women were expected to receive 3 or 4 doses of SP during pregnancy depending on when they first reported to antenatal clinics (at weeks: 12-16; 24-28; 32; and 36).

This study was approved by the Ethical Review Board at Research Institute Hospital 12 de Octubre, Madrid (Spain); the Ethical and Protocol Review Committee of University of Ghana's College of Health Sciences (Ghana) for the samples taken in Breman-Asikuma; and the Ethical Review Committee of University of Mbujimayi (DRC) for the samples taken in Mbujimayi. The study was conducted according to the guidelines laid down by the Helsinki Declaration, with written informed consent obtained for each adult participant, or in the case of children a parent or guardian of the child participant provided written informed consent on their behalf or child assent. The samples were specifically obtained for this study including infection status and analysis of polymorphisms in connection with malaria immunity that has been partially published [55]. Volunteer information was anonymized prior to analysis.

2.2. Malaria diagnosis and estimation of parasitaemia

For the diagnosis of *P. falciparum* and to estimate parasitaemia in blood samples, DNA was extracted from a DBS collected from each individual and quantitative polymerase chain reaction (qPCR) was performed to detect and quantify the presence of Pf-18S rRNA gene as previously reported elsewhere [55]. DNA was extracted from DBS collected on FTA cards using the InstaGene Whole Blood Kit (Bio-Rad), following the manufacturer's protocol with slight modifications as previously described [58]. A standard curve was prepared by serial dilutions in Phosphate buffered saline (PBS) of asynchronic *P. falciparum* Dd2 cultures [60] which maintained the mean haematocrit value of the patients (35%), thus simulating the composition of infected human blood. These samples were dried in DBS to extract DNA. To rule out DNA loss in the extraction from card compared to fresh blood, the same standard curve was made by extracting DNA directly from fresh blood. Comparison of both standard curves showed highly similar cycle threshold and slope values (Supplementary Figure 1). Thus, the standard curve with dried blood was chosen to interpolate experimental values to estimate parasitaemia in each sample. Supplementary Table 1 provides a descriptive overview of the demographic and clinical features of the study population, including age, sex, pregnancy status, country of origin, malaria diagnosis (microscopic vs submicroscopic), and parasitaemia levels.

To determine the prevalence of malaria and the intensity of infection in the population, *P. falciparum* parasitaemia was estimated in all individuals by qPCR. The parasite limit of detection (LOD) by microscopic examination of blood smears is defined as 150 parasites/μL of blood as the mean value previously established [55]. Thus, although optical microscopy was not used to quantify parasitemia, we use the terms "submicroscopic malaria" (SM) and "microscopic malaria" (MM) throughout the figures for consistency and clarity. Accordingly, samples were classified into three infection groups: non-infected (NI), submicroscopic malaria (SM or <150: 0.5-150 parasites/μL) or microscopic malaria (MM or 150+: >150 parasites/μL).

2.3. Genetic analysis of polymorphic variants

To determine the prevalence of the set of selected polymorphisms associated to protection against malaria in Africa, DNA extracted from Ghana (n = 200) and DRC (n = 224) donor samples was analysed. This set of polymorphic genes comprised *HBB* (NCBI Gene ID: 3043), *G6PD* (NCBI Gene ID: 2539), *PIEZO1* (NCBI Gene ID: 9780) and *PKLR* (NCBI Gene ID: 5313). Of the 424 participants initially included, complete genotyping was obtained for 419 individuals. In five cases, PCR amplification failed for one o more of the analyzed polymorphisms, likely due to poor DNA quality, and these samples were excluded from genotype-based analyses since not a full genotype at each allele was obtained.

2.3.1. HbS and HbC

To determine the presence of the *HbS* variant Glu6Val A>T (rs334) and *HbC* variant Glu6Lys G>A (rs33930165), a fragment of the *HBB* gene including the polymorphic position was amplified by PCR, followed by restriction fragment length polymorphism (RFLP) analysis. Each PCR reaction (60 μL) included 400 nM direct primer (5' AGTCAGGGCAGAGCCATCTA 3'), 400 nM reverse primer (5' CAGATCCCCAAAGGACTCAA 3'), 6 μL of 10X commercial reaction buffer, 200 μM deoxynucleotide triphosphates (dNTPs), 20 mU/μL of *Taq* polymerase, 45.3 μL of water and 1.5 μL of extracted DNA. Each amplification round included a negative control with water instead of DNA. Amplification on a Veriti Thermal Cycler (Applied Biosystems) followed a cycling comprising hot start at 94°C for 4 min; then 40 cycles of 10 sec at 94°C, 30 sec at 60°C and 20 sec at 72°C; and finally, 7 min at 72°C. This PCR amplification yielded a 353-base pair (bp) band that was subjected to RFLP analysis. For this, the PCR product was purified with Speedtools PCR clean-up kit (Biotools) and digested with *Dde*I and *Bse*RI enzymes separately. The design and experimental conditions of this RFLP method are defined in Supplementary Figure 2.

2.3.2. G6pd A-

The diagnosis of the G6PDA- variant (rs1050828) was determined by PCR-RFLP in a similar way to the *HBB* variants. This variant has two mutations, a primary Asn126Asp which by itself alone is the non-deficient variant G6PD A, and the Val68Met mutation on top of it. Since both mutations are necessary to generate the *G6pd A-* variant with the Val68Met mutation always attending with the Asn126Asp, the detection of the Val68Met mutation determine *G6pd A-* [61]. For this purpose, a fragment of the *G6PD* gene located on the X chromosome containing the polymorphic Val68Met position was analysed by PCR-RFLP. Each PCR reaction (40 μL) included 200 nM direct primer (5' CATCTGGTAAGTGTGTGTCCCACC 3'), 200 nM reverse primer (5' GGTCACGGGGGGGCTGGGCTGGTAAT 3'), 4 μL of 10X commercial reaction buffer, 200 μM dNTPs, 20 mU/μL *Taq* polymerase, 31.8 μL water and 1 μL of extracted DNA. Each amplification round included a negative control with water instead of DNA. Amplification on a Veriti Thermal Cycler (Applied Biosystems) followed a cycling comprising hot start at 94°C for 4 min; next 40 cycles of 45 sec at 94°C, 30 sec at 60°C and 20 sec at 72°C; and a final step of 7 min at 72°C. The resulting 382 bp fragment was purified and eluted in a volume of 20 μL for subsequent RFLP. In this case the appearance of a new restriction site for *Nla*III is diagnostic of the A- variant (see Supplementary Figure 3 for design and conditions of RFLP).

