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RAPID COMMUNICATION

Concurrent PIEZO1 activation and ATP2B4 blockade effectively reduce the risk of cerebral malaria and inhibit *in vitro* *Plasmodium falciparum* multiplication in red blood cells

Malaria caused by the *Plasmodium falciparum* parasite is responsible for more than 240 million cases per year and killed 627,000 people in 2020, mostly African children. The malaria parasite is transmitted by mosquitos belonging to the genus *Anopheles*. After an asymptomatic liver stage, the parasite is released into the bloodstream to invade red blood cells (RBCs) and replicate asexually. This erythrocytic phase is associated with a variety of clinical manifestations, including mild and severe malaria. Cerebral malaria (CM) is one of the most severe forms, characterized by the sequestration of parasitized RBCs in the small capillaries of the brain and the local development of cytokine-mediated inflammation. Genetic variants in genes encoding proteins involved in red blood cell physiology are protective factors against severe malaria, as clearly demonstrated for the sickle cell variant of hemoglobin (HbS). Rare gain-of-function mutations in Piezo1, a mechanosensitive calcium channel, are involved in hereditary xerocytosis, a disease characterized by red cell dehydration and mild hemolysis. Interestingly, RBC dehydration is associated with reduced *Plasmodium* infection *in vitro*[1] suggesting that *PIEZO1* polymorphisms may protect against malaria. Recently, a gain-of-function *PIEZO1* E756del variant (rs59446030) under positive selection in Africa, was associated with protection against severe malaria in children in Gabon.[2] However, a large case–control study in Ghana failed to replicate this association.[3] In this study, we sought to further characterize the role of this *PIEZO1* polymorphism in malaria by confirming its association in a Senegalese population and identifying the genetic interaction with polymorphisms in the *ATP2B4* gene that encodes PMCA4, the major RBC calcium pump. We prove that activation of Piezo1 modulates intracellular calcium concentration and inhibits *Plasmodium falciparum* multiplication in RBCs. Furthermore, we demonstrated the additive effect of Piezo1 activation and PMCA4 blockade on the inhibition of parasite multiplication in erythrocytes.

The E756del variant of *PIEZO1*, a 3-nucleotide deletion (GGA) in the coding region of the gene, results in the deletion of glutamic acid (Fig. 1A). Genotyping of this mutation was performed in a Senegalese population composed of 82 individuals with CM, 27 with severe malaria, 74 with mild malaria, and 77 healthy controls living in Dakar (Table S1). Genotype frequencies were consistent with Hardy–Weinberg Equilibrium. We found that the E756del variant was strongly associated with protection against CM ($P = 0.007$, odds ratio = 0.36 [0.17–0.76]) in our Senegalese population using a Fisher's exact test and logistic regression analysis (Fig. 1B). This association remained significant even when age was included as a covariate in the statistical model ($P = 0.009$, odds ratio = 0.35 [0.16–0.77]). Our results are consistent with a previous study showing that Piezo1 gain-of-function mice are protected against experimental CM.[1] In addition, we evaluated the effect of this *PIEZO1* variant in combination with the *ATP2B4* haplotype of 5 regulatory SNPs (rs11210734-rs1541252-rs1541253-rs1541254-rs1541255) (Table S1) that we previously showed to be associated with severe malaria.[4] In this study, we showed that individuals having at least one *ATP2B4* minor allele haplotype are protected against CM ($P = 0.04$, estimated odds ratio = 0.5 [0.26–0.93]) (Fig. 1C). Second, we demonstrated a combined effect between the protective

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A

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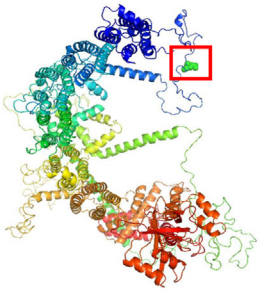


