A common polymorphism in the druggable ion channel *PIEZO1* is associated with protection from severe malaria

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1

1 **Abstract**

2 Malaria caused by the Apicomplexan parasite *Plasmodium falciparum* has served as a strong

3 evolutionary force throughout human history, selecting for red blood cell polymorphisms that

4 confer innate protection against severe disease. Recently, gain-of-function mutations in the

5 mechanosensitive ion channel *PIEZO1* were shown to ameliorate *Plasmodium* parasite growth,

6 blood-brain barrier dysfunction, and mortality in a mouse model of malaria. In humans, the gain-

7 of-function allele *PIEZO1* E756del is highly prevalent and enriched in Africans, raising the

8 possibility that it is under positive selection due to malaria. Here we used a case-control study

9 design to test for an association between *PIEZO1* E756del and malaria severity among children

10 in Gabon. We found that the E756del variant is strongly associated with protection against severe

11 malaria in heterozygotes, independent of the protection conferred by the sickle cell trait

12 (hemoglobin AS). *In vitro* experiments using donor red blood cells failed to find an effect of

13 E756del on parasite growth, suggesting this variant confers a mild channel defect and/or that its

14 protective effect may be mediated by other tissue types *in vivo*. Nonetheless, we show that Yoda1,

15 a small molecule agonist of PIEZO1, has potent antimalarial activity in both E756del and wild-

16 type red blood cells. Our findings demonstrate that *PIEZO1* is an important innate determinant of

17 malaria susceptibility in humans and holds potential as druggable host target for malaria control.

18

19 **Introduction**

20 Malaria infection due to *Plasmodium falciparum* is a major cause of childhood morbidity

21 and mortality in endemic countries. The symptoms of the disease start when the parasite invades

22 and replicates in red blood cells. Upon infection, there are three possible clinical outcomes that

23 are influenced by the host, parasite, and environmental factors: uncomplicated malaria, severe

24 malaria, or asymptomatic parasitemia. The development of asymptomatic parasitemia is largely

25 influenced by adaptive immunity that results from repeated exposures, whereas it is estimated

26 that ~25% of the clinical variation in malaria severity can be explained by innate genetic factors

2

27   that act additively (MACKINNON *et al.* 2005). A variety of studies have shown that one of the

28   strongest protective factors is heterozygosity for the hemoglobin S allele (HbAS), which leads to

29   impaired parasite proliferation at low oxygen tension (PASVOL *et al.* 1978; JALLOW *et al.* 2009;

30   TIMMANN *et al.* 2012; MALARIA GENOMIC EPIDEMIOLOGY *et al.* 2015; ARCHER *et al.* 2018). Many

31   other candidate susceptibility loci reside in genes encoding membrane or structural proteins of

32   the red blood cell (NGUETSE AND EGAN 2019). However, associations between established

33   candidate loci and malaria severity explain only a fraction of the variance in clinical outcome,

34   suggesting additional susceptibility factors have yet to be discovered (NDILA *et al.* 2018).

35   The mechanosensitive ion channel PIEZO1 was recently identified as a new candidate

36   susceptibility factor for severe malaria (MA *et al.* 2018). PIEZO1 acts as a nonselective cation

37   channel in a variety of tissues and has established roles in sensing blood flow through the

38   vasculature, cell migration and differentiation, and red blood cell volume control (LI *et al.* 2014;

39   PATHAK *et al.* 2014; RANADE *et al.* 2014; CAHALAN *et al.* 2015; WANG *et al.* 2016). Gain-of-function

40   (GOF) mutations in *PIEZO1* underlie hereditary xerocytosis, a disorder characterized by red blood

41   cell dehydration, reduced osmotic fragility and mild hemolytic anemia (ARCHER *et al.* 2014;

42   GLOGOWSKA *et al.* 2017). Previous work has shown that *P. falciparum* parasites replicate poorly

43   in severely dehydrated red blood cells, including those from patients with hereditary xerocytosis

44   (TIFFERT *et al.* 2005). The demonstration that expression of a *PIEZO1*GOF allele in mice modeled

45   the RBC abnormalities observed in hereditary xerocytosis, inhibited proliferation of *Plasmodium*

46   *berghei* ANKA parasites, and protected the mice from cerebral malaria provided some *in vivo*

47   evidence that GOF mutations in *PIEZO1* may influence susceptibility to severe malaria (MA *et al.*

48   2018).

49   Using a comparative genomics approach, a common *PIEZO1* polymorphism was

50   identified in healthy individuals of African descent that may be under positive selection and act as

51   a gain-of-function allele (MA *et al.* 2018). The mutation, E756del (rs572934641), is a 3 nucleotide

52   deletion in a coding region of the gene that includes ~60 bp of short tandem repeats. PIEZO1

3

53   E756del was classified as a gain-of-function mutant based on studies in HEK cells, where it

54   displayed a prolonged inactivation time constant after mechanical stimulation as compared to

55   wild-type PIEZO1. Results from *in vitro* assays using the human malaria parasite *P. falciparum*

56   suggested that parasite growth was impaired in RBCs from E756del heterozygotes as compared

57   to wild-type RBCs (MA *et al.* 2018). Together, these findings raise the intriguing possibility that

58   the *PIEZO1* E756del allele may be protective against severe malaria in humans.

59   Here, we screened a large case-control study group from Gabon for the *PIEZO1* E756del

60   mutation and assessed its impact on the risk of severe malaria. We found that E756del was

61   associated with protection from severe malaria in heterozygotes, suggesting that natural genetic

62   variation in *PIEZO1* is an innate determinant of malaria susceptibility in humans. This association

63   was independent of hemoglobin AS, which appears to have an epistatic interaction with *PIEZO1*

64   E756del. While wild-type and E756del red blood cells supported the intracellular growth of *P.*

65   *falciparum* equally well, we found that chemical upregulation of PIEZO1 inhibited *P. falciparum*

66   replication. PIEZO1 is a druggable ion channel that may be under selective pressure to protect

67   against severe malaria, suggesting that it holds potential as a target for a novel, host-directed

68   therapy for this ancient disease.