2.3.3. Piezo1 E756del

The *Pz_E756del* variant (rs59446030) is characterised by the deletion of a TCC triplet in a highly repetitive region in which, in the normal allele this sequence is repeated a total of eight times, and after the triplet deletion results in the *Pz_E756del* variant consisting of seven repeats instead. To characterize this variant, PCR amplification of the polymorphic fragment followed by Sanger sequencing was chosen.

The high GC content of this fragment required a specific amplification strategy for this type of sequences, characterised by a strong

secondary structure and greater resistance to denaturation of complementary strands. Thus, PCR was performed with Phusion high fidelity PCR master mix with buffer adapted to high GC conditions (Thermo Fisher Scientific). Each PCR reaction (30 µL) contained 500 nM direct primer (5' ATTTTCTCGGACCCTCTCTCCCAG 3'), 500 nM reverse primer (5' GGGACATGGGGCACAGCAGAC 3'), 15 µL of the commercial master mix containing buffer, dNTPs and polymerase, 11 µL of water and 1 µL of previously extracted DNA. A negative control with 1 µL of water instead of DNA was used. The PCR cycling programme used included hot start at 98°C for 30 sec; following with 40 cycles of 10 sec at 98°C, and 6 sec at 72°C; with a final step of 7 min at 72°C. PCR products resolved by 2% agarose gel electrophoresis systematically showed two bands: a 222 bp band containing the desired *PIEZO1* fragment and a non-specific band of about 700 bp. The PIEZO1-specific band was cut out and purified with Speedtools PCR clean-up kit (Biotools). The purified PCR product was sequenced using the Sanger method and the sequencing result analysed using Chromas software (Supplementary Figure 4).

2.3.4. Pklr

The *PKLR* gene was analysed for the presence of two polymorphic variants, Arg242His (rs141393570) and Leu241Val (rs147659527), both caused by substitution of a single nucleotide in the sequence, G>A and C>G respectively. For this, a fragment of the gene containing the two polymorphic sites was amplified by PCR and sequenced by the Sanger method. Each PCR reaction (50 µL) contained 400 nM direct primer (5' CTCTTTCCATGTCCGCAGGC 3'), 400 nM reverse primer (5' CTTGATGCCGTGTCCTTCCG 3'), 5 µL commercial 10X reaction buffer, 200 µM dNTPs, 20 mU/µL *Taq* polymerase, 40.1 µL water and 2.5 µL of extracted DNA. A negative control of amplification replacing DNA by 2.5 µL of water was used. The cycling programme included hot start at 94°C for 4 min; following with 40 cycles of 10 sec at 94°C, 30 sec at 60°C and 20 sec at 72°C; with a final step of 7 min at 72°C. The purified 251 bp PCR product was Sanger sequenced and analysed as described in Supplementary Figure 5.

2.4. Data analysis

All statistical analyses, data processing, and graphical visualizations were conducted using the R environment (version 4.1.1). The non-parametric Wilcoxon Rank Sum test was used to compare groups for variables with a non-normal distribution, while the Student's t-test was applied to variables that followed a normal distribution. Differences in the proportion of groups of infection were examined with Fisher's exact test. Crude Odds Ratio for binary parasitaemia categories were estimated using MedCalc software. Statistical significance was established at a threshold of $p \leq 0.05$.

Linkage disequilibrium (LD) analysis was performed to assess the non-random association between polymorphisms genotyped in this study. Pairwise LD values were calculated using both Lewontin's standardized disequilibrium coefficient (D′) and the squared correlation coefficient (r^2), which respectively provide measures of recombination history and allelic correlation between loci. LD estimations were carried out using Haploview v4.2 software [62].

3. Results

3.1. Frequency of malaria-protective genes in Ghana and in DRC

A total of 424 individuals from Ghana and DRC were genotyped at four genetic loci. Specifically, with respect to five alleles of *PIEZO1*, one of *G6PD*, two of *HBB*, and two of *PKLR*. (Table 1).

The allele and genotype frequencies of the minority variants were calculated and compared to the frequencies reported in the *Ensembl* database for individual continents, as well as for the entire human population. The most frequent minority variant was *Pz_E756del*, with an allele frequency near 0.2; followed by *G6pd A-* and *HbS* (0.17 and 0.11

Table 1
Allelic and genotypic frequencies of polymorphisms potentially associated with malaria protection.[a]

Polymorphism ID	Gene	Mutation	Variant	Genotypic frequency			Allelic frequency[c]			Allelic frequency in 1000 genomes/gnomAD				
				Homozyg. N	Heterozyg.	Homozyg. V	Ghana	DRC	Ghana + DRC	WORLD	AFR	AMR	EAS	EUR	SAS
rs59446030 (delCCT)	PIEZO1	E756del	Pz_E756del	0.64	0.32	0.035	0.24	0.156	0.196	0.051	0.179	0.019	0.00	0.001	0.005
rs59446030 (dupCCT)	PIEZO1	E756ins	Pz_E756ins	0.984	0.016	0.00	0.0175	0.0	0.0082	0.004	0.01	0.001	0.001	0.004	0.004
rs59446030 (del(CCT)$_2$)	PIEZO1	E755_E756del	Pz_E755_E756del	0.9953	0.0047	0.00	0.00	0.0044	0.0023	0.00	0.00	0.00	0.00	0.00	0.00
rs144777557 (delCTG)	PIEZO1	Q749del	Pz_Q749del	0.9555	0.0445	0.00	0,0275	0.0176	0.0222	0.00	0.00	0.00	0.00	0.00	0.00
rs202139830	PIEZO1	E750Q	Pz_E750Q	0.9696	0.0281	0.0023	0.02	0.0132	0.0164	0.043	0.142	0.014	0.002	0.005	0.010
rs1050828	G6PD	V68M	G6pd A-	0.737	0.1659	0.0972[b]	0.1725	0.1844	0.1727	0.038	0.135	0.013	0.00	0.00	0.00
rs334	HBB	E7V	HbS	0.7822	0.2037	0.0117	0.115	0.1123	0.1136	0.027	0.100	0.007	0.00	0.00	0.00
rs33930165	HBB	E7K	HbC	0.9668	0.0332	0.00	0.06	0.0044	0.0304	0.003	0.013	0.00	0.00	0.00	0.00
rs147689373	PKLR	E277K	PK_E277K	0.9668	0.0332	0.00	0.0076	0.0243	0.0166	0.003	0.009	0.001	0.00	0.00	0.00
rs147659527	PKLR	L241V	PK_L241V	0.9810	0.0165	0.0024	0.0229	0.0	0.0106	0.0002	0.001	0.00	0.00	0.00	0.00

