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

Short title: Elevated *P. falciparum* sexual conversion in HbAC and HbAS RBCs

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AUTHOR CONTRIBUTIONS

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analysis (statistical analysis, model fitting using GLMs and GLMMs); Elisabet Tintó-Font: methodology (SCAs); Odin Goovaerts: methodology (flow cytometry); Pieter Guetens: laboratory procedures and analysis (HBBseq and qPCR); Ana Moreno-Murillo and Erin Sauve: laboratory procedures and analysis (HBBseq); Yarno Valgaerts: laboratory procedures (IgG and IgM detection by flow cytometry); T. Edwig Traoré, D. Florence Ouedraogo, Halidou Tinto: field work in Nanoro, Burkina Faso; Ann Van de Velde: clinical evaluation of patients and coordination of blood sample collection at UZA; Alfred Cortés: supervision, methodology, writing- review and editing; Guy Caljon, supervision, methodology, writing- review and editing; Anna Rosanas-Urgell, conceptualization, resources, supervision, funding acquisition, project administration, methodology, writing- original draft, writing- review and editing, substantial revisions and conceptual input. All authors read and approved the final manuscript.

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.

AUTHOR SUMMARY

Transmission of *Plasmodium falciparum* – a malaria causing parasite- depends on the parasite's ability to form gametocytes, the stages that infect mosquitoes. In many African regions where malaria is common, hemoglobin variants such as hemoglobin S (the sickle-cell trait) and C are highly prevalent because they protect against severe disease. However, it remains unclear whether these variants also influence the parasite's early shift (*i.e.,* sexual conversion) toward gametocyte development. In our study, we combined *ex vivo* and *in vitro* sexual conversion assays to measure sexual conversion in red blood cells with different hemoglobin genotypes. We found that sexual conversion was higher in parasites growing in red blood cells carrying hemoglobin AC and AS genotypes compared to normal AA hemoglobin. These findings provide experimental support for earlier epidemiological observations reporting that individuals with these hemoglobin variants often carry more gametocytes and are more infectious to mosquitoes. We also measured antibody levels in individuals with these red blood cells and found no differences in immunity that could explain the variation in sexual conversion. Our results show that host genetic background can influence gametocyte development and may shape the infectious reservoir in high-burden settings. This knowledge is important for designing effective strategies for malaria elimination.

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

75 countries reporting a resurgence in clinical cases (1). Innovative approaches for malaria control
76 and elimination are thus urgently needed.

77 *Plasmodium* infections have exerted strong evolutionary pressure on the human genome, with
78 hemoglobin mutations providing a well-documented example (2-5). Mutations in the human
79 HBB gene, such as hemoglobin S (HbS) and hemoglobin C (HbC), result from a substitution
80 of glutamic acid at position 6 with valine in HbS and lysine in HbC. Individuals can carry
81 homozygous (HbSS or HbCC), heterozygous (HbAS or HbAC) or compound heterozygous
82 (HbSC) mutations (6-8). HBB mutations lead to significant changes in red blood cell (RBC)
83 morphology and function, influencing malaria susceptibility and clinical outcomes (5, 9, 10).
84 HbS mutations induce hemoglobin polymerization under low oxygen, causing red blood cells
85 (RBCs) to reshape into sickle forms. HbSS causes ~60-80% RBC sickling (2) with severe
86 complications including vaso-occlusion, hemolysis, and chronic anemia (11, 12) and high
87 early-life mortality with estimates ranging from 50-90% among children in low-and middle
88 income countries (13). On the other hand, HbAS carriers are typically asymptomatic (2), with
89 fewer sickled RBCs (~40%)(12, 14). HbC mutations cause hemoglobin crystallization without
90 sickling but increase blood viscosity and shorten RBC life span (15). HbCC and HbAC is
91 usually a mild and asymptomatic disorder, with HbCC occasionally causing mild chronic
92 hemolytic anemia in some individuals (2, 7, 15). HbSC carriers display moderate disease
93 severity and a mixed phenotype with ~50% sickled/crystallized and "pita-shaped" RBCs, (2,
94 11, 16).

95 While HbSS and HbCC are relatively rare (~2%), likely due to the high mortality, HbAS and
96 HbAC can reach frequencies of 10-30% in regions such as Mali and Burkina Faso, which has
97 been linked to the protection they confer against *P. falciparum* malaria (4, 5, 8, 17-20). HbAC
98 offers mild protection against severe malaria, while HbAS and HbCC provide strong protection
99 against both uncomplicated and severe malaria (5, 15, 21-23). The protective mechanisms of

100 the different HBB mutations are not fully understood (4, 24). HbS infected-RBCs (iRBCs),
101 especially in HbSS, exhibit elevated heme and HO-1−mediated oxidative stress. This causes
102 polymerization, disrupts the actin cytoskeleton, alters iRBCs morphology, and impairs PfEMP-
103 1 iRBCs surface expression (25-27). In HbAS iRBCs, this reduces cytoadherence and
104 sequestration, promoting splenic clearance and controlled parasite infection (28, 29).

105 Beyond protection from disease, HbS and HbC RBCs may influence the transmission potential
106 of *P. falciparum*. Population studies have reported conflicting evidence regarding whether
107 HbAS influences gametocyte carriage. No significant association has been reported between
108 HbAS and increased gametocyte carriage, though HbAC and HbCC show associations (30). In
109 contrast, other studies found a link between HbAS and gametocyte carriage (31, 32). Few
110 studies have also reported higher infectivity to mosquitoes in individuals carrying HbAS (30,
111 33), HbAC (30, 34) and HbCC (30) compared to wild-type HbAA iRBCs.

112 Gametocyte production is initiated by a process called sexual conversion, in which a small
113 proportion (~1%) of asexual *P. falciparum* parasites commit to sexual development at each
114 replication cycle, a proportion referred to as the sexual conversion rate (SCR), and initiate
115 gametocytogenesis (35-37). Sexual commitment is marked by epigenetic activation of the
116 master regulator *pfap2-g* (38, 39), a transcription factor that irreversibly results in sexual
117 conversion and gametocyte development (38, 39). Sexual rings circulate in blood (40-43),
118 while maturation of gametocyte stages I to IV occurs mainly in the bone marrow over ~10 days
119 (44). Mature male and female stage V gametocytes re-enter the peripheral blood (*i.e.,*
120 gametocyte carriage), and are the only parasite stages capable of infecting *Anopheles*
121 mosquitoes. Gametocyte carriage determines human to mosquito transmission and correlates
122 with infectivity to mosquitoes (45-50).

123 Elevated gametocyte carriage reported in individuals with HbAC and HbAS genotypes could
124 result from elevated sexual conversion but also prolonged infections, leading to accumulation
125 of gametocytes in peripheral blood over numerous replication cycles. Several host related
126 factors have been shown to increase sexual conversion (51), including antimalarial treatment
127 (52-54), lysophosphatidylcholine depletion (55-57), and reticulocyte-rich environments (58,
128 59). A terminal investment in transmission through increased sexual conversion (60-63), could
129 similarly be initiated by the impaired environment in HbS and HbC iRBCs (4, 29, 64-69). To
130 date, only one study has investigated the link between sexual conversion and HBB genetics,
131 showing that increased intracellular heme in HbS iRBCs promotes sexual conversion (25).
132 Another potential driver of elevated sexual conversion in HBB mutant RBCs is the host
133 immunity against *P. falciparum*. HbS and HbC mutations appear to modulate host immunity
134 against *P. falciparum* by increasing regulatory cytokines (*e.g.,* IL-10) (68)), upregulating HO-
135 1 (66), and dampening pro-inflammatory responses (*e.g.,* TNF-α, IFN-γ) (24), which both
136 protect against severe malaria and enhance parasite clearance. Moreover, HBB mutant RBCs
137 show elevated antibody levels against specific RBC antigens (24). including increased IgG
138 against asexual-stage antigens (*e.g.,* AMA1, EBA175, MSP1-3, CSP, and PSE) and RBC
139 surface antigens such as PfEMP-1 in HbAS individuals across multiple populations (70-73).,

140 Discrepancies in gametocyte carriage studies highlight the need to investigate upstream
141 processes such as sexual conversion. Demonstrating that HBB mutant RBCs (*e.g.,* HbAS and
142 HbAC) enhance *P. falciparum* sexual conversion could improve strategies to interrupt
143 transmission (74) and identify carriers as key contributors to the infectious reservoir for
144 targeted transmission-blocking interventions.

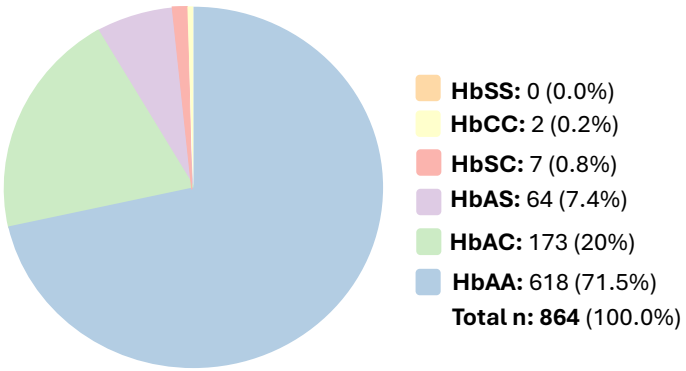
145 In this study, we investigated the impact of host HBB mutant genotypes (HbAA, HbAC and
146 HbAS, HbSS, HbCC and HbSC) on sexual conversion in *ex vivo* and *in vitro P. falciparum*

147 cultures (52, 75) and assessed levels of IgG and IgM against trophozoite- and stage I
148 gametocyte -infected RBCs (Troph-Gam-iRBCs) by flow cytometry.

149 RESULTS

150 HbAC and HbAS genotype prevalences in Nanoro, Burkina Faso

151 Using Sanger Sequencing, we genotyped the HBB gene in participants of a longitudinal cohort
152 study (*i.e., InHost* study) in Nanoro, Burkina Faso (n = 864) and determined the population
153 frequencies of HbAA, HbAC, HbCC, HbAS, HbSS, and HbSC genotypes (Fig 1). HbAA (wild-
154 type) was the most frequent (71.5%), followed by HbAC (20%), HbAS (7.7%), HbSC (0.8%),
155 and HbCC (0.2%); no HbSS individuals were identified.



156

157 **Fig 1. Genotype frequencies of the HBB gene in the study population.** Pie chart depicting the sample sizes (n)
158 and frequencies (%) of the different HBB genotypes in the study population.