69

70   **Methods**

71   *Genetic Association study design and participants*

72   Using a case-control approach, we assembled 542 samples from children aged 4-140

73   months that had been obtained in three previously published studies conducted in Lambaréné

74   and Libreville, Gabon (KUN *et al.* 1998; KALMBACH *et al.* 2006; KREMSNER *et al.* 2009). After

75   excluding samples for missing parameters, the analytic cohort consisted of 446 samples, of which

76   193 were controls with mild malaria and 253 were cases with severe malaria. All cases presented

77   with microscopically confirmed *P. falciparum* parasitemia, signs and symptoms of severe malaria

78   and no evidence of other severe diseases. Severe malaria was defined as severe anemia

4

79   (hemoglobin <50 g/l) and/or hyperparasitemia (>250,000 parasites/μl, corresponding to >10% infected

80   erythrocytes), a Blantyre coma score ≤2 and/or other facultative signs of severe malaria such as

81   cerebral malaria, convulsions, hypoglycemia, and respiratory distress. The control group was those

82   with mild malaria coming from the same geographical area as the cases. Mild malaria was defined

83   as parasitemia 1000–50,000/μl on admission, no schizontemia, circulating leukocytes containing

84   malarial pigment <50/μl, not homozygous for hemoglobin S, hemoglobin >80 g/l, platelets >50/nl,

85   leukocytes <12/nl, lactate <3 mmol/l, and blood glucose >50 mg/dl. Written informed consent for

86   each child was provided by the parents/guardians before enrollment.

87

88   *Mutation screening*

89   *PIEZO1* exon 17 was amplified by PCR using the following primers: 5′-

90   CAGGCAGGATGCAGTGAGTG-3′ (forward) and 5′-GGACATGGCACAGCAGACTG-3′

91   (reverse). Amplification reactions were performed in one batch in the same laboratory. The

92   thermal conditions were an initial denaturation (95°C, 3 min) followed by 35 cycles of 95°C for 30

93   s, 65°C for 30 s and 72°C for 1 min. The PCR was completed with a final extension step of 72°C

94   for 5 min. PCR products were visualized through electrophoresis on a 1·2% agarose gel stained

95   with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently,

96   the PCR products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany)

97   and directly used as templates for DNA sequencing (Quintara Biosciences, San Francisco, CA,

98   USA). Mutations were identified by aligning the sequences with the *PIEZO1* reference sequence

99   (NG_042229.1) using the Geneious 10.2.3 software (Auckland, New Zealand) and visually

100 reconfirmed from their electropherograms. All samples were also genotyped for the HbS

101 polymorphism (rs334) in the *HBB* gene (NG_059281.1). Briefly, we amplified exon 1 of the *HBB*

102 gene using the primer pairs: 5′-AGTCAGGGCAGAGCCATCTA-3′ (forward) and 5′-

103 GTCTCCACATGCCCAGTTTC-3′ (reverse). The PCR conditions were: initial denaturation (95°C,

104 3 min) followed by 35 cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 1 min. The amplification

5

105 was completed with a final extension step of 72°C for 5 min. The sequencing and variant detection

106 were performed as described above.

107

108 *Statistical analysis*

109 Participants with HbSS or HbSC hemoglobin genotypes were excluded from all analyses

110 due to the severity of their underlying hematologic diseases. Additionally, all primary analyses

111 were conducted only on participants without missing observations. Deviation from Hardy-

112 Weinberg equilibrium was assessed using Chi-square analysis. Descriptive statistics were

113 generated to evaluate the distributions of key characteristics by malaria severity status. The

114 Wilcoxon rank sum test and Fisher's exact test were used to determine if there were significant

115 differences in malaria severity status in continuous and categorical characteristics, respectively.

116 The primary outcome was an indicator for whether a patient had mild or severe malaria.

117 A main effects logistic regression model was fit to malaria severity status as a function of *PIEZO1*

118 E756del genotype (normal [WT/WT=reference level], heterozygous [WT/DEL], and homozygous

119 [DEL/DEL]), hemoglobin S genotype (normal HbAA, sickle cell trait HbAS), age in months, sex,

120 and the study from which the participant information was extracted. Odds ratios (ORs) and 95%

121 confidence intervals (CIs) were extracted from the logistic regression model. The estimated

122 probability of severe malaria was extracted from the model for each participant and pairwise

123 comparisons between the *PIEZO1* E756del variants and between hemoglobin S genotypes were

124 presented. P-values for the pairwise differences in *PIEZO1* E756del were calculated from Tukey's

125 HSD (honestly significant difference) post hoc test. The Hosmer-Lemeshow goodness of fit was

126 used to evaluate model fit using 10 bins.

127 In order to assess whether HbAS confounds the association between *PIEZO1* E756del

128 and malaria severity status, an additional model was fit to malaria severity status as a function of

129 *PIEZO1* E756del, HbAS, age, sex, study, and an interaction term between HbAS and *PIEZO1*

130 E756del. Due to convergence issues based on small sample size, a Bayesian logistic regression

6

131   model was used (GELMAN ANDREW AND YU-SUNG SU 2018). Similar methods to those used in the

132   main effects model were implemented. Unadjusted logistic models were also fit to assess the

133   association between seven additional *PIEZO1* polymorphisms near E756del and severe malaria.

134   Classification trees (CART) were implemented to determine whether a synergistic effect

135   of any *PIEZO1* polymorphisms existed in order to predict malaria severity status. CART is a tree-

136   based learning technique for classifying observations, which yields a decision rule by partitioning

137   the data into subsets based on variables entered into the algorithm through the minimization of

138   an error function. Two trees were grown to a maximum depth of 3 levels and minimal node size

139   of 4 using the ctree function in the party package in R (HOTHORN *et al.* 2006), and included the

140   following features, respectively: 1) all identified *PIEZO1* mutations, HbAS, age, and sex; 2) all

141   identified *PIEZO1* mutations excluding *PIEZO1* E756del, HbAS, age, and sex. To ensure the

142   validity of these models, we conducted a 5 times repeated 10-fold cross-validation (CV) for both

143   CARTs. The CV area under the curve (AUC) and 95% CIs were reported for each tree (KOHAVI

144   1995). Spearman's rank correlation test was also used in order to assess the genetic association

145   (linkage) between *PIEZO1* E756del and the other *PIEZO1* mutations.

146   As a sensitivity analysis, missing values in age and sex were imputed by chained

147   equations with predictive mean matching in order to assess the robustness of the two *PIEZO1*

148   E756del logistic regression results (VAN BUUREN AND GROOTHUIS-OUDSHOORN 2011).

149   A p-value $< 0\cdot05$ was considered statistically significant. All analyses were conducted

150   using R software v3.5.2 (DEVELOPMENT CORE TEAM 2013).