N = normal (majority allele), V = mutant variant (minority allele). WORLD = World population, AFR = Africa, AMR = America, EAS = East Asia, EUR = Europe, SAS = South Asia

[a] The calculations in the table were based on the following successfully genotyped samples: 423 out of 424 for the PIEZO1 and HBB polymorphisms, 421 for the G6PD A- polymorphism, and 418 for the PKLR polymorphism

[b] Includes homozygous females and hemizygous males

[c] Supplementary Table 2 provides full allelic and genotypic frequencies in Ghana and DRC separately.

respectively) (Table 1). All allele frequencies in our study were as high or higher than those in 1000 Genomes/gnomAD for the African population: with the exception of the *PIEZO1* allele (*Pz_E750Q*), the frequency of which was much lower than previously described in other malaria endemic populations (0.016 vs. 0.142). As expected, given that the polymorphic genes studied have been associated with malaria protection, its allele variant frequencies in Ghana and DRC were higher than those described in non-African populations (Table 1).

Of the 419 fully genotyped individuals only 129 (30.8%) had none of the variants analysed; 174 (41.5%) had a single variant, 86 (20.5%) had two variants, 27 (6.4%) had three, and 3 individuals (0.7%) had 4. The *Pz_E750Q* variant was found only in individuals carrying the most common variant of the *PIEZO1* gene in Africa, *Pz_E756del*. Thus, of the 8 individuals carrying the *Pz_E750Q* variant found, all carried the *Pz_E756del* variant simultaneously and five carried it in homozygosis, indicating a possible linkage disequilibrium (LD) between these two loci. We further explored the LD between *Pz_E756del* and *Pz_E750Q* to better understand their co-occurrence patterns in our study population. The LD analysis revealed contrasting values for the two commonly used LD measures. While the r^2 value was low ($r^2 = 0.068$), indicating a limited correlation between the two loci, the D′ value reached the maximum of 1.0, reflecting perfect relative LD. This apparent discrepancy is explained by the very low allele frequency of E750Q (0.0164) and the asymmetric distribution of the variants in our study population. Specifically, all individuals carrying the rare E750Q variant also carried E756del, but many individuals had E756del without E750Q. This scenario leads to a high D′ value, indicating that recombination between these sites is unlikely, while the low r^2 value reflects the poor predictive capacity of one variant for the presence of the other due to the frequency imbalance.

3.2. Gene variants and parasitaemia levels

The study population was analyzed according to parasitaemia levels and genotype categories. As shown in Table 2, individuals carrying all consensus normal (wild-type) variants ($n = 130$) showed the highest prevalence of high parasitaemia (≥150 parasites/μL) at 19.2% ($n = 25$), and the lowest prevalence of low parasitaemia (<150 parasites/μL) at 36.15% ($n = 47$). In contrast, among individuals carrying one or more of the studied polymorphic variants, the prevalence of high parasitaemia ranged from 0% to 15.4%, depending on the specific genotype. Similarly, when considering only infected individuals, those with a normal genotype showed a markedly higher proportion of high parasitaemia cases (34.7%) compared to those carrying protective polymorphisms, in whom the prevalence ranged between 0% and 22.6% (Fig. 1). However, the overall proportion of infected individuals (regardless of parasitaemia level) was not higher among those with a normal genotype compared to individuals carrying malaria-protective variants.

Table 2
Individuals segregated by polymorphisms and malaria infection

Polymorphism	NI	SM (<150)	MM (150+)	TOTAL
Normal	58	47	25	130
Pz_E756del	59	72	21	152
Pz_Q749del	7	10	2	19
Pz_E750Q	6	7	0	13
G6PD_A-	40	57	14	111
HbS	40	42	10	92
HbC	8	14	4	26
PK_E277K	8	5	1	14

The columns display the number of individuals categorized as free of malaria infection (NI), with submicroscopic malaria (SM), or with microscopic malaria (MM), along with the total count for each polymorphic status, noting that some individuals may carry more than one polymorphism.

3.3. The impact on parasitaemia of a host genetic variant may vary with age

In *P. falciparum* endemic areas significant immunity develops with recurrent infections; for this reason, *P. falciparum* malaria is more life-threatening in small children. When we stratified our study subjects by age (Fig. 2), we found that *HbS* heterozygotes showed significantly lower parasitaemia than individuals without this variant in every age group: and this difference was statistically significant in the most malaria vulnerable groups: under 5 and over 50 years of age (Fig. 2C), while in individuals aged 6-13 years and adults aged 14-49 years only a decreasing trend in parasitaemia was observed. Further analyses of parasitemia level in all infection cases according to the presence or absence of polymorphic variants conferring malaria protection are presented in Supplementary Figure 6.

Given the unique biological condition of pregnant women concerning malaria infection, and their receipt of antimalarial prophylaxis during pregnancy as per health policy protocols (see above in the Material and Methods section), they were analysed separately from other individuals in the same age group (Fig. 3). When no polymorphisms were present, parasitaemia levels in pregnant women were significantly lower than those in the 14–49 age group, as indicated by the lowest p-value (≤0.01). In contrast, pregnant women carrying the *Pz_E756del*, *G6pd A-*, *HbS* or *HbC* polymorphisms exhibited higher parasitaemia compared to their counterparts without any mutations, with statistically significant increases observed in *HbS* and *HbC* carriers. This pattern appears attributable to the reduced parasitaemia observed in pregnant women without polymorphisms, a reduction not seen in pregnant women carrying any of the studied variants.

3.4. Association of polymorphisms and parasitaemia level

To evaluate the potential protective effect associated with the polymorphic alleles analyzed, odds ratios (OR) were calculated. Thus, OR of 150+ was estimated for infected individuals that have a variant genotype, compared with individuals who did not have any. This analysis was conducted for each variant with a minimum of 10 infected individuals in the dataset (Table 3). Given the substantial contribution of pregnancy in modulating the level of parasitaemia (see above, Fig. 3), we performed OR analysis on all cases of infection, both overall and excluding pregnant women, as shown in Table 3 in the left and right halves, respectively. This would allow not only to reinforce the statistical significance contributed by each variant in the case of being obtained in both infection groups, but also to know its differential impact for each one, given that the different *n* numbers for each polymorphism, when segregating in different caseloads, were not always high.