159

160 Novel *ex vivo* sexual conversion assays reveal elevated sexual
161 conversion rates in HbAS and HbAC RBCs

162 To investigate the effect of HBB genotypes on *P. falciparum* sexual conversion we measured
163 next cycle SCRs of in host induced *P. falciparum* isolates collected from individuals with
164 different HBB genotypes (HbAA, n =14; HbAC, n =12; HbAS, n = 6) from the Nanoro Health
165 District, Burkina Faso, using an in-house *ex vivo* sexual conversion assay (*ev*SCA), optimized
166 and validated as described in *Supporting information file subsection "Ex vivo sexual*

167 *conversion assays (evSCAs)"*. SCRs were quantified by immunofluorescent staining (IFAs)
168 detecting the number of parasites stained positive for the Pfs16 protein (stage I gametocytes)
169 (76-78) out of the number of parasites stained positive for DNA/parasite nuclei (*i.e.,* the total
170 parasite population consisting of stage I gametocytes plus schizont stages), expressed as a
171 percentage. The experimental set-up of the *ev*SCAs and representative immunofluorescent
172 assay (IFA) images are shown in Figs 2A and 2F, respectively.
173 The SCR (median[IQR]) was 2.65% [1.72] in *P. falciparum* isolates from HbAA genotype,
174 5.05% [2.55] for HbAC and 18.40% [19.70] for HbAS genotypes. SCR increased significantly
175 in HbAC (Beta-binomial GLMM, OR[95%CI] = 1.82 [1.17-2.82], $p = 0.0032$**) and HbAS
176 (Beta-binomial GLMM, OR [95%CI] = 8.16 [4.34-15.37], $p < 0.0001$***) genotypes
177 compared to HbAA (Fig 2B and Table S1). Fold-change in SCRs (median [IQR]) of *P.*
178 *falciparum* isolates in HbAC and HbAS compared to the mean of wild-type HbAA RBCs were
179 2.04 [0.85] in HbAC and 7.07 [7.32] in HbAS, with values in HbAS RBCs significantly higher
180 than in HbAC RBCs (Kruskal-Wallis test, overall $p = 0.034$*; Fig 2C). Parasitemias did not
181 significantly differ between mutant HBB RBCs (HbAC and HbAS) and wild-type HbAA RBCs
182 (Kruskal-Wallis test, $p > 0.05$, Fig 2D); and SCRs and initial parasitemia did not correlate
183 (Spearman correlation, $\rho = 0.05$, $p > 0.05$; (Fig S1A). These results suggest that differences in
184 initial parasitemias do not have an impact on the observed elevated SCRs.
185 The proportion of RBCs infected with stage I gametocytes (extrapolated from proportion of
186 iRBCs in Giemsa-stained slides after MACS enrichment) was highest in cultures with HbAS
187 RBCs, followed by HbAC RBCs, and lowest in wild-type HbAA RBCs (Fig 2E) (same trend
188 observed as in SCRs by HBB genotype, Fig 2B). This confirms that the higher SCRs in mutant
189 HBB genotypes reflect a net increase in gametocyte production rather than a reduction in
190 asexual parasite numbers.
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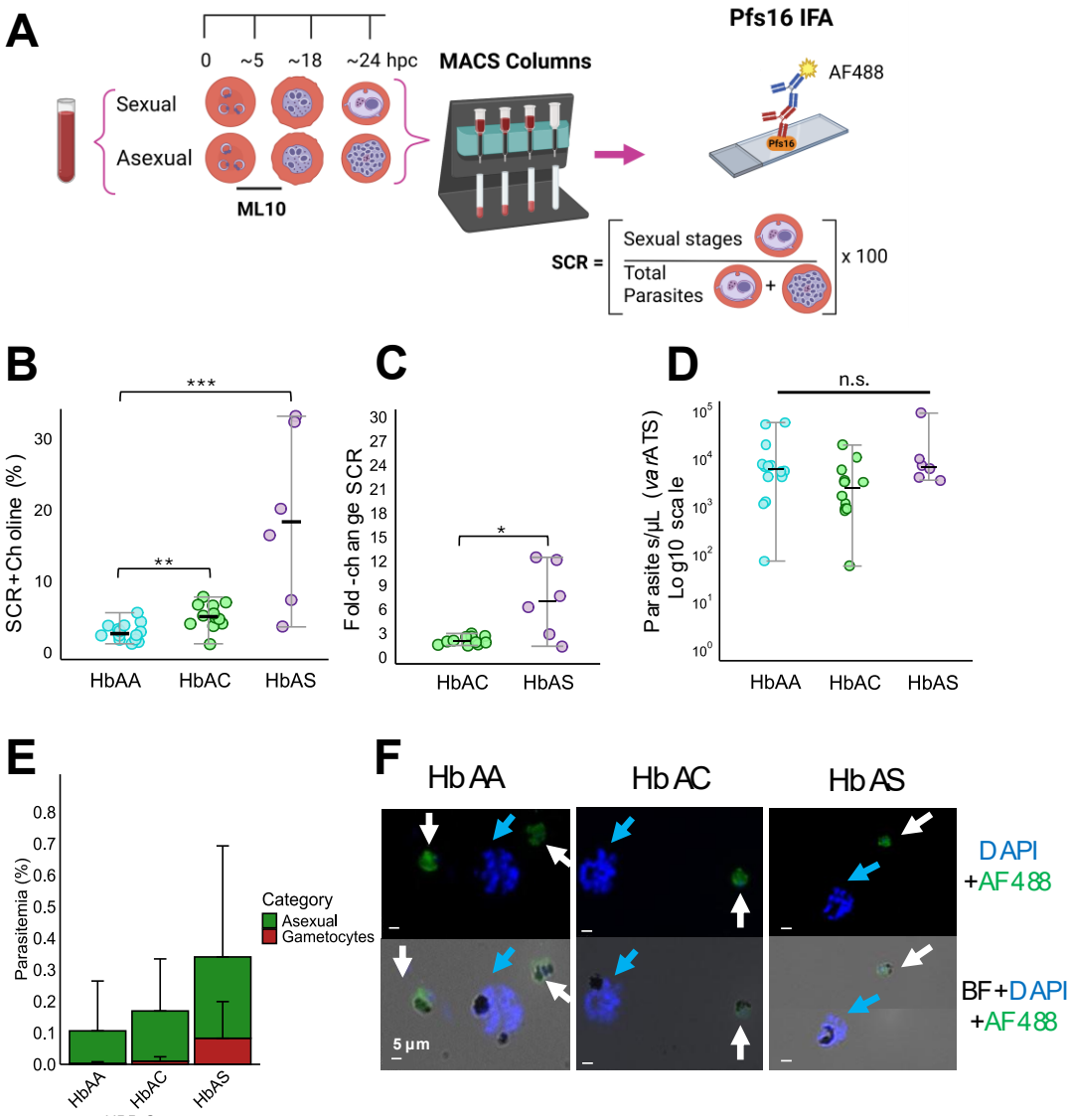

Fig 2. Effect of human hemoglobin beta (HBB) HbAA, HbAC and HbAS genotypes on sexual conversion determined with *ex vivo* sexual conversion assays (*ev*SCAs). (A) Schematic representation of the *ev*SCAs. Parasite isolates from RBCs with different HBB genotypes were cultured *ex vivo* under basal non-inducing (+Choline) conditions. ML10 was added at 11 hpi to block reinvasion. Stage I gametocytes and schizonts were enriched by MACS, and sexual conversion rates were measured by IFA using Pfs16 (sexual stages) and DAPI (total parasites) staining. **(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: 40×; 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).

215 ## *In vitro* sexual conversion assays with the *P. falciparum* transgenic
216 reporter line NF54-*gexp02-Tom*

217 To validate findings from the *ev*SCAs, we established an *in vitro* sexual conversion assay
218 (*iv*SCA) based on an assay that was previously published (52) using a *P. falciparum* transgenic
219 reporter line NF54-*gexp02-Tom* (52, 75), which carries a genome-integrated tandem Tomato
220 (tdTom) fluorescent marker under the control of the promoter of the early gametocyte marker
221 gene *gexp02*. In our assay, parasites, were allowed to invade and grow in cryopreserved non-
222 infected RBCs with different HBB genotypes from CRUN and UZA (28 endemic samples:
223 HbAA $n = 5$, HbAC $n = 5$, HbAS $n = 4$, HbSS $n = 12$, HbSC $n = 2$; and six non-endemic HbAA
224 controls, HbAA CTRL; (Fig 3A, Tables 1 and 3).We measured the SCRs by flow cytometry
225 (FC) detecting sexual stages by the expression of the red fluorescent protein TdTom under the
226 promotor of the *gexp02* gene, which is exclusively expressed in sexual stages (75), and the total
227 number of infected RBCs (iRBCs) using the DNA stain Hoechst33342. The *iv*SCA
228 experimental set-up, validated as described in *Supporting information file subsection "In vitro*
229 *sexual conversion assays (ivSCAs)"*, is illustrated in Fig 3A and representative flow cytometry
230 plots by HBB genotype are shown in Fig S2. Parasite cultures were tightly synchronized by
231 Percoll purification at the schizont stage (~40-44 hpi), followed by the addition of RBCs with
232 different HBB genotypes to allow reinvasion. Subsequently, sorbitol lysis was performed at
233 the ring stage to obtain a narrow developmental window of ~0-5 hpi (Fig 3A). During the assay,
234 choline was either maintained to repress or depleted (-Choline) to induce sexual conversion,
235 following established protocols (55, 75, 79). Choline depletion was performed at ring stage
236 (~0-5 hpi) (Fig 3A). SCRs were determined as the proportion of parasites that developed into
237 gametocytes in the cycle following exposure to RBCs with different HBB genotypes
238 (Generation 1, Fig 3A). Since sexual parasites begin expressing the TdTom reporter at ~10-15
239 hpi in Generation 1, and SCRs are typically measured at ~25-30 hpi (Generation 1, Fig 3A)

240 (75), and given the observed developmental delays in HbAC, HbAS, HbSS, and HbSC RBCs
241 compared to wild-type HbAA RBCs (Fig 3), we included an additional later timepoint (~35-
242 40 hpi) within the same cycle to ensure robust TdTom detection across all HBB genotypes.

243 **Table 1: Overview of the different experiments with the number of cryopreserved RBC samples by HBB**
244 **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**

245

246 At ~35-40 hpi (Generation 1) SCRs (median[IQR]) in wild-type HbAA RBC cultures were at
247 10.8%[0.49] at the threshold of what is considered induced (>10%). SCR was slightly but non-
248 significantly increased to 13.30%[4.71] for HbAC (Beta-binomial GLM, HbAC vs. HbAA
249 RBCs: OR[95% CI] = 1.25[0.81−1.92] , $p > 0.05$) and significantly increased to 16.5%[5.64]
250 for HbAS RBC cultures (HbAS vs. HbAA RBCs: OR[95% CI] = 1.73 [1.12−2.66], p =
251 0.0056**; Table S1). In HbSS the SCRs were significantly lower than in wild-type control
252 HbAA RBCs (6.96% [3.17], HbSS vs. HbAA (Beta-binomial GLM: OR[95% CI] = 0.64
253 [0.43−0.95], $p = 0.021$*; Table S1), while in HbSC RBC cultures (SCR of 7.99% [1.83]), there
254 was no statistically significant difference to HbAA (Beta-binomial GLM, $p > 0.05$, Table S1
255 and Fig 3B). Similarly, the fold-change in SCRs (median [IQR]) relative to wild-type fresh
256 HbAA non-endemic RBC cultures showed a trend consistent with the observations above, with
257 elevated SCRs in HbAC (1.47 [0.66]) and HbAS (2.05 [0.38]) RBCs, while remaining
258 unchanged (~1) in HbAA (1.23 [0.31]), HbSS (0.84 [0.49]), and HbSC (1.10 [0.25]) RBCs.
259 However, fold-change differences between HbAA and HBB mutant RBCs were not
260 statistically significant (Wilcoxon Rank Sum post-hoc test, $p > 0.05$; Fig 3C).

261 Conversely, at ~25-30 hpi (Generation 1), no differences were found in SCRs between HbAA
262 and HBB mutant RBCs (Kruskal-Wallis test, $p > 0.05$) (Fig S3A). Compared to ~35-40 hpi
263 (Generation 1), these results suggest that at ~25-30 hpi (Generation 1) a proportion of parasites
264 may not yet have reached the stage of TdTom expression. This is supported by the observation
265 that the SCR (median [IQR]) in HbAC and HbAS increased from 9.41%[5.14] and 15.0%[6.48]
266 at ~25-30 hpi to 13.3%[4.71] and 16.5%[5.64] at ~35-40 hpi, respectively, within Generation
267 1 (Fig 3A), indicating that parasites in these RBCs were indeed developmentally delayed.
268 In cultures in which sexual conversion had been stimulated by choline depletion (-Choline) at
269 both time points of Generation 1 (Fig 3A), no significant differences in SCRs were observed
270 between HbAA and HbAC or HbAS RBCs (Table S1 and Figs S3B and S3C). These findings
271 confirm that maximal SCR induction following choline depletion does not vary across mutant
272 HBB RBCs compared to HbAA RBCs. The significantly lower SCRs in HbSS and HbSC RBCs
273 may be explained by the partial arrest and extreme delay in development observed in these
274 cultures (Fig 3 and Table S2).

