Elevated *Plasmodium falciparum* sexual conversion in HbAC and HbAS red blood cells without altered IgG/IgM levels to trophozoite and stage I gametocyte-specific antigens

Running title: *Plasmodium falciparum*, hemoglobin beta mutations, sexual conversion and humoral immunity.

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ABSTRACT

Malaria transmission relies on the differentiation of *Plasmodium falciparum* asexual parasites into gametocytes, a process determined by sexual conversion. Mutations in the host hemoglobin beta (HBB) gene, are known to influence parasite growth, and disease outcome, yet their impact on sexual conversion remains unclear. We investigated the effect of HBB mutant genotypes on sexual conversion and humoral immunity in individuals from Nanoro, Burkina Faso. Sexual conversion rates were assessed with a newly developed *ex vivo* sexual conversion assay (*ev*SCA) in HBB genotypes HbAA, HbAC, and HbAS, and an *in vitro* SCA

(*iv*SCA) using the NF54-*gexp02-Tom* sexual conversion reporter line cultured in red blood cells (RBCs) with HbAA, HbAC, HbAS, HbSS, and HbSC genotypes. IgG and IgM responses against trophozoite- and stage I gametocyte-infected RBC surface antigens were quantified by flow cytometry in plasma from individuals with HbAA, HbAC, HbAS, HbCC, and HbSC genotypes. Growth in HBB mutant RBCs significantly increased sexual conversion rates in both *ex vivo* and *in vitro* assays, while humoral responses did not differ between HBB mutant and wild-type individuals. These results demonstrate that HBB mutations enhance sexual conversion independently of IgG- and IgM-mediated immunity, highlighting a host-genetic factor that may influence malaria transmission potential.

Keywords: *Plasmodium falciparum*, Malaria transmission, Gametocytes, Sexual conversion, Hemoglobin beta (HBB), Red blood cells, Humoral immunity.

INTRODUCTION

Plasmodium falciparum (*P. falciparum*) causes over half a million deaths annually, mostly in young children from low- and middle-income countries. Despite the substantial progress in reducing the global malaria burden during the last decade, this decline has stalled, with some countries reporting a resurgence in clinical cases [1]. These trends highlight the urgent need to develop and implement innovative approaches for malaria control and elimination.

Plasmodium infections have exerted strong evolutionary pressure on the human genome, with hemoglobin mutations providing a well-documented example [2-5]. Mutations in the human hemoglobin beta (HBB) gene, specifically those giving rise to hemoglobin S (HbS) and hemoglobin C (HbC), result from a substitution of glutamic acid at position 6 with valine in HbS and lysine in HbC. Individuals can carry homozygous (HbSS or HbCC), heterozygous (HbAS or HbAC) or compound heterozygous (HbSC) forms, with HbSC individuals inheriting one copy of each mutation [6-8]. HBB mutations lead to significant changes in red blood cell (RBC) morphology and function, influencing malaria susceptibility and clinical outcomes [5, 9, 10]. HbS mutations induce hemoglobin polymerization under low oxygen, causing RBCs to reshape into sickle forms. In individuals with HbSS, extensive sickling affects approximately 60-80% of total RBCs [2], resulting in severe complications including vaso-occlusion, hemolysis, and chronic anemia [11, 12]. Early-life mortality is also markedly elevated, with estimates ranging from 50-90% among children in low-and middle income countries [13]. On the other hand, HbAS carriers are typically asymptomatic [2], with fewer sickled RBCs (~40%) under low oxygen [12, 14]. HbC mutations cause hemoglobin crystallization without sickling but increase blood viscosity and shorten RBC life span [15]. HbCC is a mild disorder with individuals typically asymptomatic while some may develop mild chronic hemolytic anemia [15]. HbAC carriers are mostly asymptomatic [2, 7]. Compound heterozygotes (HbSC) display

25 a mixed phenotype with ~50% sickling and ~50% crystallization [2], leading to moderate
26 disease severity and altered RBC shapes, such as "pita bread" or "tricorn" forms under low
27 oxygen [11, 16].

28 While HbSS and HbCC are relatively rare (~2%), likely due to the high mortality, HbAS and
29 HbAC can reach frequencies of 10-30% in regions such as Mali and Burkina Faso, which has
30 been linked to the protection they confer against *P. falciparum* malaria [4, 5, 8, 17-20]. HbAS
31 provides strong protection against both uncomplicated and severe malaria, significantly
32 reducing hospitalization risk [5, 21-23]. HbAC confers mild protection [5], while HbCC offers
33 substantial protection [15]. The protective mechanisms of the different HBB mutations are not
34 fully understood [4, 24], but include impaired parasite growth [4, 25-31], altered RBC
35 morphology [32, 33], disrupted trafficking of *P. falciparum* erythrocyte membrane protein-1
36 (PfEMP-1) with reduced cytoadherence [24, 32, 34, 35], changes in microRNA expression [26,
37 36], and heme oxygenase-1 (HO-1) induction [37, 38]. HbS infected-RBCs (iRBCs), especially
38 in HbSS and to a lesser extent in HbAS, exhibit elevated heme levels linked to increased HO-
39 1−mediated hemoglobin digestion and oxidative stress. This disrupts the actin cytoskeleton,
40 alters iRBCs morphology, and impairs the surface expression of adhesins such as PfEMP-1 on
41 iRBCs [32, 39, 40]. In HbAS iRBCs, this altered presentation of the major adhesin PfEMP-1
42 reduces cytoadherence and vascular sequestration, promoting splenic clearance and controlled
43 parasite infection [34]. In contrast, Archer, N.M., et al., (2018) [31] proposed a mechanism in
44 which delayed parasite development in HbAS iRBCs arises from HbS-polymerization
45 following cytoadherence and sequestration of iRBCs into hypoxic microcirculatory niches (*i.e.,*
46 bone marrow and spleen), where polymer-induced oxidative stress impairs parasite
47 development.

48 Beyond protection from disease, HbS and HbC RBCs may influence the transmission potential
49 of *P. falciparum*. Host hemoglobin genetics may significantly affect gametocyte (the

50 transmissible stage of the parasite) carriage. Population studies have reported conflicting
51 evidence regarding whether HbAS influences gametocyte carriage. Gouagna, L., et. al., (2010)
52 [41] reported no significant association between HbAS and increased gametocyte carriage,
53 though they did observe associations for HbAC and HbCC. In contrast, other studies found a
54 significant link between HbAS and gametocyte carriage [42], while some reported only a
55 modest contribution of HbAS to increased gametocyte prevalence [43]. While most evidence
56 focuses on elevated gametocyte carriage, a few studies have also reported higher infectivity to
57 mosquitoes in individuals carrying HbAS [41, 44], HbAC [41, 45] and HbCC [41] HBB mutant
58 RBCs compared to wild-type HbAA RBCs.

59 Gametocyte production is initiated by a process called sexual conversion. In each asexual
60 replication cycle of *P. falciparum*, a variable but typically small proportion of parasites (~1%),
61 commit to sexual development and initiate gametocytogenesis [46-48]. The proportion of
62 parasites that commit to sexual development is referred to as sexual conversion rate (SCR).
63 Sexual commitment is marked by epigenetic activation of the master regulator *pfap2-g* [49,
64 50], a transcription factor that irreversibly results in sexual conversion and gametocyte
65 development [49, 50]. The initiation of its expression is triggered by the activation of *P.*
66 *falciparum* gametocyte development 1 (PfGDV1) protein [51], which derepresses *pfap2-g* and
67 which in turn leads to the initiation of the sexual transcriptional program, halting asexual
68 replication and inducing the expression of early gametocyte biomarkers [52, 53]. Sexual rings
69 are found in the circulating blood [52, 54-56], while maturation from gametocyte stages I to
70 IV occurs mainly in the bone marrow over ~10 days [57]. Mature male and female stage V
71 gametocytes are released back into peripheral blood (gametocyte carriage), and are the only
72 parasite stages capable of infecting *Anopheles* mosquitoes. Gametocyte carriage determines
73 human to mosquito transmission and correlates with infectivity to mosquitoes [58-63].

The elevated gametocyte carriage, that has been reported in individuals carrying the HbAC and HbAS genotype, could be explained by a number of factors amongst which elevated sexual conversion but also prolonged infection leading to gametocytes in the peripheral blood that stem from numerous replication cycles. Given the impaired parasite growth in HbS and HbC iRBCs [64], *P. falciparum* may prioritize transmission through increased sexual conversion consistent with the terminal investment hypothesis [65-68]. This theory suggests that when parasites encounter hostile conditions they increase gametocyte production to improve their chances of being transmitted. Which has been supported by the findings that several host related factors increase sexual conversion [69], including antimalarial treatment [70-72], lysophosphatidylcholine depletion [73-75], and reticulocyte-rich environments [76, 77]. So far only one study has investigated the link between sexual conversion and HBB genetics showing that increased intracellular heme in sickle-cell anemia (HbS iRBCs) forming an impaired RBC environment for parasite survival, increases sexual conversion [39]. Another potential driver of elevated sexual conversion in HBB mutant RBCs is the host immunity against *P. falciparum*. HbS and HbC mutations appear to modulate host immunity against *P. falciparum* through increased regulatory cytokines such as IL-10 [29]), upregulated HO-1 [27], and dampened pro-inflammatory responses (*e.g.,* TNF-α, IFN-γ) [24], which at the same time protect from severe symptoms of malaria that are largely due to an exacerbated immune response and improve parasite clearance. Moreover, elevated antibody levels against specific RBC antigens have been reported in individuals carrying HBB mutant RBCs [24]. In HbAS individuals, enhanced humoral responses have been observed, including increased IgG antibody levels against asexual-stage antigens (*e.g.,* AMA1, EBA175, MSP1-3, CSP, and PSE) in low-transmission areas in Burkina Faso [78], as well as elevated antibodies against RBC surface antigens such as PfEMP-1 in multiple populations [78-81]. These immune changes impair cytoadherence and

98 promote clearance of HBB mutant iRBCs, potentially creating conditions that favor sexual
99 conversion as a parasite survival strategy.

100 The discrepancies in studies assessing gametocyte carriage highlight the need to investigate
101 upstream parasite processes, such as sexual conversion, which directly determine gametocyte
102 production. Demonstrating that HBB mutant RBCs, such as HbAS and HbAC, enhance *P.*
103 *falciparum* sexual conversion could improve strategies to interrupt malaria transmission [82].
104 This would identify individuals carrying these genotypes as important contributors to the
105 infectious reservoir and emphasize their relevance for targeted transmission-blocking
106 interventions.

107 In this study, we investigated the impact of host HBB mutant genotypes on sexual conversion
108 of *P. falciparum* and whether we observe differences in IgG- and IgM-mediated humoral
109 immunity that could be a potential driver of an increase in sexual conversion in these
110 individuals. We assessed sexual conversion rates *ex vivo* in individuals with HBB genotypes
111 HbAA, HbAC and HbAS infected with *P. falciparum* from Nanoro (Burkina Faso); and *in vitro*
112 using the *NF54-gexp02-Tom* transgenic sexual conversion reporter line [53, 70], cultured in
113 (non-infected) blood from individuals with HBB genotypes HbAA, HbAC, HbAS, HbSS, and
114 HbSC. Levels of IgG and IgM against trophozoite- and stage I gametocyte -infected RBCs
115 (Troph-Gam-iRBCs) were evaluated by flow cytometry in plasma from Burkinabé individuals
116 with HBB genotypes HbAA, HbAC, HbAS, HbCC, and HbSC. Our results show that growth
117 in blood with HBB mutant genotypes increases parasite sexual conversion both in *ex vivo* and
118 *in vitro* cultures. We did not find altered levels of IgG- or IgM against trophozoite and stage I
119 gametocyte-iRBC surface antigens in individuals with HBB mutant genotypes.

120

121

MATERIALS AND METHODS

Details about reagents and tools are accessible in Supplementary Table 1.

Study design.

Sample collection for the present study was embedded in a longitudinal cohort study assessing the effect of host factors on transmission potential (*InHost* study, ethical advice reference 1261/18 and 19/06/064), conducted in the Nanoro Department, a rural, malaria-hyperendemic region in Central-West Burkina Faso (Figure 1) [83] [Characterizing the *Plasmodium falciparum* infectious reservoir asymptomatic asexual and gametocyte stage across off peak and peak transmission seasons in a rural and high-transmission setting in Burkina Faso: a longitudinal 2-year cohort study, *manuscript in preparation*]. Briefly, 871 study participants of all ages from four villages (Nanoro, Nazoanga, Séguédin and Soum) were followed for two years (March 2019 - February 2021) through six cross-sectional surveys conducted during both high and low transmission season to assess asexual and sexual parasite carriage and risk factors. Active case detection was complemented by passive case detection at peripheral health centers.

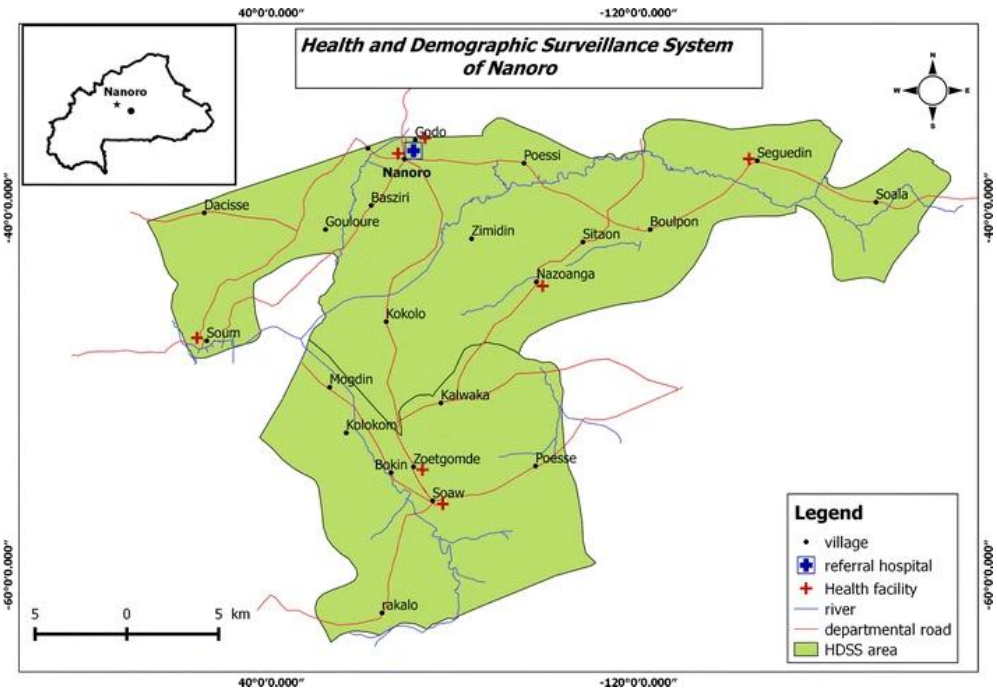


Figure 1. Map of the study area. The inset map highlights the location of the Nanoro department of Boulkiemdé Province in Central Burkina Faso, with the black dot indicating the position of the capital, Ouagadougou.

138 For this study, from all participants of the *InHost* study (*n* = 871), 864 individuals were
139 genotyped for their human hemoglobin beta (HBB) genotype (see section on "Hemoglobin beta
140 sequencing"). Participants were then selected for this sub-cohort based on their genotype. A
141 venous blood sample was collected from all selected participants. Samples were screened for
142 *P. falciparum (Pf)* infection. Samples from *Pf*-infected individuals were used to perform *ex*
143 *vivo* sexual conversion assays (*ev*SCAs) and samples from non-infected individuals were
144 preserved to perform *in vitro* sexual conversion assays (*iv*SCAs). We included all individuals
145 carrying the HBB genotypes HbAC (*n* = 72), HbAS (*n* = 50), HbCC (*n* = 2), HbSC (*n* = 7), and
146 a random subset of individuals with the HbAA genotype (*n* = 94) (Supplementary Figure 1).
147 In total, 225 individuals were listed (age groups "5 - 19 years" [*n* = 166] and "more than 20
148 years" [*n* = 59]). Listed individuals were visited by a nurse at home (Figure 1) and were invited
149 to the Clinical Research Unit of Nanoro (CRUN) for screening and enrolment into the study.
150 Population screening for *P. falciparum* infections and enrolment took place over a two-month
151 period (August-September 2022).
152 At CRUN, the eligibility was assessed based on inclusion and exclusion criteria for the *ev*SCAs
153 (*Pf*-infected individuals) and *iv*SCAs (non-infected individuals). Inclusion criteria consisted
154 of: being enrolled in the main *InHost* study, a confirmed *P. falciparum* malaria infection (for
155 the *ev*SCAs) or a confirmed negative *P. falciparum* malaria infection (for the *iv*SCAs), willing
156 to participate in this study and undergo additional sampling, and signed informed consent.
157 Exclusion criteria consisted of: mixed infection with other *Plasmodium* species (only for *Pf*-
158 infected individuals in the *ev*SCAs), antimalarial treatment within the last two weeks, signs of
159 severe malaria (*e.g.* prostration), severe chronic illness besides hemoglobinopathies (*e.g.,*
160 cancer, heart disease, kidney or liver disorders, HIV/AIDS, etc.), and planning to leave the
161 study region during the research period. From 162 listed individuals, a total of 161 participants
162 were selected as eligible and were included within this sub-cohort [with age groups "5-19

163 years" (n = 122) and "more than 20 years" (n = 39); with HBB genotypes HbAA (n = 70),
164 HbAC (n = 58), HbAS (n = 24), HbCC (n = 2), HbSC (n = 7)] (Supplementary Figure 1). A 15
165 ml venous blood sample was collected from each participant. After sample collection, blood
166 samples were screened for *P. falciparum* infections (see paragraph on "*P. falciparum*
167 diagnosis"). Samples with confirmed *P. falciparum* infection (*Pf*-infected individuals, *Pf*+) and
168 parasitemia by light microscopy (LM) ≥ 0.05% (*i.e.* ≥ 2500 parasites/µl) were processed for
169 *ev*SCAs at CRUN (Table 1). Blood samples negative for *P. falciparum* infection were
170 processed and preserved for *iv*SCAs conducted at the Institute of Tropical Medicine (ITM),
171 Antwerp (Table 1, see section on "*iv*SCAs"). *Pf*-infected individuals, as detected by LM and
172 confirmed with *P. falciparum var* gene acidic terminal sequence (*var*ATS) quantitative PCR
173 (qPCR), were treated with Artemether-Lumefantrine (AL) following national guidelines and
174 were asked to return three days later to confirm infection clearance.
175 Sample size was calculated based on a power analysis assuming an expected effect size of 50%,
176 a 5% significance level, and 90% power. This sample size was considered sufficient to detect
177 differences in sexual conversion by HBB genotype, based on effect sizes reported in previous
178 studies [76]. To account for potential variability in HBB genotype groups, a minimum of 8
179 participants per HBB genotype group was required. To ensure sufficient sampling and
180 successful assays per HBB genotype group, the sample size was upscaled to a maximum of 15
181 participants per HBB genotype group.
182 To increase the number of HbSC and HbSS blood samples for *iv*SCA, additional blood samples
183 were collected from patients with hemoglobinopathies (HbSS and HbSC) visited at the
184 University Hospital of Antwerp (UZA), Belgium. A total of 13 adults (aged 19-37 years),
185 routinely followed at UZA's Hematology Unit, were invited to donate a blood sample with
186 exclusion criteria of fever. The inclusion period was between October 2023 and April 2024.
187 **Table 1: Number of samples (n) per *P. falciparum* (*Pf*) infection status at the time of sample collection and**
188 **HBB genetics of the donor, for the** *ex vivo* sexual conversion assays **(*ev*SCAs),** *in vitro* sexual conversion assays

189 (***iv*SCAs) and Immunoglobulin (Ig) detection assays**. Note: *Pf*+ samples used in the *ev*SCAs must have a
190 parasitemia ≥ 0.05% (*i.e.,* ≥ 2500 parasites/µl), as the established limit of accurate assessment of sexual conversion
191 with these assays. Abbreviations: Hb, hemoglobin; CRUN, Clinical Research Unit in Nanoro; ITM, Institute of
192 Tropical Medicine in Antwerp; UZA, University Hospital in Antwerp.
193

	HbAA	HbAC	HbAS	HbSC	HbCC	HbSS	Total
***Ev*SCAs**							
Pf+ (CRUN, total n)	14	12	6	-	-	-	32
***Iv*SCAs**							
Pf- (total n)	5	5	4	2	-	12	28
CRUN (n)	5	5	4	1	-	-	
UZA (n)	-	-	-	1	-	12	
Ig detection							
Pf+ (CRUN)	13	13	13	3	1	-	43
Pf- (CRUN)	5	5	5	2	1	-	18
Total n (*Pf*+ and *Pf-)*	18	18	18	5	2	-	61

194

195 ***Plasmodium falciparum* diagnosis.**

196 *P. falciparum* malaria diagnosis was done by RDT using the Bioline™ malaria antigen P.f®
197 test (05FK50, Abbott) (recommended by the National malaria Control Programme (NMCP) in
198 Burkina Faso), following the manufacturer instructions. In addition, malaria parasites were
199 detected and quantified by LM following standard protocols at CRUN [84]. In brief, blood
200 smears were stained with Giemsa and examined until 200 white blood cells were counted.
201 Parasitemia was calculated based on the number of asexual parasites per white blood cells,
202 expressed as asexual parasites per microliter of blood, assuming a white blood cell count of
203 8,000 per microlite. The presence of gametocytes and other *P. falciparum* species (*P. ovale*
204 *and P. malariae*) was assessed in all positive blood smears [85]. Blood samples that tested
205 positive for *P. falciparum* by both RDT and LM were classified as *P. falciparum*-infected.
206 Samples that were negative by LM (regardless of the RDT result), were identified as non-
207 infected (Table 1 and Supplementary Figure 1). Negative infection with *P. falciparum* was
208 confirmed by *var*ATS qPCR, using five µL of DNA (extracted with QIAamp DNA Mini Kit,
209 Qiagen Germany), as previously described [83, 86] (Supplementary Figure 1). The limit of
210 detection of the *var*ATS-based qPCR was 0.1 parasite/µL (in our hands), with samples showing
211 Ct > 39.7 considered negative. DNA from the *P. falciparum* 3D7 strain served as a positive

212 control, while negative controls included DNA from non-infected human blood and a no-
213 template control (NTC).