These analyses showed that variants *Pz_E756del*, *G6pd A-* and *HbS* significantly decreased the likelihood of 150+ malaria in all infected cases but also excluding the contribution of those pregnant women (OR values: 0.481 and 0.437; 0.452 and 0.326; and 0.414 and 0.208, respectively). OR values for individuals with two variants versus none showed clear-cut significant differences in both infection groups (OR values: 0.323 and 0.367), strengthening their contribution towards additive protection when more than one protective mutation accumulated. The additive impact of more than one variant versus only one was statistically significant when analysed in all infection cases (OR value: 0.409), probably because when removing infected pregnant women *n* value decreased. Perhaps also the effect of the large number of pregnancy cases that generally decrease parasitaemia levels (as discussed below) contributes to the fact that the effect of one variant versus none was clearly observed when removing infected pregnant women (OR value: 0.451) from all infection cases (OR value: 0.789).

Since *G6pd A-* was the only polymorphism analyzed that is X-linked, the heterozygotes are all female (unlike the other autosomal-linked ones), so the male and female G6PD status *vs.* parasitemia was also plotted separately. In addition, G6PD heterozygotes (females) are

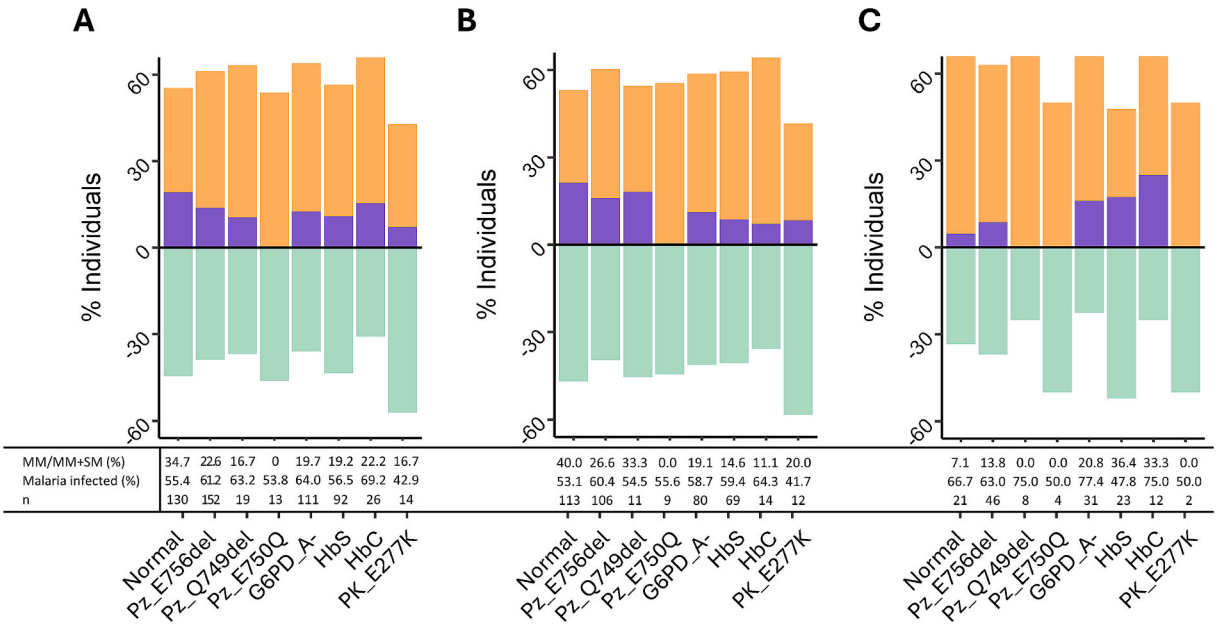

Fig. 1. Malaria infection status according to the presence of genetic variants. The percentage of individuals classified as non-infected (green), with submicroscopic malaria (orange), or with microscopic malaria (purple) is shown for each genotype group. Only variants observed in at least 10 individuals are included. The total number of individuals (*n*) analyzed within each genotype group and the corresponding percentages of microscopic malaria over all infections (MM / MM+SM) and total malaria infection (any parasitaemia) are indicated below the bars. (A) All study participants. (B) Non-pregnant individuals only. (C) Pregnant women only.

unique in potentially having red cell mosaicism, and so we explored *G6pd A-* protection as well not pooling hemi- and homozygotes. These analyses separating G6PD status by gender or zygosis (see distribution in Supplementary Figure 7) rendered significance in homo/hemizygosis when pregnant women were removed from all infected cases (OR value: 0.239), suggesting protection. Identically, when *G6pd A-* heterozygotes and hemizygotes/homozygotes were pooled together, the protection effect increased significancy (OR value: 0.326).

When a similar analysis was carried out by comparing non-infected with <150, we found not statistically significant ORs. This suggests that protection is not against getting infected, but against higher levels of parasitaemia.

3.5. Cumulative effect of malaria resistance by coinheritance of variants

In order to evaluate the overall cumulative effect of multiple protective genes, in terms of protection against malaria, we summed the mutations present in each individual in the population ($n = 428$). We then applied Student's t-test to both the <150 (SM) and the 150+ (MM) groups (Fig. 4). When we compared the number of variants in *P. falciparum* infected versus non-infected individuals there was no significant difference. However, the <150 (SM) parasitemia group had a significantly higher number of variants (mean 1.17) than those with 150+ parasitemia (mean 0.81). The difference had a *p*-value of 0.0064).

Moreover, by using Fisher's exact test we found that there was no difference in the rate of infection, regardless of the number of protective variants (Fig. 5). However, the ratio of individuals infected with MM (150+) was significantly lower in carriers of two or more variants, compared with those with a single variant (14.9% *vs*. 29.2%) (Fig. 5). Furthermore, a significant inverse correlation was observed between the number of mutations and parasitaemia ($R = -0.13$; p-value $= 0.036$). These results suggest that the inheritance of several but different protective polymorphic allelic variants in malaria-endemic populations exerts an additive effect on infection resistance leading to milder infections.