151

152   *Blood sample collection and genotyping for in vitro parasite growth assays*

153   Subjects who self-identified as African or African-American were recruited to donate blood

154   at the Stanford Clinical Translational Research Unit. All participants and/or their parents gave

155   informed consent according to a protocol approved by the Stanford University IRB (#40479).

156   Whole blood samples were drawn into CPDA tubes and processed within 48 hours to remove

157 serum and separate the buffy coat and red blood cells. Red blood cells were washed and stored

158 in RPMI-1640 medium (Sigma) supplemented with 25 mM HEPES, 50 mg/L hypoxanthine, 2.42

159 mM sodium bicarbonate media at 4°C for up to 48 hours, and then cryopreserved in human AB+

160 serum and glycerol at -80°C. Genomic DNA was isolated from buffy coats using a DNeasy Blood

161 and Tissue Kit (Qiagen). *PIEZO1* exon 17 was amplified by PCR of genomic DNA using the

162 primers described above and Q5 polymerase in the following reaction conditions: 98°C for 30

163 seconds initial denaturation followed by 35 cycles of 98°C for 10 seconds, 70°C for 20 seconds,

164 and 72°C for 20 seconds, followed by a 2 minute final extension at 72°C. PCR products were

165 visualized on an agarose gel and sent for Sanger sequencing at Elim Bio (Hayward, CA). The

166 *PIEZO1* E756 genotypes were manually determined from chromatograms examined using

167 FinchTV software (Geospiza Inc). All genotypes were assessed by two independent researchers.

168

169 *P. falciparum* culture and growth assays

170   *P. falciparum* strain 3D7 is a laboratory-adapted strain that was obtained from the Walter

171 and Eliza Hall Institute (Melbourne, Australia) and was routinely cultured in human erythrocytes

172 obtained from the Stanford Blood Center at 2% hematocrit in RPMI-1640 supplemented with 25

173 mM HEPES, 50 mg/L hypoxanthine, 2.42 mM sodium bicarbonate and 4.31 mg/ml Albumax

174 (Invitrogen) at 37°C in 5% CO_2 and 1% O_2. Parasite growth assays were initially performed using

175 fresh erythrocytes and then repeated after cryopreservation and thawing. Schizont-stage

176 parasites were isolated using a MACS magnet (Miltenyi) and added at 0.5% initial parasitemia to

177 previously cryopreserved wild-type or E756del heterozygous erythrocytes that had been washed

178 and resuspended in complete RPMI with bicarbonate and Albumax as above. Assays were

179 performed at 0.5% hematocrit in a volume of 100 μl per well in 96-well plates. For drug treatments,

180 erythrocytes were incubated in the indicated concentrations of Yoda-1 (Sigma) for three hours at

181 1% hematocrit and washed in complete RPMI three times before adding to assay plates.

182 Parasitemias were determined on day 0, day 1 (24 hours) and day 3 (72 hours) by staining with

183   SYBR Green 1 nucleic acid stain (Invitrogen, ThermoFisher Scientific, Eugene, OR, USA) at

184   1:2000 dilution in PBS/0.3% BSA for 20 minutes, followed by flow cytometry analysis on a

185   MACSQuant flow cytometer (Miltenyi).

186   For each genetic background (WT versus E756del heterozygote), assays were performed

187   in technical duplicates using seven biological replicates (N=7) from unrelated donors. The percent

188   parasitemia relative to control was determined for each biological replicate by normalizing the

189   parasitemia on day 3 at each drug concentration relative to that in the absence of drug. Then, the

190   mean of normalized day 3 parasitemia and S.E.M. was calculated for each genetic background

191   at each drug concentration. Dose response inhibition curves (log inhibitor vs response) were

192   generated in PRISM 8 Version 8.0.2 (159) and the top of the curves were constrained to equal

193   100 to calculate IC50 for Yoda-1. Log IC50 of the two curves were compared statistically using

194   the extra-sum-of-squares F test.

195

196   **Results**

197   *The PIEZO1 E756del variant is associated with protection from severe malaria*

198   To determine if the *PIEZO1* E756del allele influences malaria susceptibility, we collected

199   542 DNA samples from three well-characterized malaria study cohorts from Gabon (KUN *et al.*

200   1998; KALMBACH *et al.* 2006; KREMSNER *et al.* 2009). Out of the 542 samples, 8 were excluded

201   for HbSS or HbSC genotypes to minimize confounding effects of severe hematologic disease. A

202   further 88 were excluded due to missing values in sex (n=78) and failure to amplify *PIEZO1*

203   E756del (n=10). Therefore, our analytic cohort consisted of 446 samples. All samples were from

204   Gabonese children between the ages of 4-140 months; 193 (43%) participants had mild malaria

205   (controls) and 253 (57%) had severe malaria (cases). Cases of severe malaria had severe

206   anemia, hyperparasitemia, signs of cerebral malaria, hypoglycemia and/or respiratory distress in

207   addition to microscopically-confirmed parasitemia. Controls with mild malaria had parasitemia and

9

208   fever with absence of severe signs. Their baseline demographics are summarized in Table 1.

209   While cases and controls were similar in terms of sex (45% vs 46% male, respectively), those

210   with severe malaria on average were younger and had higher parasite densities. Differences by

211   study are also summarized (Table S1).

Table 1. Baseline demographics

		Malaria status		
Baseline characteristic	**Overall (n=446)**	**Mild (n=193)**	**Severe (n=253)**	**P-value**
Sex, n (%)				
Male	201 (45%)	88 (46%)	113 (45%)	0.92
Female	245 (55%)	105 (54%)	140 (55%)	
Age in months, median (range)	35 (4-140)	43 (8-140)	29 (4-133)	<0.001
Parasite density (parasite/μL)[1], median (range)	35,000 (20-1,544,880)	15,000 (588-434,074)	108,518 (20-1,544,880)	<0.001
Study, n (%)				
Kremsner et al.	195 (44%)	49 (25%)	146 (58%)	
Kun et al.	195 (44%)	98 (51%)	97 (38%)	<0.001
Kalmbach et al.	56 (12%)	46 (24%)	10 (4%)	