214 **Sample collection and processing.**

215 At CRUN, a 15 ml venous blood sample was collected in an Acid Citrate Dextrose (ACD) tube
216 and was immediately transported to the laboratory for processing. Samples were centrifuged
217 (1,648g for 5 minutes) and washed two times with RPMI to isolate the RBC pellet. RBC pellets
218 from *P. falciparum*-infected blood samples were immediately put in culture for the *ev*SCAs
219 (see section on "*Ex vivo* sexual conversion assays (*ev*SCAs)". RBC pellets from non-infected
220 samples were cryopreserved (1:3 with 3% sorbitol and 0.65% NaCl) and stored directly at -
221 80°C until shipment to ITM, where preserved in liquid nitrogen until use in *iv*SCAs. Two
222 hundred microliters of whole blood were used for DNA extraction and confirmation of *P.*
223 *falciparum* infection by *var*ATS qPCR (see section on "*P. falciparum* diagnosis). At UZA, 15-
224 20 ml of non-infected venous blood samples were collected in EDTA tubes and immediately
225 transported at 4°C to ITM for processing, done as described for CRUN. The resulting RBC
226 pellets were cryopreserved (1:3 with 3% sorbitol and 0.65% NaCl) by direct snap-freezing in
227 liquid nitrogen for subsequent *iv*SCAs. Additionally, ABO and Rhesus D blood groups were
228 determined with agglutination tests (BIO-RAD, REF: 801320, 801345, 802032).

229 **Hemoglobin beta sequencing (HBBseq).**

230 In samples collected at CRUN, PCR amplification of the HBB gene (NCBI_Gene:3043) was
231 performed to identify single nucleotide polymorphisms (SNPs) following the PCR conditions
232 of *Wittwer, C.T., et al., Clin Chem.(2003)*[87], excluding the High Resolution Melting (HRM)
233 step, and the PCR mix from the standard protocol for *glurp* genotyping [88]. Primer pairs
234 (sequences: [HBB-fwd: GGGTTGGCCAATCTACTCCC and HBB-rev: TCAAGCGTCCCA-
235 TAGACTCAC]) were designed to amplify a 599 bp product. PCR reactions were performed
236 in a total volume of 25 µl, containing 2.5 µl of DNA template, 10X Buffer B, 5mM of each

237 dNTP, 0.3 µM of each primer, 25 mM of $MgCl_2$, and FIREPol® DNA Polymerase (Solis
238 Biodyne, Ref: 01-01-00500). Thermal cycling conditions were as follows: an initial
239 denaturation at 94°C for 6 minutes, followed by 40 cycles of 60.6°C for 30 seconds, and 72°C
240 for 1 minute, with a final extension at 72°C for 10 seconds. After Sanger sequencing,
241 chromatograms were analysed using BioEdit 7.2. (Informer Technologies) to identify the wild-
242 type and the mutant HBB codons (Table 2). In samples collected at UZA, the HBB genotype
243 sequences were determined by capillary hemoglobin electrophoresis at UZA [89].

244 **Table 2: HBB codons and genotypes identified by Sanger sequencing analysis of the HBB gene (HBB-seq).**

HBB allele	Mutation	HBB codon
HbA (wild type)	-	GAG
HbS (mutant)	***c.20A>T = E (Glu)> V (Val)***	GTG
HbC (mutant)	***c.19G>A = E (Glu) > K (Lys)***	AAG
HbSC (double mutant)	***HbS mutation (Glu→Val) + HbC mutation (Glu→Lys)***	AAG + GTG [90]

245

246 ***Ex vivo* sexual conversion assays (*ev*SCAs).**

247 *Optimization and validation*

248 We developed a new *ex vivo* sexual conversion assay (*ev*SCA; Figure 4A) to measure sexual
249 conversion in freshly collected field isolates even with low parasite densities. The assay is
250 based on the maturation of ring-stage parasites present in circulating blood to schizonts and
251 stage I gametocyte, followed by their quantification using an immunofluorescence assay (IFA)
252 detecting parasite DNA and the gametocyte-specific protein *Pfs16*. The sexual conversion rate
253 is defined as the proportion of gametocytes stage I among all parasites. For method
254 optimization we tested: 1) parasite purification methods for performance and field feasibility,
255 and 2) the minimal detectable parasite density of the infection and culture volume where we
256 could accurately quantify sexual conversion by IFAs. For optimization purposes, we used mock

257 cultures of the transgenic reporter line NF54-*gexp02-Tom* [53, 70] (see section on "*iv*SCAs"
258 for details).
259 For parasite purification, we compared the performance of the magnetic-activated cell
260 separation (MACS) LD and LS columns (Miltenyi Biotec) using NF54-*gexp02-Tom* parasites
261 cultured in a 10 ml volume, at 3% hematocrit (Hct), and ~11% parasitemia, containing mixed
262 parasite stages of ring, trophozoite, schizonts and gametocyte stage I. In brief, ~255-300 µl of
263 iRBC pellet was diluted 1:4 with culture medium (final volume: ~1,020-1,200 µl) to reduce
264 viscosity and facilitate efficient separation, and passed through the columns. We performed
265 two elution steps with culture medium: the first elution (6 ml) containing non-infected RBCs
266 and ring stages; and a second elution (6ml) with the bound fraction after removing the column
267 from the magnet yielding hemozoin-containing stage I gametocytes and schizonts. The first
268 and second elution were then pelleted (1'648 g, 5 min centrifugation). From the purified RBC
269 pellets, 3 µl (LD column) and 1.5 µl (LS column) were used to prepare thin blood smears to
270 quantify the percentage of parasite stages by LM, and to confirm the presence of ring stages
271 (first elution) and gametocytes stage I and schizonts (second elution). As results showed that
272 purification was better with MACS LD column, we next investigated the limit of detectable
273 parasitemia using MACS LD columns.
274 To determine minimal detectable parasitemia, we cultured NF54-*gexp02-Tom* parasites at 3%
275 Hct in 10 and 20 ml volumes, until a parasitemia of ~1%. Then, we performed a 10-fold serial
276 dilution to mimic parasite density ranges from ~1% to ~0.001%. Starting with ~8.5 ml of
277 culture at ~1% parasitemia (corresponding to ~255 µl-300 µl RBC pellet) from a 10 ml culture,
278 10-fold dilutions were prepared by mixing decreasing volumes of culture with non-infected
279 RBCs, until reaching a parasitemia of ~0.001% (keeping the final volume of RBC pellet fixed
280 at 255 µl). For a 20 ml culture, the respective volumes were doubled (510 µl of RBC pellet).
281 Then, for each condition (dilution point) RBC pellets were diluted 1:4 with culture medium

282 (end volume of ~1'020 µl) and purified with MACS LD columns. A thin blood smear per
283 condition was prepared from 3 µl of the purified RBC pellet without dilution, following of
284 IFAs to detect Pf16 to determine minimal detectable parasitemia.
285 Additionally, we assessed whether we could cryopreserve parasite isolates and perform sexual
286 conversion assays later on without losing sensitivity compared to the use of fresh isolates. For
287 this purpose, we compared the estimated minimal detectable parasitemia between matched
288 fresh and cryopreserved cultures (3% Hct, 20 ml culture). Cryopreservation and thawing was
289 done as described for the *iv*SCA. Volumes and parasitemias used for cryopreservation were
290 matched to the fresh culture samples (1% parasitemia, 600 µl RBC pellet, 100 % Hct). The
291 entire RBC pellet recovered after thawing was immediately put in culture following the same
292 procedure described before. Lastly, we aimed to achieve a homogeneous distribution of iRBCs
293 in a monolayer of non-infected RBCs in the thin blood smears prepared after MACS, to ensure
294 reliable comparison of sexual conversion across smears, independently of parasitemia. To
295 achieve this, we tested different dilutions of the MACS-purified iRBC pellet in non-infected
296 RBCs and PBS, in fresh mock cultures (3% hematocrit, 20 ml) purified by MACS with 10-fold
297 serial dilutions of parasitemia ranging from ~1% to ~0.01%, following the procedure described
298 above. The final *ev*SCA protocol was validated using *P. falciparum*-infected clinical samples
299 at CRUN, Nanoro (Burkina Faso).
300 Results indicated that the minimal detectable parasitemia could be as low as ~0.05% in patient
301 samples using a 20 ml fresh culture (instead of 0.01% with mock samples), which we evaluated
302 to be superior to using cryopreserved cultures or a 10 ml fresh cultures. The most effective
303 strategy for diluting the MACS-purified RBC pellet was a 1:2 dilution with non-infected RBCs,
304 followed by resuspension in PBS at a 1:2 ratio.

305 *Ex vivo sexual conversion assays in fresh P. falciparum field isolates.*

306 *P. falciparum*-infected venous blood samples (15 ml) collected from individuals carrying
307 different HBB genotypes (Table 1; HbAA n = 14, HbAC n = 12, HbAS n = 6; total n = 32)
308 were cultured *ex vivo* to measure sexual conversion. Cultures at 3% Hct and 20 ml flask volume
309 were prepared by mixing 600 µl of *P. falciparum*-infected RBC pellet with 20 ml of RPMI
310 1640 medium containing 0.5% Albumax II (Gibco, 11021-029) and 2 mM choline chloride
311 (Sigma-Aldrich, C7527) [53, 70]. Parasites at the ring stage were incubated in a gas chamber
312 containing a lit candle inside a 37°C incubator under static conditions until ~24 hours post-
313 culture (hpc). At this time, sexually committed rings present in the venous blood sample are
314 expected to have developed into stage I gametocytes expressing Pf16, while asexual parasites
315 will have progressed to the schizont stage (Figure 4A). During culture, at ~5 hpc, ML10 (80
316 nM), a cGMP-dependent protein kinase inhibitor, was added to prevent schizont rupture and
317 reinvasion [50, 91]. Giemsa-stained smears were examined by LM to confirm that most
318 parasites had reached either stage I gametocytes or schizonts stages. At ~24 hpc, parasites were
319 harvested and purified using MACS LD columns (Miltenyi Biotec). In brief, cultured parasites
320 are centrifuged (1,648 g, 5 min) and pelleted infected-RBCs (iRBCs) were diluted 1:4 with
321 culture medium, loaded onto an LD-MACS column, and washed four times with 1 ml medium
322 to separate non-infected RBCs and ring-stage-infected RBCs (first elution). The column was
323 then removed from the magnet, and stage I gametocytes and schizonts were recovered by
324 gravity elution, followed by gentle plunger flushing twice with 3 ml medium (second elution).
325 Recovered parasites were pelleted (1,648 g, 5 min), resuspended 1:2 with non-infected RBCs,
326 and diluted 1:2 in PBS. From the second elution, four thin blood smears (3 µl each) were
327 prepared and left to air-dry; three were used for on-site IFAs, and one was Giemsa-stained for
328 LM to verify MACS enrichment.

329 *Immunofluorescence assays (IFAs).*

330 IFAs were performed at CRUN (Burkina Faso) as previously described [50, 53, 70], with
331 modifications. Slides were stained in batches of four to eight cultures with three slides per
332 culture, to optimize processing and staining consistency. Primary antibody mouse anti-*Pfs16*
333 (1:400, kindly provided by R. Sauerwein and T. Bousema, Radboud University) [92] and
334 secondary antibody goat anti-mouse IgG (H+L) Alexa Fluor 488 (1:1000; Abcam, ab150113)
335 were used, with incubations and washes under static conditions. Slides were mounted with
336 Aqueous Fluoroshield (Abcam, ab104139) containing DAPI (0.0002%) and stored at 4°C until
337 shipment to ITM (Belgium). At ITM, slides were visualized within six months of preparation
338 on an ECHO Revolve epifluorescence microscope (ECHO, San Diego, CA) to determine SCRs
339 (Figure 4F). Analysis was blinded to HBB genotype. Stage I gametocytes were identified as
340 *Pfs16*-positive cells (green) and counted among a minimum of 1,000 DAPI-positive cells (blue)
341 per slide (stage I gametocytes and schizonts). Slides with fewer than 1,000 DAPI-positive cells
342 were excluded from further analysis during the first reading. For each culture (n = 35), three
343 of the four prepared slides were stained by IFA and examined by a primary reader. Slides from
344 three cultures were excluded due to insufficient DAPI-positive cells. From the remaining
345 cultures (n = 32) a random subset of ~10% (4/32), with three IFA slides per culture, was
346 examined by a second reader to assess inter-reader reliability via intraclass correlation
347 coefficient (ICC) analysis, based on a two-way random effects model, absolute agreement,
348 single measures [ICC(2,1) [93]]. For each IFA slide, SCRs were calculated as the proportion
349 of *Pfs16*-positive parasites among total DAPI-positive cells. Across the three replicate IFA
350 slides per culture, the mean SCR (±SD) was computed and used to calculate and report the
351 median SCR [IQR] by HBB genotype (Figures 4A and 4B), statistical differences were assess
352 as described for proportions in "Data analysis" section. Fold-change in SCR for HbAC and
353 HbAS was calculated relative to HbAA by dividing each mutant genotype's median SCR by

354 that of the wild-type (Figure 4C). Statistical analysis was performed as described in the ”Data
355 analysis” section: Differences in initial parasitemia across HBB genotypes (continuous non-
356 parametric variables) (Figure 4B-D) and potential confounding effect of initial parasitemia on
357 SCRs (non-parametric correlation across continuous data) (Supplementary Figure 2A).
358 Stable *Pfs16* fluorescence was confirmed by the counting of ≥1 stage I gametocyte per 10
359 fields. For each culture, the three replicate IFA slides were analysed to quantify SCRs. The
360 mean, minimum and maximum SCR were calculated across replicates. To assess inter-slide
361 reproducibility (technical repeatability) of SCR measurements, an ICC (2,1) analysis (two-way
362 random effects, single measures, consistency) was performed using SCR values from the three
363 replicate slides per culture (n = 32). Counts of stage I gametocytes and total parasites were
364 recorded for each IFA slide and summarized as median (Q1-Q3). From 5 out of 32 cultures
365 (15.6%) specificity controls were performed by staining one slide per culture without primary
366 antibody (negative controls). Stage V gametocytes (also express *Pfs16*), identified by their
367 shape, were excluded from the counting as they are a product of sexual conversion occurring
368 >10 days prior to the sexual commitment cycle under investigation. Giemsa-stained slides from
369 each *ev*SCA were examined by LM to estimate parasite purification by MACS and confirm
370 approximate parasite age/stage to assure parasites were sufficiently mature to express *Pfs16*
371 (starts expression at ~20 hpi [50]).

372 ***In vitro* sexual conversion assays (*iv*SCAs).**
373 *Determination of RBC integrity after thawing across HBB genotypes.*
374 Cryopreserved non-infected RBC samples, collected at CRUN and UZA and stored as
375 described in section on “Sample collection and processing,” were thawed at ITM. In total, 28
376 cryopreserved endemic RBC samples were used (HbAA $n = 5$, HbAC $n = 5$, HbAS $n = 4$, HbSS
377 $n = 12$, HbSC $n = 2$; Table 1 and 3). A total of six HbAA non-endemic samples, four fresh and
378 two cryopreserved non-endemic HbAA RBC samples, were used in the experiments as controls

379 (*i.e.,* HbAA CTRL). One fresh HbAA RBC sample was included per experiment to calculate
380 fold-changes in SCR (see sub-section on "*In vitro* sexual conversion assays"). Thawing was
381 performed using a three-step protocol, sequentially adding three volumes of 3.5% NaCl
382 solution (10x the RBC volume each time), followed by RPMI washes and centrifugation (1,648
383 g, 5 min) as per standard procedure [94]. The *in vitro* sexual conversion assay (*iv*SCA) used
384 here is based on the assay that was previously published (*Portugaliza et al., eLife (2020)* [70]);
385 the difference is that now, NF54-*gexp02-Tom P. falciparum* parasites [53, 70], are allowed to
386 invade and grow in RBCs with different HBB genotypes (Figure 5A).
387 To validate the experimental set-up, we first evaluated RBC integrity and recovery after
388 thawing across a subset of RBC samples with different genotypes ($n = 1$ for each cryopreserved
389 endemic RBC sample from CRUN: HbAA, HbAC, and HbAS), compared to fresh non-
390 endemic HbAA RBCs from ITM). Since RBCs with mutant HBB genotypes are more fragile
391 and lysis-prone, hemolysis was assessed across HBB genotypes on day 0 to evaluate whether
392 the thawing procedure led to RBC lysis and at days 0, 3 and 7 to assess for how long the RBCs
393 could be used for culture after thawing. The recovered RBC pellet volume (µl) at day 0 (after
394 thawing) was measured using a pipette and the number of RBCs per milliliter was assessed
395 using the hemocytometer [Scepter™ 3.0 Handheld Cell Counter, Millipore® Cat:
396 PHCC30000]) on day 0, 3, and 7 (after thawing). The mean recovered RBC pellet volumes at
397 day 0 (after thawing) were at least 30 µl after dilution with RPMI to 50% Hct in the subset of
398 RBC samples with different genotypes, the minimum required volume to initiate culture wells.
399 RBC counts per milliliter decreased by day 7 across all HBB genotypes and thawed RBCs at
400 day 0 appeared morphologically distressed by LM right after the thawing procedure; therefore,
401 we used RBCs three days after thawing for the *iv*SCAs.
402 Finally, as the cryopreserved RBC samples collected at CRUN and the UZA were stored in
403 differently (CRUN: at -80°C before transfer to liquid nitrogen at ITM, three months after

404 sample collection; UZA: directly snap-frozen in liquid nitrogen at ITM) we assessed if this
405 difference has an impact on RBC lysis by comparing the recovered RBC pellet volume (µl) in
406 three donor-matched non-endemic HbAA control samples, preserved according to either the
407 CRUN or UZA protocol, or used fresh. The two matched cryopreserved samples, one stored
408 using the CRUN protocol and the other using the UZA protocol, showed comparable yields of
409 recovered RBC pellet volume (~400 µl), whereas the matched fresh HbAA sample yielded
410 ~4,000 µl. However, SCRs did not differ between cryopreserved and fresh samples, indicating
411 that neither differences in post-cryopreservation storage nor cryopreservation itself biased SCR
412 outcomes in our assay.