4. Discussion

Malaria has been regarded as a major force in shaping the human genome in the recent history of humankind [2]. Over the past decades, numerous studies have uncovered several polymorphisms in genes that are important in erythrocyte structure and function and that confer innate resistance to malaria, apparently reducing lethality before reproductive age, and have thus been positively selected in malaria-endemic populations [2,63]. We have investigated in two populations from malaria hyperendemic regions -Breman-Asikuma in Ghana and Mbujimayi in the DRC [64,65]- three classic polymorphic genes: *HbS* [66], *HbC* [21], *G6pd A-* [67]; in addition to the *Pz_E756del* variant of the *PIEZO1* gene, recently linked to protection against severe malaria [42]. Additionally, we examined two other *PIEZO1* variants (rs144777557 and rs202139830) not yet associated with malaria, and two *PKLR* gene variants (E277K and L241V: rs147689373 and rs147659527), which are common in African populations [51,68], but whose role in human malaria remains uncertain. Then, we have proceeded to test the aggregate association of these polymorphic variants with the severity of *P. falciparum* infection.

The high selective pressure exerted by *P. falciparum* on these two populations is dramatically illustrated by the fact that 69% of the people tested had at least one of the variant genes studied, and 27% had two or more. Each one of the allelic variants in *PIEZO1*, *G6PD* and *HBB* had a much higher prevalence in the study populations than in malaria-free regions, thus confirming previous data [2,4,63].

Of the variants analysed, *Pz_E756del* was the most prevalent, with a 19% allele frequency, very similar to that reported from other malaria-endemic regions [42,43,69]. In a mouse model Ma *et al.* [42] have found that this variant ameliorates experimental cerebral malaria and its associated mortality by diminishing parasite growth. A protective effect against severe malaria has been recently reported in Gabonese children [43]. Our results also indicate that heterozygotes for the *Pz_E756del* polymorphic variant are less likely to develop 150+ malaria compared to non-carriers: thus, the protective effect appears to consist in controlling severity. The precise mechanism of this protection is not yet clear, although dehydration of the erythrocyte [42,45], reduced export of virulence factors [43] and/or iron overload [46] in individuals

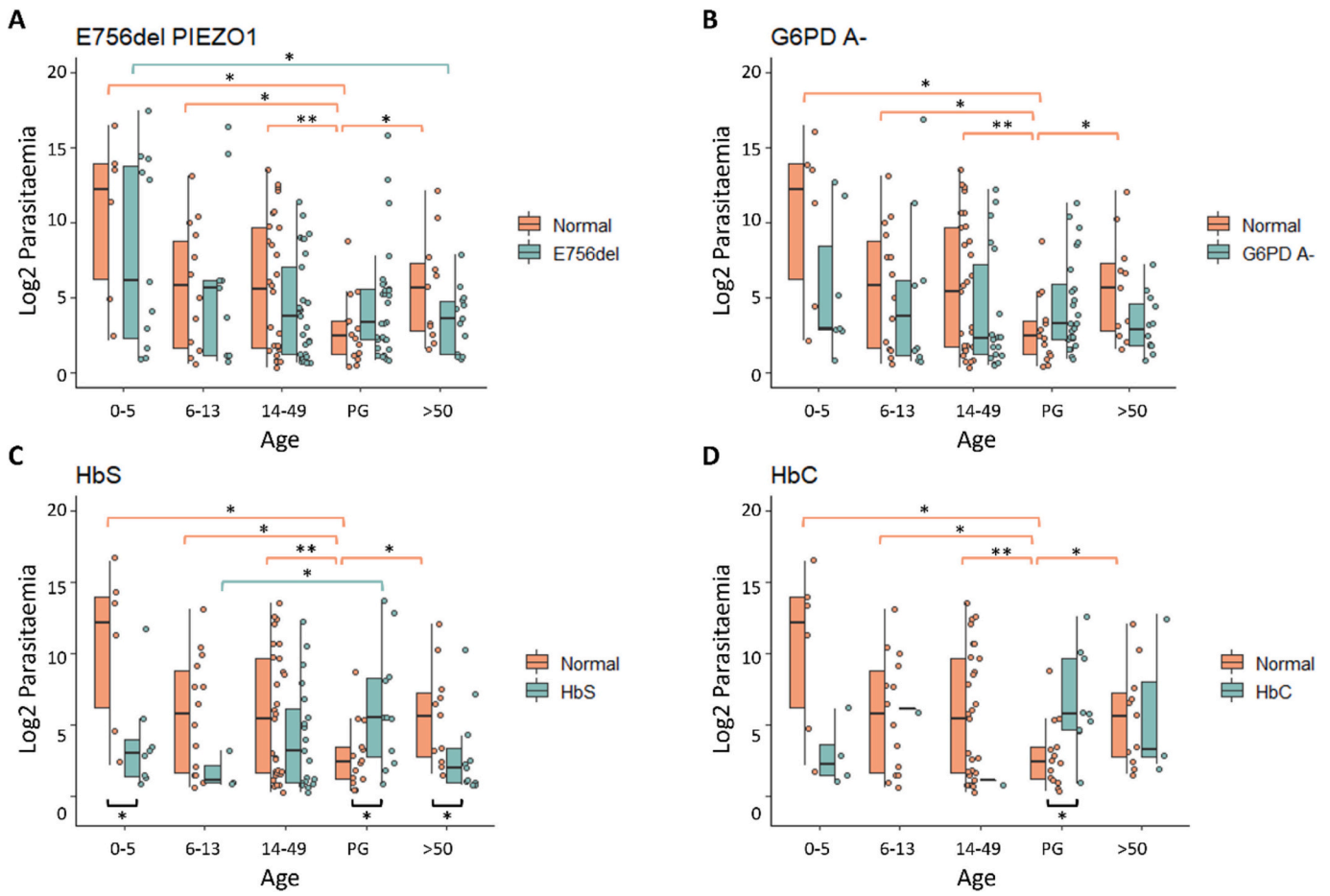

Fig. 2. Parasitaemia level according to the presence or absence of polymorphic variants conferring malaria protection by age group and pregnancy. Parasitaemia level was compared between individuals carrying any of the variants conferring malaria protection and those carrying none of these variants (label as "Normal"). Parasitaemia according to variant: A, *Pz_E756del*; B, *G6pd A-*; C, *HbS*; D, *HbC*. Significant differences obtained by *Wilcoxon* test are indicated by asterisks (*). *, *p*-value ≤ 0.05; **, *p*-value ≤ 0.01. Differences by age or pregnancy group (PG) are indicated at the top and differences between mutants and non-mutants are indicated at the bottom of the graph. Each box plot shows the distribution of parasitaemia level for each group (the central bar indicates the median, the edges of the boxes Q1 and Q3 and the whiskers extend to the minimum and maximum values not exceeding Q1/Q3 $\pm$ 1.5 x IQR).

carrying *Pz_E756del* have been reported. In homozygosis *Pz_E756del* increases serum iron parameters in an age-dependent manner [46], and the frequency of homozygotes we have observed is similar to that in other studies [43,70,71]. Even if there is a homozygous disadvantage, we think it does not qualify for a balanced polymorphism. Indeed, *Pz_E756del* does not significantly modify hematological or clinical parameters in patients with sickle cell anemia or HbSC disease [70,71]. Research to determine the mechanism by which this polymorphism reduces the severity of *P. falciparum* infection might contribute to the search for new anti-malarials.