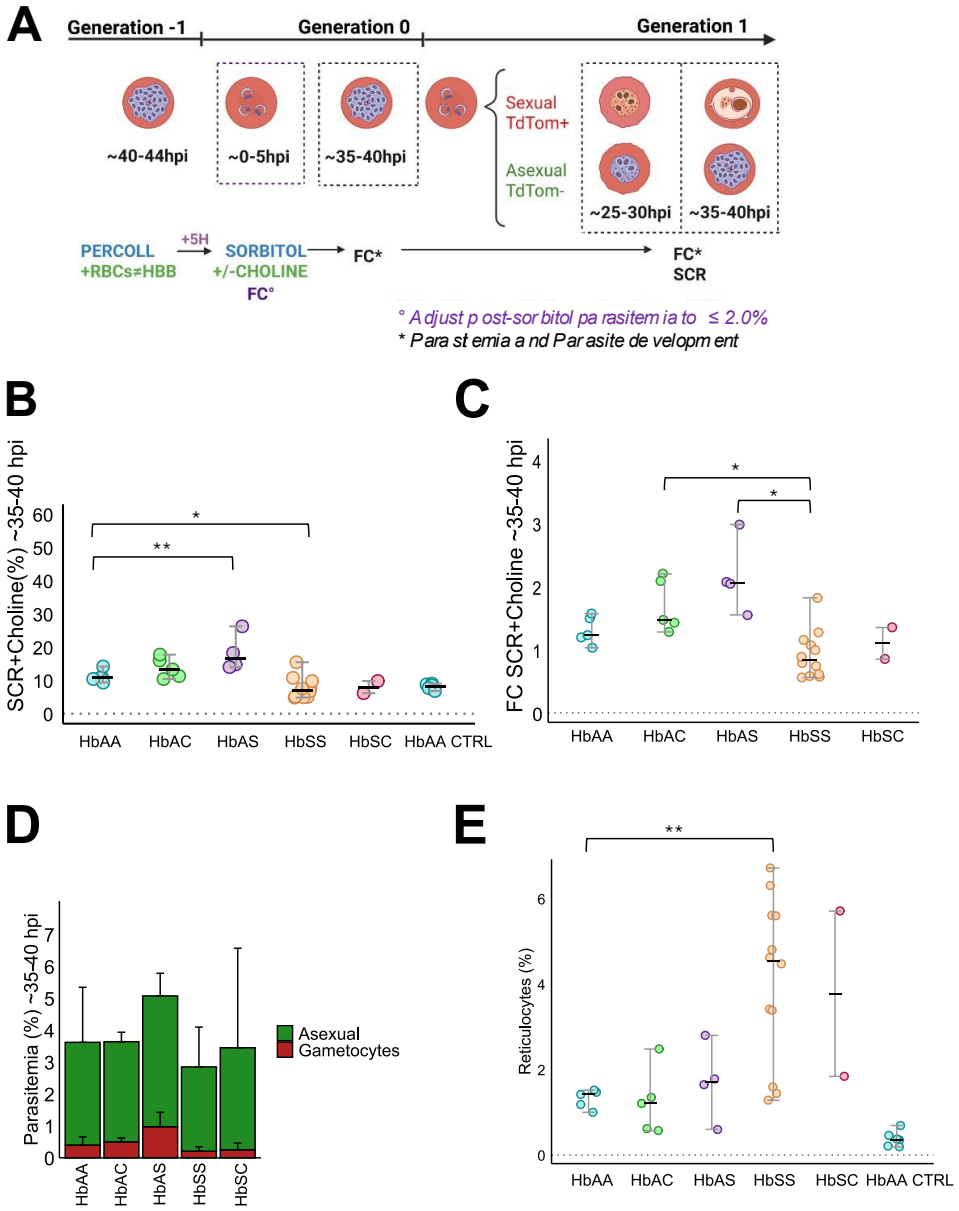

Fig 3. Effect of human hemoglobin beta (HBB) HbAA, HbAC and HbAS genotypes on sexual conversion determined with *in vitro* sexual conversion assays (*iv*SCAs). (A) Experimental design of *iv*SCAs using the NF54-*gexp02-Tom P. falciparum* line. Tightly synchronized late-stage schizonts (Generation -1) 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 **(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 [GLM] with Bonferroni 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).

292

293 Differences in SCRs could not be explained by variations in initial parasitemia at ~0-5 hpi
294 (Generation 0, all cultures were started with a parasitemia below 2.0%), as no significant
295 differences were found between HBB genotypes (Beta-binomial GLM, $p > 0.05$; Table S1) and
296 no correlation was observed between SCR and parasitemia (Spearman $\rho = 0.10$, $p > 0.05$; Fig
297 S1B). Analysis of the proportion of RBCs infected with asexual parasites and stage I
298 gametocytes by HBB genotype has shown that total gametocytemia (determined by FC at ~35-
299 40 hpi, Generation 1) was highest in cultures with HbAS RBCs, followed by HbAC RBCs, and
300 lowest in wild-type HbAA RBCs, while levels in HbSS and HbSC RBCs were similar to or
301 lower than those in HbAA RBCs (Fig 3D). Total gametocytemia at ~25-30 hpi (Generation 1)
302 followed the same dynamics (Fig S3E). This confirms that the higher SCRs in HbAS and
303 HbAC reflect enhanced gametocyte production rather than diminished asexual parasite
304 numbers.
305 Since reticulocytes have been proposed to enhance SCRs (58), and individuals with mutant
306 HBB genotypes often exhibit anemia-associated reticulocytosis, we assessed reticulocyte
307 percentages across genotypes. Including all HBB groups (HbAA, HbAC, HbAS, HbSS, and
308 HbSC), we observed significant differences in reticulocyte percentage with the highest median
309 percentages in HbSS compared to HbAA RBCs (Beta-binomial GLM, OR [95%CI] = 2.56
310 [1.20−5.44], $p = 0.008$**, Table S1 and Fig 3E). No correlation between reticulocyte
311 percentage and SCR was found (Spearman $\rho = 0.10$, $p > 0.05$; Fig S3F), reinforcing the
312 conclusion that higher SCRs are due to intrinsic properties of mutant RBCs rather than
313 reticulocyte abundance. Additionally, to control for hemolysis during FC experiments, we
314 assessed RBC counts (RBCs/µl) across HBB genotypes at various timepoints, as shown in
315 Table S3. No significant differences were observed at any of the time points, indicating that

316 mutant HBB RBCs did not undergo hemolysis during the course of the experiment (Kruskal-
317 Wallis test, overall $p > 0.05$).

318 Red blood cells with mutant HBB genotypes lead to delayed 319 parasite development

320 *P. falciparum* growing in HbAS RBCs exhibits developmental delay/arrest before DNA
321 replication when growing under low oxygen conditions (≤ 5% O_2, as present in the bone
322 marrow and spleen, and in standard *P. falciparum* cultures as used for the *iv*SCA).
323 Developmental delay/arrest in a particular HBB genotype would affect the time at which sexual
324 conversion can be measured *in vitro.* In NF54-*gexp02-Tom* parasites, grown in HbAA RBCs,
325 TdTom signal can be detected from ~10-15 hpi until ~96-101 hpi in the cycle after exposure
326 (Generation 1), and sexual conversion is usually measured at ~25-30 hpi (Generation 1) when
327 parasites are large rings/early trophozoites (52, 75). If an HBB mutant genotype causes a delay
328 in development, TdTom might not yet be expressed at ~25-30 hpi (Generation 1).
329 To determine whether parasite stage development of NF54-*gexp02-Tom* parasites differs
330 between RBCs with wild-type HbAA and mutant HBB genotypes (HbAC, HbAS, HbSS,
331 HbSC), we first quantified the percentage of rings, trophozoites and early/late schizonts in a
332 set of RBC samples with the different HBB genotypes (HbAA, $n = 1$; HbAC, $n = 1$; HbAS, n
333 $= 2$; HbSS, $n = 2$; HbSC, $n = 2$) using LM (Fig 4A). Parallel analysis of the proportion of late
334 stages (schizonts) in the same samples by flow cytometry (FC) (gating strategy in Fig 4C)
335 revealed consistent patterns between the two approaches. We therefore applied FC to the full
336 set of samples (HbAA, $n = 5$; HbAC, $n = 5$; HbAS, $n = 4$; HbSS, $n = 12$; HbSC, $n = 2$; and
337 fresh non-endemic HbAA CTRLs, $n = 6$, Fig 4B), enabling accurate detection of genotype-
338 specific developmental delays.
339 We found that parasites in HbAA RBCs progressed normally from Generation 0 to 1 with no
340 apparent delay. In HbAC RBCs, development was slightly delayed (~3 hours delay vs. HbAA

341 at Generation 0, ~35-40 hpi) but without arrest, whereas in HbAS RBCs a more pronounced
342 delay was observed (~10 hours delay) and partial developmental arrest in one sample (Fig 4
343 and Table S2). HbSS and HbSC RBCs presented a similar delay to HbAS (~ 9 and ~16 hours
344 delay, respectively) with a persistent population of schizonts, trophozoites, and late rings across
345 all timepoints (Fig 4A), consistent with the high frequency of partially arrested/delayed cultures
346 (67% in HbSS and 50% in HbSC) (Table S2). The (partial) arrest/extreme delay in these
347 cultures potentially makes them unsuitable for SCR measurement. Given the observed
348 developmental delays in all mutant genotypes, measuring sexual conversion at a single
349 timepoint (~25-30 hpi, Generation 1) may not be accurate. To capture all *gexp02-Tom-*
350 expressing sexual parasites (75), including those with delayed development, SCRs were
351 additionally quantified at ~35-40 hpi (Generation 1) (+ 20 hours post-expression of *gexp02*
352 should account for the 3-15 h delay measured). Detailed analysis is further described in
353 *Supporting information file in sub-section "Assessment of parasite development in RBCs with*
354 *different HBB genotypes".*

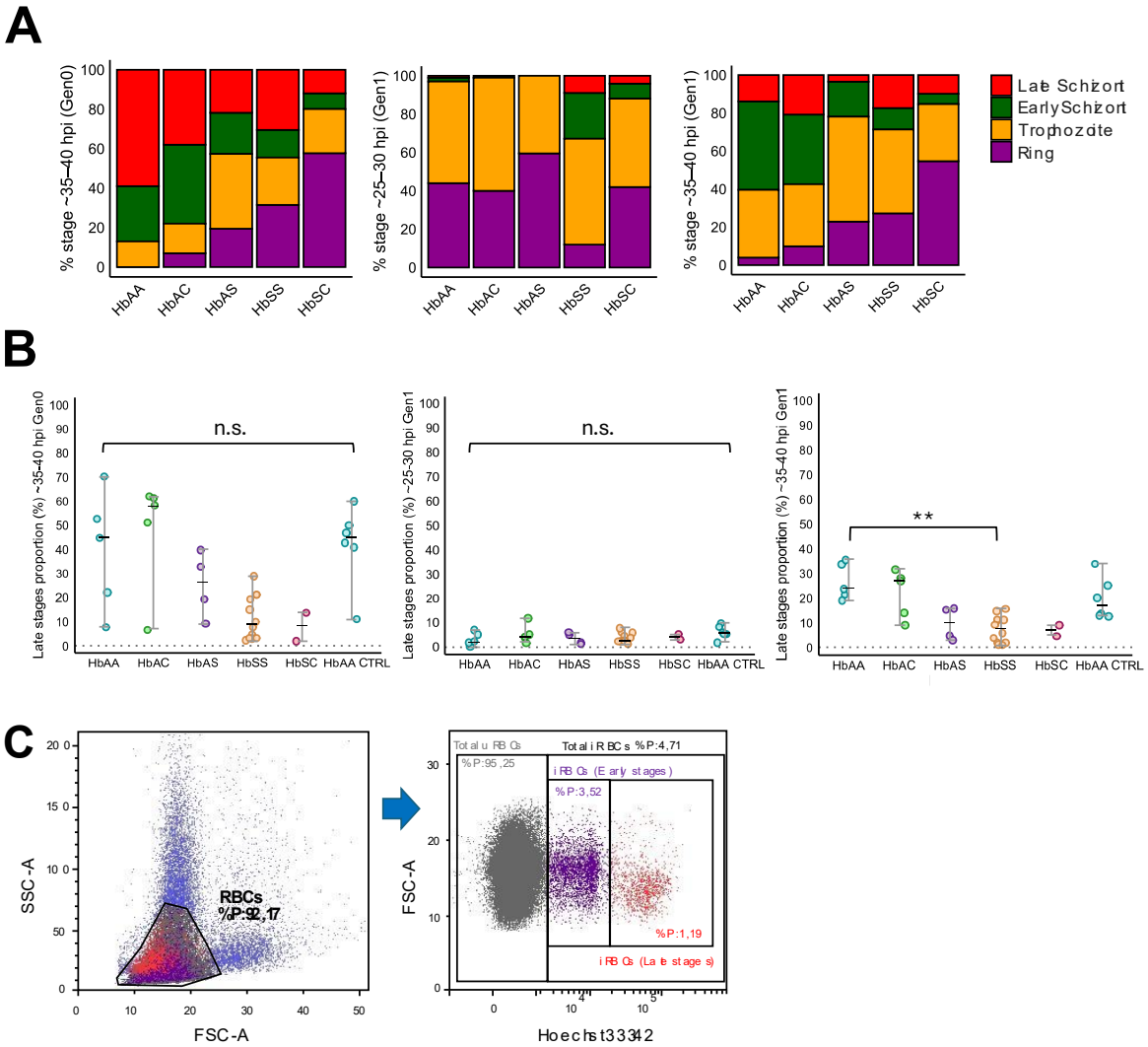

Fig 4. *P. falciparum* parasite development in red blood cells (RBCs) with different HBB genotypes. (A) Microscopy-based quantification of parasite stages expressed as percentages of a total of 100 counted parasites per sample. Y-axis: percentage of parasite stages (%); X-axis: HBB groups. Sample size 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). 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 [GLM] with Bonferroni 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).