Continuous and categorical variables were compared across malaria status using the Wilcoxon rank sum test and Fisher's exact test, respectively. [1]n=44 excluded due to zero values.
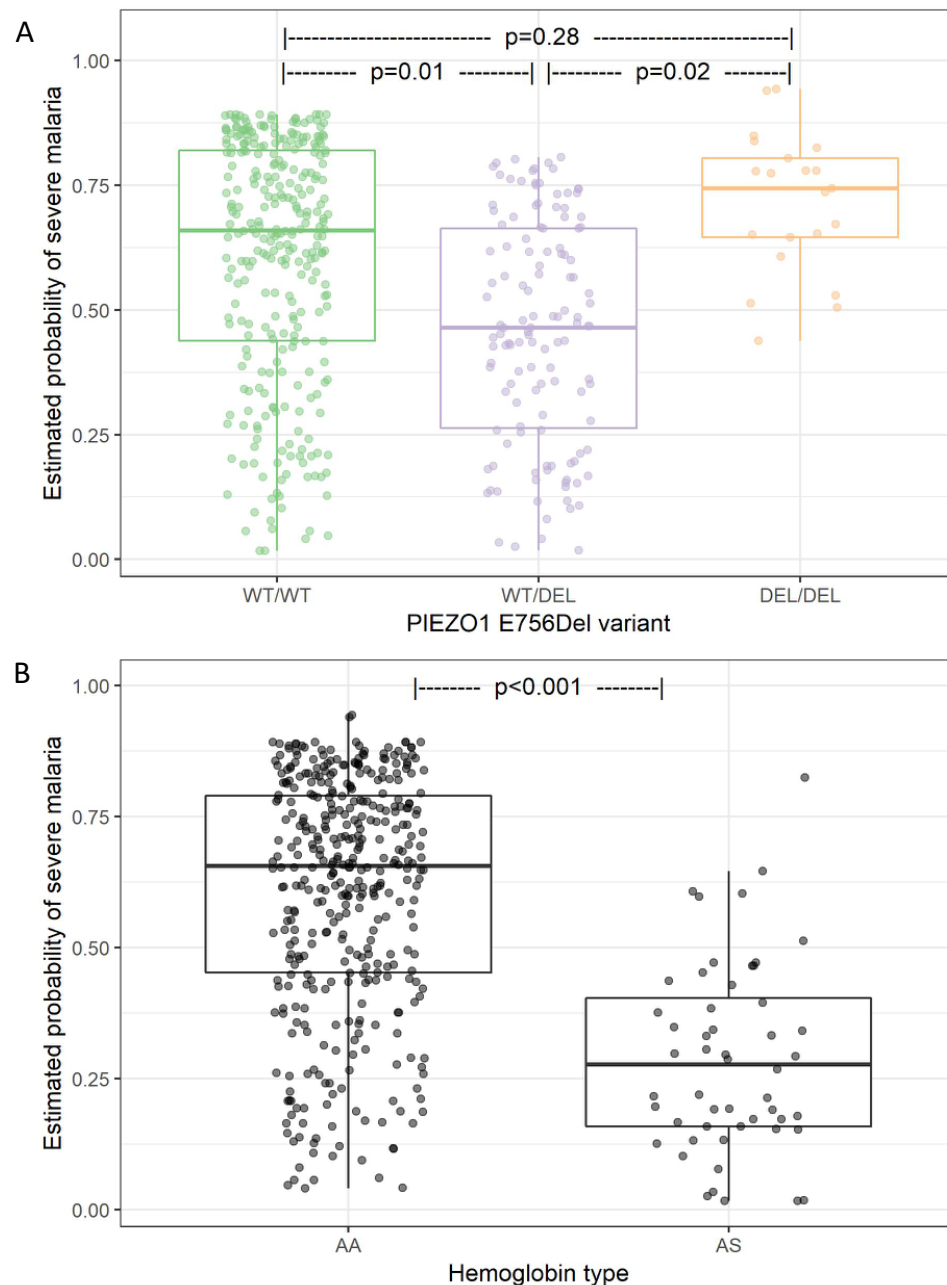
212

213   The *PIEZO1* E756del variant was common among the study population, with a

214   heterozygote prevalence of 36% in controls and 23% in cases (minor allele frequency=0.19). The

215   variant was in Hardy-Weinberg equilibrium in the controls but not in the cases. We used a logistic

216   regression model to predict the probability of severe malaria as a function of *PIEZO1* genotype,

217   HbAS status, age, sex and study for each participant. The odds of severe malaria in those with

218   the heterozygous E756del genotype (WT/DEL) were half the odds of those with the homozygous

219   wild-type genotype (OR 0·50, 95% CI 0·31-0·81; p=0·005), suggesting that having one copy of

220   *PIEZO1* E756del is protective against severe disease (Table 2 and Table S2). Homozygous wild-

221   type subjects at E756 (WT/WT) had a significantly higher predicted probability of severe malaria

222   compared to E756del heterozygous (WT/DEL) individuals (median 67% vs. 46%, p=0·01) (Figure

223   1A). These results suggest that the E756del variant confers protection for heterozygotes even as

224 they age and develop increased adaptive immunity to malaria. In contrast, participants

225 homozygous mutant for E756del (DEL/DEL) had a high probability of severe malaria compared

226 to heterozygotes (p=0.02), suggesting that harboring two copies of this mutant allele negates the

227 protective effect observed in carriers (Figure 1A). While participants with the homozygous mutant

228 genotype (DEL/DEL) had over twice the odds of severe malaria compared to those who were

229 homozygous wild-type, this association was not statistically significant, likely due to the low overall

230 frequency of the DEL/DEL genotype (OR 2·26, 95% CI 0·82-6·94, p=0·28) (Table 2 and Figure

231 1A).