413 **Table 3: Overview of the different experiments with the number of cryopreserved RBC samples by HBB**
414 **genotype.**

	Experiments				
Sample by HBB genotype	**1**	**2**	**3**	**4**	**Total n**
HbAA (CRUN)	-	1	3	1	5
HbAC (CRUN)	1	2	1	1	5
HbAS (CRUN)	2	1	1	-	4
HbSS (UZA)	-	2	2	8	12
HbSC (CRUN/UZA)	-	2	-	-	2
Total n° samples	**3**	**8**	**7**	**10**	**28**

415

416 *In vitro sexual conversion assays.*

417 The transgenic *P. falciparum* reporter line NF54-*gexp02-Tom* [53, 70], which carries a
418 genome-integrated tandem Tomato (tdTom) fluorescent marker under the control of the
419 promoter of the early gametocyte marker gene *gexp02*, was used to assess the impact of RBCs
420 with different HBB genotypes on sexual conversion *in vitro*. To minimize bias due to prior
421 culture duration, all cultures were initiated from parasites of the same cryopreserved batch of
422 an expanded culture. *In vitro* sexual conversion assays (*iv*SCAs) were performed as follows
423 (Figure 5A). NF54-*gexp02-Tom* parasites were routinely maintained with parasitemias of less
424 than 2 % in O+ RBCs (3% Hct) in RPMI 1640-based medium supplemented with 0.5%

425 Albumax II (Gibco, ref: 11021-029) and 2 mM choline chloride (+Choline; Sigma-Aldrich,
426 C7527), analogous to LysoPC supplementation [51, 53, 73]. Cultures were incubated at 37°C
427 with shaking conditions (70 rpm) in a gas mixture (5% O_2, 5.5% CO_2, balance N_2).
428 To obtain cultures of a well-defined age window, we used Percoll/sorbitol synchronization
429 (Figure 5A). Percoll-purified schizonts (~40-44 hpi, Generation -1) were used to establish fresh
430 cultures with thawed cryopreserved RBCs (day 3 post thawing) with different HBB genotypes
431 (exposure, Generation 0), now cultured in static conditions. Approximately 5 hours later, when
432 parasites have reinvaded the new RBCs, cultures were subjected to 5% D-sorbitol lysis [50,
433 53] to obtain cultures of a defined 0-5 hpi age window. Post-sorbitol initial parasitemia was
434 then measured by flow cytometry, and parasites were cultured in two parallel subcultures (~200
435 µl final volume) per sample. One sub-culture was maintained in +Choline medium (non-
436 inducing sexual conversion conditions), and the other in choline-free medium (-Choline;
437 inducing conditions). Cultures were maintained under these respective conditions for two
438 generations (48h, until ~35-40 hpi in Generation 1, Figure 5A, hpi calculations are based on a
439 44 h replication cycle empirically established for this parasite line in our lab). In each
440 experiment we tested at least RBCs with two different HBB genotypes. Samples with different
441 HBB genotypes were used in the experiments if the recovered RBC pellet volume after thawing
442 was ≥ 30 µl after dilution with RPMI to 50% Hct. From a total of 20 cryopreserved non-infected
443 RBC samples from CRUN that were confirmed to be negative for *P. falciparum* by *var*ATS
444 qPCR (HbAA [n = 7], HbAC [n = 6], HbAS [n = 5], and HbSC [n = 2], Supplementary Figure
445 1), a total of 15 samples (HbAA [n = 5], HbAC [n = 5], HbAS [n = 4], and HbSC [n = 1],
446 Supplementary Figure 1) had a RBC pellet volume after thawing of ≥ 30 µl after dilution with
447 RPMI to 50% Hct (with mean recovered RBC pellet volumes after thawing in HbAA, 178 µl
448 (range: 50−596 µl); HbAC, 111 µl (range: 30−320 µl); HbAS, 246 µl (range: 64−630 µl);
449 HbSC, 558 µl (range: 35−1080 µl); and HbSS, 600 µl (range: 300−1120 µl). These samples

450 were included within the *iv*SCAs, in addition to non-infected, cryopreserved RBC samples
451 from the UZA HbSS [*n* = 12], and HbSC [*n* = 1], Supplementary Figure 1). Total numbers of
452 samples tested per HBB genotype and experiment are shown in Table 3 (HbAA [*n* = 5], HbAC
453 [*n* = 5], HbAS [*n* = 4], HbSS [*n* = 12], and HbSC [*n* = 2]). Flow cytometry (FC) analysis was
454 used to measure parasitemia, assess parasite stage development (*i.e.,* proportion of parasites
455 reaching the intraerythrocytic schizont stage or late-stage proportions), and quantify sexual
456 conversion at defined time points during the assay (Figure 5A). Parasitemia was determined at
457 ~0-5 hpi (rings), together with (1) the proportion of schizont stages at ~35-40 hpi in Generation
458 0 and 1, and (2) sexual conversion rates (SCRs) at ~25-30 hpi (large rings/trophozoites) and
459 ~35-40 hpi (schizonts) in Generation 1 (Figure 5A). The standard timepoint to measure sexual
460 conversion with this parasite line is ~25-30 hpi to ensure detection of all sexually committed
461 parasites, as TdTomato-expression under the *gexp02* promoter starts around ~10-15 hpi [53].
462 The ~35-40 hpi timepoint was included at Generation 1 to account for potential delayed stage
463 development in mutant HBB genotypes (see section on "Assessment of parasite stage
464 development in RBCs with different HBB genotypes"). Four independent experiments were
465 conducted, with RBC samples included in each experiment detailed in Table 3. Each
466 experiment included a fresh HbAA blood sample to control the effect of RBC storage after
467 cryopreservation.
468 Samples for FC analysis (using a CytoFLEX LX flow cytometer, Beckman Coulter) were
469 prepared by resuspending parasite cultures (1:3) in PBS containing 1% BSA, followed by
470 staining with Hoechst 33342 (1:500, BD Pharmingen) in a final volume of 100 µl per well.
471 Samples were then incubated at 37°C for 30 minutes. To measure parasitemia and proportion
472 of schizont stages, FC analysis (with 100,000 events collected per sample) was set to detect
473 Hoechst 33342 (350 nm laser; 450/40 filter). RBCs were gated by plotting side scatter area
474 (SSC-A) versus forward scatter area (FSC-A), with singlets selected using FSC-H versus FSC-

475 A. Parasites were identified within the singlet gate by Hoechst fluorescence. Hoechst-positive
476 events were defined as exceeding background levels from unstained negative controls. To
477 quantify SCRs, Hoechst fluorescence was combined with the fluorescent reporter TdTomato
478 (tdTom) (561 nm laser; 610/20 filter) that is integrated in the NF54-*gexp02-Tom* line to
479 distinguish sexual (Hoechst+/tdTom+) from asexual (Hoechst+/tdTom-) parasites. SCRs were
480 calculated as the proportion of Hoechst+/tdTom+ events among all Hoechst+ events. Samples
481 were analyzed with FlowLogic™ version 8.7 (Inivai Technologies™).
482 Sexual conversion rates (SCRs) were expressed as a percentage (median [IQR]) per HBB
483 genotype (Figure 5B, Supplementary Figures 2A, 2B and 2C). Fold-changes in SCRs were
484 calculated per experiment (Table 3) by dividing the SCR of each cryopreserved mutant RBC
485 sample by the SCR of a fresh, non-endemic HbAA RBC control sample. (Figure 5C,
486 Supplementary Figure 3D). Statistical analysis was performed as described in the ”Data
487 analysis” section: differences in SCRs and post-sorbitol initial parasitemia across HBB
488 genotypes tested as described for proportions, differences in fold-change as described for
489 continuous data across categories (Figure 5B-D; Supplementary Figure 3A-E). Although post-
490 sorbitol initial parasitaemia’s were adjusted to ≤ 2% to avoid over-induction of SCRs, we
491 assessed potential confounding of SCRs by initial parasitemia with Spearman correlation
492 (Supplementary Figure 2B). Differences in median RBC counts/µl across HBB genotypes at
493 timepoints ~35-40 hpi (Generation 0), and ~25-30 hpi and ~35-40 hpi (Generation 1) were
494 assessed to control for hemolysis as described for continuous non-parametric variables across
495 categorical groups in ”Data analysis” section (Supplementary Table 3).
496 To exclude confounding factors potentially associated with higher SCR and differences in HBB
497 genotype we assessed reticulocyte counts and ABO and Rhesus D blood groups. Reticulocyte
498 percentages were measured for all RBC samples on the same day as Percoll synchronization
499 (Generation -1, Figure 5A). Briefly, RBC suspensions were mixed 1:1 with reticulocyte stain

500 (Sigma-Aldrich), incubated 15 min at room temperature, and used to prepare a thin blood smear
501 for LM estimation of reticulocyte percentage. Differences in reticulocyte percentages across
502 all HBB genotypes (Figure 5E) were assessed as described for proportions in "Data analysis"
503 section. A correlation analysis of SCRs and reticulocyte percentages in HbAA, HbAC and
504 HbAS genotypes was performed using Spearman correlation (Supplementary Figure 3F)
505 (HbSS and HbSC genotypes were excluded for this analysis as they had extremely lowered
506 SCR compared to HbAC and HbAS but high reticulocyte counts). We determined the ABO
507 and Rhesus D blood groups in all samples using agglutination tests with Seraclone™ Anti-A,
508 Anti-B, and Anti-D (RH1) reagents (Bio-Rad). ABO and Rhesus D blood group distributions
509 across HBB genotypes were evaluated using Pearson's χ^2 test.
510
511 *Assessment of parasite stage development in RBCs with different HBB genotypes.*
512 To determine whether parasite development across HBB genotypes exhibited delays or arrest
513 in specific HBB genotypes as described by Archer, N.M., et al. (2018) [31], parasite stage
514 development was evaluated during the *iv*SCAs (Figure 5A) at ~35-40 hpi (Generation 0) and
515 ~25-30 hpi/~35-40 hpi (Generation 1) using LM and FC to determine whether parasite
516 development across HBB genotypes was comparable or exhibited delays or arrest in specific
517 HBB genotypes. Delayed or arrested parasite development in the *iv*SCAs would impact SCR
518 measurements since NF54-*gexp02-Tom* parasites would start expressing *gexp02-Tom* later.
519 This would require measuring sexual conversion at a later time point to assure reliable
520 outcomes of the SCR measurements. The NF54-*gexp02-Tom* line typically has an
521 intraerythrocytic cycle of ~44 hpi in HbAA RBCs, and starts to express *gexp02-Tom* in
522 Generation 1 from ~10-15 hpi until ~96-101 hpi [53]. However, environmental changes (*e.g.,*
523 change from shaking to static incubation) might extend the cycle to ≥ 48 hpi.

524 The proportion of rings, trophozoites and early/late schizonts was quantified at ~35-40 hpi in
525 Generation 0 and ~25-30 hpi/~35-40 hpi in Generation 1 by LM of Giemsa-stained thin blood
526 smears (100 parasite counts per sample; Figure 4A) in a subset of RBC samples (HbAA n = 1;
527 HbAC n = 1; HbAS n = 2; HbSS n = 2; HbSC n = 2). FC was used as described earlier to
528 determine the percentage of "late stages". For this a distinct population of cells were gated with
529 increased Hoechst 33342 fluorescence (increased DNA content due to multiplication of the
530 nuclei in schizont stages), within the Hoechst+ cells (Figure 4B). Upon confirming consistent
531 results between LM and FC after screening the same samples by FC, FC was subsequently used
532 in all *iv*SCA samples (n = 28: HbAA n = 5; HbAC n = 5; HbAS n = 4; HbSS n = 12; HbSC n
533 = 2; and fresh non-endemic HbAA CTRLs n = 6) to determine the proportion of late-stages
534 (*i.e.,* schizonts) by HBB genotype (Figure 4B and 4C). Differences in proportion of late-stages
535 across HBB genotypes (*i.e.,* schizonts) were evaluated as described for proportions in "Data
536 analysis" section (Figure 4B).
537 From LM data we calculated: (1) the percentage of rings, trophozoites and early/late schizonts
538 (Figure 4A), (2) the mean parasite age using the age in hpi [95] of each stage (rings,
539 trophozoites, schizonts) and the observed proportions; and (3) the developmental delay (in
540 hours) by subtracting mean parasite age of the mutant genotypes from the wild type HbAA
541 genotype. Finally, for each genotype, we compared the developmental delay between
542 Generations 0 and 1 by calculating the difference in mean parasite age at ~35-40 hpi between
543 both generations. Then, the development of each independent NF54-*gexp02-Tom* culture was
544 classified as: *expected development*, *delayed development* or *(partially) arrested/extremely*
545 *delayed development* by assessing several parameters obtained from FC data: (1) parasite
546 multiplication rates (PMRs), calculated as the ratio of schizont parasitemia at ~35-40 hpi in
547 Generation 1 to that at ~35-40 hpi in the preceding cycle (Generation 0; Figure 5A), (2) late-
548 stage proportions at three timepoints (~35-40 hpi Generation 0; ~25-30 hpi Generation 1; ~35-

549 40 hpi Generation 1), and (3) fold-changes in late-stage proportions between timepoints
550 (Supplementary Table 2). To account for potential developmental delays or arrest, sexual
551 conversion was also assessed at a later time point than usual (at ~35-40 hpi in addition to ~25-
552 30 hpi [53], Generation 1; Figure 5A).

553 **Flow cytometry assays to assess IgG and IgM immunity against *P. falciparum*.**

554 To study the IgG and IgM levels against *P. falciparum* in individuals with different HBB
555 genotypes by flow cytometry, we selected endemic human plasma samples from the *InHost*
556 longitudinal cohort study. We classified samples as "endemic" or "non-endemic" based on the
557 country of origin. An "endemic" country has consistent, stable *P. falciparum* transmission,
558 while a "non-endemic" country has no local transmission, with infections typically imported
559 [96]. As the expected variation and magnitude of potential differences among HBB genotypes
560 were unknown, we based the sample size on the minimum of 8 individuals per HBB genotype
561 used in the *ev*SCAs and *iv*SCAs. A maximum of 15-20 individuals per genotype was included
562 to ensure sufficient sampling. Selection criteria included equal representation of the most
563 frequent genotype groups (HbAA [n = 18], HbAC [n = 18], HbAS [n = 18], HbCC [n = 2], and
564 HbSC [n = 5]), *P. falciparum (Pf)* infection status with a large proportion of active *P.*
565 *falciparum* infections (*Pf*+; n = 10-15) and a smaller proportion of non-infected individuals
566 (*Pf*−; n = 2-5), age range (1-19 years to exclude maternal antibodies), and homogeneous
567 distribution amongst the ethnicity (all Mossi) and village (Nanoro, Nazoanga, Soum, and
568 Séguédin) groups. Table 1 shows the number of participants per HBB genotype included in the
569 assays. The *P. falciparum* transgenic line NF54-*gexp02*-Tom [53, 70] was cultured under the
570 same conditions as described in the *iv*SCAs section and Figure 5A, with the following
571 modifications: (1) parasites were cultured only in wild-type HbAA fresh RBCs and (2) flow
572 cytometry assays were performed on early trophozoite/stage I gametocyte-infected RBCs

573 (Troph-Gam-iRBCs) at ~20-25 hpi (Generation 1), following the protocol described in
574 *Dantzler et al., 2019* [97] with the adaptations described below.
575 Human plasma samples were pre-diluted in PBS with 1% Bovine Serum Albumin (BSA, Sigma
576 Aldrich) and heparin (2'500U; Sigma Aldrich) (PBS-BSA-Hep) at dilutions of 1:5000 (IgG)
577 and 1:100 (IgM). *In vitro* generated Troph-Gam-iRBCs (25-30hpi; Generation 1) were pre-
578 diluted to 0.6% Hct in PBS-BSA-Hep. Pre-diluted human plasma samples were incubated at a
579 1:10 dilution with pre-diluted Troph-Gam-iRBCs, in separate 96-well plates for IgG and IgM
580 assays for 1.5 hours at room temperature. After 2 washes with PBS-BSA-Hep, secondary
581 antibodies were added to the Troph-Gam-iRBCs for 30 minutes at 4°C at 0.6% hematocrit.
582 AlexaFluor488 (AF488) mouse anti-human IgG (Jackson ImmunoResearch Europe) and
583 PerCP/Cy5.5® mouse anti-human IgM (Abcam) were used at final dilutions of 1:640 and 1:320
584 in PBS-BSA-Hep for the IgG and IgM assays, respectively. After 2 washes with PBS-BSA-
585 Hep, the DNA stain Hoechst33342 (BD Pharmingen) was added to the Troph-Gam-iRBCs at
586 a final dilution of 1:5000 in PBS-BSA-Hep at 0.6% hematocrit for 30 minutes at 37°C. To
587 verify that the Troph-Gam-iRBCs measured contained exclusively red blood cells (no white
588 blood cells), in each batch pre-diluted Troph-Gam-iRBCs (to 0.6% hematocrit in PBS-BSA-
589 Hep) were stained with APC anti-human CD236 (Glycophorin C) Recombinant Antibody
590 (Biolegend®) in triplicate at a 1:40 final dilution (Supplementary Figure 5C). Immunostained
591 samples were analyzed on the flow cytometer CytoFLEX LX (Beckman Coulter), collecting
592 100,000 cells per culture. We analyzed the median percentage of total, asexual and sexual-
593 infected red blood cells (Troph-Gam-iRBCs) that were positive for IgG and IgM binding to
594 surface antigens on the Troph-Gam-iRBCs (*i.e.,* α-Troph-Gam-iRBCs , α- Troph-iRBCs and
595 α-Gam-iRBCs IgG & IgM). Samples were considered positive for IgG or IgM if the levels of
596 α-Troph-Gam-iRBCs IgG & IgM exceeded that of a naïve non-endemic plasma from a
597 volunteer (negative control with HbAA genotype with no prior *P. falciparum* infection, from a

598 non-endemic country [*i.e.,* Belgium] collected at the clinic of the Institute of Tropical Medicine
599 [ITM] in Antwerp). Three non-endemic samples from an acute *Pf*-infected traveler collected
600 at the ITM clinic at day 0, 21 and 23 of *P. falciparum* diagnosis (*i.e.,* acute *Pf-infected* non-
601 endemic plasma D0, D21 and D23) were used as positive controls. A schematic of the gating
602 strategy to measure the surface reactivity of asexual (tdTom-) and sexual (tdTom+) Troph-
603 Gam-iRBCs (Hoechst33342+) to IgG (α-Troph-iRBCs IgG: Hoechst33342+/tdTom-/AF488+
604 and α-Gam-iRBCs IgG: Hoechst33342+/tdTom+/ AF488+) or IgM (α-Troph-iRBCs IgM:
605 Hoechst33342+/tdTom-/PerCP-Cy5.5.+ and α-Gam-iRBCs IgM (Hoechst33342+/tdTom+/
606 PerCP-Cy5.5.+) in the analyzed endemic human plasma samples is represented in
607 Supplementary Figure 5B and 5C.
608 To determine the optimal plasma dilution factor, a pool of five endemic and hyperimmune
609 plasma samples from *Pf*-infected individuals in a sub-cohort within the *InHost* study (*i.e., Pf*-
610 infected hyperimmune endemic plasma pool of symptomatic individuals with high *P.*
611 *falciparum* parasitemia ranging from 45,667 to 175,000 parasites per µl) [71] was titrated for
612 the IgG and IgM assay separately. For the IgM assay, four additional plasma samples were
613 titrated (*i.e.,* "naïve non-endemic plasma" and "acute *Pf*-infected non-endemic plasma at D0,
614 D21 and D23") (Supplementary Figure 5A).
615 FlowLogic™ Software version 8.7 (Inivai Technologies™) was used to analyze the flow
616 cytometry data. The α-Troph-Gam-iRBCs IgG (Hoechst33342+/tdTom-/+/AF488+) and α-
617 Troph-Gam-iRBCs IgM (Hoechst33342+/tdTom-/+/PerCP-Cy5.5.+) levels for each sample
618 were expressed as total event counts and percentage of positive cells, and the signal as median
619 fluorescence intensity (MFI) per HBB genotype group. The α-Troph-Gam-iRBCs IgG and IgM
620 levels and signal were compared by HBB genotype group for the total Troph-Gam-iRBCs, and
621 the asexual or sexual Troph-Gam-iRBCs (Figure 7 and Supplementary Figure6). To correct for
622 the background signal from the non-infected RBCs in the IgG and the IgM channel, the MFI

623 of all samples (including the positive and the negative controls) was expressed as a ΔMFI. For
624 the asexual and sexual Troph-Gam-iRBCs, ΔMFI was calculated as ΔMFI (AF488+/PerCP-
625 Cy5.5.+) = ("MFI IgG+/IgM+ of asexual [tdTom-]/sexual [tdTom+] Troph-iRBCs
626 [Hoechst33342+]") - ("MFI IgG+/IgM+ of non-infected RBCs [tdTom-/Hoechst 33342-]")
627 within each plasma sample. For the total Troph-iRBCs, ΔMFI was calculated as ΔMFI
628 (AF488+/PerCP-Cy5.5.+) = ("MFI IgG+ or IgM+ iRBCs [Hoechst33342+]") - ("MFI IgG+ or
629 IgM+ of non-infected RBCs [Hoechst33342-]" of trophozoite stages incubated with PBS-BSA-
630 Hep, instead of plasma).

631 **Data analysis.**

632 Statistical analyses were performed in RStudio software version 2025.09.1+401(©2025 Posit
633 Software; RRID:SCR_000432), with p-values ($p < 0.05$) considered significant. The statistical
634 analysis type was chosen based on the nature of the variables: proportions based on counts
635 were assessed using binomial and beta-binomial generalized linear models (GLM) and mixed
636 models (GLMM) with Bonferroni adjustment for pairwise comparisons. We chose beta-
637 binomial models due to (possible) violations of the assumptions of the binomial model
638 (overdispersion), this was borderline acceptable in *ev*SCA but not in *iv*SCA. Random factors
639 were used to account for technical replicaets. To test for the relevance of other factors we also
640 modelled the initial parasitemia (*ev*SCA & *iv*SCA), and reticulocyte percentage (*iv*SCA). To
641 address the liberal parametric Wald Z estimates and confidence intervals, we also computed
642 more conservative Bayesian estimates and credible intervals using a Markov chain Monte
643 Carlo (MCMC) approach when model specification and convergence allowed. GLM(M)
644 analysis results are reported in Supplementary Table 7.
645 Continuous variables were first assessed for normality using the Shapiro-Wilk test to guide the
646 choice between parametric and non-parametric methods. Continuous non-parametric variables
647 across categorical groups with ≥2 levels were analysed using the Kruskal-Wallis test, and when

648 significant, this was followed by Wilcoxon rank sum post-hoc tests for pairwise comparisons
649 with Holm correction. For normally distributed variables, one-way ANOVA was applied, with
650 Bartlett's F-test used to assess homogeneity of variances, followed by Bonferroni post-hoc tests
651 for pairwise comparisons if ANOVA was significant. Associations between categorical
652 variables were evaluated using Pearson's χ^2 test. Continuous non-parametric variables were
653 correlated using Spearman correlation.
654 In the *ev*SCAs and the *iv*SCAs, risk analyses stratified by HBB genotype were conducted to
655 examine associations with demographic and clinical variables (*e.g.,* SCRs, gender, age, village,
656 ethnicity, …). An additional risk analysis for factors associated with elevated basal SCRs
657 included the same covariates with the addition of HBB genotype (Supplementary Table 4 for
658 the *ev*SCAs, and Supplementary Table 5 for the *iv*SCAs).
659
660 **Ethical approvals.**
661 The *InHost* study, a longitudinal cohort in Burkina Faso, was approved by the ethical review
662 board of the Comité d'Éthique pour la Recherche en Santé (CERS: 2018/10/131) in Burkina
663 Faso, and by the IRB-ITM (1261/18) and IRB-UZA (19/06/064) in Belgium. The amendment
664 for our study nested in the *InHost* study was approved by CERS (N°2022000140/MSHP/
665 MESRI/CERS), the IRB at ITM (1261/18), and the IRB at UZA (3347) in Belgium. Ethical
666 approval for additional sample collection at the Hematology Unit at UZA was granted by the
667 IRBs at ITM (1538/21) and UZA (3215).