374 **HBB genotypes are uniquely associated with enhanced SCRs**
375 **compared to other host factors**

376 To assess whether factors other than the mutant HBB genotypes influenced SCRs, we
377 performed a risk analysis of additional variables (*i.e.,* gender, village, ethnicity, age,
378 parasitemias, etc.) stratified by HBB genotype group, in the study population in the *ev*SCAs (n
379 = 32). This showed that only SCRs differ amongst the HBB genotypes HbAA, HbAC and
380 HbAS (Kruskal-Wallis test, overall $p < 0.001$***; Table S4).

381 For the same aim, we performed a risk analysis of factors in the study population in the *iv*SCAs
382 (n = 34) that may impact sexual conversion rates (SCRs) in addition to the HBB mutant
383 genotypes (Table 2). Our analysis indicated that HBB genotypes and gender are a risk factor
384 for enhanced SCRs (Fisher's exact test, overall $p < 0.01$** and $p < 0.05$*, respectively). The
385 percentage of reticulocytes was negatively associated with SCRs, with the lowest SCR and
386 highest percentage of reticulocytes in HbSS and HbSC RBCs (Spearman $\rho = -0.50$, $p <$
387 0.01**). The remaining factors did not show any impact on SCRs (Fisher's exact test and
388 Kruskal-Wallis test, $p > 0.05$).

389 We also explored which factors are potentially associated with HBB genotype (Table S5). We
390 noted that the percentage of reticulocytes is the highest in the HBB mutant genotypes HbSS
391 and HbSC, followed by HbAS, HbAC and HbAA (Kruskal-Wallis, overall $p <0.001$***).
392 Additional factors such as age, gender, village and ethnicity showed statistically significant
393 differences in their distribution across HBB genotype categories (Fisher's exact test, overall p
394 < 0.05), whereas blood groups did not (Fisher's exact test, $p > 0.05$).

395

Table 2. Risk Analysis of factors for elevated sexual conversion rates (SCRs) at 35-40 hpi (Generation 1), in the study participants within the *in vitro* sexual conversion assays (*n* = 34). Data from *P. falciparum* non-infected samples collected in the Nanoro Department, Burkina Faso and the UZA (Belgium). Statistical tests performed are shown at the bottom of the figure with correspondent *p*-values (with significant *p*-values as: $p < 0.05$*, $p < 0.01$**, $p < 0.001$***). Abbreviations: HBB, Hemoglobin beta; SCR, Sexual conversion rates; hpi, hours post-invasion; IQR, Interquartile range.

	SCR 35-40 hpi	***p*$**
***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

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

We analyzed IgG and IgM levels against *P. falciparum* in individuals with different HBB genotypes using flow cytometry, in selected endemic human plasma samples from the *InHost* longitudinal cohort study (see sub-section on *"Materials and methods, Study design"*). Detailed information on assay optimization and validation is provided in the *Supporting information file subsection IgG and IgM detection assays by flow cytometry).*

For robust ΔMFI values a cut-off of a minimum number of event counts per category was set to 200 events (Figs S4A and S4C). In each assay, positive controls (three acute *P. falciparum-infected* non-endemic plasma from a traveller at D0, D21 and D23) and a negative control ("naïve non-endemic plasma") were analyzed. The "naïve non-endemic plasma" sample consistently did not reach the 200 event counts threshold, confirming its suitability as a negative control. The negative control values served as a baseline for the levels of α-Troph-Gam-iRBCs IgG & IgM and were lower than those of the endemic plasma samples of all HBB genotypes (Fig 5). Endemic plasma samples were considered positive for IgG or IgM when both the event count and the levels of α-Troph-Gam-iRBCs IgG and IgM exceeded those observed in the "naïve non-endemic plasma". All endemic plasma samples exhibited event counts below the threshold of 200 for the levels of total α-Troph-Gam-iRBCs IgM (Hoechst33342+/PerCP-Cy5.5.+) and α-Troph-iRBCs IgM (Hoechst33342+/ tdTom-/PerCP-Cy5.5.+). The number of α-Gam-iRBCs IgG (Hoechst33342+/tdTom+/AF488+) and IgM (Hoechst33342+/tdTom+/PerCP-Cy5.5.+) was below 200 events for all endemic plasma samples ($n = 61$). Thirteen samples (21%) had no detected (*i.e.,* zero) events for α-Gam-iRBCs IgM. Based on these parameters, the MFI could only be evaluated for total and α-Troph-iRBCs IgG in the statistical analyses (Figs S5B and S5D).

448 Importantly, the events count in α-Troph-Gam-iRBCs and α-Troph-iRBCs IgM of the positive
449 controls at D21 and D23 exceeded both the event counts observed in the "naïve non-endemic
450 plasma" and the threshold of 200 events, confirming that our flow cytometry assay can detect
451 α-Troph-Gam-iRBCs IgM (Fig S5C). The time course of the "acute *P. falciparum*-infected
452 non-endemic plasma at D0, D21 and D23" generally followed the expected IgG (D0 < D21 <
453 D23) and IgM (D0 < D21 > D23) dynamics in α-Troph-Gam-iRBCs IgG & IgM after a *P.*
454 *falciparum* infection (80, 81).
455 We found no significant differences in the levels of α-Troph-Gam-iRBCs IgG & IgM (% of
456 cells/ΔMFI data are only considered robust for α-Troph-Gam-iRBCs IgG, reaching the
457 minimum threshold of 200 events; Fig 5) (Beta-binomial GLM, $p > 0.05$; Table S1). When
458 stratifying the Troph-Gam-iRBCs in asexual and sexual iRBCs, there was no significant
459 difference in the levels of α-asexual and α-sexual iRBCs IgG & IgM in endemic plasma from
460 individuals carrying different HBB genotypes in either subgroup (Fig 5) (Beta-binomial GLM,
461 $p > 0.05$; Table S1). We detect low levels or absence of α-Gam-iRBCs IgG & IgM, and low
462 levels or absence of IgM against Troph-Gam-iRBCs, Troph-iRBCs and Gam-iRBCs in the
463 plasma of the endemic individuals, as compared to high levels of IgG against Troph-Gam-
464 iRBCs and Troph-iRBCs (Figs 5 and S5).
465 The evaluation of variables in the study population ($n = 61$) such as: *P. falciparum* infection
466 status at the time of sample collection, parasitemia (parasites per µl), presence of gametocytes
467 in blood, age, gender and village; stratified by HBB genotype (HbAA [$n = 18$], HbAC [$n = 18$],
468 HbAS [$n = 18$], HbCC [$n = 2$], and HbSC [$n = 5$]) revealed no significant differences between
469 the categories (Fisher's exact test and Kruskal-Wallis test; $p > 0.05$) (Table S6). In our study
470 population, there is a homogeneous distribution of individuals across the categorical variables,
471 with no observed sampling bias that could affect further analysis.
472

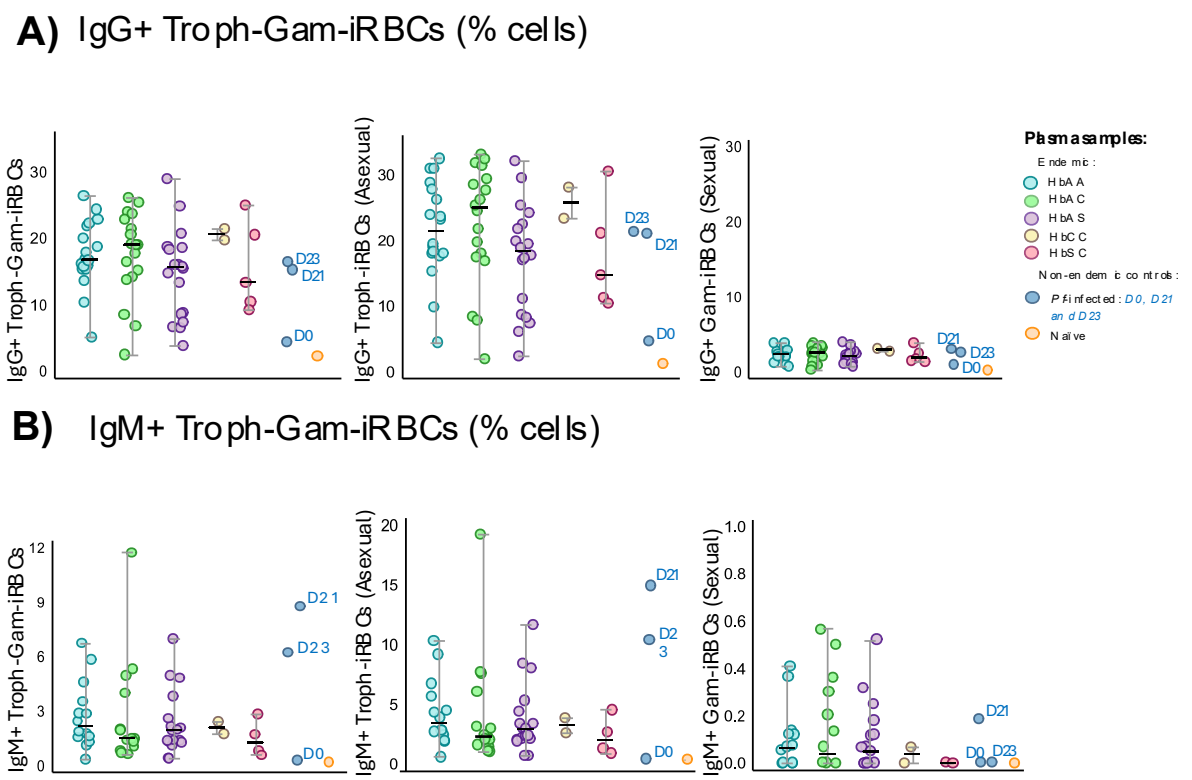

Fig 5. IgG and IgM mediated humoral immune response against *P. falciparum* in plasma samples from individuals with different human hemoglobin beta (HBB) genotypes. Data from *P. falciparum* infected (n = 44) and non-infected (n = 17) plasma samples collected at the Clinical Research Unit (CRUN) in Nanoro, Burkina Faso (total n = 61). **(A)** Percentage of α-Troph-Gam-iRBCs IgG: total (left), asexual (middle) and sexual (right). **(B)** Percentage α-Troph-Gam-iRBCs IgM: total (left), asexual (middle) sand sexual (right). Positive controls ("*Pf*-infected non-endemic" *i.e.,* three acute *Pf*-infected non-endemic plasma samples from a traveller at D0, D21, and D23) and a negative control *i.e.,* "Naïve non-endemic": naïve plasma from a non-endemic volunteer with HbAA genotype) are shown as individual dots per sample. The Y-axis range differs between graphs for visualization. No significant differences were found (Beta-binomial generalized linear models [GLM] with Bonferroni adjustment for pairwise comparisons, $p > 0.05$). **Abbreviations:** IgG and IgM, Immunoglobulin G and M; Troph-Gam-iRBCs, trophozoite- and gametocyte-infected red blood cells; *Pf, P. falciparum*. D: Day(s).