232

Table 2. Associations with malaria severity

Characteristic	Malaria status		Odds ratio (95% CI)
	Mild (n=193)	Severe (n=253)	
PIEZO1 E756Del			
WT/WT	118 (40%)	180 (60%)	reference
WT/DEL	69 (54%)	58 (46%)	0.50** (0.31, 0.81)
DEL/DEL	6 (29%)	15 (71%)	2.26 (0.82, 6.94)
Hemoglobin type			
AA	156 (40%)	238 (60%)	reference
AS	37 (71%)	15 (29%)	0.27*** (0.13, 0.52)
Age			0.98*** (0.97, 0.99)
Male	88 (44%)	113 (56%)	0.70 (0.45, 1.08)

Note: * p < 0.05, ** p < 0.01, *** p < 0.001. Percentages are out of the row totals. Model also adjusted for study in which the data was collected. The Hosmer-Lemeshow goodness of fit test suggested the model fit was appropriate (X^2=11.91, p-value=0.16).
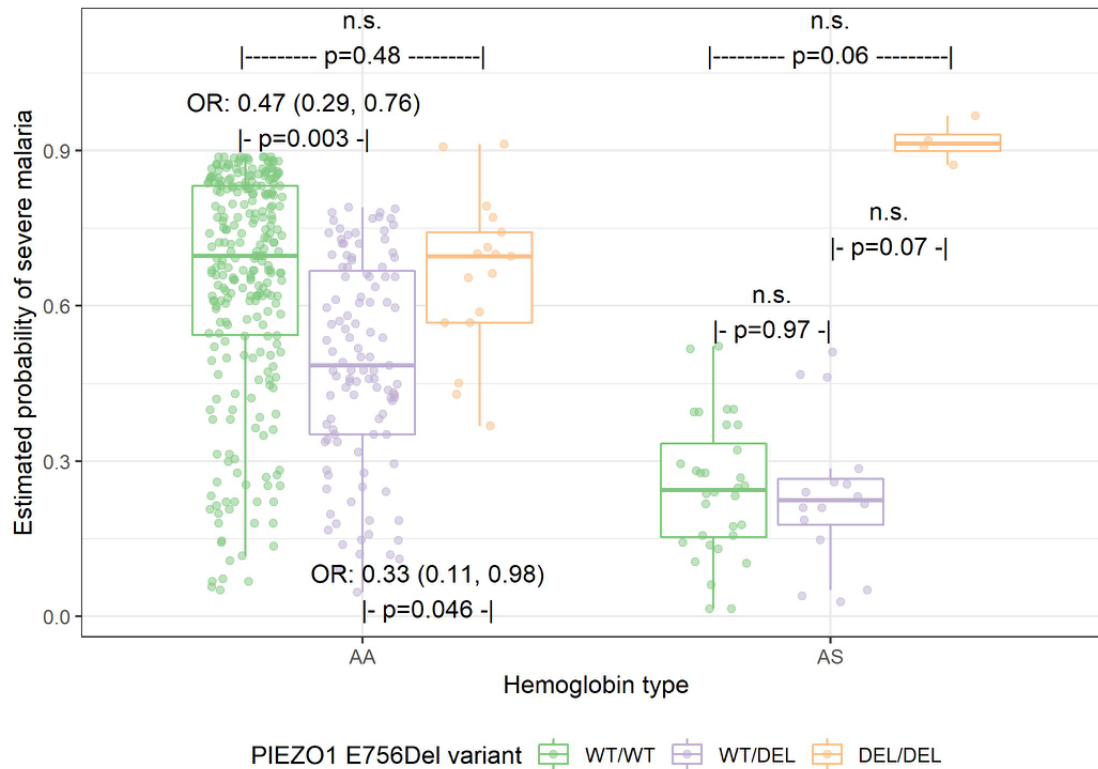
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Figure 1. Association of malaria severity with *PIEZO1* and hemoglobin genotypes. Estimated probability of severe malaria extracted from the model presented in Table 2 by (**A**) *PIEZO1* E756 genotype and (**B**) hemoglobin beta genotype. P-values for pairwise differences in *PIEZO1* were calculated using Tukey's HSD post hoc test. Model adjusted for age, sex, and study.

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238   *Association between HbAS and protection from severe malaria*

239   As expected, we also found that the sickle cell trait polymorphism (HbAS) was significantly

240   associated with mild malaria in our study population (HbAS vs HbAA, OR 0·27, 95% CI 0·13-0·52;

241   p<0·001) (Table 2). Children with HbAS had a significantly lower predicted probability of severe

242   malaria compared to children with normal hemoglobin (HbAA) (28% vs 66%; p<0·001) (Figure

243   1B), an effect approximately twice as strong as that of *PIEZO1* E756del in our study population.

244   This result is consistent with published literature on the protective effect of the sickle cell trait for

245   severe malaria (MANGANO *et al.* 2015; UYOGA *et al.* 2019).

246

247   *Interplay between PIEZO1 E756del and HbS on malaria severity*

248   Because of the high frequencies of both the *PIEZO1* E756del variant and HbAS in the

249   study population, we sought to assess whether HbAS confounds the association found for

250   E756del heterozygotes and malaria susceptibility by refitting the main effects model with the

251   addition of an interaction term for HbAS and E756del. For subjects with HbAA, the E756del

252   homozygous wild-type genotype (WT/WT) was associated with a significantly higher probability

253   of severe malaria compared to the E756 heterozygous genotype (WT/DEL) (OR 2·09, 95% CI

254   1·28-3·41; p=0·003, Figure 2). In contrast, in subjects with HbAS, heterozygosity for E756del did

255   not alter susceptibility to severe malaria compared to E756 wild-type (p=0·97, Figure 2 and Table

256   S3). These results suggest that the heterozygous *PIEZO1* E756del genotype reduces the risk of

257   severe malaria in individuals with normal hemoglobin, but that effect is masked by the strong

258   protective effect of HbAS in those with sickle cell trait.

13

Figure 2. Association between *PIEZO1* and malaria severity by hemoglobin type. n.s. = not statistically significant. A Bayesian logistic regression model was fit to malaria severity status as a function of hemoglobin type, *PIEZO1*, and their interaction. Overall interaction effect of p=0.08. P-values for pairwise differences between *PIEZO1* within each hemoglobin type were calculated using Tukey's HSD post hoc test. Model also adjusted for age, sex, and study. The Hosmer-Lemeshow goodness of fit test suggested the model fit was appropriate (X^2=6.03, p-value=0.64).