668 **Role of funding source.**
669 The funding source was not involved in the study design, data collection and analysis,
670 manuscript preparation, or the decision to submit the work for publication. The funding sources
671 are the Belgium, Directorate-General Development Cooperation and Humanitarian Aid (DGD)

and the Research Foundation – Flanders (FWO) with "PhD fellowship strategic basic research" (1S74321N) and "Research project grant" (G067823N).

RESULTS

HbAC and HbAS genotypes prevalences in Nanoro Department, Burkina Faso.

With Sanger Sequencing, we genotyped the hemoglobin beta (HBB) gene and measured the population frequencies of the different HBB genotypes (HbAA, HbAC, HbCC, HbAS, HbSS, and HbSC) of all the participants of a longitudinal cohort study (*i.e.,* InHost study) in Nanoro, Burkina Faso (n = 864) (Figure 2). The wild-type genotype HbAA was the most frequent, observed in 71.5% of the individuals. The distribution of the mutant genotypes was as follows: HbAC was present in 20%, HbAS in 7.7%, HbSC in 0.8%, and HbCC in 0.2% of the study participants. No individuals were identified with the HbSS genotype.

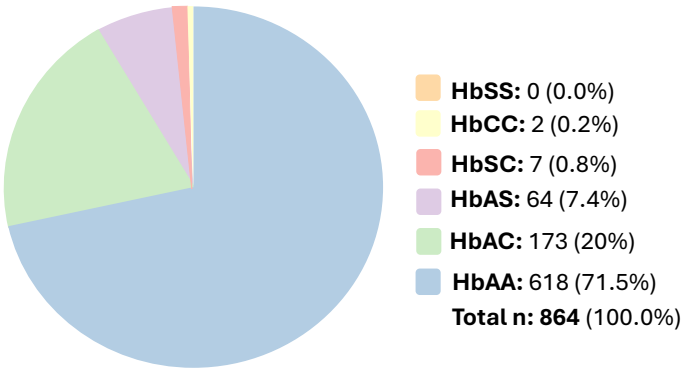


Figure 2. Genotype frequencies of the HBB gene in the study population. Pie chart depicting the sample sizes (n) and frequencies (%) of the different HBB genotypes in the study population.

Novel *ex vivo* sexual conversion assays reveal elevated parasite sexual conversion rates in individuals with mutant HbAS and HbAC genotypes compared to wildtype HbAA.

Ex vivo sexual conversion assays (evSCAs): assay optimization and validation

To ensure robustness and feasibility of the *ex vivo* sexual conversion assays (*ev*SCAs) in a field setting using *P. falciparum* isolates with varying parasitaemia, we first optimized key assay

692 parameters using mock cultures of the NF54-*gexp02-Tom* transgenic line [53]. First,
693 comparison of MACS LD versus LS columns for parasite enrichment demonstrated that the
694 MACS LD column was more efficient, yielding a larger final RBC pellet (~8 µl versus ~1.5 µl
695 with LS) and a higher proportion of enriched parasites (~75% versus ~58% with LS), and was
696 therefore selected for all subsequent experiments. Second, we evaluated detection sensitivity
697 across a range of parasitemias (from ~1% until ~0.001%), in two culture volumes (10-20 ml),
698 and assessed the impact of parasite cryopreservation. Using fresh infected RBCs, the minimal
699 detectable parasitemia was 0.1% for 10 ml cultures (~3 million parasites) and 0.01% for 20 ml
700 cultures (~600,000 parasites). In contrast, for 20 ml cultures derived from cryopreserved
701 iRBCs, the detection threshold increased to 0.1% (~6 million parasites), because parasitemia
702 after cryopreservation decreased drastically (from 1 % to ~0.17%). Based on these findings,
703 the optimal setup for subsequent assays consisted of 20 ml parasite cultures using fresh RBCs.
704 Lastly, to achieve a homogeneous distribution of iRBCs in a monolayer of non-infected RBCs,
705 we tested different dilutions of the MACS-enriched iRBC pellet in non-infected RBCs and
706 PBS. The final approach used a two-step dilution: first, a 1:2 dilution with fresh non-infected
707 RBCs, followed by a 1:2 dilution with 1x PBS. The final protocol was validated using patient
708 *P. falciparum* samples at CRUN, Nanoro (Burkina Faso), confirming its feasibility under field
709 conditions and that sexual parasites could be reliably detected at parasitemias as low as 0.05%
710 (instead of 0.01% with mock samples). Out of 32 clinical samples for which the *ev*SCA was
711 performed successfully, 13 (~41%) had a starting parasitemia between 0.05% and 0.08%; the
712 remaining samples presented with starting parasitemias ranging from 0.08% to 1% (n = 19
713 [59%]).
714 Inter-reader reliability was assessed by re-reading three IFA slides per culture in 12.5% of the
715 total cultures (4/32) by a second reader. Intraclass correlation coefficient (ICC) analysis of the
716 resulting sexual conversion rates (SCRs), demonstrated very strong agreement between readers

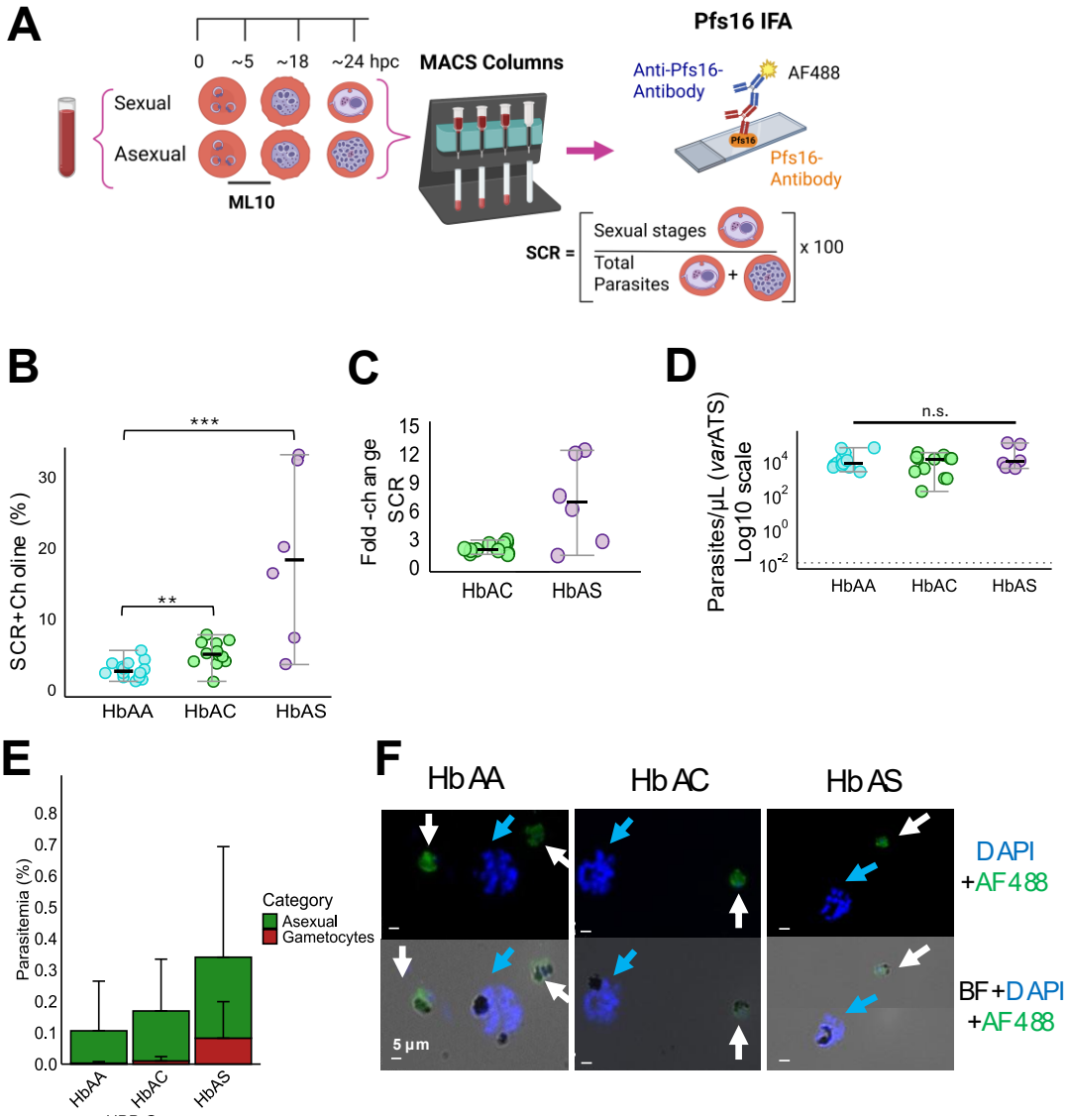
717 with an ICC(2,1) of 0.96 (95% CI: 0.543-0.998, $p = 0.0055$**). We observed consistent *Pfs16*
718 fluorescence across the IFA smears, with a median of 44 stage I gametocytes per smear (IQR:
719 28-64) and an average of 19 stage I gametocytes per 10 fields, confirming that each slide
720 contained at least one visible stage I gametocyte per 10 fields. A median of 1,009 total parasites
721 per smear (IQR: 1,009-1,039), and of 20 counted fields per smear (range: 14-32) were
722 observed. Inter-smear reproducibility of SCRs between the three IFA slide replicates was
723 assessed with Intraclass Correlation Coefficient (ICC) analysis (two-way random effects,
724 single measures, consistency). SCR measurements showed good inter-replicate consistency,
725 with an ICC of 0.79 (95% CI: 0.663-0.884; $p < 0.001$***), indicating reliable quantification of
726 sexual conversion across replicate smears from the same parasite isolate. SCR measurements
727 demonstrated good inter-replicate consistency, with an ICC(2,1) of 0.792 (95% CI: 0.663–
728 0.884; $p < 0.001$), indicating reliable quantification of sexual conversion across technical
729 replicates (smears) from the same parasite culture. Finally, Giemsa-stained replicate smears of
730 the IFA samples were examined by LM, confirming a MACS enrichment efficiency of ~70%
731 (when comparing to a Giemsa-stained slide performed prior to MACS enrichment) and
732 confirmed that ~54% of parasites observed post MACS were late schizonts with a parasite age
733 of ~44-48 hpi [50]. This ensured that parasites were sufficiently matured for sexual stages to
734 express Pfs16.

735 *Ex vivo sexual conversion rates (SCRs).*

736 To investigate the effect of HBB genotypes on *P. falciparum* sexual conversion we measured
737 sexual conversion rates (SCRs) of *P. falciparum* isolates collected from individuals with
738 different HBB genotypes (HbAA with n =14, HbAC with n =12 and HbAS with n = 6; total n
739 = 32) using an in-house *ex vivo* sexual conversion assay (*ev*SCA). Sexual conversion rates
740 (SCRs) were quantified by immunofluorescent staining (IFAs) detecting the number of
741 parasites stained positive for the *Pfs16* protein (stage I gametocytes) [92, 98, 99] out of the

742 number of parasites stained positive for DNA/parasite nuclei (*i.e.,* the total parasite population
743 consisting of stage I gametocytes plus schizont stages), expressed as a percentage. The
744 experimental set-up of the *ev*SCAs and representative immunofluorescent assay (IFA) images
745 are shown in Figure 3A and 3F, respectively.
746 In non-inducing culture conditions (+Choline) the SCR (median[IQR]) was 2.65%[1.72] in *P.*
747 *falciparum* isolates from HbAA genotype, 5.05%[2.55] for HbAC and 18.40%[19.70] for
748 HbAS genotypes. SCR increased significantly in HbAC (Beta-binomial GLMM, OR[95%CI]
749 = 1.82 [1.17-2.82], p = 0.0032** and HbAS (Beta-binomial GLMM, OR[95%CI] = 8.16
750 [4.34-15.37], p = < 0.0001***) genotypes compared to HbAA (Figure 3B and Supplementary
751 Table 7). We calculated the fold change in SCRs (median [IQR]) of *P. falciparum* isolates in
752 HbAC and HbAS compared to wild-type HbAA RBCs. The fold change was 2.04 [0.85] in
753 HbAC and 7.07 [7.32] in HbAS, with values in HbAS RBCs significantly higher than in HbAC
754 RBCs (Kruskal-Wallis test, overall p = 0.034*; Figure 3C). We investigated initial parasitemias
755 in relation to SCRs to assess whether high SCRs could be attributed to differences in initial
756 parasitemia levels (*e.g.,* higher versus lower initial parasitemias). Parasitemias did not
757 significantly differ between mutant HBB RBCs (HbAC and HbAS) and wild-type HbAA RBCs
758 (Kruskal-Wallis test, p > 0.05, Figure 3D). Correlation analysis between SCRs and initial
759 parasitemia was not significant (Spearman correlation, ρ = 0.05, p > 0.05; (Supplementary
760 Figure 2A). These results suggest that differences in initial parasitemias do not have an impact
761 on the observed elevated SCRs.
762 We assessed whether higher SCR in parasite isolates with AS and AC RBCs are due to lower
763 asexual parasite densities, reducing the denominator of the SCR thus inflating SCR without
764 increased counts of sexual parasites. Analysis of the proportion of red blood cells infected with
765 asexual parasites and stage I gametocytes by HBB genotype (determined by IFAs at ~24 hpc,),
766 showed that the number of red blood cells infected with stage I gametocytes was clearly higher

767 in cultures with HbAS RBCs, followed by HbAC RBCs, compared to cultures with wild-type
768 HbAA RBCs (Figure 3E) (same trend observed in SCRs by HBB genotype, Figure 3B). The
769 proportion of infected red blood cells was extrapolated from Giemsa stained slides after MACS
770 enrichment (Figure 3E). This confirms that the higher SCRs observed are attributable to the



771 effect of HBB genotype, reflecting a net increase in gametocyte production rather than a
772 reduction in asexual parasite numbers.

773 **Figure 3. Effect of human hemoglobin beta (HBB) HbAA, HbAC and HbAS genotypes on sexual conversion**
774 **determined with *ex vivo* sexual conversion assays (*ev*SCAs). (A)** Schematic representation of the *ev*SCAs.
775 Parasite isolates from RBCs with different HBB genotypes were cultured *ex vivo* under basal non-inducing
776 (+Choline) conditions. ML10 was added at 11 hpi to block reinvasion. Stage I gametocytes and schizonts were
777 enriched by MACS, and sexual conversion rates were measured by IFA using Pf16 (sexual stages) and DAPI
778 (total parasites) staining. *Created with BioRender.* **(B-C)** Dot plots show the median (bold line), quartiles and
779 range indicated by whiskers. **(B)** Sexual conversion rates (SCRs) by HBB genotype determined with IFA. Sample

size n per group: HbAA n = 14, HbAC n = 12 and HbAS n = 6; total n = 32. Data presented as the median and IQR of three independent technical IFA replicates. **(C)** Fold-change SCRs in HbAC and HbAS genotypes relative to HbAA. **(D)** Initial parasitemia at 0 hpc (parasites per µl; log10 scale) by HBB genotype determined with *var*ATS qPCR. **(E)** Proportion of asexual and gametocyte stages, after MACS enrichment, within the initial parasitemia by HBB genotype. **(F)** Representative IFA images of parasites in HbAA, HbAC and HbAS RBCs. Top row with DAPI (all parasites; blue arrows: schizonts) and AF488 (white arrows: stage I gametocytes) overlay and bottom row including brightfield (BF) channel to visualize the parasite's hemozoin. Objective: 40X; Scale bar: 5µm. Images were captured with the Revolve (ECHO) camera. **Statistical tests: B,** Beta-binomial generalized linear mixed models (GLMM) with Tukey adjustment for pairwise comparisons; **D,** Kruskal-Wallis test (p-values: bold solid line), followed by the Wilcoxon Rank Sum post-hoc test with Holm correction (p-values: normal solid line) when significant. Significant p-values: $p < 0.05$*, $p < 0.01$**, $p < 0.001$***; and non-significant p-values as: ns. **Abbreviations:** Hpc, hours post culture; ML10, cGMP-dependent protein kinase inhibitor; hpi, hours post-invasion; AF, Alexa Fluor; DAPI, DNA stain 4',6-diamidino-2-phenylindole; *Pfs16 antibody: Kindly provided by R. Sauerwein and T. Bousema, Radboud University*; *var*ATS qPCR, var gene acidic terminal sequence (*var*ATS) quantitative PCR (qPCR).

***In vitro* observations of *Plasmodium falciparum* parasites growing in RBCs with different HBB genotypes**

Red blood cells with mutant HBB genotypes lead to delayed parasite development.

Archer, N.M., et al. (2018) [31] has shown that under low oxygen conditions (≤ 5% O_2, as present in the bone marrow and spleen, as well as in standard *P. falciparum* cultures as used for the *iv*SCA) *P. falciparum* in HbAS RBCs exhibit developmental arrest/delay before DNA replication. Developmental delay/arrest in a particular HBB genotype would affect the time at which sexual conversion can be measured *in vitro.* In NF54-*gexp02-Tom* parasites, grown in HbAA RBCs, TdTomato signal can be detected from ~10-15 hpi until ~96-101 hpi in the cycle after exposure (Generation 1), and sexual conversion is usually measured at ~25-30 hpi (Generation 1) when parasites are large rings/early trophozoites [53, 70]. If a HBB mutant genotype would cause a delay in development TdTomato might not yet be expressed at ~25-30 hpi (Generation 1).

To determine whether parasite stage development of NF54-*gexp02-Tom* parasites differs between RBCs with wild type HbAA and mutant HBB genotypes (HbAC, HbAS, HbSS, HbSC), we first quantified the percentage of rings, trophozoites and early/late schizonts in a set of RBC samples with the different HBB genotypes (total n = 8: HbAA n = 1, HbAC n = 1, HbAS n = 2, HbSS n = 2, HbSC n = 2) using LM (Figure 4A). Parallel analysis of the proportion

815 of late stages (schizonts) in the same samples by flow cytometry (FC) (gating strategy in Figure
816 4C) revealed consistent patterns between the two approaches. We therefore applied FC to the
817 full set of samples (total n = 28: HbAA n = 5, HbAC n = 5, HbAS n = 4, HbSS n = 12, HbSC
818 n = 2; and fresh non-endemic HbAA CTRLs n = 6, Figure 4B), enabling accurate detection of
819 genotype-specific developmental delays.
820 With LM, we quantified the proportions of rings, trophozoites and early/late schizonts at ~35-
821 40 hpi in Generation 0 and at ~25-30 hpi/~35-40 hpi in Generation 1 (*iv*SCA set-up in Figure
822 5A). At Generation 0 (~35-40 hpi, Figure 4A left graph), parasites in wild-type HbAA RBCs
823 reached the expected mean parasite age of ~43 hpi, closely matching the ~44 hpi life cycle,
824 with parasite stage distribution dominated by schizonts (87%), with no rings and 13%
825 trophozoites. In contrast, mutant HBB RBCs showed clear developmental delays: mean
826 parasite ages were ~40 hpi (HbAC), ~33 hpi (HbAS), ~34 hpi (HbSS), and ~28 hpi (HbSC),
827 corresponding to a developmental delays of ~3-15 hours. These delays were reflected in
828 parasite stage distributions, with a marked shift toward earlier stages, increased proportions of
829 trophozoites and rings relative to HbAA: HbAC (+7% rings, +2% trophozoites, -9% schizonts),
830 HbAS (+20% rings, +25% trophozoites, -45% schizonts), HbSS (+32% rings, +11%
831 trophozoites, -43% schizonts), and HbSC showed the most pronounced delay (+58% rings,
832 +9% trophozoites, -67% schizonts). At Generation 1 (~35-40 hpi, Figure 4A right graph), mean
833 parasite ages were further reduced compared to Generation 0, indicating continued
834 developmental delays and prolonged life cycles. Even in HbAA, mean parasite age was ~38
835 hpi (*i.e.,* ~6 hours younger than the expected ~44 hpi cycle), with parasite stage proportions
836 shifting toward earlier stages (60% schizonts versus 87% in Generation 0). Developmental
837 delays relative to HbAA persisted in mutant genotypes: ~2 hours in HbAC, ~9 in HbAS, ~7 in
838 HbSS, and ~13 in HbSC, again supported by parasite stage distributions showing a shift
839 towards earlier stages and reduced schizont proportions (+6% rings, -4% trophozoites, -2%

840 schizonts in HbAC; +19% rings, +19% trophozoites, -38% schizonts in HbAS; +23% rings,
841 +8% trophozoites, -31% schizonts in HbSS; +50% rings, -6% trophozoites, -44% schizonts in
842 HbSC). Thus, while HbAA parasites displayed a modest extension of the life cycle (*i.e.,* ~1 to
843 6-hour delay), mutant HBB RBCs consistently showed more pronounced delays and an
844 accumulation of early parasite stages (rings/trophozoites), most pronounced and severe in
845 HbSC RBCs. Together, these findings demonstrate stable genotype-specific developmental
846 delays across successive generations 0 and 1. At ~25-30 hpi (Generation 1) (first timepoint for
847 SCR measurement), parasites in HbAA RBCs are expected to be at the large ring/trophozoite
848 stage (reference), which is indeed observed for the majority of parasites (> 95%) (Figure 4A,
849 middle graph). A similar stage distribution was observed in HbAC and HbAS RBCs, with 24%
850 large rings (within 40% total ring stages) and 59% trophozoites in HbAC, and 48% large rings
851 (within 59% total ring stages) and 41% trophozoites in HbAS. In HbSS and HbSC RBCs, large
852 rings and trophozoite stages were still detectable but in lower proportions than in HbAA, with
853 8% large rings (within 12% total ring stages) and 55% trophozoites in HbSS, and 25% large
854 rings (within 42% total ring stages) and 46% trophozoites in HbAS. Notably, in HbSS and
855 HbSC RBCs schizont stages (potentially persisting from Generation 0) were also observed at
856 this timepoint. However, this stage distribution pattern in HbSS and HbSC mutant RBCs
857 persists across all measured timepoints, suggesting that although some (delayed) progression
858 occurs, a subset of parasites may remain developmentally arrested.