Further analysis of the levels of α-Troph-Gam-iRBCs IgG & IgM in the study population by "*P. falciparum* infection status", revealed that the levels of α-Troph-Gam-iRBCs IgG & IgM were higher in the *P. falciparum*-infected individuals (n = 44, median[IQR] for IgG: 17.90[6.30], and for IgM: 2.03[3.34]) compared to the non-infected individuals (n = 17, median[IQR] for IgG: 9.10[9.66], and for IgM: 1.56[1.27]) (Beta-binomial GLM, OR[95%CI] = 1.81 [1.35−2.43], p = 0.001** [for IgG]; and OR[95%CI] = 1.51 [1.01−2.23], p = 0.043* [for IgM]) (Fig S7). These results confirm the expected IgM dynamics, where IgM levels are

492 highest during acute or recent infections (*P. falciparum*-infected individuals) compared to past
493 infections (non-infected individuals) (80, 81).
494 Stratification analysis of the levels of α-Troph-Gam-iRBCs IgG & IgM by age-group (*i.e.,*
495 younger age: "1 to 4 years" compared to older age: "5 to 19 years") in *P. falciparum*-infected
496 compared to non-infected individuals, showed that both *P. falciparum*-infected and non-
497 infected individuals with younger age had lower levels of α-Troph-Gam-iRBCs IgG (*Pf*-
498 infected n = 22 and median[IQR]: 17.90[7.23]; non-infected n = 15 and median[IQR]:
499 9.30[10.80]) than those with an older age (*P. falciparum*-infected n = 22 and median
500 [IQR]:18.60[5.64]; non-infected n = 2 and median[IQR]: 12.60[3.69]) (Welch's ANOVA, p =
501 0.082 [IgG]; Kruskal-Wallis test p = 0.38 [IgM]). *P. falciparum*-infected individuals with
502 younger age had slightly higher levels of α-Troph-Gam-iRBCs IgM (n = 22, median [IQR]:
503 2.19[2.33]) than those with an older age (n = 22, median [IQR]: 1.87[3.04]) (Kruskal-Wallis
504 test, p = 0.94). In contrast, the levels of α-Troph-Gam-iRBCs IgM in non-infected individuals
505 with younger age were slightly lower (n = 15, median[IQR]: 1.56[1.12]) than those with an
506 older age (n = 2, median [IQR]: 2.18[0.00]) (Kruskal-Wallis test, p = 0.16).
507 Together these results (the positive controls exceeding the 200 events threshold for α-Troph-
508 Gam-iRBCs IgM and IgG, the time course dynamic of α-Troph-Gam-iRBCs IgM and IgG in
509 the acute *P. falciparum*-infected non-endemic plasmas), show that the flow cytometry assay
510 can accurately detect Troph-iRBCs IgM levels despite the low level or absence in endemic
511 individuals (Fig 5). The increase of IgG with age (*i.e.,* exposure in endemic individuals) and
512 decrease of IgM with age gives insight into the reason for the low α-Troph-Gam-iRBCs IgM
513 levels in the endemic plasma (exposure leads to a build-up of immunity that replaces low-
514 affinity IgM by an affinity-matured IgG response) (81-85).
515

516 # DISCUSSION

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

525 Amoah et al., (2020) (86) analyzed the effect of HBB genotype on SC of field isolates from
526 Ghanaian children aged 6-15 years with uncomplicated malaria, and reported a non-significant
527 trend of decreasing SCRs from HbAA to HbAC and HbAS. The discrepancy with our results
528 is most likely methodological, because quantifying SC in natural infections is challenging as
529 parasites in freshly collected blood include morphologically indistinguishable sexual and
530 asexual rings. The authors used prolonged culture (≥4 days) to distinguish sexual stages from
531 asexual forms, similarly to Usui et al., (2019) (41). The limitation of this approach is the timing
532 mismatch where SCRs are measured as the ratio of stage II gametocytes on day 4 (D4) by the
533 total parasitemia on day 0 (D0). This can underestimate SCR because parasites contributing to
534 the denominator (D0) are counted before culture adaptation affects viability, while the
535 numerator (D4) includes only gametocytes that survive and mature after several days in *ex vivo*
536 conditions, during which parasite loss can occur. Likewise, limitations have been reported
537 when quantifying low SCRs (< 0.3%) in low-density natural infections by LM (41). For
538 instance, at 0.05% parasitemia and 0.3% SCRs, they would be detecting five gametocytes
539 among 30,000 RBCs. This approach provides limited sensitivity and resolution and could
540 explain why up to 24% of samples were reported as gametocyte negative.

541 We addressed these limitations using an adapted *ev*SCA to quantify next cycle sexual
542 conversion (39). We measure whether in-host exposure in individuals with RBCs with different
543 HBB genotypes leads to stimulation of sexual commitment. We quantify the in-host effect by
544 assessing the subsequent sexual conversion (next cycle, *ex vivo*) as number of stage I
545 gametocytes (gamI) per number of schizonts and gamI (overall parasites) 24 hours post culture
546 (hpc). Quantification is done by immunofluorescence, gamI detected by Pfs16 and overall
547 parasites by DAPI (87). Inclusion of PKG inhibitor ML10 prevented reinvasion, allowing
548 numerator and denominator measurements for the SCR at the same time point, while
549 enrichment using LD columns improved sensitivity. This approach reliably quantified sexual
550 conversion in infections with densities as low as 0.05% (~2,500 parasites/µl) and detected a
551 minimum of 38 stage I gametocytes per sample, with no gametocyte-negative cultures.

552 We validated *ex vivo* findings using an *iv*SCA with a *gexp02-reporter* line (75), allowing early
553 detection of sexual rings. While our SCR results in *iv*SCA were broadly consistent with those
554 of Flage, B., et al., (2023) (25) for HbAA and HbAS RBCs (median SCRs: 10.8% vs. 10% in
555 HbAA; 16.5% vs. 15% in HbAS), the results are not directly comparable due to clear
556 methodological differences in how SCR was measured. In our assay, SCR is defined and
557 measured as the proportion of *gexp02*-expressing parasites among total parasites at the next
558 cycle conversion (generation 1), while sexual parasites were quantified at generation 1 and total
559 parasitemia at generation 0 in (25), making direct comparison impossible.

560 We observed a partial developmental delay in parasites infecting HbAS and HbAC RBCs, and
561 a more pronounced arrest or extreme delay in HbSS and HbSC under low-oxygen conditions
562 (5% O_2), both by LM and FC, consistent with the findings of Archer, N.M., et al., (2018) (29).
563 Later time-point measurements (~35-40 hpi) increased SCRs in endemic HbAA, HbAC, and
564 HbAS RBCs compared with ~25-30 hpi, suggesting delayed sexual marker expression and thus
565 underestimation of SCR at the earlier time point due to developmental delay. Of note, parasites

566 grown in control non-endemic HbAA RBCs showed stable SCRs across both time points. The
567 observed differences between endemic and non-endemic HbAA RBCs may reflect unmeasured
568 host genetic factors (*e.g.,* blood group variants or other erythrocytic polymorphisms). In HbSS
569 and HbSC RBCs, we observe SCRs similar to HbAA and lower than HbAS and HbAC,
570 consistently across both measurement time points. This contrasts with findings in (25), who
571 reported markedly higher SCR in HbSS RBCs (35% vs. 6.96% in our study) and no
572 developmental delay at day 2 of culture, although this was not quantitatively assessed. As noted
573 above, differences in the timing and approach used to quantify SCR make the results not
574 comparable between studies.
575 Under choline-depleted conditions, SCRs in HbSS and HbSC increased modestly (from <10%
576 to 10-20%) but remained below levels in HbAA, HbAC, and HbAS RBCs (>30-40%). This is
577 likely due to severe developmental delay causing under-detection of sexually committed rings
578 at the measured time points rather than inhibited sexual commitment. Overall, our data
579 indicates that accurately assessing SC in HbSS and HbSC RBCs is challenging with this
580 approach due to developmental delay. Measuring sexual commitment one cycle earlier, for
581 instance through *GDV1* activation, might circumvent this issue, although this is experimentally
582 demanding because *GDV1* is expressed transiently and at low levels. Given the very low
583 prevalence of HbSS and HbSC genotypes in malaria-endemic populations, the added value of
584 such complex assays for transmission studies is likely limited.

585 We evaluated potential confounding factors that were previously to SC (52, 58, 59, 88-92),
586 including reticulocyte abundance, parasitemia, and ABO blood group. No significant
587 differences in reticulocyte percentages were observed among HbAA, HbAC, and HbAS RBCs,
588 and reticulocyte levels did not correlate with SCRs at 35-40 hpi. Similarly, neither baseline
589 parasitemia nor ABO blood group influenced SCR in either the *ev*SCA or *iv*SCA. Together,

590 these results indicate that the observed increase in SC is specifically associated with HBB
591 genotype rather than these other host-related variables.

592 The increase in SC in RBCs with HBB mutant genotypes *in vitro*, in the absence of plasma and
593 white blood cells, points to RBC-intrinsic effects rather than host immune responses or soluble
594 plasma factors. HbS and HbC RBCs exhibit elevated levels of free heme (93), linked to
595 increased oxidative stress that leads to polymerization of HbS under low oxygen conditions
596 (94), and delays parasite development (29). The oxidative stress inhibits actin polymerization
597 in infected HbS and HbC RBCs (27), which alters protein trafficking and PfEMP1 display (66,
598 69). This reduces cytoadherence (28), and membrane flexibility (95). Oxidative stress also
599 modifies RBC micro RNAs that can hybridize with parasite mRNAs (65), directly affecting
600 parasite development and metabolism, and increasing gametogenesis (25). In (25), authors
601 showed that heme directly induces SC. Taken together, these findings suggest that the effect
602 of HbAC and HbAS RBCs on SC *in vitro* is likely driven by oxidative stress caused by free
603 heme within these RBCs, mediated by their specific miRNA profiles. The stronger effect *ex*
604 *vivo*, particularly in HbAS (7.07- vs ~2.05- fold *in vitro*), probably reflects additional *in vivo*
605 factors such as parasite genetic diversity (41, 87), or host factors not captured *in vitro* (87, 96).

606 To further investigate potential *in vivo* influences, we investigated the humoral immune
607 response in the different HBB genotypes. Reduced antigen presentation, cytoadherence, and
608 increased splenic clearance (28) could plausibly lead to an altered immune response in HbAC
609 and HbAS. However, epidemiological studies examining immunity in individuals with HbAS
610 and HbAC genotypes have yielded conflicting results. Older children with HbAS have been
611 shown to be more protected against the establishment of parasitemia, suggesting an acquired
612 mechanism of protection (67). In contrast, in (97) no differences were found in antibody
613 responses between individuals with HbAA, HbAS, and HbAC genotypes against their panel of
614 491 immunoreactive *P. falciparum* antigens. More recent studies have shown that serological

615 responses tend to be lower in individuals with HbAC and HbAS when focusing on specific
616 surface antigens (98, 99). In a cross-sectional study conducted in Ghana about circulating
617 inflammatory cytokines, has shown that expression levels of CXCL10, TNF-α, CCL2, IL-8,
618 and IL-6 varied by hemoglobin genotype (100).
619 Given that differences in serological responses appear specific to certain surface antigens, we
620 chose to measure IgG and IgM levels against intact infected RBCs. This approach enables the
621 assessment of immune responses to native surface antigens, minimizing potential artifacts
622 arising from non-native protein folding, glycosylation, or conformation. We focused on
623 trophozoite-infected RBCs (25-30 hpi), as surface antigen presentation (*i.e.,* PfEMP1
624 expression) begins around 16 hpi and plateaus at approximately 24 hpi (101). Moreover,
625 commitment to SC occurs between 17-37 hpi (39, 52), suggesting that any antibody-mediated
626 induction of sexual commitment would need to occur at this stage.
627 We compared IgG and IgM responses to trophozoite- and gametocyte-infected RBCs (Troph-
628 Gam-iRBCs) using plasma samples from Burkinabé individuals carrying HbAA, HbAC,
629 HbAS, HbCC, and HbSC genotypes, assessed by flow cytometry. Our analyses revealed no
630 significant differences in IgG or IgM levels against Troph-Gam-iRBCs across genotypes. IgM
631 responses, as well as responses against gametocyte-infected RBCs, were generally low or
632 absent. Therefore, we could not confirm genotype-dependent differences in humoral responses
633 to these parasite stages and consider it unlikely that antibodies directly drive the differential
634 SC observed in these individuals. Nonetheless, this does not exclude the possibility that
635 genotype-specific antibody responses to later parasite stages, particularly during
636 gametocytogenesis, as suggested by recent studies on gametocyte antigenicity (102, 103),
637 could influence transmission potential, either by promoting clearance of developing
638 gametocytes in the human host or by inducing gametocyte sterility within the mosquito vector
639 (104).