259

260   For subjects with HbAA, the odds of severe malaria in those with the E756del

261   heterozygous mutation (WT/DEL) were one third the odds of those with the homozygous mutation

262   (OR 0·33, 95% CI 0·11-0·98; p=0·046, Figure 2). While this association was also apparent within

263   individuals with HbAS, a difference in the risk of severe malaria failed to reach statistical

264   significance between the two mutations (p=0·07). This result may suggest that the homozygous

265   mutant E756del genotype alters RBC physiology in a way that abolishes the protective effect of

266   HbAS, and may act with it synergistically to generate an unfavorable environment for controlling

267   malaria infection. However, these results would need to be replicated in larger studies.
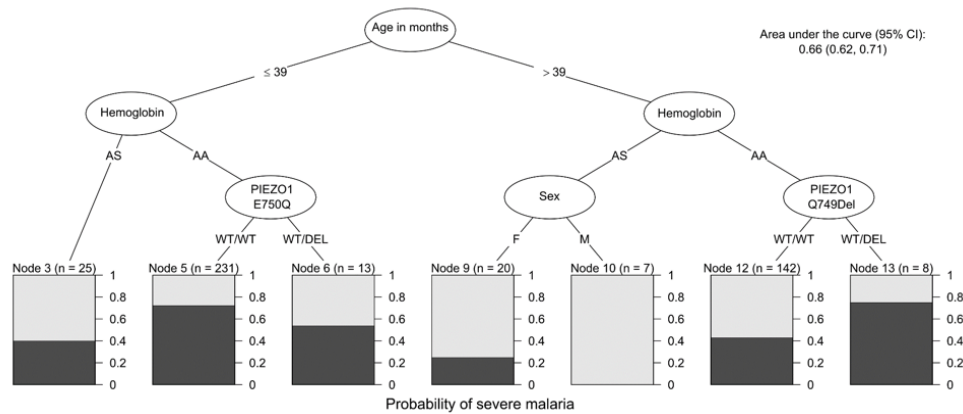
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268   *Analysis of other proximal PIEZO1 polymorphisms*

269   In addition to E756del, we identified seven other single nucleotide polymorphisms and

270   insertion-deletions within the ~160 bp region we sequenced, which ranged in frequency from 0.4%

271   to 7.9% (Table S4). Even though these mutations are in close proximity to E756del, none of them

272   were significantly associated with severe malaria individually. To determine whether any of these

273   *PIEZO1* variants act synergistically with hemoglobin type, sex or age to influence malaria

274   susceptibility in the absence of E756del, we used classification trees (CART) (Figure 3A). For

275   subjects with HbAA, when E756del was excluded, heterozygosity for E750Q appeared protective

276   in younger children, whereas heterozygosity for Q749del was associated with an increased

277   probability of severe malaria in older children. However, correlation testing showed E750Q and

278   Q749del were significantly correlated with E756del, suggesting that these two variants may be

279   serving as a proxy for E756del in predicting malaria severity (results not shown). This hypothesis

280   is further strengthened when the E756del polymorphism is included in the analysis, as no other

281   *PIEZO1* variants were predictive of disease severity in the presence of E756del (Figure 3B).

282   Together, these results suggest that E756del is the causal variant in this region, and while two of

283   the polymorphisms appeared to predict malaria severity, these may be serving as surrogates for

284   E756del.

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288

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A

B
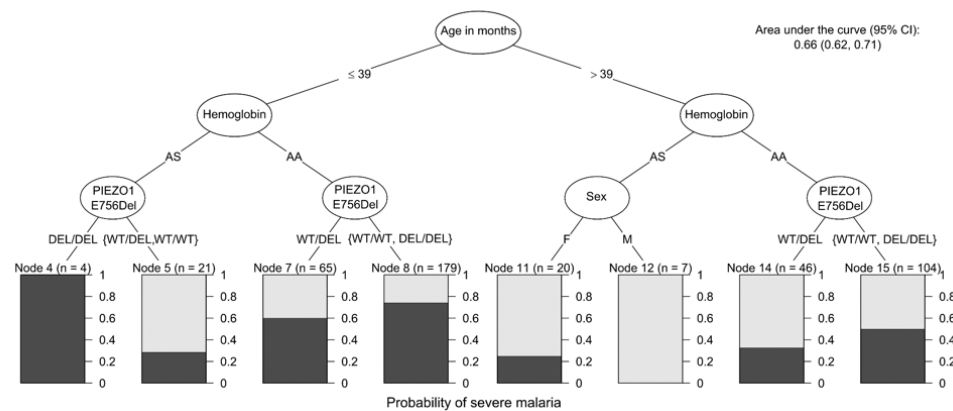

Figure 3. Classification trees predicting severe malaria with various possible predictors included. (A) Possible predictors: all *PIEZO1* variants except for E756Del, hemoglobin type, sex, and age. (B) Possible predictors: all *PIEZO1* variants, hemoglobin type, sex, and age. The higher the black bars, the higher the likelihood of severe malaria.

290

291   *Effect of PIEZO1 E756del on in vitro growth of P. falciparum*

292   Previous work suggested that human red blood cells heterozygous for the *PIEZO1*
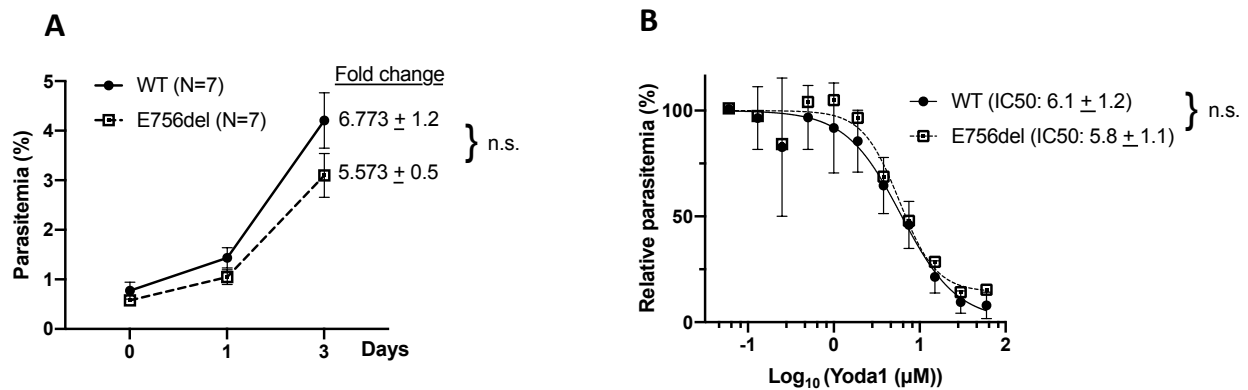
293   E756del mutation are less supportive of *P. falciparum in vitro* growth than wild-type red blood

294   cells (MA *et al.* 2018). To further investigate whether impaired intracellular growth could be a

295   mechanism by which E756del protects against severe malaria, we identified local donors of

16

296   African descent who are wild-type or heterozygous at *PIEZO1* E756del and used their red blood

297   cells in *P. falciparum* growth assays. To our surprise, we found that *P. falciparum* replicated

298   equally well in the wild-type versus E756del heterozygous erythrocyte samples (p=0.37, Figure

299   4A). Similar trends were observed when the cells were used freshly or after cryopreservation.