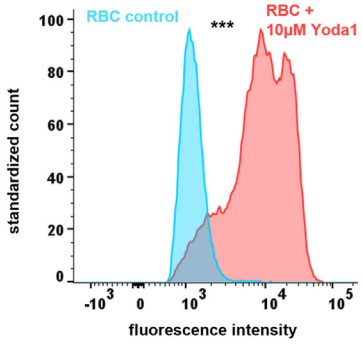
B

Variant	Minor allele	Protective genotype	Ctrl (n=77)	MM (n=74)	SM (n=27)	CM (n=82)	Odd ratio (95% CI) Ctrl vs MM	Odd ratio (95% CI) Ctrl vs SM	Odd ratio (95% CI) Ctrl vs CM
rs59446030 (GGA> -)	E756Del	E756/E756Del	28 (36%)	18 (24%)	9 (33%)	14 (17%)	0.56 (0.28 - 1.14)	0.88 (0.35 - 2.21)	**0.36** (0.17 - 0.76)**

C

Variant present	Ctrl	CM	Odd ratio (95% CI)	P-value (two-tailed)
PIEZO1				
Risk genotypes (DEL/DEL + WT/WT)	49	68	1	-
Protective genotype (WT/DEL)	28	14	0.36 (0.17-0.76)	0.007
ATP2B4				
Risk haplotype (Major/Major)	26	45	1	-
Protective haplotypes (Major/Minor + Minor/Minor)	49	42	0.50 (0.26-0.93)	0.04
Combination				
Risk genotypes PIEZO1/haplotype ATP2B4	17	33	1	-
Protective genotypes PIEZO1/haplotype ATP2B4	17	5	0.15 (0.05-0.48)	0.0009
Interaction PIEZO1 × ATP2B4			0.28 (0.13-0.61)	0.001

D



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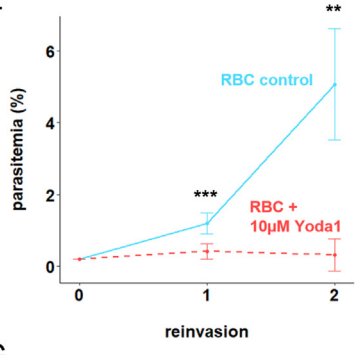

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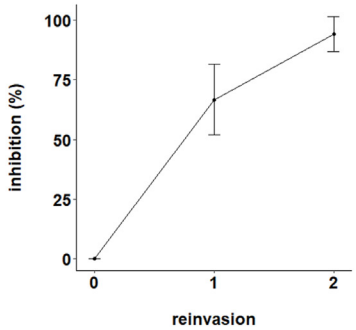

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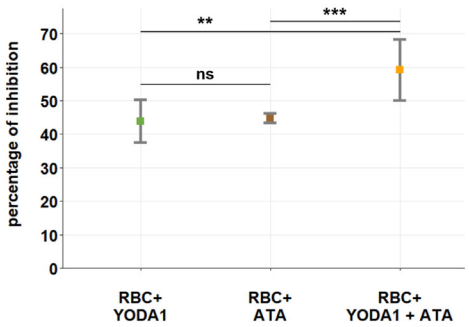

Figure 1 The combination of genetic variants in the *ATP2B4* and *PIEZO1* genes protects against cerebral malaria by altering calcium homeostasis that inhibits *P. falciparum* reinvasion. **(A)** Description of the E756Del mutation of *PIEZO1*. The E756Del mutation (rs59446030) is a 3-base pair DNA deletion in a GGA repeat and results in a deletion of a glutamate (E) amino acid. The ancestral genotype consists of 8 repeats of the GGA motif, compared to 7 repeats in E756Del mutation. Genotyping was performed