640 Overall, this study robustly demonstrates elevated sexual conversion of *P. falciparum* parasites
641 in HbAS and HbAC RBCs both *in vitro* and *ex vivo*. These findings further support the view
642 that *P. falciparum* can adjust its reproductive investment in response to stress-related cues. In
643 mutant HBB RBCs, elevated levels of free heme can induce developmental delay and thereby
644 compromise survival within the human host; increased investment in sexual conversion may
645 thus represent a parasite survival strategy (25, 29, 63).

646 Previous studies have shown that microenvironmental factors such as LysoPC depletion,
647 nutrient limitation and exposure to antimalarial drugs can modulate malaria transmission by
648 changing the rate of parasite's SC. How factors such as free heme, are being translated into a
649 signal received by epigenetic regulators controlling the sexual commitment gene *ap2-g* or *gdv1*
650 remains an open question (25, 39).
651 At the population level, HBB mutant genotypes have been linked to increased gametocyte
652 carriage and higher mosquito infectivity. In Burkina Faso, it has been reported a two- to four-
653 fold higher infection rates in HbAS, HbAC, and HbCC carriers (30). Similar trends were
654 observed in Mali and Cameroon, where HbAC and HbAS individuals carried more
655 gametocytes and were more infectious to mosquitoes than HbAA (33, 34). These population-
656 level associations are consistent with our findings, and suggest that increased SC in mutant
657 HBB RBCs may contribute to enhanced transmission potential.

658 MATERIALS AND METHODS

659 Details about reagents and tools are accessible in Table S7.

660 Study design

661 Sample collection for the present study was embedded in a longitudinal cohort study assessing
662 the effect of host factors on transmission potential (*InHost* study, ethical advice reference
663 1261/18 and 19/06/064), conducted in the Nanoro Department, a rural, malaria-hyperendemic

region in Central-West Burkina Faso (Fig 6) (105) [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-up 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.

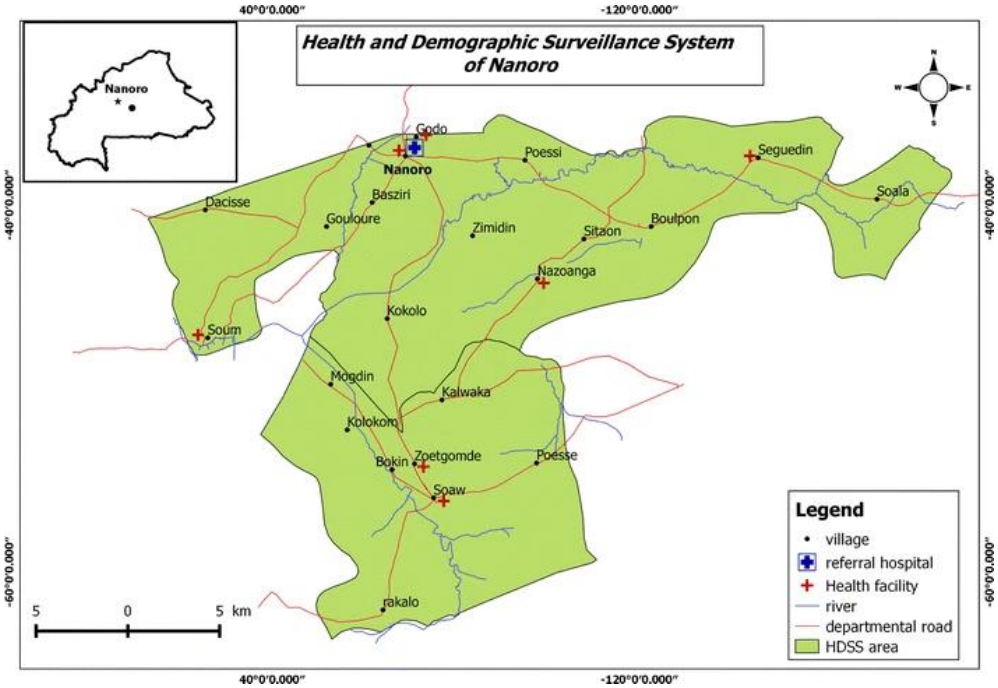

Fig 6. Map of the study area. The inset map highlights the location of the Nanoro department of Boulkiemdé Province in Central Burkina Faso, with the black dot indicating the position of the capital, Ouagadougou.

For this study, from all participants of the *InHost* study (n = 871), 864 individuals were genotyped for their human HBB genotype (see section on "Hemoglobin beta sequencing"). Participants were then selected for this sub-cohort based on their genotype. A venous blood sample was collected from all selected participants, and screened for *P. falciparum* infection. Samples from *P. falciparum*-infected individuals were used to perform *ex vivo* sexual conversion assays (*ev*SCAs) and samples from non-infected individuals were preserved to

682 perform *in vitro* sexual conversion assays (*iv*SCAs). We included all individuals carrying the
683 HBB genotypes HbAC (n = 72), HbAS (n = 50), HbCC (n = 2), HbSC (n = 7), and a random
684 subset of individuals with the HbAA genotype (n = 94) (Fig S6). In total, 225 individuals were
685 listed (age groups "5 - 19 years" [n = 166] and "more than 20 years" [n = 59]). Listed
686 individuals were visited by a nurse at home and were invited to the Clinical Research Unit of
687 Nanoro (CRUN, Burkina Faso) for screening and enrolment into the study. Population
688 screening for *P. falciparum* infections and enrolment took place over a two-month period
689 (August-September 2022).
690 At CRUN, the eligibility was assessed based on inclusion and exclusion criteria for the *ev*SCAs
691 (*P. falciparum*-infected individuals) and *iv*SCAs (non-infected individuals). Inclusion criteria
692 consisted of: being enrolled in the main *InHost* study, a confirmed *P. falciparum* malaria
693 infection (for the *ev*SCAs) or a confirmed negative *P. falciparum* malaria infection (for the
694 *iv*SCAs), willing to participate in this study and undergo additional sampling, and signed
695 informed consent. Exclusion criteria consisted of: mixed infection with other *Plasmodium*
696 species (only for *P. falciparum*-infected individuals in the *ev*SCAs), antimalarial treatment
697 within the last two weeks, signs of severe malaria (*e.g.* prostration), severe chronic illness
698 besides hemoglobinopathies (*e.g.,* cancer, heart disease, kidney or liver disorders, HIV/AIDS,
699 etc.), and planning to leave the study region during the study period. Of the 225 listed
700 individuals, 162 were screened at CRUN, of whom 161 participants were selected as eligible
701 and were included within this sub-cohort (Fig S6). A 15 ml venous blood sample was collected
702 from each participant. After sample collection, blood samples were screened for *P. falciparum*
703 infections (see section on "*P. falciparum* diagnosis"). Samples with confirmed *P. falciparum*
704 infection and parasitemia by light microscopy (LM) ≥ 0.05% (*i.e.* ≥ 2500 parasites/µl) were
705 processed for *ev*SCAs at CRUN (Table 3). Blood samples negative for *P. falciparum* infection
706 were processed and preserved for *iv*SCAs conducted at the Institute of Tropical Medicine

707 (ITM), Antwerp (Table 3, see section on "*iv*SCAs"). *P. falciparum*-infected individuals, as
708 detected by LM and confirmed with *P. falciparum var* gene acidic terminal sequence (*var*ATS)
709 quantitative PCR (qPCR), were treated with Artemether-Lumefantrine (AL) following national
710 guidelines and were asked to return three days later to confirm infection clearance.
711 Sample size was calculated based on a power analysis assuming an expected effect size of 50%,
712 a 5% significance level, and 90% power. This sample size was considered sufficient to detect
713 differences in sexual conversion by HBB genotype, based on effect sizes reported in previous
714 studies (58). To account for potential variability in HBB genotype groups, a minimum of 8
715 participants per HBB genotype group was required. To ensure sufficient sampling and
716 successful assays per HBB genotype group, the sample size was upscaled to a maximum of 15
717 participants per HBB genotype group.
718 To increase the number of HbSC and HbSS blood samples for *iv*SCA, additional blood samples
719 were collected from patients with hemoglobinopathies (HbSS and HbSC) visited at the
720 University Hospital of Antwerp (UZA), Belgium. A total of 13 adults (aged 19-37 years),
721 routinely followed at UZA's Hematology Unit, were invited to donate a blood sample with
722 exclusion criteria of fever. The inclusion period was between October 2023 and April 2024.

723 **Table 3: Number of samples (n) per *P. falciparum* infection status at the time of sample collection and HBB**
724 **genetics of the donor, for the *ex vivo* sexual conversion assays (*ev*SCAs), *in vitro* sexual conversion assays**
725 **(*iv*SCAs) and Immunoglobulin (Ig) detection assays.** Note: *P.falciparum-infected (Pf+)* samples used in the
726 *ev*SCAs must have a parasitemia ≥ 0.05% (*i.e.,* ≥ 2500 parasites/µl), as the established limit of accurate assessment
727 of sexual conversion with these assays. Abbreviations: *Pf, P. falciparum*, Hb, hemoglobin; CRUN, Clinical
728 Research Unit in Nanoro; ITM, Institute of Tropical Medicine in Antwerp; UZA, University Hospital in Antwerp.
729

	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

730 ## *Plasmodium falciparum* diagnosis

731 *P. falciparum* malaria diagnosis was done by RDT using the Bioline™ malaria antigen P.f®
732 test (05FK50, Abbott) (recommended by the National malaria Control Programme (NMCP) in
733 Burkina Faso), following the manufacturer instructions. In addition, malaria parasites were
734 detected and quantified by LM following standard protocols at CRUN (106). In brief, venous
735 blood smears were stained with Giemsa and examined until 200 white blood cells were
736 counted. Parasitemia was calculated based on the number of asexual parasites per white blood
737 cells, expressed as asexual parasites per microliter of blood, assuming a white blood cell count
738 of 8,000 per microliter. The presence of gametocytes and other *Plasmodium* species (*P. ovale*
739 *and P. malariae*) was assessed in all positive blood smears (107). Blood samples that tested
740 positive for *P. falciparum* by both RDT and LM were classified as *P. falciparum*-infected.
741 Samples that were negative by LM (regardless of the RDT result), were identified as non-
742 infected (Table 3 and Fig S6). Negative infection with *P. falciparum* was confirmed by *var*ATS
743 qPCR, using five µl of DNA (extracted with QIAamp DNA Mini Kit, Qiagen Germany), as
744 previously described (105, 108) (Fig S6). The limit of detection of the *var*ATS qPCR was 0.1
745 parasite/µl (in our hands), with samples showing Ct > 39.7 considered negative. DNA from the
746 *P. falciparum* 3D7 strain served as a positive control, while negative controls included DNA
747 from non-infected human blood and a no-template control (NTC).

748 ## Sample collection and processing

749 At CRUN, a 15 ml venous blood sample was collected in an Acid Citrate Dextrose (ACD) tube
750 and the residual blood in the needle was used for *P. falciparum* diagnosis by RDT and LM (see
751 section on "*P. falciparum* diagnosis"). The sample was then immediately transported to the
752 laboratory for processing. Samples were centrifuged (1,648 g for 5 minutes) and washed two
753 times with RPMI to isolate the RBC pellet. RBC pellets from *P. falciparum*-infected blood
754 samples were immediately put in culture for the *ev*SCAs (see section on "*Ex vivo* sexual

755 conversion assays (*ev*SCAs)". RBC pellets from non-infected samples were cryopreserved (1:3
756 dilution in cryopreservation solution containing 3% sorbitol and 0.65% NaCl) and stored
757 directly at -80°C until shipment to ITM, where preserved in liquid nitrogen until use in *iv*SCAs.
758 Two hundred microliters of whole blood were used for DNA extraction and confirmation of *P.*
759 *falciparum* infection by *var*ATS qPCR (see section on "*P. falciparum* diagnosis). At UZA, 15-
760 20 ml of non-infected venous blood samples were collected in EDTA tubes and immediately
761 transported at 4°C to ITM for processing, done as described at CRUN. The resulting RBC
762 pellets were cryopreserved (1:3 dilution in cryopreservation solution containing 3% sorbitol
763 and 0.65% NaCl) by direct snap-freezing in liquid nitrogen for subsequent *iv*SCAs.
764 Additionally, ABO and Rhesus D blood groups were determined with agglutination tests (BIO-
765 RAD, REF: 801320, 801345, 802032).