Previous data have suggested possible linkage between *Pz_E750Q* and *Pz_E756del* [43]. Our findings indicate, for the first time, that the two variants are indeed in linkage disequilibrium. All individuals carrying *Pz_E750Q* in their genome also carried *Pz_E756del* and a high proportion of them, 62.5%, did so in homozygosis. Given that we had few cases of one variant without the other, we cannot say which one is more protective against malaria, or whether it is precisely their combined presence that is protective.

Extensive evidence indicates that *HbAS*, the sickle cell trait, facilitates clearance of infected red blood cells [12–14] and reduces malarial cytoadherence and rosette formation [72] while activating a NK-cell memory subset contributing to a enhanced IFN-γ-mediated immune response to control of parasite density [73]. Malaria infected erythrocytes carrying *HbC* (AC or CC) also reduce their capacity for cytoadherence and rosette formation due to an abnormal arrangement of the parasite protein PfEMP1 responsible for host membrane remodelling and sequestration in the microvasculature [25]. As for the *G6pd A-* variant, which causes G6PD deficiency in Africa [27,28,74], it has been shown to increase erythrocyte oxidative stress, triggering increased phagocytosis of parasitised erythrocytes [36,37]. Our estimates show a high frequency of these three variants in malaria-endemic regions, with 11% of *HbS,* 3% *HbC* and 17% *G6pd A-* allele frequency. In agreement with previously reported protective effects [9,11,31,75], our study shows that *HbS* and *G6pd A-* carriers are less likely to develop high parasitaemia malaria, confirming the resistance offered by these variants against severe infection. Regarding HbC we did not observe a statistically significant reduction in the prevalence of submicroscopic malaria among the eighteen individuals with *HbAC* compared to those without any protective variant. This result is not surprising, because protection is primarily in C/C homozygotes [22].

Polymorphic variants at the *PKLR* locus have a higher prevalence in the two African populations we have studied, compared to than that described in non-malaria-endemic regions [51,76]. However, in our study the number of *P. falciparum*-infected individuals with these *PKLR* genotypes was too small for assessing malaria protection.

In both Ghana and DRC, given the increased threat posed by malaria during pregnancy [77], health policies dictate that pregnant women, in the interval from week 12 to week 36 received 3 or 4 doses of SP. Therefore, the data on parasitemia are difficult to interpret.