859 Complementarily, we quantified the proportions of late stages (schizonts) using FC at
860 ~35-40 hpi in Generation 0 and at ~25-30 hpi and ~35-40 hpi in Generation 1 (*iv*SCA set-up
861 in Figure 5A). At ~35-40 hpi in Generation 0 and 1 we expect the majority of parasites to be at
862 schizont stage.In Generation 1, we confirmed significantly different proportions of late stages
863 (schizonts), between HbAA and HbSS RBCs (Beta-binomial GLMM, OR[95% CI] = 0.13
864 [0.03−0.60], p = 0.0026**; Figure 4B, right graph and Supplementary Table 7), whereas in

865 Generation 0 no significances were found (Wilcoxon Rank Sum post-hoc test; $p > 0.05$; Figure
866 4B, left graph). In mutant HbAS and HbSC RBCs median numbers of late stages are lower
867 than in HbAA RBCs at those time points but no significant difference was detected (Wilcoxon
868 Rank Sum post-hoc test and Beta-binomial GLMM; $p > 0.05$; Figure 4B, left and right graph).
869 These findings support our earlier LM-based observations, pointing toward a slight
870 developmental delay in parasites within HbAS and HbSC RBCs not prone to impact SCR
871 measurements with the NF54-*gexp02-Tom* line and a significant delay in HbSS RBCs that
872 could impact SCR measurements. At ~25-30 hpi (Generation 1), parasites in all HBB
873 genotypes are predominantly at early stages (rings/trophozoites), as expected, indicated by low
874 proportions of late stages (Figure 4B, middle graph). At this timepoint, no significant
875 differences were observed between genotypes (Kruskal Wallis test, $p > 0.05$). The progression
876 from predominantly late stages at ~35-40 hpi in Generation 0 to early stages at ~25-30 hpi
877 (Generation 1) to late stages in at ~35-40 hpi in Generation 1 shows that in HbAA, HbAC and
878 HbAS RBCs there was no complete arrest of parasite development. Notably, in mutant HbSS
879 and HbSC RBCs at ~25-30 hpi (Generation 1), a proportion of late stages was detected by LM
880 that is absent in other HBB genotypes (Figure 4A, middle graph), yet this population is not
881 detected by FC (Figure 4B, middle graph). This may suggest that the observed schizonts might
882 be underdeveloped (potentially containing less DNA and thus not being detected by FC as late
883 stages).
884 Additionally, we evaluated the parasite multiplication rate (PMR) by HBB genotype,
885 comparing schizonts at ~35-40 hpi in Generation 0 and Generation 1 to confirm parasite
886 development across cycles. All HBB genotypes had a median [IQR] PMR greater than 1
887 between Generation 0 and Generation 1, with median PMRs smaller in HBB mutant genotypes
888 in comparison to HbAA: HbAA, 3.5 [2.0]; HbAC, 2.0 [1.0]; HbAS, 3.5 [1.5]; HbSS, 4.0 [2.0];

889 and HbSC, 2.0 [1.0] (Kruskal-Wallis test, $p > 0.05$). These findings show that parasites are able
890 to multiply across all genotypes.
891 As the proportions of late stages showed high IQRs per genotype at the different time points,
892 we further investigated whether delay or arrest was more pronounced in certain cultures -
893 taking into account that other host factors may vary from participant donating the RBCs apart
894 from HBB genotype. We analysed several parameters obtained by FC: the proportion of late
895 stages at three timepoints (~35-40 hpi at Generation 0; ~25-30 hpi at Generation 1; and ~35-
896 40 hpi at Generation 1), and fold change of late stages from one time point to the next
897 (Supplementary Table 2), as well as the PMR. We defined three developmental classes based
898 on the comparison of the median and interquartile ranges of each parameter in parasites
899 growing in each group of mutant HBB RBCs compared to the HbAA RBCs: *expected*
900 *development* (≤ one parameter outside the HbAA reference range), *delayed development* (2-3
901 parameters outside the HbAA reference range); (*partially) arrested/extremely delayed*
902 development (≥ 4 parameters outside the HbAA reference range). In HbAA (5/5 [100%]) and
903 HbAA CTRL RBCs (6/6 [100%], all cultures exhibited the expected developmental pattern. In
904 contrast, the HBB mutant RBCs showed variable patterns: in HbAC, 40% (2/5) showed
905 expected development, and 60% (3/5) showed delayed development; in HbAS, 50% (2/4)
906 showed expected development, 25% (1/4) delayed development, and 25% (1/4) (partial)
907 arrest/extremely delayed development; in HbSS, none of the samples showed expected
908 development, 33% (4/12) showed delayed development, and 67% (8/12) exhibited (partial)
909 arrest/extremely delayed development. In HbSC RBCs, none of the samples showed expected
910 development, 50% (1/2) showed delayed development, and the remaining 50% (1/2) (partial)
911 arrest/extremely delayed development (Supplementary Table 2).
912 In summary, parasites in HbAA RBCs progressed normally from Generation 0 to 1 with no
913 apparent delay. In HbAC RBCs, development was slightly delayed but without arrest, whereas

914 in HbAS RBCs, partial developmental arrest was observed in one sample. In HbSS and HbSC
915 RBCs, a persistent population of schizonts, trophozoites, and late rings was seen across all
916 timepoints (Figure 4A), consistent with the high frequency of partially arrested/delayed
917 cultures (67% in HbSS and 50% in HbSC) (Supplementary Table 2). The (partial)
918 arrest/extreme delay in these cultures potentially makes them unsuitable for SCR measurement.
919 Given the observed developmental delays in all mutant genotypes, measuring sexual
920 conversion at a single timepoint (~25-30 hpi, Generation 1) may not be accurate. To capture
921 all *gexp02-Tom*-expressing sexual parasites [53], including those with delayed development,
922 sexual conversion rates (SCRs) were additionally quantified at ~35-40 hpi (Generation 1) (+
923 20 hours post-expression of *gexp02* should account for the 3-15 h delay measured).

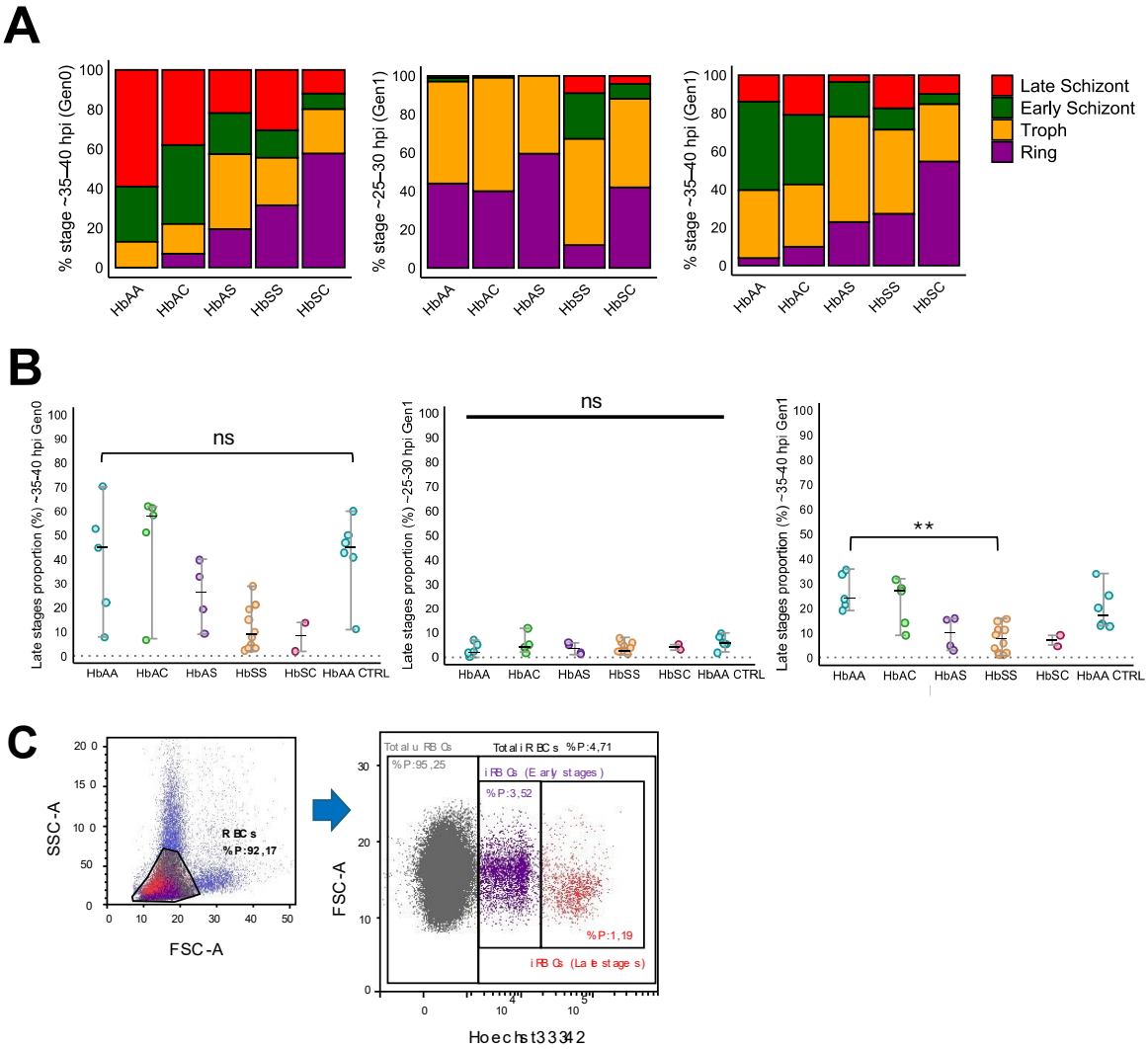


Figure 4. ***P. falciparum*** **parasite development in red blood cells (RBCs) with different HBB genotypes. (A)** Microscopy-based quantification of parasite stages expressed as percentages of a total of 100 counted parasites per sample. Y-axis: percentage of parasite stages (%); X-axis: HBB groups. Sample size n per group: HbAA n=1, HbAC n = 1 and HbAS n = 2, and HbSS n = 2 and HbSC n =2; total n = 8. Timepoints shown are ~35-40 hpi (Generation 0; left graph), ~25-30 hpi (Generation 1; middle graph) and ~35-40 hpi (Generation 1; right graph). **(B)** Dot plots showing the proportion of late stages (*i.e.,* schizont stages) on the total infected RBCs by flow cytometry (Y-axis) by HBB genotype groups (X-axis). Timepoints shown are ~35-40 hpi (Generation 0), ~25-30 hpi (Generation 1) and ~35-40 hpi (Generation 1). Datapoints show the median (bold-line) proportion of late stages and the interquartile range (IQR). Sample size n per group: endemic RBC samples with HbAA n = 5, HbAC n = 5 and HbAS n = 4, and HbSS n = 12 and HbSC n = 2, total n=28; and non-endemic fresh RBC samples with "HbAA CTRL" n = 6. **(C)** Representative flow cytometry plots showing the gating strategy for the detection of late parasite stages. Left: total RBCs gated in the SSC-A versus FSC-A plot. Right: within this population, uninfected RBCs (uRBCs) and infected RBCs (iRBCs) are identified, with iRBCs further gated into early and late parasite stages. **Statistical tests**:(B, right graph) Beta-binomial generalized linear mixed models (GLMM) with Tukey adjustment for pairwise comparisons; (B, left and middle graph) Kruskal-Wallis test with the Wilcoxon Rank Sum post-hoc test. Significant p-values are shown as: $p < 0.05$*, $p < 0.01$**, $p < 0.001$***; non-significant p-values as: ns. Overall p-values are presented with a bold solid line, and p-values after pairwise comparisons with a normal solid line.. **Abbreviations:** Hpi, hours post-invasion; SSC-A, Side Scatter-Area; FSC-A, Forward Scatter-Area; CTRL, control sample (fresh HbAA RBCs).

944 .
945
946 *In vitro sexual conversion assays (ivSCAs): RBC integrity after thawing across HBB*
947 *genotypes.*

948 To establish *in vitro* sexual conversion assays (*iv*SCAs) in the *P. falciparum* NF54-*gexp02-*
949 *Tom* reporter line in cryopreserved, non-infected RBCs with different HBB genotypes (Figure
950 5A), we first assessed RBC integrity after thawing across a subset of RBC samples with
951 different genotypes (*n* = 1 for each cryopreserved endemic RBC sample from CRUN: HbAA,
952 HbAC, and HbAS), compared to fresh non-endemic HbAA RBCs from ITM). RBC lysis was
953 evaluated by measuring RBC counts per ml at days 0, 3 and 7 (after thawing) to assess for how
954 long after thawing RBCs can be used in cultures. RBC counts/ml at day 0, 3 and 7 were: fresh
955 non-endemic HbAA RBCs, 3.61e+09, 9.65e+09 and 3.35e+09; cryopreserved HbAA RBCs,
956 1.06e+10, 1.73e+09 and 3.31e+09; cryopreserved HbAC RBCs, 4.62e+09; 5.41e+09 and
957 2.67e+09; and cryopreserved HbAS RBCs, 7.07e+10, 1.10e+10 and 3.35e+09. RBC counts
958 decreased by day 7 across all HBB genotypes and thawed RBCs at day 0 appeared
959 morphologically distressed by LM right after the thawing procedure; thus, only thawed RBCs
960 at day 3 were used in the *iv*SCAs.
961 Lastly, as the cryopreserved RBC samples were stored differently (CRUN: at -80°C before
962 transfer to liquid nitrogen at ITM, three months after sample collection; UZA: directly snap-
963 frozen in liquid nitrogen at ITM), we evaluated whether this difference in storage had an impact
964 on RBC lysis by comparison of the recovered RBC pellet volume (µl) in three donor-matched
965 non-endemic HbAA control samples, preserved according to either the CRUN or UZA
966 protocol, or used fresh. No differences were found in the two cryopreserved samples as these
967 yielded 200 µl of recovered RBC pellet (at 100% Hct) after thawing from an input
968 cryopreserved volume of RBCs at 100% Hct of 500µl (40% of RBC pellet recovery after
969 cryopreservation), compared to 1,000 µl of RBCs at 100% Hct obtained in fresh RBCs.
970 Importantly, sexual conversion rates (SCRs) were similar across conditions: 8.5% in fresh

971 HbAA RBCs, and 7.7% versus 6.8% in the cryopreserved HbAA RBCs with storage according
972 to CRUN and UZA, respectively. The results suggest that neither cryopreservation nor
973 subsequent storage methods affected RBC integrity after thawing or sexual conversion rates
974 (SCRs) in the *iv*SCA.

975 *In vitro sexual conversion rates (SCRs).*

976 To validate findings from the *ev*SCAs, we cultured the *P. falciparum* transgenic line NF54-
977 *gexp02*-Tom [53, 70] *in vitro* in RBCs with different HBB genotypes. A total of 28
978 cryopreserved endemic RBC samples used in the *iv*SCA including HbAA (n = 5), HbAC (n =
979 5), HbAS (n = 4), HbSS (n = 12), and HbSC (n = 2) (Table 1 and Supplementary Figure 1). An
980 overview of the number of RBC samples by HBB genotype by performed experiment is given
981 in Table 3. A total of six HbAA non-endemic samples, four fresh and two cryopreserved non-
982 endemic HbAA RBC samples, were used in the experiments as controls (*i.e.,* HbAA CTRL).
983 We measured the sexual conversion rates (SCRs) by flow cytometry (FC) detecting sexual
984 stages by the expression of the red fluorescent protein TdTomato under the promotor of the
985 gexp02 gene, which is exclusively expressed in sexual stages [53], and the total number of
986 infected RBCs (iRBCs) using the DNA stain Hoechst33342. The *iv*SCA experimental set-up
987 is illustrated in Figure 5A and representative flow cytometry plots by HBB genotype are shown
988 in Supplementary Figure 4. Parasite cultures were routinely maintained in choline-
989 supplemented Albumax-based medium (+Choline) to mimic the natural repression of sexual
990 conversion observed in healthy human serum. Parasite cultures were tightly synchronized by
991 Percoll purification at the schizont stage (~40-44 hpi), followed by the addition of RBCs with
992 different HBB genotypes to allow reinvasion. Subsequently, sorbitol lysis was performed at
993 the ring stage to obtain a narrow developmental window of ~0-5 hpi (Figure 5A). During the
994 assay, choline was either maintained to repress or depleted (-Choline) to induce sexual
995 conversion, following established protocols [51, 53, 73]. Choline depletion was performed at

996 ring stage (~0-5 hpi) (Figure 5A). SCRs were determined as the proportion of parasites that
997 developed into gametocytes in the cycle following exposure (Generation 1, Figure 5A). Since
998 sexual parasites begin expressing the TdTomato reporter at ~10-15 hpi in Generation 1, and
999 SCRs are typically measured at ~25-30 hpi (Generation 1, Figure 5A) [53], we included an
1000 additional later timepoint (~35-40 hpi) within the same cycle to ensure robust TdTomato
1001 detection across all HBB genotypes. This was particularly important given observations of
1002 developmental delays (in HbAC, HbAS, HbSS, and HbSC RBCs) compared to wild-type
1003 HbAA RBCs (Figure 4).
1004 At ~35-40 hpi (Generation 1) in cultures supplemented with choline (+Choline), the SCR
1005 (median[IQR]) increased from 10.8%[0.49] in wild-type HbAA RBC cultures to 13.30%[4.71]
1006 for HbAC and 16.5%[5.64] for HbAS RBC cultures (Beta-binomial GLM, HbAC v.s. HbAA
1007 RBCs: OR[95% CI] = 1.25 [0.81−1.92] , $p > 0.05$; HbAS v.s. HbAA RBCs: OR[95% CI] =
1008 1.73 [1.12−2.66], $p = 0.0056$**; Supplementary Table 7). In HbSS and HbSC RBC cultures,
1009 the SCRs appeared visually lower than in wild-type control HbAA RBCs (6.96% [3.17] and
1010 7.99% [1.83], respectively), with statistically significant differences in HbSS vs. HbAA (Beta-
1011 binomial GLM: OR[95% CI] = 0.64 [0.43−0.95], $p = 0.021$*; Supplementary Table 7) but not
1012 significant in HbSC vs. HbAA (Beta-binomial GLMM, $p > 0.05$, Supplementary Table 7 and
1013 Figure 5B). Similarly, the fold change in SCRs (median [IQR]) relative to wild-type fresh
1014 HbAA non-endemic RBC cultures showed a trend consistent with the observations above, with
1015 elevated SCRs in HbAC (1.47 [0.66]) and HbAS (2.05 [0.38]) RBCs, while remaining
1016 unchanged (~1) in HbAA (1.23 [0.31]), HbSS (0.84 [0.49]), and HbSC (1.10 [0.25]) RBCs.
1017 However, differences between HbAA and HBB mutant RBCs were not statistically significant
1018 (Wilcoxon Rank Sum post-hoc test, $p = 0.039$*; Figure 5C).
1019 Conversely, in cultures supplemented with choline (+Choline) at ~25-30 hpi (Generation 1),
1020 no differences were found in SCRs between HbAA and RBCs with mutant HBB genotypes

1021 (Kruskal-Wallis test, $p > 0.05$) (Supplementary Figure 3A). Compared to ~35-40 hpi
1022 (Generation 1), these results suggest that at ~25-30 hpi (Generation 1) a proportion of parasites
1023 may not yet have reached the stage of TdTomato expression. This is supported by the
1024 observation that the SCR (median [IQR]) in HbAC and HbAS increased from 9.41%[5.14] and
1025 15.0%[6.48] at ~25-30 hpi to 13.3%[4.71] and 16.5%[5.64] at ~35-40 hpi, respectively, within
1026 Generation 1 (Figure 5A), indicating that parasites in these RBCs were indeed developmentally
1027 delayed.
1028 In contrast, at ~35-40 hpi (Generation 1, Figure 5A) in cultures in which sexual conversion had
1029 already been stimulated by choline depletion (-Choline), SCRs were induced (>10%) in HbAA,
1030 HbAC, HbAS, and HbSS RBCs but not in HbSC RBCs (~10%). No significant differences in
1031 SCRs were observed between HbAA vs. HbAC RBCs (Beta-binomial GLM, OR[95%CI] =
1032 1.02 [0.66−1.59], $p > 0.05$) and HbAA vs. HbAS RBCs (Beta-binomial GLM, OR[95%CI] =
1033 1.06 [0.66−1.69], $p > 0.05$) (Supplementary Table 7 and Supplementary Figure 3C). Similar
1034 results were obtained at ~25-30 hpi (Generation 1) in choline depleted cultures showing no
1035 significant differences in SCRs between HbAA vs. HbAC RBCs (Beta-binomial GLM,
1036 OR[95%CI] = 1.03 [0.65−1.63], $p > 0.05$) and HbAA vs. HbAS (Beta-binomial GLM,
1037 OR[95%CI] = 1.28 [0.81−2.03], $p > 0.05$) RBCs) (Supplementary Table 7 and Supplementary
1038 Figure 3B). These findings in choline depleted cultures confirm that maximal SCR induction
1039 following choline depletion does not vary across mutant HBB RBCs compared to HbAA
1040 RBCs..