genotype of *PIEZO1* and the protective haplotype of *ATP2B4* ($P = 9.10-4$, odds ratio = 0.15 [0.05—0.48]) (Fig. 1C). We also identified a significant genetic interaction between *ATP2B4* and *PIEZO1* variants using logistic regression analysis ($P = 1.10-3$ (odds ratio = 0.28 [0.13—0.61]) (Fig. 1C). For individuals heterozygous and homozygous for the haplotype with minor *ATP2B4* alleles, the odds ratio was 0.5. In contrast, for individuals with both the protective heterozygous genotype for *PIEZO1* and the protective *ATP2B4* haplotype, the odds ratio was 0.15, indicating stronger protection against CM. *ATP2B4* encodes PMCA4, a calcium pump, and E756Del is a gain-of-function mutation that increases the activity of the mechanosensitive cationic channel Piezo1, both of which are expressed on the surface of RBCs. To assess *in vitro* the effect of Piezo1 activation on intracellular calcium, we incubated human RBCs from a healthy blood group O donor provided by the local blood bank (Etablissement Français du Sang) with Yoda 1, a specific activator of Piezo1. As shown in Figure 1D, in each of the two experiments performed, cells treated with Yoda1 had a 10-fold increased calcium concentration ($P < 0.0001$) compared with cells without Yoda1. Similarly, a meta-analysis considering MFI, SD, and sample size showed increased calcium concentration in Yoda1-treated cells ($P < 0.001$). Yoda1 (in the micromolar range) causes increased opening of Piezo1 in response to mechanical stimulation, thus allowing massive calcium entry. In the RBC, increased intracellular calcium activates the GARDOS channel, resulting in potassium efflux, water loss, and cellular dehydration.[5] Piezo1 is an important channel for maintaining calcium homeostasis and RBC volume. Then, using synchronized mature stages parasites (*Plasmodium falciparum* 3D7 strain) incubated in the presence of 10 μM of Yoda1 for three days, we showed that after two invasions, parasitemia increased from 0.2% to 5% in the control culture without Yoda1 while it dropped to 0.32% in the culture with Yoda1 ($P = 0.002$) (Fig. 1E). As shown in Figure 1F, the percentage of inhibition reached 94% after two invasions on day 3, confirming that the increase in intracellular calcium in erythrocytes prevents parasite multiplication. We thus demonstrated *in vitro* that parasite multiplication is significantly inhibited by the chemical activation of Piezo1 in agreement with a previous study. We have previously shown that the *ATP2B4* minor allele haplotype is associated with a decrease in enhancer activity and, consequently a decrease in the long PMCA4a and PMCA4b transcripts, the two isoforms expressed mainly in the brain and red blood cells respectively.[4] This decrease in *ATP2B4* expression leads to increased intracellular calcium[4] probably due to a functional impairment of active calcium extrusion. To evaluate the effect of PMCA4 inhibition on parasite growth, experiments were performed in the presence of ATA (aurintricarboxylic), a specific inhibitor of PMCA4. At 100 μM of ATA, we observed approximately 44%

by PCR amplification using the forward primer 5′-CAGGCAGGATGCAGTGAGTG-3′ and the reverse primer 5′-GGACATGGCACAGCAGACTG-3′, followed by Sanger sequencing. The chromatogram shows the result of three observed genotypes, homozygous E756, heterozygous E756/E756Del, and homozygous E756Del. **(B)** Association of the *PIEZO1* mutation with cerebral malaria in the Senegalese population-based study. A total of 260 individuals were genotyped, including 77 healthy individuals (Control, Ctrl), 74 subjects with mild malaria (MM) characterized by symptoms such as headache, fever, nausea, 27 cases of severe malaria (SM) without neurological symptoms, and 82 cases of cerebral malaria (CM) characterized by deep coma (GCS <9). We performed the statistical analysis with IBM SPSS Statistics. Fisher's exact test was used to calculate *P*-values without any covariate ($P = 0.006$). **(C)** Association of *PIEZO1* mutation and *ATP2B4* regulatory variants with cerebral malaria. *P*-values and odds ratios were calculated using logistic regression to assess the association of *PIEZO1* and *ATP2B4* genetic variants separately or in combination. Considering the combination of the protective genotype of *PIEZO1* with the protective haplotypes of *ATP2B4*, the association was highly significant by both Fisher's exact test and logistic regression. **(D)** Visualization of calcium concentration in red blood cells without (blue) and with (pink) Yoda 1 treatment. Culture of red blood cells from healthy donor blood was performed for 24 h in the presence of the agonist Yoda1 at a final concentration of 10 μM. Intracellular calcium was measured by flow cytometry with Fluo-4. Fluorescence intensity was estimated on 20,000 labeled red blood cells. Analyzing each of the two experiments performed with Yoda1-treated cells showed a significant increase in calcium concentration compared with cells without Yoda1 ($P < 0.0001$). **(E)** Estimation of *in vitro* parasitemia without (blue) and with (pink) Yoda1. *P. falciparum* (3D7 strain) culture was synchronized by sorbitol treatment. 24 h later, the percentage of parasitemia was brought to 0.2% and the parasites were cultured in the presence of 10 μM of Yoda1 for 2 reinvasions. Parasitemia was determined by flow cytometry, using SYBR Green to label the parasites, after 24 h and 72 h of culture in the presence of Yoda1. A paired sample *t*-test was performed to assess the impact of Yoda1 on the parasite. During the first and second reinvasion, the percentage of parasitemia was lower in the presence of Yoda1 ($P = 0.0007$ and $P = 0.002$, respectively). **(F)** Inhibition of parasite invasion. Percentage inhibition of parasite invasion was estimated on days 1 and 3 of infected red blood cells culture in the presence of 10 μM Yoda1. **(G)** *In vitro* inhibition of parasite invasion using ATA (aurintricarboxylic acid), a specific inhibitor of PMCA4 (the plasma membrane calcium ATPase 4) encoded by *ATP2B4* and/or Yoda1 an activator of PIEZO1. A culture of *P. falciparum* (3D7 strain) was synchronized by sorbitol treatment and incubated 24 h later in the presence of either Yoda1 at 1.8 μM (green), ATA at 100 μM (brown), or both ATA at 100 μM and Yoda1 at 1.8 μM (orange). After 24 h of treatment, the parasitemia of each culture was determined by flow cytometry, using SYBR Green to label the parasites. Student's *t*-test was used to calculate *P*-values. Stronger inhibition was observed by combining Yoda1 and ATA ($P = 0.001$ and $P < 0.001$ compared to Yoda1 and ATA, respectively). No statistical difference was observed between Yoda1 and ATA (*P*-value = 0.254). **(H)** Conformation of the human PIEZO1 protein. Representation of the monomer of human PIEZO1 constructed by sequence homology with mice (Uniprot KB - E2JF22) using the SWISS-MODEL. The result was visualized on PyMOL. Position E756 is shown in the red frame. The region containing this mutation in humans is not conserved in mice, which does not allow us to establish a 3D structure of this region by sequence homology. Data are represented ± standard deviation. $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant.