766 Hemoglobin beta sequencing (HBBseq)

767 In samples collected at CRUN, PCR amplification of the HBB gene (NCBI_Gene:3043) was
768 performed to identify single nucleotide polymorphisms (SNPs) using PCR conditions adapted
769 from (109) and (110). Primer pairs were designed to amplify a 599 bp product (Table S7). PCR
770 reactions were performed in a total volume of 25 µl, containing 2.5 µl of DNA template, 10×
771 Buffer B, 5 mM of each dNTP, 0.3 µM of each primer, 25 mM of $MgCl_2$, and FIREPol® DNA
772 Polymerase (Solis Biodyne, Ref: 01-01-00500). Thermal cycling conditions were as follows:
773 an initial denaturation at 94°C for 6 minutes, followed by 40 cycles of 60.6°C for 30 seconds,
774 and 72°C for 1 minute, with a final extension at 72°C for 10 seconds. After Sanger sequencing,
775 chromatograms were analysed using BioEdit 7.2. (Informer Technologies) to identify the wild-
776 type and the mutant HBB codons (Table 4). In samples collected at UZA, the HBB genotype
777 sequences were determined by capillary hemoglobin electrophoresis at UZA as described
778 elsewhere (111).
779

780 **Table 4. 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 (112)

781

782 # *Ex vivo* sexual conversion assays (*ev*SCAs)

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

784 *P. falciparum*-infected venous blood samples (15 ml) collected from individuals carrying
785 different HBB genotypes (Table 3; HbAA n = 14, HbAC n = 12, HbAS n = 6; total n = 32)
786 were cultured *ex vivo* to measure sexual conversion rates (SCRs), assay optimization and
787 validation described in *Supporting information file*. Cultures at 3% Hct and 20 ml volume were
788 prepared by mixing 600 µl of *P. falciparum*-infected RBC pellet with 20 ml of RPMI 1640
789 medium containing 0.5% Albumax II (Gibco, 11021-029) and 2 mM choline chloride (Sigma-
790 Aldrich, C7527) (52, 75). Parasites at the ring stage were incubated using candle jar method
791 under static conditions until ~24 hours post-culture (hpc). At this time, sexually committed
792 rings present in the venous blood sample are expected to have developed into stage I
793 gametocytes expressing Pfs16, while asexual parasites will have progressed to the schizont
794 stage (Fig 2A). During culture, at ~5 hpc, ML10 (80 nM), a cGMP-dependent protein kinase
795 inhibitor, was added to prevent schizont rupture and reinvasion (39, 113). Giemsa-stained
796 smears were examined by LM to confirm that most parasites had reached either stage I
797 gametocytes or schizonts stages. At ~24 hpc, parasites were harvested and purified using
798 MACS LD columns (Miltenyi Biotec). In brief, cultured parasites are centrifuged (1,648 g, 5
799 min) and pelleted infected-RBCs (iRBCs) were diluted 1:4 with culture medium, loaded onto

800 an LD-MACS column, and washed four times with 1 ml medium to separate non-infected
801 RBCs and ring-stage-infected RBCs (first elution). The column was then removed from the
802 magnet, and stage I gametocytes and schizonts were recovered by gravity elution, followed by
803 gentle plunger flushing twice with 3 ml medium (second elution). Recovered parasites were
804 pelleted (1,648 g, 5 min), resuspended 1:2 with non-infected RBCs, and diluted 1:2 in PBS.
805 From the second elution, four thin blood smears (3 µl each) were prepared and left to air-dry;
806 three were used for on-site IFAs, and one was Giemsa-stained for LM to verify MACS
807 enrichment.

808 **Immunofluorescence assays (IFAs)**

809 IFAs were performed at CRUN (Burkina Faso) as previously described (39, 52, 75), with
810 modifications. Slides were stained in batches of four to eight cultures with three slides per
811 culture, to optimize processing and staining consistency. Primary antibody mouse anti-Pfs16
812 (1:400, kindly provided by R. Sauerwein and T. Bousema, Radboud University) (76) and
813 secondary antibody goat anti-mouse IgG (H+L) Alexa Fluor 488 (1:1000; Abcam, ab150113)
814 were used, with incubations and washes under static conditions. Slides were mounted with
815 Aqueous Fluoroshield (Abcam, ab104139) containing DAPI (0.0002%) and stored at 4°C until
816 shipment to ITM (Belgium). At ITM, slides were visualized within six months of preparation
817 on an ECHO Revolve epifluorescence microscope (ECHO, San Diego, CA) to determine SCRs
818 (Fig 2F). Analysis was blinded to HBB genotype. Stage I gametocytes were identified as Pfs16-
819 positive cells (green) and counted among a minimum of 1,000 DAPI-positive cells (blue) per
820 slide (stage I gametocytes and schizonts). Slides with fewer than 1,000 DAPI-positive cells
821 were excluded from further analysis during the first reading. For each culture (n = 35), three
822 of the four prepared slides were stained by IFA and examined by a primary reader. Slides from
823 three cultures were excluded due to insufficient DAPI-positive cells. From the remaining
824 cultures (n = 32) a random subset of ~10% (4/32), with three IFA slides per culture, was

825 examined by a second reader to assess inter-reader reliability via intraclass correlation
826 coefficient (ICC) analysis, based on a two-way random effects model, absolute agreement,
827 single measures [ICC(2,1) (114)]. For each IFA slide, SCRs were calculated as the proportion
828 of Pfs16-positive parasites among total DAPI-positive cells. Across the three replicate IFA
829 slides per culture, the mean SCR (±SD) was computed and used to calculate and report the
830 median SCR [IQR] by HBB genotype (Figs 4A and 4B), statistical differences were assess as
831 described for proportions in "Data analysis" section. Fold-change in SCR for HbAC and HbAS
832 was calculated relative to HbAA by dividing each mutant genotype's median SCR by that of
833 the wild-type (Fig 2C). Statistical analysis was performed as described in the "Data analysis"
834 section: Differences in initial parasitemia across HBB genotypes (continuous non-parametric
835 variables) (Figs 2B-D) and potential confounding effect of initial parasitemia on SCRs (non-
836 parametric correlation across continuous data) (Fig S1A).
837 Stable Pfs16 fluorescence was confirmed by the counting of ≥1 stage I gametocyte per 10
838 fields. For each culture, the three replicate IFA slides were analyzed to quantify SCRs. The
839 mean, minimum and maximum SCR were calculated across replicates. To assess inter-slide
840 reproducibility (technical repeatability) of SCR measurements, an ICC (2,1) analysis (two-way
841 random effects, single measures, consistency) was performed using SCR values from the three
842 replicate slides per culture (n = 32). Counts of stage I gametocytes and total parasites were
843 recorded for each IFA slide and summarized as median (Q1-Q3). From 5 out of 32 cultures
844 (15.6%) specificity controls were performed by staining one slide per culture without primary
845 antibody (negative controls). Stage V gametocytes (also express Pfs16), identified by their
846 shape, were excluded from the counting as they are a product of sexual conversion occurring
847 >10 days prior to the sexual commitment cycle under investigation. Giemsa-stained slides from
848 each *ev*SCA were examined by LM to estimate parasite purification by MACS and confirm

849 approximate parasite age/stage to assure parasites were sufficiently mature to express Pfs16
850 (starts expression at ~20 hpi (39)).

851 *In vitro* sexual conversion assays (*iv*SCAs)

852 *In vitro* sexual conversion assays (*iv*SCAs) were performed as follows (Fig 3A). NF54-*gexp02-*
853 *Tom* parasites (52, 75) were routinely maintained with parasitemias of less than 2 % in O+
854 RBCs (3% Hct) in RPMI 1640-based medium supplemented with 0.5% Albumax II (Gibco,
855 ref: 11021-029) and 2 mM choline chloride (+Choline; Sigma-Aldrich, C7527), analogous to
856 LysoPC supplementation (55, 75, 79). Cultures were incubated at 37°C with shaking conditions
857 (70 rpm) in a gas mixture (5% O_2, 5.5% CO_2, balance N_2). To minimize bias due to prior culture
858 duration, all cultures were initiated from parasites of the same cryopreserved batch of an
859 expanded culture.
860 To obtain cultures of a well-defined age window, we used Percoll/sorbitol synchronization (Fig
861 3A). Percoll-purified schizonts (~40-44 hpi, Generation -1) were used to establish fresh
862 cultures with thawed cryopreserved RBCs (day 3 post thawing) with different HBB genotypes
863 (exposure, Generation 0), now cultured in static conditions. Thawing of RBCs was performed
864 using a three-step protocol, sequentially adding three volumes of 3.5% NaCl solution (10× the
865 RBC volume each time), followed by RPMI washes and centrifugation (1,648 g, 5 min) as per
866 standard procedure (115). Approximately 5 hours later, when parasites have reinvaded the new
867 RBCs, cultures were subjected to 5% D-sorbitol lysis (39, 75) to obtain cultures of a defined
868 0-5 hpi age window. Post-sorbitol initial parasitemia was then measured by flow cytometry,
869 and parasites were cultured in two parallel subcultures (~200 µl final volume) per sample. One
870 sub-culture was maintained in +Choline medium (non-inducing sexual conversion conditions),
871 and the other in choline-free medium (-Choline; inducing conditions). Cultures were
872 maintained under these respective conditions for two generations (48 hpi, until ~35-40 hpi in
873 Generation 1, Fig 3A, hpi calculations are based on a 44 hours replication cycle empirically

874 established for this parasite line in our lab). In each experiment we tested RBCs with at least
875 two different HBB genotypes. Samples with different HBB genotypes were used in the
876 experiments if the recovered RBC pellet volume after thawing was ≥ 30 µl after dilution with
877 RPMI to 50% Hct. From a total of 20 cryopreserved non-infected RBC samples from CRUN
878 that were confirmed to be negative for *P. falciparum* by *var*ATS qPCR, 15 samples had a RBC
879 pellet volume ≥ 30 µl after thawing and dilution in RPMI to 50% Hct (Fig S6). The mean
880 recovered RBC pellet volumes after thawing were: HbAA, 178 µl (range: 50−596 µl); HbAC,
881 111 µl (range: 30−320 µl); HbAS, 246 µl (range: 64−630 µl); HbSC, 558 µl (range: 35−1080
882 µl); and HbSS, 600 µl (range: 300−1120 µl). These samples were included within the *iv*SCAs,
883 in addition to non-infected, cryopreserved RBC samples from the UZA HbSS and HbSC (Fig
884 S6). Total numbers of samples tested per HBB genotype and experiment are shown in Table 1.
885 Flow cytometry (FC) analysis was used to measure parasitemia, assess parasite stage
886 development (*i.e.,* proportion of parasites reaching the intraerythrocytic schizont stage or late-
887 stage proportions), and quantify sexual conversion at defined time points during the assay (Fig
888 3A). Parasitemia was determined at ~0-5 hpi (rings), together with (1) the proportion of
889 schizont stages at ~35-40 hpi in Generation 0 and 1, and (2) sexual conversion rates (SCRs) at
890 ~25-30 hpi (large rings/trophozoites) and ~35-40 hpi (schizonts) in Generation 1 (Fig 3A). The
891 standard timepoint to measure sexual conversion with this parasite line is ~25-30 hpi to ensure
892 detection of all sexually committed parasites, as TdTom-expression under the *gexp02* promoter
893 starts around ~10-15 hpi (75). The ~35-40 hpi timepoint was included at Generation 1 to
894 account for potential delayed stage development in mutant HBB genotypes (see section on
895 "Assessment of parasite stage development in RBCs with different HBB genotypes"). Four
896 independent experiments were conducted, with RBC samples included in each experiment
897 detailed in Table 1. Each experiment included a fresh HbAA blood sample to control the effect
898 of RBC storage after cryopreservation.