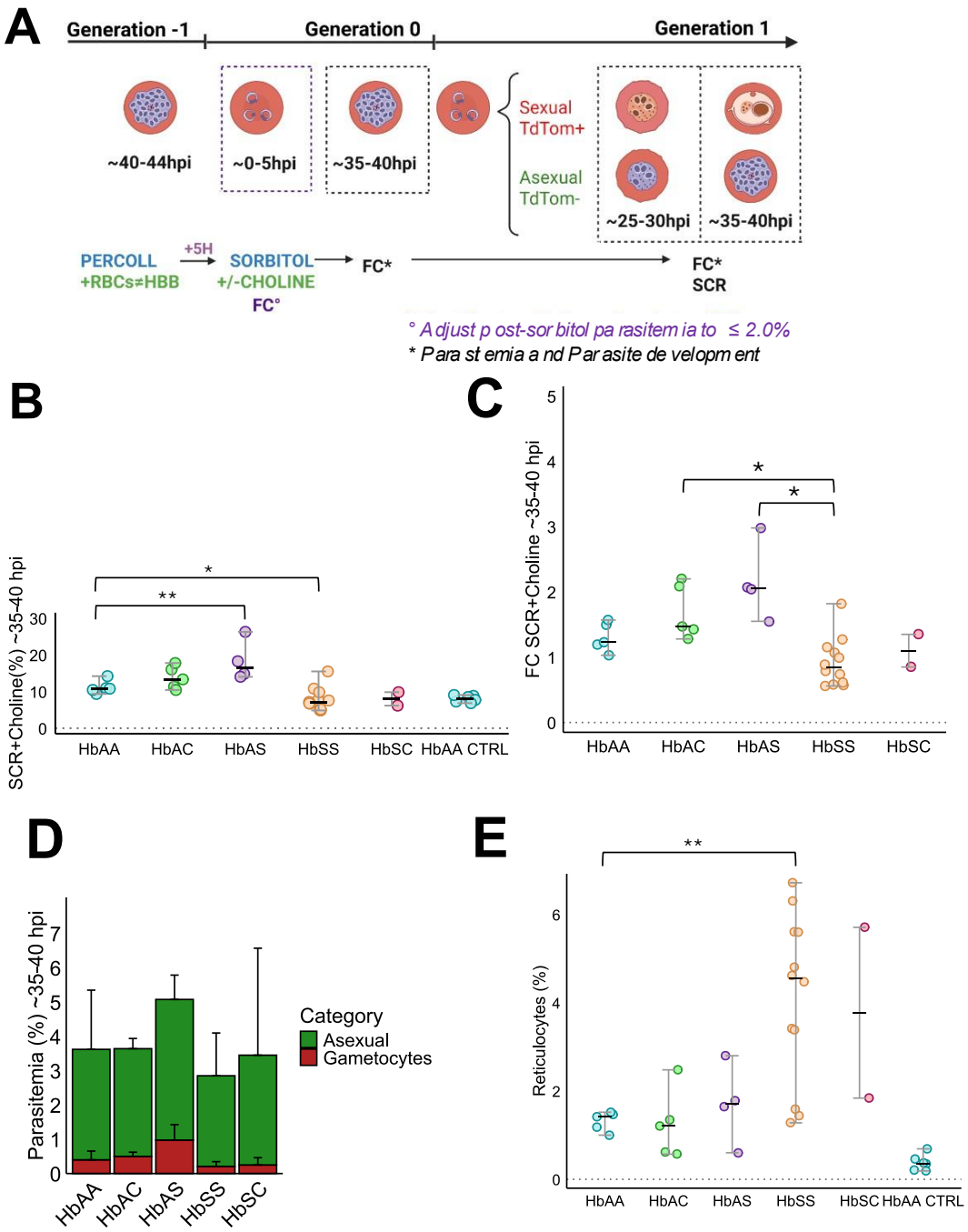


Figure 5. Effect of human hemoglobin beta (HBB) HbAA, HbAC and HbAS genotypes on sexual conversion determined with *in vitro* sexual conversion assays (*iv*SCAs). (A) Experimental design of *iv*SCAs using the NF54-*gexp02-Tom P. falciparum* line. Tightly synchronized late-stage schizonts (Generation -1) were allowed to reinvade RBCs of different HBB genotypes, and ring stages (~0-5 hpi) were then cultured under both non-inducing (+Choline) and inducing (-Choline) conditions. Parasite development was assessed by flow cytometry (FC) at timepoints: ~35-40 hpi (Generation 0) and ~25-30 hpi/~35-40 hpi (Generation 1). Sexual conversion rates (SCRs) were assessed FC at ~25-30 hpi and ~35-40 hpi in Generation 1. *Created with BioRender.* **(B, C and E)** Dot plots show the median (bold line), quartiles and range indicated by whiskers. **(B)** Sexual conversion rates (SCRs) by HBB genotype determined with FC at ~35-40 hpi (Generation 1), in +Choline cultures. Sample size n per group: endemic RBC samples with HbAA $n = 5$, HbAC $n = 5$ and HbAS $n = 4$, and HbSS $n = 12$ and HbSC $n = 2$, total n=28; and non-endemic fresh RBC samples with "HbAA CTRL" $n = 6$. **(C)** Fold-change SCRs in cryopreserved HbAA, HbAC, HbAS, HbSS and HbSC genotypes relative to fresh HbAA RBCs. **(D)** Proportion of asexual and

gametocyte stages and total parasitemia by HBB genotype at ~35-40 hpi (Generation 1), in +Choline cultures. **(F)** Reticulocytes percentage by HBB genotype. **Statistical tests:** (B and E), Beta-binomial generalized linear mixed models (GLMM) with Tukey adjustment for pairwise comparisons; (C) Kruskal-Wallis test with the Wilcoxon Rank Sum post-hoc test with Holm correction. Significant p-values are shown as: $p < 0.05$*, $p < 0.01$**, $p < 0.001$***; non-significant p-values as: ns. Overall p-values are presented with a bold straight line. **Abbreviations:** Hpi, hours post-invasion; CTRL, control sample (fresh HbAA RBCs)

Notably, at both ~25-30 hpi and ~35-40 hpi (Generation 1), SCRs in HbSS and HbSC RBCs remained below 10% (not induced) in choline supplemented cultures, appearing visually lower than wild-type HbAA with only significant differences in HbSS vs. HbAA at 35-40hpi (Beta-binomial GLMM; OR[95% CI] = 0.64 [0.43−0.95] $p = 0.021$*, Supplementary Table 7 and Supplementary Figure 3A,Figure 5B). In contrast, in choline-depleted cultures, SCRs in HbSS and HbSC RBCs (10-20%), were lower at ~35-40 hpi than the >30% observed in HbAC and HbAS RBCs (Supplementary Figure 3C). The lower SCRs in HbSS and HbSC RBCs may be explained by the partial arrest and extreme delay in development observed in these cultures (Figure 4 and Supplementary Table 2).

We analyzed initial parasitemia across HBB genotypes to determine whether it contributed to variation in SCRs. Differences in SCRs could not be explained by variations in initial parasitemia at ~0-5 hpi (Generation 0), as no significant differences were found between HBB genotypes (Beta-binomial GLM, $p > 0.05$; Supplementary Table 7) and no significant correlation was observed between SCR and parasitemia (Spearman $\rho = 0.10$, $p > 0.05$; Supplementary Figure 2B). All cultures were started with a parasitemia below 2.0%. Next, we examined whether higher SCRs in a particular HBB genotype were not inflated by lower asexual parasitemia. Analysis of the proportion of RBCs infected with asexual parasites and stage I gametocytes by HBB genotype has shown that total gametocytemia (determined by FC at ~35-40 hpi (Generation 1) was clearly higher in cultures with HbAS RBCs, followed by HbAC RBCs, compared to wild-type HbAA RBCs, while levels in HbSS and HbSC RBCs were similar to or lower than those in HbAA RBCs (Figure 5D). Total gametocytemia at ~25-

1082 30 hpi (Generation 1) followed the same dynamics (Supplementary figure 3E). This confirms
1083 that the higher SCRs in HbAS and HbAC reflect enhanced gametocyte production rather than
1084 diminished asexual parasite numbers.
1085 Since reticulocytes have been proposed to enhance SCRs [76], and individuals with mutant
1086 HBB genotypes often exhibit anemia-associated reticulocytosis, we assessed reticulocyte
1087 percentages across genotypes. Including all HBB groups (HbAA, HbAC, HbAS, HbSS, and
1088 HbSC), we observed significant differences in reticulocyte percentage with the highest median
1089 percentages in HbSS compared to HbAA RBCs (Beta-binomial GLMM, OR[95%CI] = 2.56
1090 [1.20−5.44], p = 0.008**, Supplementary Table 7 and Figure 5E). Importantly, SCR
1091 differences in HbAC and HbAS RBCs could not be attributed to reticulocyte percentage. A
1092 non-significant correlation between reticulocyte percentage and SCR was found (Spearman ρ
1093 =0.10, p > 0.05; Supplementary Figure 3F) , reinforcing the conclusion that higher SCRs are
1094 due to intrinsic properties of mutant RBCs rather than reticulocyte abundance. Additionally, to
1095 control for hemolysis during FC experiments, we assessed RBC counts (RBCs/µl) across HBB
1096 genotypes at various timepoints, as shown in Supplementary Table 3. No significant
1097 differences were observed at any of the time points, indicating that mutant HBB RBCs did not
1098 undergo hemolysis during the course of the experiment (Kruskal-Wallis test, overall $p > 0.05$).
1099 *HBB genotypes are uniquely associated with enhanced SCRs compared to other host factors.*
1100 To assess whether factors other than the mutant HBB genotypes influenced SCRs, we
1101 performed a risk analysis of additional variables (*i.e.,* gender, village, ethnicity, age,
1102 parasitaemias, etc.) stratified by HBB genotype group, in the study population in the *ev*SCAs
1103 (n = 32). This showed that only SCRs differ amongst the HBB genotypes HbAA, HbAC and
1104 HbAS (Kruskal-Wallis test, overall $p < 0.001$***; Supplementary Table 4). These results
1105 suggest that mutant HBB genotypes (HbAC and HbAS), compared to the wild type HbAA,
1106 resulted in induction of the *P. falciparum* sexual conversion.

1107 For the same aim, we performed a risk analysis of factors in the study population in the *iv*SCAs
1108 (n = 34) that may potentially impact sexual conversion rates (SCRs) in addition to the HBB
1109 mutant genotypes (Table 4). Our analysis indicated that HBB genotypes and gender are a risk
1110 factor for enhanced SCRs (Fisher's exact test, overall $p < 0.01$** and $p < 0.05$*, respectively).
1111 The percentage of reticulocytes was negatively associated with SCRs, with the lowest SCR and
1112 highest percentage of reticulocytes in HbSS and HbSC RBCs (Spearman $\rho = -0.50$, $p < 0.01$**).
1113 Blood groups and other factors (village and ethnicity) did not show any impact on SCRs
1114 (Fisher's exact test and Kruskal-Wallis test, $p > 0.05$). We also explored which factors are
1115 potentially associated with HBB genotype (Supplementary Table 5). We noted that the
1116 percentage of reticulocytes is the highest in the HBB mutant genotypes HbSS and HbSC,
1117 followed by HbAS, HbAC and HbAA (Kruskal-Wallis, overall p <0.001***). Additional
1118 factors such as age, gender, village and ethnicity showed statistically significant differences in
1119 their distribution across HBB genotype categories (Fisher's exact test, overall p <0.05),
1120 whereas blood groups did not (Fisher's exact test, $p > 0.05$).

1121 **Table 4. Risk Analysis of factors for elevated sexual conversion rates (SCRs) at 35-40 hpi (Generation 1),**
1122 **in the study participants within the *in vitro* sexual conversion assays (n = 34).** Data from *P. falciparum* non-
1123 infected samples collected in the Nanoro Department, Burkina Faso and the UZA (Belgium). Statistical tests
1124 performed are shown at the bottom of the figure with correspondent p-values (with significant p-values as: $p <$
1125 0.05*, $p < 0.01$**, $p < 0.001$***). Abbreviations: HBB, Hemoglobin beta; SCR, Sexual conversion rates; hpi,
1126 hours post-invasion; IQR, Interquartile range.

	SCR +Choline 35-40 hpi	$p^{\$}$
+Choline n(%)	28 (100.0)	NA
HBB Genotype (median (IQR))		<0.01**
HbAA	10.8 (0.49)	
HbAC	13.30 (4.71)	
HbAS	16.50 (5.64)	
HbSS	6.96 (3.17)	
HbSC	7.99 (1.83)	
ABO blood group (median (IQR))		0.15
O+	7.57 (7.86)	
A+	8.80 (2.52)	
B+	17.80 (3.48)	
A-	9.34 (2.87)	
B-	10.90 (3.77)	

Gender (median (IQR))			<0.05*
Female	11.30 (5.04)		
Male	7.57 (4.09)		
Village (median (IQR))			0.30
Nanoro	11.30 (3.75)		
Nazoanga	15.40 (0.60)		
Soum	0 (0)		
Séguédin	0 (0)		
Ethnicity (median (IQR)) 0.40			
Gourounsi	17.80 (0.00)		
Mossi	12.30 (4.14)		
	Median (IQR)	rho°	*p*°
Reticulocytes (percentage)	1.50 (5.63)	-0.50	<0.01**
Age	27.00 (30.00)	-0.18	0.37

Significant p-values ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***).
$Categorical variables test: Fisher's exact test.
$Continuous variables test: Kruskal Wallis test and Post-Hoc analysis with Wilcoxon Rank Sum Post-hoc Test with Holm correction.
° Continuous variables : Spearman correlation test

IgG and IgM detection assays by Flow cytometry: Demographics of study population and assay validation

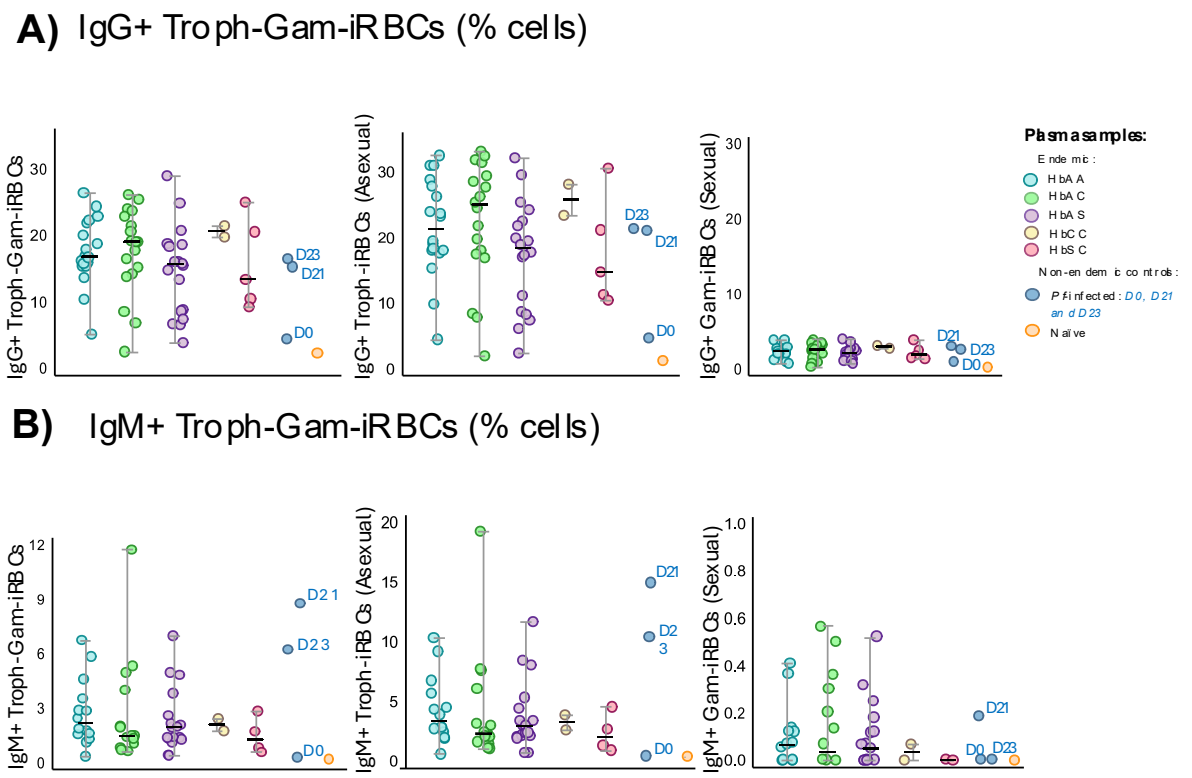
The evaluation of variables in the study population (n = 61) such as: *P. falciparum* infection status at the time of sample collection, parasitemia (parasites per µl), presence of gametocytes in blood, age, gender and village; stratified by HBB genotype (HbAA [n = 18], HbAC [n = 18], HbAS [n = 18], HbCC [n = 2], and HbSC [n = 5]) revealed no significant differences between the categories (Fisher's exact test and Kruskal-Wallis test; $p > 0.05$) (Supplementary Table 6). In our study population, there is a homogeneous distribution of individuals across the categorical variables, with no observed sampling bias that could affect further analysis.

The plasma titration curves showed a clear dose-response dynamic for IgG detection with the "*Pf*-infected hyperimmune endemic plasma pool", while for IgM detection the measured percentage of positive cells was low even at the lowest plasma dilution. Therefore, four additional plasma samples were titrated for IgM detection (*i.e.,* "naïve non-endemic plasma" and "acute *Pf-infected* non-endemic plasma at D0, D21 and D23"). While the "naïve non-endemic plasma" remained under the threshold of the "hyperimmune *Pf*-infected endemic plasma pool", the "acute *Pf*-infected non-endemic plasma at D21 and D23" exceeded the values

1143 of both the "*Pf*-infected hyperimmune endemic plasma pool" and the "naïve non-endemic
1144 plasma", and showed a dose-response dynamic. Due to the generally low levels of IgM
1145 measured against early trophozoite/stage I gametocyte-infected RBCs (α-Troph-Gam-iRBCs
1146 IgM), we chose the optimal plasma dilution with highest percentage of positive cells detected
1147 for IgM (1:320), while for IgG detection we used the dilution in the slope (1:640)
1148 (Supplementary Figure 5A).

1149 For robust ΔMFI values a cut-off of a minimum number of event counts per category was set
1150 to 200 events (Supplementary Figure 5A and 5C). In each assay, positive controls (three acute
1151 *Pf-infected* non-endemic plasma from a traveler at D0, D21 and D23) and a negative control
1152 ("naïve non-endemic plasma") were analyzed. The "naïve non-endemic plasma" sample
1153 consistently did not reach the 200 event counts threshold, confirming its suitability as a
1154 negative control. The negative control values served as a baseline for the levels of α-Troph-
1155 Gam-iRBCs IgG & IgM and were lower than those of the endemic plasma samples of all HBB
1156 genotypes (Figure 7). Endemic plasma samples were considered positive for IgG or IgM when
1157 both the event count and the levels of α-Troph-Gam-iRBCs IgG and IgM exceeded those
1158 observed in the "naïve non-endemic plasma". All endemic plasma samples exhibited event
1159 counts below the threshold of 200 for the levels of total α-Troph-Gam-iRBCs IgM
1160 (Hoechst33342+/PerCP-Cy5.5.+) and α-Troph-iRBCs IgM (Hoechst33342+/ tdTom-/PerCP-
1161 Cy5.5.+). The number of α-Gam-iRBCs IgG (Hoechst33342+/tdTom+/ AF488+) and IgM
1162 (Hoechst33342+/ tdTom+/PerCP-Cy5.5.+) was below 200 events for all endemic plasma
1163 samples (n=61). Thirteen samples (21%) had no detected (*i.e.,* zero) events for α-Gam-iRBCs
1164 IgM. Based on these parameters, the MFI could only be evaluated for total and α-Troph-iRBCs
1165 IgG in the statistical analyses (Supplementary Figure 6B and 6D).
1166 Importantly, the events count in α-Troph-Gam-iRBCs and α-Troph-iRBCs IgM of the positive
1167 controls at D21 and D23 exceeded both the event counts observed in the "naïve non-endemic

1168 plasma" and the threshold of 200 events, confirming that our flow cytometry assay can detect
1169 α-Troph-Gam-iRBCs IgM (Supplementary Figure 6C). The time course of the "acute *Pf*-
1170 infected non-endemic plasma at D0, D21 and D23" generally followed the expected IgG (D0
1171 < D21 < D23) and IgM (D0 < D21 > D23) dynamics in α-Troph-Gam-iRBCs IgG & IgM after
1172 a *P. falciparum* infection [100, 101].