300   These results suggest that while the E756del mutation may confer gain-of-function kinetics (MA

301   *et al.* 2018), at least in heterozygous RBCs, the phenotype is not strong enough to impair

302   intracellular growth of *P. falciparum in vitro*.

303   To determine if chemical activation of the PIEZO1 ion channel could inhibit intracellular

304   growth of *P. falciparum* in RBCs, we used the small synthetic molecule Yoda1, which was recently

305   identified as a specific agonist of both human and mouse PIEZO1 (SYEDA *et al.* 2015). We

306   observed a dose-dependent effect on *P. falciparum* growth in both wild-type and E756del RBCs,

307   with an IC50 of ~6 µM (Figure 4B). This IC50 concentration is similar to that required for Yoda1

308   to induce PIEZO1-specific Ca^{2+} responses in HEK cells or mouse RBCs (CAHALAN *et al.* 2015;

309   SYEDA *et al.* 2015). These results demonstrate that Yoda1 has antimalarial activity and point to a

310   critical role for erythrocyte PIEZO1 in promoting the development of *P. falciparum* in red blood

311   cells. The observation that the antimalarial potency of Yoda1 is similar in the wild-type and

312   E756del genetic backgrounds provides additional evidence that the E756del mutation likely

313   confers only a mild gain-of-function phenotype in heterozygous RBCs.

A

B

Figure 4. *P. falciparum* growth is preserved in E756del erythrocytes but a small molecule agonist of PIEZO1 has antimalarial activity. (**A**) Growth curves for *P. falciparum* strain 3D7 in WT or *PIEZO1* E765del heterozygous erythrocytes. Each point represents mean raw parasitemia of N=7 donors for each genetic background, and error bars represent SEM. Each sample was run with two technical replicates. "Fold change" indicates the average fold difference ($\pm$SEM) in parasitemia on day 3 relative to day 0. n.s., not significant, p-value=0.3725. (**B**) *P. falciparum* strain 3D7 parasitemia as a function of Yoda1 concentration, as determined by flow cytometry after 3 days of drug treatment. The relative parasitemia was calculated for each biological replicate (N=7 for each genetic background) by normalizing the day 3 parasitemia at each drug concentration (two technical replicates) relative to the parasitemia in the absence of drug. Yoda1 IC50 $\pm$ SEM (μM) for WT or E765del erythrocytes are shown in parenthesis. n.s., not significant, p-value=0.4639. SEM=Standard error of mean.

314

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Discussion

316

317   In this work, we sought to determine the clinical relevance of a recent study demonstrating

318   a survival benefit for *Plasmodium*-infected mice carrying a *PIEZO1* gain-of-function allele (MA *et*

319   *al.* 2018). In the *PIEZO1*GOF mouse model, the RBCs were dehydrated, intracellular parasite

320   growth was impaired, and the animals were protected from developing cerebral malaria. We

321   assessed the human *PIEZO1* mutation E756del, which was identified as a gain-of-function allele

322   based on its prolonged inactivation time constant after mechanical stimulation in HEK cells (MA

323   *et al.* 2018). Using samples from Gabonese patients with severe or uncomplicated malaria, we

324   found that *PIEZO1* E756del is significantly associated with protection against severe malaria.

325   Children heterozygous for E756del had a significantly lower predicted probability of severe

326   malaria compared to those with wild-type *PIEZO1*, even when controlling for age, sex, and HbAS

327   status. As E756del is present at high frequencies in African populations (MA *et al.* 2018), these

328   results suggest that it may be a major determinant of innate resistance to severe malaria. Whether

329   this advantage is extended to all severe malaria sub-phenotypes remains to be investigated in

330   the context of a larger study.

18

331   Given the strong protective effect of *PIEZO1* E756del on severe malaria in our study, its

332   absence from GWAS candidate lists is particularly notable. The sensitivity of GWAS studies for

333   malaria in Africa are limited by several factors, including weak linkage disequilibrium and

334   population stratification (JALLOW *et al.* 2009), as well as technical limitations in assessing complex

335   or repetitive regions via microarrays or deep sequencing. The *PIEZO1* E756del mutation is a

336   three nucleotide deletion within a region of short tandem repeats, making it difficult to detect by

337   high-throughput methods. In our study, accurate genotyping of this locus required Sanger

338   sequencing and manual alignments. These studies on *PIEZO1* highlight the benefit of using a

339   combination of sequencing methods for the identification of host factors that may influence

340   susceptibility to malaria.

341   The finding that E756del is protective against severe malaria only in heterozygotes is

342   reminiscent of the protective effect of HbAS, where heterozygotes with sickle cell trait are

343   protected from malaria-related mortality but homozygotes with sickle cell disease are not (AMBE

344   *et al.* 2001; AIDOO *et al.* 2002; KOMBA *et al.* 2009). Although GOF mutations in *PIEZO1* are a

345   recognized cause of hereditary xerocytosis (GLOGOWSKA *et al.* 2017), to our knowledge the

346   E756del allele has not been implicated in any hematologic disease (MA *et al.* 2018). If E756del is

347   truly a GOF allele it seems likely that homozygous mutant cells would be dehydrated and display

348   altered permeability, predisposing to hemolytic anemia and severe symptoms upon infection with

349   *P. falciparum*. Given the high allele frequency of E756del in our Gabonese study population

350   (19%), understanding how homozygosity for this mutation affects PIEZO1 channel function, RBC

351   hydration status and *in vitro* susceptibility to *P. falciparum* are important open questions.

352   We did not observe any influence of co-inheritance of HbAS and E756del heterozygosity

353   on malaria susceptibility, suggesting that having one E756del allele does not contribute any

354   additional effect to the already powerful protection conferred by HbAS. In contrast, subjects with

355   HbAS who are homozygous mutant for E756del had an extremely high predicted probability of

356   severe malaria. While this association was not statistically significant, it highlights the need for

357　further research on *PIEZO1* E756del, as it suggests that the protective effect of HbAS on malaria

358　can be subverted by structural or permeability abnormalities found in homozygous E756del RBCs.