Pregnant women carrying *HbS* or *HbC* exhibited higher parasitemia

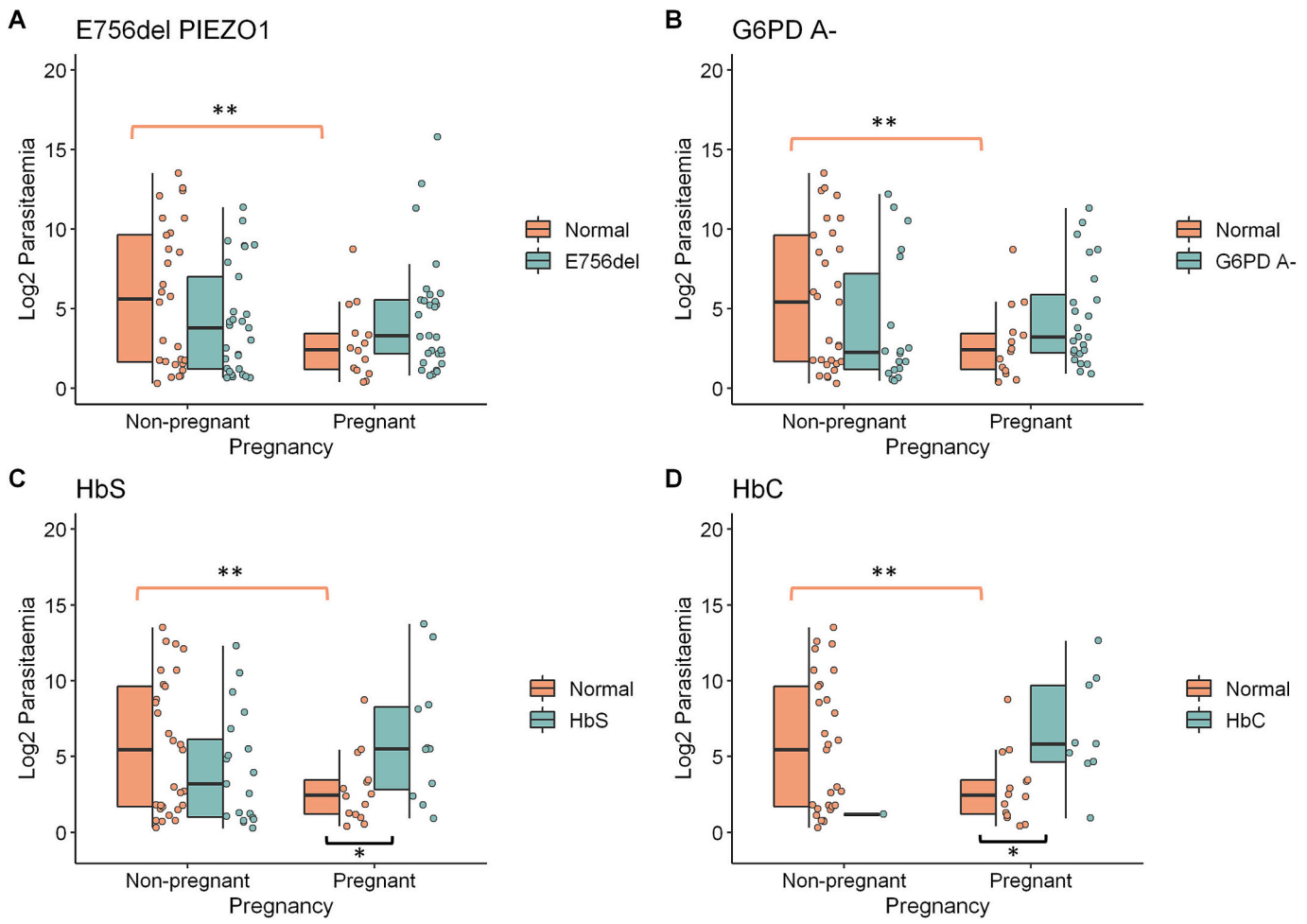

Fig. 3. Parasitaemia level according to the presence or absence of polymorphic variants conferring malaria protection in pregnancy. Parasitaemia level was compared between individuals carrying any of the variants conferring malaria protection and those carrying none of these variants (label as "Normal"). Parasitaemia according to variant: A, *Pz_E756del*; B, *G6pd A-*; C, *HbS*; D, *HbC*. Significant differences obtained by *Wilcoxon* test are indicated by asterisks (*). *, *p*-value ≤ 0.05; **, *p*-value ≤ 0.01. Differences by pregnancy group (non-pregnant *vs.* pregnant) are indicated at the top and differences between mutants and non-mutants are indicated at the bottom of the graph. Each box plot shows the distribution of parasitaemia level for each group (the central bar indicates the median, the edges of the boxes Q1 and Q3 and the whiskers extend to the minimum and maximum values not exceeding Q1/Q3 ± 1.5 x IQR).

Table 3
Odds ratio of developing 150+ malaria in subjects with variant genotypes vs. no variant genotype.

	All infection				Non-pregnant infection			
Genotype	*n**	OR	95% CI	*P*-value	*n**	OR	95% CI	*P*-value
Normal	71				57			
Pz_E756del	82	0.481	0.234 – 0.991	**0.0473**	58	0.437	0.197 – 0.973	**0.043**
Pz_Q749del	12	0.368	0.075 – 1.813	0.2191	6**	NC	NC	NC
G6PD A-	71	0.452	0.211 – 0.967	**0.0408**	47	0.326	0.133 – 0.798	**0.014**
G6PD A- hetero	42	0.502	0.207-1.214	0.126	20	0.458	0.146-1.434	0.180
G6PD A- homo/hemi	29	0.383	0.130-1.128	0.0817	27	0.239	0.073-0.782	**0.018**
HbS	49	0.414	0.173 – 0.990	**0.0474**	38	0.208	0.071 – 0.612	**0.004**
HbC	18	0.526	0.156 – 1.769	0.2989	9**	NC	NC	NC
Pz_E756del + G6PD A-	22	0.291	0.0783-1.0783	0.0647	15	0.211	0.043-1.026	0.054
Pz_E756del + HbS	18	0.230	0.049 – 1.082	0.0629	13	0.25	0.051 – 1.233	0.089
HbS + G6PD A-	14	0.142	0.0175-1.146	0.0669	6**	NC	NC	NC
1 variant *vs.* full normal	110/71	0.789	0.418-1.488	0.464	85/57	0.451	0.219-0.928	**0.030**
>1 variant *vs.* full normal	67/71	0.323	0.141-0.740	**0.008**	38/57	0.367	0.143-0.939	**0.036**
>1 variant *vs.* 1 variant	67/110	0.409	0.186-0.898	**0.026**	38/85	0.813	0.323-2.045	0.659

OR = Odds ratio; OR was calculated by comparing individuals carrying the mutation with individuals who did not carry any of the mutations studied (full normal). OR was not calculated (NC) in those genotypes with a n value below 10 (indicated by **). Two analyses for each genotype were performed including or not pregnant women (left and right sections, respectively). Heterozygous individuals were included for each variant except for *G6pd A-* where three statuses where considered: all carriers, homozygous + hemizygous and heterozygous individuals. P values <0.05 are shown in bold. (*) In the lowest three rows where two groups are compared, the column indicating sample size (n) shows the number of individuals at each of the group compared separated by a dash (e.g., 110/71).

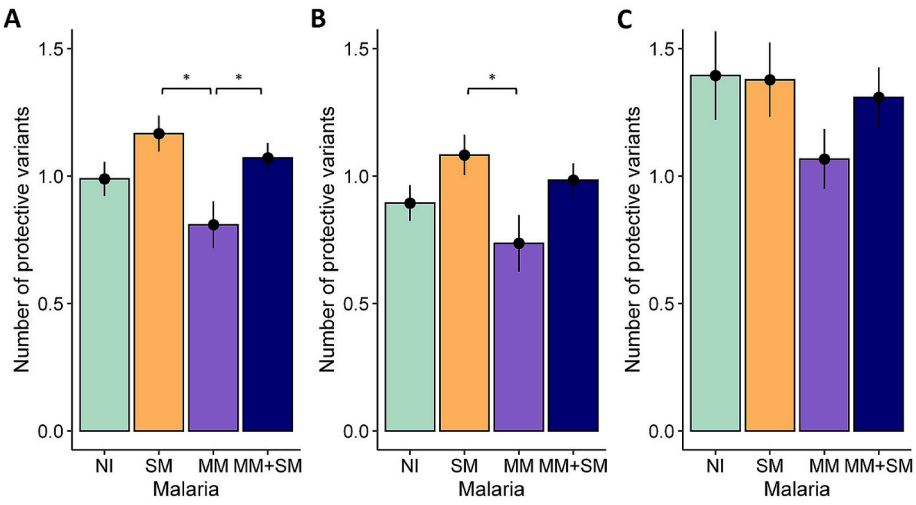

Fig. 4. Type of malaria infection according to the number of potentially protective variants. Malaria infection status *vs.* number of accumulated variants in the individuals sharing such status. Significant differences obtained by *Student's t*-test are indicated by asterisk (*), *p*-value $\leq$ 0.05. NI, non-infected; SM, submicroscopic malaria; MM, microscopic malaria. (A) All study participants. (B) Non-pregnant individuals only. (C) Pregnant women only.