1173
1174 **Figure 7. IgG and IgM mediated humoral immune response against *P. falciparum* in plasma samples from**
1175 **individuals with different human hemoglobin beta (HBB) genotypes.** Data from *P. falciparum* infected (*n* =
1176 44) and non-infected (*n* = 17) plasma samples collected at the Clinical Research Unit (CRUN) in Nanoro, Burkina
1177 Faso (total *n* = 61). Data points show the total percentage of IgG-positive or IgM-positive trophozoite- and
1178 gametocyte- infected red blood cells (α-Troph-Gam-iRBCs IgG & IgM) for each HBB genotype group, with
1179 median (bold line), quartiles and range indicated by whiskers. **(A)** Percentage of α-Troph-Gam-iRBCs IgG: total
1180 (left), asexual (middle) and sexual (right). **(B)** Percentage α-Troph-Gam-iRBCs IgM: total (left), asexual (middle)
1181 sand sexual (right). Positive controls ("*Pf*-infected non-endemic" *i.e.,* three acute *Pf*-infected non-endemic plasma
1182 samples from a traveller at D0, D21, and D23) and a negative control *i.e.,* "Naïve non-endemic": naïve plasma
1183 from a non-endemic volunteer with HbAA genotype) are shown as individual dots per sample. The Y-axis range
1184 differs between graphs for visualization. No significant differences were found (Kruskal-Wallis with Wilcoxon
1185 Rank Sum post-hoc for non-parametric variables, one-way ANOVA with Bonferroni post-hoc for parametric
1186 variables, $p > 0.05$). **Abbreviations:** IgG and IgM, Immunoglobulin G and M; Troph-Gam-iRBCs, trophozoite-
1187 and gametocyte-infected red blood cells; *Pf, P. falciparum*. D: Day(s).
1188

1189 **Comparison of IgG and IgM levels in plasma from different HBB genotypes living in a**
1190 **malaria endemic region by flow cytometry.**

1191 In endemic human plasma samples ($n = 61$) from individuals carrying different HBB genotypes
1192 living in Nanoro (Burkina Faso), a malaria endemic region, we found no significant differences
1193 in the levels of α-Troph-Gam-iRBCs IgG & IgM (% of cells/MFI data only reliable for α-
1194 Troph-Gam -iRBCs IgG) (Kruskal-Wallis with Wilcoxon Rank Sum post-hoc and one-way
1195 ANOVA with Bonferroni post-hoc, $p > 0.05$) (Figure 7). When stratifying the Troph-Gam-
1196 iRBCs in asexual and sexual iRBCs, there was no significant difference in the levels of α-
1197 asexual and α-sexual iRBCs IgG & IgM in endemic plasma from individuals carrying different
1198 HBB genotypes in either subgroup (% of cells/MFI data only reliable for α- Troph-iRBCs IgG)
1199 Kruskal-Wallis with Wilcoxon Rank Sum post-hoc and one-way ANOVA with Bonferroni
1200 post-hoc, $p > 0.05$) (Figure 7).

1201 We detect low levels or absence of α-Gam-iRBCs IgG & IgM, and low levels or absence of
1202 IgM against Throph-Gam-iRBCs, Troph-iRBCs and Gam-iRBCs in the plasma of the endemic
1203 individuals, as compared to high levels of IgG against Throph-Gam-iRBCs and Troph-iRBCs
1204 (Figure 7 and Supplementary Figure 6).

1205 Further analysis of the levels of α-Troph-Gam-iRBCs IgG & IgM in the study population by
1206 "*P. falciparum (Pf)* infection status", revealed that the levels of α-Troph-Gam-iRBCs IgG &
1207 IgM were higher in the *Pf*-infected individuals ($n = 44$, median[IQR] for IgG: 17.90[6.30], and
1208 for IgM: 2.03[3.34]) compared to the *Pf* non-infected individuals ($n = 17$, median[IQR] for
1209 IgG: 9.10[9.66], and for IgM: 1.56[1.27]) (Kruskal-Wallis test with Wilcoxon Rank Sum post-
1210 hoc test, $p = 0.0063$** [for IgG]; and $p > 0.05$ [for IgM]) (Supplementary Figure 7). These
1211 results confirm the expected IgM dynamics, where IgM levels are highest during acute or recent
1212 infections (*Pf*-infected individuals) compared to past infections (*Pf* non-infected individuals)
1213 [100, 101].

1214 Stratification analysis of the levels of α-Troph-Gam-iRBCs IgG & IgM by age-group (*i.e.,*
1215 younger age: "1 to 4 years" compared to older age: "5 to 19 years") in *Pf*-infected compared to
1216 *Pf* non-infected individuals, showed that both *Pf*-infected and *Pf* non-infected individuals with
1217 younger age had lower levels of α-Troph-Gam-iRBCs IgG (*Pf*-infected n = 22 and
1218 median[IQR]: 17.90[7.23]; *Pf* non-infected n=15 and median[IQR]: 9.30[10.80]) than those
1219 with an older age (*Pf*-infected n = 22 and median[IQR]:18.60[5.64]; *Pf* non-infected n = 2 and
1220 median[IQR]: 12.60[3.69]) (One-way ANOVA with Bonferroni post-hoc test [IgG]; Kruskal-
1221 Wallis with Wilcoxon Rank Sum post-hoc test [IgM], all $p > 0.05$). *Pf*-infected individuals with
1222 younger age had slightly higher levels of α-Troph-Gam-iRBCs IgM (n = 22, median[IQR]:
1223 2.19[2.33]) than those with an older age (n = 22, median[IQR]: 1.87[3.04]) (Kruskal-Wallis
1224 with Wilcoxon Rank Sum post-hoc test, $p > 0.05$). In contrast, the levels of α- Troph-Gam-
1225 iRBCs IgM in *Pf* non-infected individuals with younger age were slightly lower (n = 15,
1226 median[IQR]: 1.56[1.12]) than those with an older age (n = 2, median[IQR]: 2.18[0.00])
1227 (Kruskal-Wallis with Wilcoxon Rank Sum post-hoc test, $p > 0.05$).
1228 Together these results (the positive controls exceeding the 200 events threshold for α-Troph-
1229 Gam-iRBCs IgM and IgG, the dynamic of α-Troph-Gam-iRBCs IgM and of IgG in the time
1230 course of the acute *Pf*-infected non-endemic plasma), show that the flow cytometry assay can
1231 accurately detect Troph-iRBCs IgM levels despite the low level or absence in endemic
1232 individuals (Figure 7). The increase of IgG with age (*i.e.,* exposure in endemic individuals) and
1233 decrease of IgM with age gives insight into the reason for the low α-Troph-Gam-iRBCs IgM
1234 levels in the endemic plasma (exposure leads to a build-up of immunity that replaces low-
1235 affinity IgM by an affinity-matured IgG response) [101-105].
1236

1237 **DISCUSSION**

1238 Our study demonstrates that *P. falciparum* sexual conversion (SC) is significantly influenced
1239 by host hemoglobin genotype. Both *ex vivo* and *in vitro* assays showed that parasites growing
1240 in RBCs carrying HBB mutations HbAS and HbAC had higher sexual conversion rates than
1241 those in HbAA. This genotype-dependent modulation of sexual conversion was accompanied
1242 by a developmental delay in parasites within HbS- and HbC-infected RBCs but was not
1243 associated with differences in IgG or IgM responses against trophozoite- and early gametocyte-
1244 infected RBC surface antigens. Together, these findings indicate that host RBC intrinsic
1245 properties, rather than humoral immunity, underly altered parasite reproductive strategies in
1246 HBB mutant backgrounds.

1247 Amoah et al., (2020) [106] analyzed the effect of HBB genotype on SC of field isolates from
1248 Ghanaian children aged 6-15 years with uncomplicated malaria, and reported a non-significant
1249 trend of decreasing sexual conversion rates (SCRs) from HbAA to HbAC and HbAS. The
1250 discrepancy with our results is most likely methodological, because quantifying sexual
1251 conversion in natural infections is challenging as parasites in freshly collected blood include
1252 morphologically indistinguishable sexual and asexual rings. The authors (similarly to Usui et
1253 al., (2019) [55]) used prolonged culture (≥4 days) to distinguish sexual stages from asexual
1254 forms. The limitation of this approach is the timing mismatch where SCRs are measured as the
1255 ratio of stage II gametocytes on day 4 (D4) by the total parasitemia on day 0 (D0). This can
1256 underestimate SCR because parasites contributing to the denominator (D0) are counted before
1257 culture adaptation affects viability, while the numerator (D4) includes only gametocytes that
1258 survive and mature after several days in *ex vivo* conditions, during which parasite loss can
1259 occur. Similarly, Usui et al., (2019) [55] faced limitations when quantifying low SCRs (<0.3%)
1260 in low-density natural infections by LM. For instance at 0.05% parasitemia and 0.3% SCRs,
1261 they would be detecting five gametocytes among 30,000 RBCs. This approach provides limited

1262 sensitivity and resolution and could explain why up to 24% of samples were reported as
1263 gametocyte negative.

1264 We addressed these limitations using an adapted *ev*SCA to quantify next cycle sexual
1265 conversion [50]. We measure whether in-host exposure in individuals with RBCs with different
1266 HBB genotypes leads to stimulation of sexual commitment. We quantify the in-host effect by
1267 assessing the subsequent sexual conversion (next cycle, *ex vivo*) as number of stage I
1268 gametocytes (gamI) per number of schizonts and gamI (overall parasites) 24 hours post culture
1269 (hpc). Quantification is done by immunofluorescence, gamI detected by Pfs16 and overall
1270 parasites by DAPI [107]. Inclusion of PKG inhibitor ML10 prevented reinvasion, allowing
1271 numerator and denominator measurements for the SCR at the same time point, while
1272 enrichment using LD columns improved sensitivity. This approach reliably quantified sexual
1273 conversion in infections with densities as low as 0.05% (~2,500 parasites/µl) an detected a
1274 minimum of 38 stage I gametocytes per sample, with no gametocyte-negative cultures.

1275 We validated *ex vivo* findings using an *iv*SCA with a *gexp02-reporter* line [53], allowing early
1276 detection of sexual rings. While our SCR results in *iv*SCA were broadly consistent with those
1277 of Flage, B., et al., (2023) [39] for HbAA and HbAS RBCs (median SCRs: 10.8% vs. 10% in
1278 HbAA; 16.5% vs. 15% in HbAS), the results are not directly comparable due to clear
1279 methodological differences in how SCR was measured. In our assay, SCR is defined and
1280 measured as the proportion of *gexp02*-expressing parasites among total parasites at the next
1281 cycle conversion (generation 1), while Flage, B., et al., (2023) [39] quantified sexual parasites
1282 at generation 1 and total parasitaemia at generation 0 making direct comparison impossible.

1283 We observed a partial developmental delay in parasites infecting HbAS and HbAC RBCs, and
1284 a more pronounced arrest or extreme delay in HbSS and HbSC under low-oxygen conditions
1285 (5% O_2), both by microscopy and flow cytometry, consistent with the findings of Archer, N.M.,

1286 et al., (2018) [31]. Later time-point measurements (~35-40 hpi) increased SCRs in endemic
1287 HbAA, HbAC, and HbAS RBCs compared with ~25-30 hpi, suggesting delayed sexual marker
1288 expression and thus underestimation of SCR at the earlier time point due to developmental
1289 delay. Of note, parasites grown in control non-endemic HbAA RBCs showed stable SCRs
1290 across both time points. The observed differences between endemic and non-endemic HbAA
1291 RBCs may reflect unmeasured host genetic factors (*e.g.,* blood group variants or other
1292 erythrocytic polymorphisms). In HbSS and HbSC RBCs, we observe SCRs similar to HbAA
1293 and lower than HbAS and HbAC, consistently across both measurement time points. This
1294 contrasts with Flage, B., et al., (2023) [39], who reported markedly higher SCR in HbSS RBCs
1295 (6.96% vs. 35% in Flage, B., et al., 2023) and no developmental delay at day 2 of culture,
1296 although this was not quantitatively assessed. As noted above, differences in the timing and
1297 approach used to quantify SCR make the results not comparable between studies.
1298 Under choline-depleted conditions, SCRs in HbSS and HbSC increased modestly (from <10%
1299 to 10-20%) but remained below levels in HbAA, HbAC, and HbAS RBCs (>30-40%). This is
1300 likely due to severe developmental delay causing under-detection of sexually committed rings
1301 at the measured time points rather than inhibited sexual commitment. Overall, our data
1302 indicates that accurately assessing sexual conversion in HbSS and HbSC RBCs is challenging
1303 with this approach due to developmental delay. Measuring sexual commitment one cycle
1304 earlier, for instance through *GDV1* activation, might circumvent this issue, although this is
1305 experimentally demanding because *GDV1* is expressed transiently and at low levels. Given the
1306 very low prevalence of HbSS and HbSC genotypes in malaria-endemic populations, the added
1307 value of such complex assays for transmission studies is likely limited.

1308 We evaluated potential confounding factors that could influence sexual conversion as they
1309 were previously linked to SC [76, 77] [70, 108-112] , including reticulocyte abundance,
1310 parasitemia, and ABO blood group. No significant differences in reticulocyte percentages were

1311 observed among HbAA, HbAC, and HbAS RBCs, and reticulocyte levels did not correlate with
1312 SCRs at 35-40 hpi. Similarly, neither baseline parasitemia nor ABO blood group influenced
1313 SCR in either the *ev*SCA or *iv*SCA. Together, these results indicate that the observed increase
1314 in sexual conversion is specifically associated with HBB genotype rather than these other host-
1315 related variables.

1316 The increase in SC in RBCs with HBB mutant genotypes *in vitro*, in the absence of plasma and
1317 white blood cells, points to RBC-intrinsic effects rather than host immune responses or soluble
1318 plasma factors. HbS and HbC RBCs exhibit elevated levels of free heme [113], linked to
1319 increased oxidative stress that leads to polymerization of HbS under low oxygen conditions
1320 [114], and delays parasite development [31]. The oxidative stress inhibits actin polymerization
1321 in infected HbS and HbC RBCs [32], which alters protein trafficking and PfEMP1 display [27,
1322 30]. This reduces cytoadherence [34], and membrane flexibility [33]. Oxidative stress also
1323 modifies RBC micro RNAs that can hybridize with parasite mRNAs [26], directly affecting
1324 parasite development and metabolism, and increasing gametogenesis [39]. Flage, B., et al.,
1325 (2023) [39] showed that heme directly induces sexual conversion. Taken together, these
1326 findings suggest that the effect of HbAC and HbAS RBCs on SC *in vitro* is likely driven by
1327 oxidative stress caused by free heme within these RBCs, mediated by their specific miRNA
1328 profiles. The stronger effect *ex vivo*, particularly in HbAS (7.07- vs ~2.05- fold *in vitro*),
1329 probably reflects additional *in vivo* factors such as parasite genetic diversity [55, 107], or host
1330 factors not captured *in vitro* [115, 116].

1331 To further investigate potential *in vivo* influences, we looked into the humoral immune
1332 response in the different HBB genotypes. Reduced antigen presentation, cytoadherence, and
1333 increased splenic clearance [34] could plausibly lead to an altered immune response in HbAC
1334 and HbAS. However, epidemiological studies examining immunity in individuals with HbAS
1335 and HbAC genotypes have yielded conflicting results. Gong et al. (2012) [28] reported that

1336 older children with HbAS were more protected against the establishment of parasitemia,
1337 suggesting an acquired mechanism of protection. In contrast, Tan et al. (2011) [117] found no
1338 differences in antibody responses between individuals with HbAA, HbAS, and HbAC
1339 genotypes against their panel of 491 immunoreactive *P. falciparum* antigens. More recent
1340 studies have shown that serological responses tend to be lower in individuals with HbAC and
1341 HbAS when focusing on specific surface antigens [118, 119]. In a cross-sectional study
1342 conducted in Ghana, Harp et al., (2020) [120] investigated circulating inflammatory cytokines
1343 and observed that expression levels of CXCL10, TNF-α, CCL2, IL-8, and IL-6 varied by
1344 hemoglobin genotype.
1345 Given that differences in serological responses appear specific to certain surface antigens, we
1346 chose to measure IgG and IgM levels against intact infected RBCs. This approach enables the
1347 assessment of immune responses to native surface antigens, minimizing potential artifacts
1348 arising from non-native protein folding, glycosylation, or conformation. We focused on
1349 trophozoite-infected RBCs (25-30 hpi), as surface antigen presentation (*i.e.,* PfEMP1
1350 expression) begins around 16 hpi and plateaus at approximately 24 hpi [121]. Moreover,
1351 commitment to sexual conversion occurs between 17-37 hpi [50, 70], suggesting that any
1352 antibody-mediated induction of sexual commitment would need to occur at this stage.
1353 We compared IgG and IgM responses to trophozoite- and gametocyte-infected RBCs (Troph-
1354 Gam-iRBCs) using plasma samples from Burkinabé individuals carrying HbAA, HbAC,
1355 HbAS, HbCC, and HbSC genotypes, assessed by flow cytometry. Our analyses revealed no
1356 significant differences in anti–Troph-Gam-iRBC IgG or IgM levels across genotypes. IgM
1357 responses, as well as responses against gametocyte-infected RBCs, were generally low or
1358 absent. Therefore, we could not confirm genotype-dependent differences in humoral responses
1359 to these parasite stages and consider it unlikely that antibodies directly drive the differential
1360 SC observed in these individuals. Nonetheless, this does not exclude the possibility that

1361 genotype-specific antibody responses to later parasite stages –particularly during
1362 gametocytogenesis, as suggested by recent studies on gametocyte antigenicity [97, 122]– could
1363 influence transmission potential, either by promoting clearance of developing gametocytes in
1364 the human host or by inducing gametocyte sterility within the mosquito vector [123].

1365 Overall, this study robustly demonstrates elevated sexual conversion of *P. falciparum* parasites
1366 in HbAS and HbAC RBCs both *in vitro* and *ex vivo*. These findings further support the view
1367 that *P. falciparum* can adjust its reproductive investment in response to stress-related cues. In
1368 mutant HBB RBCs, elevated levels of free heme can induce developmental delay and thereby
1369 compromise survival within the human host; increased investment in sexual conversion may
1370 thus represent a parasite survival strategy [31, 39, 68].

1371 Previous studies have shown that microenvironmental factors such as LysoPC depletion,
1372 nutrient limitation and exposure to antimalarial drugs can modulate malaria transmission by
1373 changing the rate of parasite's sexual conversion. How factors such as free heme, are being
1374 translated into a signal received by epigenetic regulators controlling the sexual commitment
1375 gene ap2-g or gdv1 remains an open question [39, 50].
1376 At the population level, HBB mutant genotypes have been linked to increased gametocyte
1377 carriage and higher mosquito infectivity. In Burkina Faso, Gouagna et al. (2010) [41]reported
1378 two- to fourfold higher infection rates in HbAS, HbAC, and HbCC carriers. Similar trends were
1379 observed in Mali and Cameroon, where Gonçalves et al. (2017) [45] and Ngou et al. (2023)[44]
1380 found that HbAC and HbAS individuals carried more gametocytes and were more infectious
1381 to mosquitoes than HbAA. These population-level associations are consistent with our
1382 findings, and suggest that increased sexual conversion in mutant HBB RBCs may contribute
1383 to enhanced transmission potential.

ACKNOWLEDGEMENTS

We are grateful to Robert W Sauerwein and Teun Bousema (Radboud University, The Netherlands) for the anti-Pfs16 monoclonal antibody. We thank Núria Casas-Vila (ISGlobal) for the help setting the *ex vivo* and *in vitro* sexual conversion assays, and Delwendé Florence Ouédraogo (CRUN) for support during the field work at CRUN. This work was supported by the Research Foundation - Flanders (FWO) with scholarship "PhD fellowship strategic basic research" to Yasmina Drissi-El Boukili under grant number 1S74321N and "Research project grant" (G067823N), funding of the Malariology Unit (to Anna Rosanas-Urgell) and the Belgium, Directorate-General Development Cooperation and Humanitarian Aid (DGD).

AUTHOR CONTRIBUTIONS

Yasmina Drissi-El Boukili, Conceptualization, Resources, Funding acquisition, Formal analysis, Investigation and laboratory procedures, Methodology, Writing- original draft, Writing- review and editing; Vera Kühne, Methodology, Writing- review and editing, Substantial revisions and conceptual input; Pieter Moris, Formal analysis (statistical analysis, model fitting using GLMMs); Elisabet Tintó-Font, Methodology (SCAs); Odin Goovaerts, Methodology (flow cytometry); Pieter Guetens, laboratory procedures and analysis (HBBseq and qPCR); Ana Moreno-Murillo; laboratory procedures and analysis (HBBseq); Yarno Valgaerts, laboratory procedures (IgG and IgM detection by flow cytometry); H. Magloire Natama, Supervision, Writing- review and editing; Alfred Cortés, Supervision, Writing- review and editing; Guy Caljon, Supervision, Writing- review and editing; Anna Rosanas-Urgell, Conceptualization, Resources, Supervision, Funding acquisition, Writing- original draft, Project administration, Writing- review and editing, Substantial revisions and conceptual input. All authors read and approved the final manuscript.