359　Therefore, future studies would need to further validate these findings. A recent analysis of

360　associations between E756del and clinical parameters in sickle cell disease (SCD) patients

361　showed that the *PIEZO1* E756del variant was associated with increased RBC density and

362　dehydration in HbSS cells, providing an additional example of a potential phenotypic interplay

363　between these genes (ILBOUDO *et al.* 2018).

364　　　　The hypothesis that *PIEZO1* may influence malaria susceptibility arose from the

365　observation that *P. falciparum* parasites grow poorly in dehydrated red blood cells and the

366　knowledge that hereditary xerocytosis, a disorder characterized by red blood cell dehydration, is

367　often caused by GOF mutations in *PIEZO1* (TIFFERT *et al.* 2005; ANDOLFO *et al.* 2013; ARCHER *et*

368　*al.* 2014). Although our results show that *PIEZO1* E756del is protective against severe malaria in

369　heterozygotes, the mechanism of protection is not yet known. In the mouse model, expression of

370　a *PIEZO1* GOF allele in the hematopoietic lineage was protective against cerebral malaria and

371　appeared to impair growth of *P. berghei* ANKA parasites, at least during the initial phase of

372　infection (MA *et al.* 2018). The same study suggested that *P. falciparum* growth was impaired in

373　human RBCs carrying an E756del allele (MA *et al.* 2018). However, in our experiments we did not

374　observe a significant *P. falciparum* growth defect in *PIEZO1* E756del heterozygous RBCs. *P.*

375　*falciparum* grew equally well in wild-type versus E756del-carrying RBCs, regardless of whether

376　the cells were fresh or had been previously cryopreserved. These results demonstrate that

377　heterozygosity for *PIEZO1* E756del does not in itself hinder *P. falciparum* invasion or growth in

378　red blood cells. While the reason for the discrepant findings is not clear, it is possible that time

379　from donation, processing variability and/or other technical issues confounded the conclusions of

380　the previous study.

381　　　　Our studies indicate that while *P. falciparum* can grow normally in human RBCs carrying

382　a *PIEZO1* E756del allele, the PIEZO1 agonist Yoda1 had potent antimalarial activity. Presumably

383 the changes in ionic permeability induced by Yoda1 create an inhospitable environment for normal

384 parasite development. Since the drug assay results demonstrate that strong activation of PIEZO1

385 in RBCs can inhibit *P. falciparum* growth, one explanation for the lack of a phenotype in untreated

386 E756del heterozygous RBCs could be that E756del confers only a mild channel defect. Indeed,

387 out of two candidates and one established *PIEZO1* GOF mutation tested, E756del displayed the

388 shortest channel inactivation time (MA *et al.* 2018). As the experiments measuring channel

389 kinetics were performed using ectopic expression in HEK cells, it is possible that the channel

390 phenotype of E756del in heterozygous RBCs is even more mild. This conclusion is further

391 supported by our findings showing that wild-type and E756del RBCs were equally sensitive to

392 Yoda1's antimalarial activity. Previous work in mice showed that Yoda1 is specific for PIEZO1, as

393 it induced cation permeability and cellular dehydration in wild-type but not PIEZO1-null mouse

394 RBCs (CAHALAN *et al.* 2015). Bioinformatic analyses aimed at identifying mechanosensitive

395 channels in pathogenic protozoa did not identify any homologues of *PIEZO1* in Apicomplexan

396 parasites, making it unlikely that Yoda1 could be acting on a parasite-encoded target (PROLE AND

397 TAYLOR 2013). In our experiments, we further minimized this possibility by pre-incubating the

398 RBCs in Yoda1 for several hours and then washing the cells extensively before infecting with

399 parasites.

400 Given that *in vitro* growth of *P. falciparum* in RBCs is unaffected by PIEZO1 E756del, how

401 might this common polymorphism protect from severe malaria? As with many life-threatening

402 systemic infections, the symptoms of severe malaria are in large part caused by the body's

403 extreme response to overwhelming infection by a microorganism. Additionally, the adhesive

404 properties of *P. falciparum*-infected RBCs enable them to sequester in the deep microvasculature

405 and adhere to uninfected RBCs, exacerbating organ dysfunction. As *PIEZO1* is expressed in

406 many tissues including the vascular endothelium and immune cells, it is possible that

407 dysregulation of its channel activity has effects on vascular tone, cell permeability and/or signaling

408 during malaria infection that are independent of any effects on RBCs. Alternatively, E756del may

409   alter *P. falciparum* virulence properties rather than intracellular growth, such as the ability of the

410   parasite to export cytoadherence proteins to the RBC plasma membrane. This type of mechanism

411   has previously been proposed to explain the malaria-protective nature of the hemoglobin C

412   mutation (FAIRHURST *et al.* 2005).

413   Our findings show a significant association between *PIEZO1* E756del and protection from

414   severe *P. falciparum* malaria. Together with the previous elegant mechanistic work on mouse

415   *PIEZO1* GOF mutations and *P. berghei* ANKA, these data firmly establish PIEZO1 as an

416   important host susceptibility factor for malaria. Though the list of innate susceptibility determinants

417   for severe malaria includes a range of factors such as hemoglobin variants, enzymes, membrane

418   proteins and immune-related molecules (APINJOH *et al.* 2013; NGUETSE AND EGAN 2019), PIEZO1

419   is unique because of its potential to influence *P. falciparum* pathogenesis on multiple levels

420   (including its asexual replication in RBCs and ability to mediate cerebral malaria), and because it

421   is considered a druggable molecule (SYEDA *et al.* 2015). The demonstration that chemical

422   activation of RBC PIEZO1 impairs *P. falciparum* growth *in vitro,* combined with the discovery that

423   the E756del allele is associated with protection from severe malaria in patients, suggests that

424   PIEZO1 has potential as a compelling target for a host-directed therapy for malaria.

425

426

427   **Author contributions**

428   CNN, experimental design, acquisition, analysis and interpretation of data, and writing the

429   manuscript; NP, analysis and interpretation of data and editing the manuscript; BS, acquisition

430   and analysis of data and editing the manuscript; ERE, conception and experimental design,

431   sample acquisition, data analysis and interpretation and editing the manuscript; PGK, TPV,

432   sample acquisition, experimental design, and editing the manuscript; ESE, conception and

433   experimental design, analysis and interpretation of data, provision of resources and supervision,

434   and writing the manuscript. All authors read and approved the final manuscript.

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Declaration of interests

437   We declare no competing interests.

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Acknowledgments

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