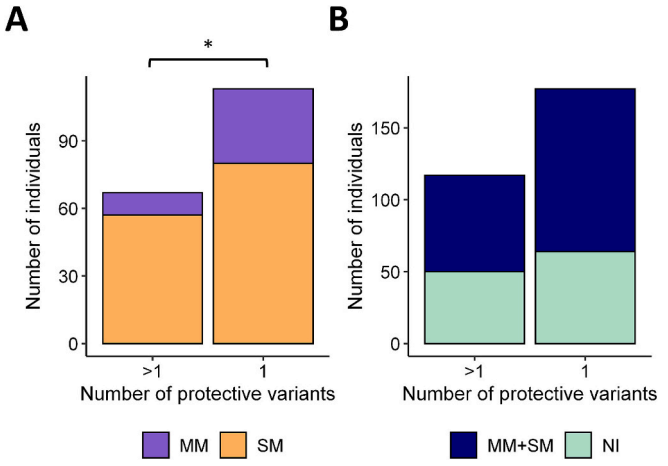

Fig. 5. Prevalence of malaria infection according to the number of potentially protective variants. A and B, carriers of one or more than one variant *vs.* proportion of malaria infection status. Significant differences obtained by *Fisher's* test are indicated by asterisk (*), *p*-value $\leq$ 0.05. NI, non-infected; SM, submicroscopic malaria; MM, microscopic malaria.

levels than those without variants, suggesting these haemoglobinopathies may not provide malaria protection during pregnancy and might even increase parasitemia post-infection. This effect could be influenced by IPTp, which promotes submicroscopic infections [78], as seen in the lowest submicroscopic malaria levels among variant-free pregnant women, potentially due to SP prophylaxis. Similar findings in prior studies [79–81] indicate that *HbAS* may not protect against placental malaria, possibly due to reduced expression of the VAR2CSA protein on parasitized *HbAS* erythrocytes [81]. Furthermore, recently a high impact of anaemia among malaria infected pregnant women carrying simultaneously *HbAS* and *G6pd A-* has been reported [82]. In line with these findings, our results suggest that the immunological environment of pregnancy, the use of IPTp, and possibly other pregnancy-specific factors (e.g., altered parasite sequestration patterns, immune modulation, or drug-parasite-host interactions) might modify the expected protective effects of these variants against parasitemia. This observation also aligns with previous research on negative and positive epistatic interactions between malaria-protective polymorphisms that may critically influence malaria susceptibility, either attenuating [53] or enhancing [54] the protective effects depending on an additional context. Further research is needed to explore parasite immune evasion in pregnant women through integrated analyses of humoral responses, genetic polymorphisms, and clinical outcomes. However, our study's inability to assess placental malaria limits conclusions about its role in the observed parasitemia increases.

Despite the high prevalence of these polymorphic variants in African regions, few studies have addressed the complex question of the phenotype manifested when two or more of them are inherited together in the same individual. The synergistic effect of different polymorphisms has been demonstrated in other diseases [83,84] or in the case of the *G6pd A-* variant, which requires two simultaneous and synergistic mutations to generate the deficient phenotype [35]. However, so far, no study has been able to demonstrate the additive or synergistic effect of polymorphisms located in different genes [43,82,85,86] that provide resistance to severe malaria. Our study, in which eight polymorphic variants were analysed simultaneously, has revealed that carrying more than one variant-gene conferring malaria protection in the same individual may have an additive or synergistic effect. Our results show that those infected with submicroscopic malaria accumulate a higher number of malaria resistance gene variants in their genome compared to those with high parasitaemia malaria. We also observed that the prevalence of malaria with high parasite load is lower in individuals inheriting two or more variants compared to those carrying only one. These results, together with the inverse correlation observed between the number of variants carried by an individual and the parasitaemia achieved, lead us to conclude that the accumulation of inherited polymorphic variants conferring malaria protection in the genome of the same individual provides additive or synergistic effect in terms of their resistance antimalarial effect.

Our data highlight the relevance of jointly analysing the polymorphisms associated with malaria resistance that coexist in the same population, as a high proportion of individuals in endemic populations carry two or more polymorphic variants. Furthermore, if these polymorphisms are analysed independently, we may make the mistake of considering normal or non-mutated individuals as those carrying other variants, which could mask a significant malaria protection exerted by that polymorphic variant. This overlooked effect on a protective mutational admixture may lead to unexpected or unnoticed results such described in some publications where no association between malaria protection (severe or uncomplicated) and polymorphic variant were observed or even with the inheritance of a given variant in homo-, hetero- or hemizygosis, as might be the case in some studies of malaria

protection by G6PD [31,87].

In summary, our findings indicate that the polymorphic variants *Pz_E756del*, *HbS* and *G6pd A-* collectively confer protection against high parasitemia malaria in populations where the disease is endemic. This protection manifests as a reduction in parasitemia levels rather than prevention of infection. Moreover, we show that the coinheritance of multiple protective variants is prevalent in malaria-endemic regions, with individuals carrying two or more variants experiencing lower parasitemia compared to those with one or none. Additionally, our results suggest a potential linkage disequilibrium between the *Pz_E750Q* and *Pz_E756del* variants of the *PIEZO1* gene, highlighting the need for further research into the mechanistic interactions of these protective variants. It is also clinically important to note that the pattern of genetic protection may be altered in pregnant women, with HbC and HbS carriers exhibiting significantly higher parasitemia levels. This observation may be linked to the effects of intermittent preventive treatment during pregnancy commonly administered in malaria-endemic regions, as well as the unique immune responses to malaria during pregnancy.

5. Conclusion

Our investigation into genetic resistance to malaria has uncovered a high prevalence of common polymorphic variants that offer protection in endemic regions. A key and integrated aspect of our study is the simultaneous analysis of coexisting polymorphisms within the same population, highlighting that a significant proportion of individuals carry two or more protective variants, whose effects appear to be cumulative. This finding has potential implications for malaria epidemiology. Previous studies have demonstrated that certain polymorphisms, such as the *HbAS*, reduce malaria morbidity and delay the onset of the first infection episode, while paradoxically increasing transmission. The potential impact of inheriting multiple protective polymorphisms on malaria transmission remains uncertain. However, the observed reduction in parasitemia among individuals carrying multiple variants, compared to those with one or none, could contribute to lower detection rates, resulting in untreated cases. This, in turn, may enhance disease transmission by maintaining a reservoir of asymptomatic carriers with subpatent infections

CRediT authorship contribution statement

Paloma Abad: Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis. **Susana Pérez-Benavente:** Methodology, Investigation. **Sara Pérez-Luz:** Writing – original draft, Supervision, Formal analysis. **Julius N. Fobil:** Writing – review & editing, Resources, Methodology, Investigation. **Batisseur Kitenge Luyenga:** Resources, Investigation, Data curation. **André Kazadi Mukendi:** Resources, Investigation, Data curation. **Antonio Puyet:** Writing – review & editing, Resources, Project administration, Data curation. **Amalia Diez:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Lucio Luzzatto:** Writing – review & editing, Supervision, Formal analysis, Data curation. **Isabel G. Azcárate:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Conceptualization. **José M. Bautista:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

We declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2025.101051.

Data availability

Data will be made available on request.

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