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REFERENCES

1. WHO, *World malaria report 2024: Addressing inequity in the global malaria response.* 2024.
2. Hannemann, A., et al., *The Properties of Red Blood Cells from Patients Heterozygous for HbS and HbC (HbSC Genotype).* Anemia, 2011. **2011:248527.** (2090-1275 (Electronic)).
3. Haldane, J.B., *Suggestions as to quantitative measurement of rates of evolution.* Evolution, 1949. **3**(1): p. 51-6.
4. Taylor, S.M., C. Cerami, and R.M. Fairhurst, *Hemoglobinopathies: slicing the Gordian knot of Plasmodium falciparum malaria pathogenesis.* PLoS Pathog, 2013. **9**(5): p. e1003327.
5. Taylor, S., C. Parobek, and R. Fairhurst, *Haemoglobinopathies and the clinical epidemiology of malaria: A systematic review and meta-analysis.* The Lancet infectious diseases, 2012. **12**: p. 457-68.
6. Friedman, M.J., *Erythrocytic mechanism of sickle cell resistance to malaria.* Proc Natl Acad Sci U S A., 1978. **Apr;75(4):1994-7.** (0027-8424 (Print)).
7. Agarwal, A., et al., *Hemoglobin C associated with protection from severe malaria in the Dogon of Mali, a West African population with a low prevalence of hemoglobin S.* (0006-4971 (Print)).
8. Modiano, D., et al., *Haemoglobin C protects against clinical Plasmodium falciparum malaria.* (0028-0836 (Print)).
9. Mackinnon, M.J., et al., *Heritability of malaria in Africa.* (1549-1676 (Electronic)).
10. de Mendonça, V.R., M. Goncalves Ms Fau - Barral-Netto, and M. Barral-Netto, *The host genetic diversity in malaria infection.* (1687-9694 (Electronic)).
11. Lawrence, C., R.L. Fabry Me Fau - Nagel, and R.L. Nagel, *The unique red cell heterogeneity of SC disease: crystal formation, dense reticulocytes, and unusual morphology.* (0006-4971 (Print)).
12. Rees, D.C., T.N. Williams, and M.T. Gladwin, *Sickle-cell disease.* The Lancet, 2010. **376**(9757): p. 2018-2031.
13. Grosse, S.D., et al., *Sickle cell disease in Africa: a neglected cause of early childhood mortality.* (1873-2607 (Electronic)).
14. Bunn Hf Fau - Noguchi, C.T., et al., *Molecular and cellular pathogenesis of hemoglobin SC disease.* (0027-8424 (Print)).
15. Zaabi., B.K.S.K.J.E.A., *Hemoglobin C Disease*. Last Update: May 29, 2023.: Treasure Island (FL): StatPearls Publishing; 2025 Jan-.
16. Ballas, S.K., et al., *Clinical, hematological, and biochemical features of Hb SC disease.* American Journal of Hematology, 1982. **13**(1): p. 37-51.
17. Wellems, T.E. and R.M. Fairhurst, *Malaria-protective traits at odds in Africa?* Nature Genetics, 2005. **37**(11): p. 1160-1162.
18. Natama, H.M., et al., *Genetic variation in the immune system and malaria susceptibility in infants: a nested case-control study in Nanoro, Burkina Faso.* (1475-2875 (Electronic)).
19. Simpore, J., et al., *Modification in the frequency of Hb C and Hb S in Burkina Faso: an influence of migratory fluxes and improvement of patient health care.* (0363-0269 (Print)).

1452 20. Bougouma, E.C., et al., *Haemoglobin variants and Plasmodium falciparum malaria in children*
1453 *under five years of age living in a high and seasonal malaria transmission area of Burkina*
1454 *Faso.* Malaria Journal, 2012. **11**(1): p. 154.
1455 21. May, J., et al., *Hemoglobin variants and disease manifestations in severe falciparum malaria.*
1456 (1538-3598 (Electronic)).
1457 22. Williams, T.N., et al., *An immune basis for malaria protection by the sickle cell trait.* (1549-
1458 1676 (Electronic)).
1459 23. Williams, T.N., et al., *Sickle cell trait and the risk of Plasmodium falciparum malaria and other*
1460 *childhood diseases.* (0022-1899 (Print)).
1461 24. Gong, L., et al., *Biochemical and immunological mechanisms by which sickle cell trait protects*
1462 *against malaria.* Malaria Journal, 2013. **12**(1): p. 317.
1463 25. Kaur, J. and R. Hora, *'2TM proteins': an antigenically diverse superfamily with variable*
1464 *functions and export pathways.* PeerJ, 2018. **6**: p. e4757.
1465 26. Chen, S.-Y., et al., *The Genomic Analysis of Erythrocyte microRNA Expression in Sickle Cell*
1466 *Diseases.* PLOS ONE, 2008. **3**(6): p. e2360.
1467 27. Fairhurst, R.M., et al., *Abnormal display of PfEMP-1 on erythrocytes carrying haemoglobin C*
1468 *may protect against malaria.* (1476-4687 (Electronic)).
1469 28. Gong, L., et al., *Evidence for both innate and acquired mechanisms of protection from*
1470 *Plasmodium falciparum in children with sickle cell trait.* (1528-0020 (Electronic)).
1471 29. Lelliott, P.M., et al., *The influence of host genetics on erythrocytes and malaria infection: is*
1472 *there therapeutic potential?* (1475-2875 (Electronic)).
1473 30. Fairhurst, R.M., C.D. Bess, and M.A. Krause, *Abnormal PfEMP1/knob display on Plasmodium*
1474 *falciparum-infected erythrocytes containing hemoglobin variants: fresh insights into malaria*
1475 *pathogenesis and protection.* Microbes Infect, 2012. **14**(10): p. 851-62.
1476 31. Archer, N.M., et al., *Resistance to Plasmodium falciparum in sickle cell trait erythrocytes is*
1477 *driven by oxygen-dependent growth inhibition.* Proceedings of the National Academy of
1478 Sciences, 2018. **115**(28): p. 7350-7355.
1479 32. Cyrklaff, M., et al., *Hemoglobins S and C interfere with actin remodeling in Plasmodium*
1480 *falciparum-infected erythrocytes.* Science, 2011. **334**(6060): p. 1283-6.
1481 33. Fröhlich, B., et al., *Hemoglobin S and C affect biomechanical membrane properties of P.*
1482 *falciparum-infected erythrocytes.* (2399-3642 (Electronic)).
1483 34. Cholera, R., et al., *Impaired cytoadherence of Plasmodium falciparum-infected erythrocytes*
1484 *containing sickle hemoglobin.* (1091-6490 (Electronic)).
1485 35. Carlson, J., et al., *Human cerebral malaria: association with erythrocyte rosetting and lack of*
1486 *anti-rosetting antibodies.* (0140-6736 (Print)).
1487 36. LaMonte, G., et al., *Translocation of sickle cell erythrocyte microRNAs into Plasmodium*
1488 *falciparum inhibits parasite translation and contributes to malaria resistance.* (1934-6069
1489 (Electronic)).
1490 37. Ferreira, A., et al., *Sickle hemoglobin confers tolerance to Plasmodium infection.* (1097-4172
1491 (Electronic)).
1492 38. Taylor, S.M. and R.M. Fairhurst, *Malaria parasites and red cell variants: when a house is not*
1493 *a home.* (1531-7048 (Electronic)).
1494 39. Flage, B., et al., *Heme promotes sexual conversion of Plasmodium falciparum in human*
1495 *erythrocytes.* Frontiers in Malaria, 2023. **1**.
1496 40. Vasquez, M., M. Zuniga, and A. Rodriguez, *Oxidative Stress and Pathogenesis in Malaria.*
1497 (2235-2988 (Electronic)).
1498 41. Gouagna, L.C., et al., *Genetic variation in human HBB is associated with Plasmodium*
1499 *falciparum transmission.* Nat Genet, 2010. **42**(4): p. 328-31.
1500 42. Andolina, C., et al., *Plasmodium falciparum gametocyte carriage in longitudinally monitored*
1501 *incident infections is associated with duration of infection and human host factors.* Scientific
1502 Reports, 2023. **13**(1): p. 7072.

43. Lawaly, Y.R., et al., *Heritability of the human infectious reservoir of malaria parasites.* PloS one, 2010. **5**(6): p. e11358-e11358.
44. Ngou, C.M., et al., *Influence of the sickle cell trait on Plasmodium falciparum infectivity from naturally infected gametocyte carriers.* BMC Infectious Diseases, 2023. **23**(1): p. 317.
45. Gonçalves, B.P., et al., *Hemoglobin variants shape the distribution of malaria parasites in human populations and their transmission potential.* Scientific reports, 2017. **7**(1): p. 14267-14267.
46. Meibalan, E. and M. Marti, *Biology of Malaria Transmission.* Cold Spring Harb Perspect Med, 2017. **7**(3).
47. Bousema, T. and C. Drakeley, *Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination.* Clin Microbiol Rev, 2011. **24**(2): p. 377-410.
48. Ngotho, P., et al., *Revisiting gametocyte biology in malaria parasites.* FEMS Microbiology Reviews, 2019. **43**(4): p. 401-414.
49. Llorà-Batlle, O., et al., *Conditional expression of PfAP2-G for controlled massive sexual conversion in <em>Plasmodium falciparum</em&gt.* Science Advances, 2020. **6**(24): p. eaaz5057.
50. Bancells, C., et al., *Revisiting the initial steps of sexual development in the malaria parasite Plasmodium falciparum.* Nat Microbiol, 2019. **4**(1): p. 144-154.
51. Filarsky, M., et al., *GDV1 induces sexual commitment of malaria parasites by antagonizing HP1-dependent gene silencing.* Science (New York, N.Y.), 2018. **359**(6381): p. 1259-1263.
52. Prajapati, S.K., et al., *The transcriptome of circulating sexually committed Plasmodium falciparum ring stage parasites forecasts malaria transmission potential.* Nature Communications, 2020. **11**(1): p. 6159.
53. Portugaliza, H.P., et al., *Reporter lines based on the gexp02 promoter enable early quantification of sexual conversion rates in the malaria parasite Plasmodium falciparum.* Scientific Reports, 2019. **9**(1): p. 14595.
54. Farid, R., et al., *Initiation of gametocytogenesis at very low parasite density in Plasmodium falciparum infection.* The Journal of Infectious Diseases, 2017. **215**(7): p. 1167-1174.
55. Usui, M., et al., *Plasmodium falciparum sexual differentiation in malaria patients is associated with host factors and GDV1-dependent genes.* Nature Communications, 2019. **10**(1): p. 2140.
56. Pelle, K.G., et al., *Transcriptional profiling defines dynamics of parasite tissue sequestration during malaria infection.* Genome Medicine, 2015. **7**(1): p. 19.
57. Venugopal, K.A.-O., et al., *Plasmodium asexual growth and sexual development in the haematopoietic niche of the host.* (1740-1534 (Electronic)).
58. Churcher, T.S., et al., *Predicting mosquito infection from Plasmodium falciparum gametocyte density and estimating the reservoir of infection.* (2050-084X (Print)).
59. Bousema, T. and C. Drakeley, *Determinants of Malaria Transmission at the Population Level. LID - 10.1101/cshperspect.a025510 [doi] LID - a025510.* (2157-1422 (Electronic)).
60. Bradley, J.A.-O., et al., *Predicting the likelihood and intensity of mosquito infection from sex specific Plasmodium falciparum gametocyte density. LID - 10.7554/eLife.34463 [doi] LID - e34463.* (2050-084X (Electronic)).
61. Barry, A., et al., *Higher gametocyte production and mosquito infectivity in chronic compared to incident Plasmodium falciparum infections.* Nature Communications, 2021. **12**(1): p. 2443.
62. Barry, A., *Mosquitoes infecting children & children infecting mosquitoes in Burkina Faso: Production and infectivity of malaria transmission stages*. 2022, Radboud Universiteit Nijmegen. p. 196.
63. Ahmad, A., et al., *Infectivity of patent Plasmodium falciparum gametocyte carriers to mosquitoes: establishing capacity to investigate the infectious reservoir of malaria in a low-transmission setting in The Gambia.* (1878-3503 (Electronic)).

64. !!! INVALID CITATION !!! [4, 25-31].
65. Carter R Fau - Miller, L.H. and L.H. Miller, *Evidence for environmental modulation of gametocytogenesis in Plasmodium falciparum in continuous culture.* (0042-9686 (Print)).
66. Pollitt, L.C., et al., *Competition and the evolution of reproductive restraint in malaria parasites.* Am Nat, 2011. **177**(3): p. 358-67.
67. Carter, L.M., et al., *Stress and sex in malaria parasites: Why does commitment vary?* Evol Med Public Health, 2013. **2013**(1): p. 135-47.
68. Schneider, P., et al., *Adaptive plasticity in the gametocyte conversion rate of malaria parasites.* PLOS Pathogens, 2018. **14**(11): p. e1007371.
69. Josling, G.A., K.C. Williamson, and M. Llinás, *Regulation of Sexual Commitment and Gametocytogenesis in Malaria Parasites.* (1545-3251 (Electronic)).
70. Portugaliza, H.P., et al., *Artemisinin exposure at the ring or trophozoite stage impacts Plasmodium falciparum sexual conversion differently.* eLife, 2020. **9**: p. e60058.
71. Portugaliza, H.P., et al., *Plasmodium falciparum sexual conversion rates can be affected by artemisinin-based treatment in naturally infected malaria patients.* (2352-3964 (Electronic)).
72. Thommen, B.T., et al., *Revisiting the Effect of Pharmaceuticals on Transmission Stage Formation in the Malaria Parasite Plasmodium falciparum.* (2235-2988 (Electronic)).
73. Brancucci, N.M.B., et al., *Lysophosphatidylcholine Regulates Sexual Stage Differentiation in the Human Malaria Parasite Plasmodium falciparum.* Cell, 2017. **171**(7): p. 1532-1544.e15.
74. Neveu, G., D. Beri, and B.F. Kafsack, *Metabolic regulation of sexual commitment in Plasmodium falciparum.* (1879-0364 (Electronic)).
75. Harris, C.T., et al., *Sexual differentiation in human malaria parasites is regulated by competition between phospholipid metabolism and histone methylation.* (2058-5276 (Electronic)).
76. Trager, W., et al., *Plasmodium falciparum: enhanced gametocyte formation in vitro in reticulocyte-rich blood.* Exp Parasitol, 1999. **91**(2): p. 115-8.
77. Birget, P.L.G., et al., *Phenotypic plasticity in reproductive effort: malaria parasites respond to resource availability.* Proc Biol Sci, 2017. **284**(1860).
78. Verra, F., et al., *Haemoglobin C and S Role in Acquired Immunity against Plasmodium falciparum Malaria.* PLOS ONE, 2007. **2**(10): p. e978.
79. Marsh, K., et al., *Antibodies to blood stage antigens of Plasmodium falciparum in rural Gambians and their relation to protection against infection.* (0035-9203 (Print)).
80. Diatta, A.M., et al., *Relationship of binding of immunoglobulin G to Plasmodium falciparum-infected erythrocytes with parasite endemicity and antibody responses to conserved antigen in immune individuals.* (1071-412X (Print)).
81. Cabrera, G., et al., *The sickle cell trait is associated with enhanced immunoglobulin G antibody responses to Plasmodium falciparum variant surface antigens.* (0022-1899 (Print)).
82. Rabinovich, R.N., et al., *malERA: An updated research agenda for malaria elimination and eradication.* (1549-1676 (Electronic)).
83. Natama, H.M., et al., *Malaria incidence and prevalence during the first year of life in Nanoro, Burkina Faso: a birth-cohort study.* Malaria Journal, 2018. **17**(1): p. 163.
84. WHO, *WHO. Basic malaria microscopy: part I. Learner's guide. .* 2010. **2nd ed.**
85. Natama, H.A.-O.X., et al., *Malaria incidence and prevalence during the first year of life in Nanoro, Burkina Faso: a birth-cohort study.* (1475-2875 (Electronic)).
86. Hofmann, N., et al., *Ultra-sensitive detection of Plasmodium falciparum by amplification of multi-copy subtelomeric targets.* PLoS Med, 2015. **12**(3): p. e1001788.
87. Wittwer, C.T., et al., *High-Resolution Genotyping by Amplicon Melting Analysis Using LCGreen.* Clinical Chemistry, 2020. **49**(6): p. 853-860.
88. WHO, M.a., *Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations.*, ed. I.c.o.b.M.f.M.V.a.c.b.t.W.H. Organization. 2008, Amsterdam, The Netherlands.

89. Rasouli Ghahfarokhi, S.M., F. Asadi, and N. Obeidi, *Use of capillary electrophoresis for detection of hemoglobinopathies in individuals referred to health centers in Masjed-Soleiman.* Iranian Journal of Blood and Cancer, 2017. **9**: p. 89-92.
90. da Guarda, C.C., et al., *Sickle cell disease: A distinction of two most frequent genotypes (HbSS and HbSC).* (1932-6203 (Electronic)).
91. Baker, D.A.-O., et al., *A potent series targeting the malarial cGMP-dependent protein kinase clears infection and blocks transmission.* (2041-1723 (Electronic)).
92. Moelans, I.I.M.D., *Pfs16, a potential vaccine candidate against the human malaria parasite Plasmodium falciparum (Dissertation).* PhD Thesis University of Nijmegen, 1995: p. 154 p.
93. *Updated guidelines on selecting an intraclass correlation coefficient for interrater reliability, with applications to incomplete observational designs*. 2024, American Psychological Association: US. p. 967-979.
94. EVIMalaR, M.A., *METHODS IN MALARIA RESEARCH* 2013. **Sixth Edition**
95. Silamut, K., et al., *A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain.* (0002-9440 (Print)).
96. WHO, *World malaria report 2024*. 2024.
97. Dantzler, K.W., et al., *Naturally acquired immunity against immature Plasmodium falciparum gametocytes.* Sci Transl Med, 2019. **11**(495).
98. Bruce, M.C., et al., *Cellular location and temporal expression of the Plasmodium falciparum sexual stage antigen Pfs16.* (0166-6851 (Print)).
99. Shabani, S. and L.R. Serbus, *Pfs16: A Key Parasitophorous Vacuole Membrane Protein Crucial for Malaria Parasite Development and Transmission.* (1875-8355 (Electronic)).
100. Boyle, M.J., et al., *IgM in human immunity to Plasmodium falciparum malaria.* Science Advances, 2019. **5**(9): p. eaax4489.
101. Walker, M.R., et al., *Acquisition and decay of IgM and IgG responses to merozoite antigens after Plasmodium falciparum malaria in Ghanaian children.* (1932-6203 (Electronic)).
102. Stanisic, D.I., et al., *Acquisition of antibodies against Plasmodium falciparum merozoites and malaria immunity in young children and the influence of age, force of infection, and magnitude of response.* (1098-5522 (Electronic)).
103. Rogier, E., et al., *Antibody dynamics in children with first or repeat Plasmodium falciparum infections.* (2296-858X (Print)).
104. Akpogheneta, O.J., et al., *Duration of naturally acquired antibody responses to blood-stage Plasmodium falciparum is age dependent and antigen specific.* (1098-5522 (Electronic)).
105. Chelimo, K., et al., *Antibodies to Plasmodium falciparum antigens vary by age and antigen in children in a malaria-holoendemic area of Kenya.* (0891-3668 (Print)).
106. Amoah, L.E., et al., *Comparative analysis of asexual and sexual stage Plasmodium falciparum development in different red blood cell types.* Malaria Journal, 2020. **19**(1): p. 200.
107. Stewart Lindsay, B., et al., *Plasmodium falciparum Sexual Commitment Rate Variation among Clinical Isolates and Diverse Laboratory-Adapted Lines.* Microbiology Spectrum, 2022. **10**(6): p. e02234-22.
108. Talman, A.M., et al., *Influence of chemotherapy on the Plasmodium gametocyte sex ratio of mice and humans.* Am J Trop Med Hyg, 2004. **71**(6): p. 739-44.
109. Uneke, C.J., *Plasmodium falciparum malaria and ABO blood group: is there any relationship?* (0932-0113 (Print)).
110. Wolofsky, K.T., et al., *ABO blood groups influence macrophage-mediated phagocytosis of Plasmodium falciparum-infected erythrocytes.* (1553-7374 (Electronic)).
111. Afoakwah, R., et al., *Relative Susceptibilities of ABO Blood Groups to Plasmodium falciparum Malaria in Ghana.* (1687-9104 (Print)).
112. Cserti-Gazdewich, C., et al., *ABO In Morbidity and Mortality of Plasmodium Falciparum malaria.* Blood, 2010. **116**(21): p. 666-666.

1655 113. Sheng, K., M. Shariff, and R.P. Hebbel, *Comparative Oxidation of Hemoglobins A and S.*
1656 Blood, 1998. **91**(9): p. 3467-3470.
1657 114. Uzunova, V.V., et al., *Free heme and the polymerization of sickle cell hemoglobin.* (1542-0086
1658 (Electronic)).
1659 115. Smalley, M.E. and J. Brown, *Plasmodium falciparum gametocytogenesis stimulated by*
1660 *lymphocytes and serum from infected Gambian children.* Trans R Soc Trop Med Hyg, 1981.
1661 **75**(2): p. 316-7.
1662 116. Stewart, L.B., et al., *Plasmodium falciparum Sexual Commitment Rate Variation among*
1663 *Clinical Isolates and Diverse Laboratory-Adapted Lines.* (2165-0497 (Electronic)).
1664 117. Tan, X., et al., *Hemoglobin S and C heterozygosity enhances neither the magnitude nor*
1665 *breadth of antibody responses to a diverse array of Plasmodium falciparum antigens.* (1537-
1666 6613 (Electronic)).
1667 118. Bailey, R.D., et al., *Children with hemoglobin C or S trait have low serologic responses to a*
1668 *subset of malaria variant surface antigens.* (1532-2742 (Electronic)).
1669 119. Asare, K.K., et al., *Immune responses to P falciparum antibodies in symptomatic malaria*
1670 *patients with variant hemoglobin genotypes in Ghana.* BMC Immunology, 2024. **25**(1): p. 14.
1671 120. Harp, K.O., et al., *Hemoglobin Genotypes Modulate Inflammatory Response to Plasmodium*
1672 *Infection.* (1664-3224 (Electronic)).
1673 121. Jensen, A.A.-O., Y.A.-O. Adams, and L.A.-O. Hviid, *Cerebral Plasmodium falciparum malaria:*
1674 *The role of PfEMP1 in its pathogenesis and immunity, and PfEMP1-based vaccines to prevent*
1675 *it.* (1600-065X (Electronic)).
1676 122. Ngotho, P., et al., *Reversible host cell surface remodelling limits immune recognition and*
1677 *maximizes transmission of Plasmodium falciparum gametocytes.*
1678 bioRxiv, 2024: p. 2024.04.30.591837.
1679 123. Bousema, T., et al., *The Dynamics of Naturally Acquired Immune Responses to Plasmodium*
1680 *falciparum Sexual Stage Antigens Pfs230 & Pfs48/45 in a Low Endemic Area in Tanzania.*
1681 PLOS ONE, 2010. **5**(11): p. e14114.

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