parasite inhibition, and similarly, we observed 45% inhibition in the presence of Yoda1 at 1.8 μM (Fig. 1G). Interestingly, when ATA and Yoda1 were added simultaneously, we achieved significantly stronger inhibition of 59% (Fig. 1G) compared with Yoda1 ($P = 0.001$) or ATA ($P < 0.001$), supporting the additive effect of PIEZO1 and ATP2B4 variants. To date, no human structure of Piezo1 has been established, and the region containing the E756Del mutation is not conserved in the mouse model, making it impossible to establish the impact of this mutation on the 3D conformation of the protein (Fig. 1H). However, we can assume that the E756Del mutation alters the secondary structure of Piezo1, resulting in a gain of function. Furthermore, the *PIEZO1* mutation and *ATP2B4* regulatory variants may play a key role in the protection against CM through the biological function of these genes in other cell types such as T cells, B cells, and astrocytes. Indeed, the increase of intracellular calcium is one of the activation signals of the T cells promoting the elimination of the parasite. In addition, the increase in intracellular calcium in astrocytes leads to a reduction in the production of pro-inflammatory cytokines and chemokines such as IL-1β, TNFα, and CXCL1 and thus may protect against CM. Moreover, PIEZO1 appears to play an important role in B cell responses to membrane-associated antigens.

In conclusion, we confirmed that the Piezo1 gain-of-function mutation is associated with protection against CM in a Senegalese population. Our results also indicate that the combination of the *PIEZO1* mutation and the *ATP2B4* SNPs further protects individuals against CM probably because they disrupt cell calcium homeostasis. Indeed, the activation of Piezo1 and the alteration of PMCA4 simultaneously affect parasite multiplication *in vitro* because of increased intracellular calcium.

Ethics declaration

The Comité d'Ethique de la Recherche de l'Université Cheikh Anta Diop de Dakar has approved the protocols. Each participant, their parents, or legal guardians for any minors before inclusion has received written or verbal information in their native language and given written consent.

Author contributions

SM and PR designed and supervised the project. MA genotyped the E756Del variant, and SN and MT participated in *ATP2B4* genotyping. AT extracted the DNA samples and performed whole genome amplification. AT, FT, and BM were involved in the recruitment, the follow-up of Senegalese individuals, and the collection and interpretation of biological data, under AD supervision. BP performed cytometry experiments for intracellular calcium levels and parasite growth rate estimation. MA, PR, and SM performed statistical analyses and interpreted the results. MA and SM wrote the paper. SM and PR supervised the whole research and provided guidance. All authors read the manuscript and approved the final version before submitting the article.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.02.029.

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