899 Samples for FC analysis (using a CytoFLEX LX flow cytometer, Beckman Coulter) were
900 prepared by resuspending parasite cultures (1:3) in PBS containing 1% BSA, followed by
901 staining with Hoechst 33342 (1:500, BD Pharmingen) in a final volume of 100 µl per well.
902 Samples were then incubated at 37°C for 30 minutes. To measure parasitemia and proportion
903 of schizont stages, FC analysis (with 100,000 events collected per sample) was set to detect
904 Hoechst 33342 (350 nm laser; 450/40 filter). RBCs were gated by plotting side scatter area
905 (SSC-A) versus forward scatter area (FSC-A), with singlets selected using FSC-H versus FSC-
906 A. Parasites were identified within the singlet gate by Hoechst fluorescence. Hoechst-positive
907 events were defined as exceeding background levels from unstained negative controls. To
908 quantify SCRs, Hoechst fluorescence was combined with the fluorescent reporter TdTom (561
909 nm laser; 610/20 filter) that is integrated in the NF54-*gexp02-Tom* line to distinguish sexual
910 (Hoechst+/tdTom+) from asexual (Hoechst+/tdTom-) parasites. SCRs were calculated as the
911 proportion of Hoechst+/tdTom+ events among all Hoechst+ events.
912 SCRs were expressed as a percentage (median [IQR]) per HBB genotype (Figs 3B and 2A-C).
913 Fold-changes in SCRs were calculated per experiment (Table 1) by dividing the SCR of each
914 cryopreserved mutant RBC sample by the SCR of a fresh, non-endemic HbAA RBC control
915 sample. (Figs 3C and S3D). Statistical analysis was performed as described in the ”Data
916 analysis” section: differences in SCRs and post-sorbitol initial parasitemia across HBB
917 genotypes tested as described for proportions, differences in fold-change as described for
918 continuous data across categories (Figs 3B-D and S3A-E).
919 Reticulocyte percentages were measured for all RBC samples on the same day as Percoll
920 synchronization (Generation -1, Fig 3A). Briefly, RBC suspensions were mixed 1:1 with
921 reticulocyte stain (Sigma-Aldrich), incubated 15 min at room temperature, and used to prepare
922 a thin blood smear for LM estimation of reticulocyte percentage. We determined the ABO and

923 Rhesus D blood groups in all samples using agglutination tests with Seraclone™ Anti-A, Anti-
924 B, and Anti-D (RH1) reagents (Bio-Rad).

925 Assessment of parasite stage development in RBCs with different 926 HBB genotypes

927 Parasite stage development was evaluated during the *iv*SCAs (Fig 3A) at ~35-40 hpi
928 (Generation 0) and ~25-30 hpi/~35-40 hpi (Generation 1) using LM and FC. The proportion of
929 rings, trophozoites and early/late schizonts was quantified at ~35-40 hpi in Generation 0 and
930 ~25-30 hpi/~35-40 hpi in Generation 1 by LM of Giemsa-stained thin blood smears (100
931 parasite counts per sample; Fig 4A) in a subset of RBC samples (HbAA n = 1; HbAC n = 1;
932 HbAS n = 2; HbSS n = 2; HbSC n = 2). FC was used as described earlier to determine the
933 percentage of "late stages". For this a distinct population of cells were gated with increased
934 Hoechst 33342 fluorescence (increased DNA content due to multiplication of the nuclei in
935 schizont stages), within the Hoechst+ cells (Fig 4B). Upon confirming consistent results
936 between LM and FC after screening the same samples by FC, FC was subsequently used in all
937 *iv*SCA samples (n = 28: HbAA n = 5; HbAC n = 5; HbAS n = 4; HbSS n = 12; HbSC n = 2;
938 and fresh non-endemic HbAA CTRLs n = 6) to determine the proportion of late-stages (*i.e.,*
939 schizonts) by HBB genotype (Figs 4B and 4C).
940 From LM data we calculated: (1) the percentage of rings, trophozoites and early/late schizonts
941 (Fig 4A), (2) the mean parasite age using the age in hpi (116) of each stage (rings, trophozoites,
942 schizonts) and the observed proportions; and (3) the developmental delay (in hours) by
943 subtracting mean parasite age of the mutant genotypes from the wild-type HbAA genotype.
944 Finally, for each genotype, we compared the developmental delay between Generations 0 and
945 1 by calculating the difference in mean parasite age at ~35-40 hpi between both generations.
946 Then, the development of each independent NF54-*gexp02-Tom* culture was classified as:

947 *expected development*, *delayed development* or *(partially) arrested/extremely delayed*
948 *development* (see detailed analysis in *Supporting information file)*.

949 Flow cytometry assays to assess IgG and IgM immunity against *P.* 950 *falciparum*

951 To study the IgG and IgM levels against *P. falciparum* in individuals with different HBB
952 genotypes by flow cytometry, we selected endemic human plasma samples from the *InHost*
953 longitudinal cohort study. We classified samples as "endemic" or "non-endemic" based on the
954 country of origin. An "endemic" country has consistent, stable *P. falciparum* transmission,
955 while a "non-endemic" country has no local transmission, with infections typically imported
956 (117). As the expected variation and magnitude of potential differences among HBB genotypes
957 were unknown, we based the sample size on the minimum of 8 individuals per HBB genotype
958 used in the *ev*SCAs and *iv*SCAs. A maximum of 15-20 individuals per genotype was included
959 to ensure sufficient sampling. Selection criteria included equal representation of the most
960 frequent genotype groups (HbAA [n = 18], HbAC [n = 18], HbAS [n = 18], HbCC [n = 2], and
961 HbSC [n = 5]), *P. falciparum* infection status with a large proportion of active *P. falciparum*
962 infections (n = 10-15) and a smaller proportion of non-infected individuals (n = 2-5), age range
963 (1-19 years to exclude maternal antibodies), and homogeneous distribution amongst the
964 ethnicity (all Mossi) and village (Nanoro, Nazoanga, Soum, and Séguédin) groups. Table 3
965 shows the number of participants per HBB genotype included in the assays. The *P. falciparum*
966 transgenic line NF54-*gexp02*-Tom (52, 75) was cultured under the same conditions as
967 described in the *iv*SCAs section and Fig 3A, with the following modifications: (1) parasites
968 were cultured only in wild-type HbAA fresh RBCs and (2) flow cytometry assays were
969 performed on early trophozoite/stage I gametocyte-infected RBCs (Troph-Gam-iRBCs) at ~20-
970 25 hpi (Generation 1), following the protocol reported in (102) with the adaptations described
971 below.

972 Human plasma samples were pre-diluted in PBS with 1% Bovine Serum Albumin (BSA, Sigma
973 Aldrich) and heparin (2,500U; Sigma Aldrich) (PBS-BSA-Hep) at dilutions of 1:5000 (IgG)
974 and 1:100 (IgM). *In vitro* generated Troph-Gam-iRBCs (25-30hpi; Generation 1) were pre-
975 diluted to 0.6% Hct in PBS-BSA-Hep. Pre-diluted human plasma samples were incubated at a
976 1:10 dilution with pre-diluted Troph-Gam-iRBCs, in separate 96-well plates for IgG and IgM
977 assays for 1.5 hours at room temperature. After 2 washes with PBS-BSA-Hep, secondary
978 antibodies were added to the Troph-Gam-iRBCs for 30 minutes at 4°C at 0.6% hematocrit.
979 AlexaFluor488 (AF488) mouse anti-human IgG (Jackson ImmunoResearch Europe) and
980 PerCP/Cy5.5® mouse anti-human IgM (Abcam) were used at final dilutions of 1:640 and 1:320
981 in PBS-BSA-Hep for the IgG and IgM assays, respectively. After 2 washes with PBS-BSA-
982 Hep, the DNA stain Hoechst33342 (BD Pharmingen) was added to the Troph-Gam-iRBCs at
983 a final dilution of 1:5000 in PBS-BSA-Hep at 0.6% hematocrit for 30 minutes at 37°C. To
984 verify that the Troph-Gam-iRBCs measured contained exclusively red blood cells (no white
985 blood cells), each batch pre-diluted Troph-Gam-iRBCs (to 0.6% hematocrit in PBS-BSA-Hep)
986 were stained with APC anti-human CD236 (Glycophorin C) Recombinant Antibody
987 (Biolegend®) in triplicate at a 1:40 final dilution (Fig S4C). Immunostained samples were
988 analyzed on the flow cytometer CytoFLEX LX (Beckman Coulter), collecting 100,000 cells
989 per culture. We analyzed the median percentage of total, asexual and sexual-infected red blood
990 cells (Troph-Gam-iRBCs) that were positive for IgG and IgM binding to surface antigens on
991 the Troph-Gam-iRBCs (*i.e.,* α-Troph-Gam-iRBCs , α- Troph-iRBCs and α-Gam-iRBCs IgG &
992 IgM). Samples were considered positive for IgG or IgM if the levels of α-Troph-Gam-iRBCs
993 IgG & IgM exceeded that of a naïve non-endemic plasma from a volunteer (negative control
994 with HbAA genotype with no prior *P. falciparum* infection, from a non-endemic country [*i.e.,*
995 Belgium] collected at the clinic of the Institute of Tropical Medicine [ITM] in Antwerp). Three
996 non-endemic samples from an acute *P. falciparum*-infected traveller collected at the ITM clinic

997 at day 0, 21 and 23 of *P. falciparum* diagnosis (*i.e.,* acute *P. falciparum-infected* non-endemic
998 plasma D0, D21 and D23) were used as positive controls. A schematic of the gating strategy
999 to measure the surface reactivity of asexual (tdTom-) and sexual (tdTom+) Troph-Gam-iRBCs
1000 (Hoechst33342+) to IgG (α-Troph-iRBCs IgG: Hoechst33342+/tdTom-/AF488+ and α-Gam-
1001 iRBCs IgG: Hoechst33342+/tdTom+/ AF488+) or IgM (α-Troph-iRBCs IgM:
1002 Hoechst33342+/tdTom-/PerCP-Cy5.5.+ and α-Gam-iRBCs IgM (Hoechst33342+/
1003 tdTom+/PerCP-Cy5.5.+) in the analyzed endemic human plasma samples is represented in Figs
1004 S4B and S4C.
1005 To determine the optimal plasma dilution factor, a pool of five endemic and hyperimmune
1006 plasma samples from *P. falciparum*-infected individuals in a sub-cohort within the *InHost*
1007 study (*i.e., P. falciparum*-infected hyperimmune endemic plasma pool of symptomatic
1008 individuals with high *P. falciparum* parasitemia ranging from 45,667 to 175,000 parasites per
1009 µl) (53) was titrated for the IgG and IgM assay separately. For the IgM assay, four additional
1010 plasma samples were titrated (*i.e.,* "naïve non-endemic plasma" and "acute *P. falciparum*-
1011 infected non-endemic plasma at D0, D21 and D23") (Fig S4A).
1012 During flow cytometry analysis, α-Troph-Gam-iRBCs IgG (Hoechst33342+/tdTom-
1013 /+/AF488+) and α-Troph-Gam-iRBCs IgM (Hoechst33342+/tdTom-/+/PerCP-Cy5.5.+) levels
1014 for each sample were expressed as total event counts, percentage of positive cells, and median
1015 fluorescence intensity (MFI) per HBB genotype group. The α-Troph-Gam-iRBCs IgG and IgM
1016 levels and signal were compared by HBB genotype group for the total Troph-Gam-iRBCs, and
1017 the asexual or sexual Troph-Gam-iRBCs (Figs 5 and S5). To correct for the background signal
1018 from the non-infected RBCs in the IgG and the IgM channel, the MFI of all samples (including
1019 the positive and the negative controls) was expressed as a ΔMFI. For the asexual and sexual
1020 Troph-Gam-iRBCs, ΔMFI was calculated as ΔMFI (AF488+/PerCP-Cy5.5.+) = ("MFI
1021 IgG+/IgM+ of asexual [tdTom-]/sexual [tdTom+] Troph-iRBCs [Hoechst33342+]") - ("MFI

1022 IgG+/IgM+ of non-infected RBCs [tdTom-/Hoechst 33342-]") within each plasma sample. For
1023 the total Troph-iRBCs, ΔMFI was calculated as ΔMFI (AF488+/PerCP-Cy5.5.+) = ("MFI IgG+
1024 or IgM+ iRBCs [Hoechst33342+]") - ("MFI IgG+ or IgM+ of non-infected RBCs
1025 [Hoechst33342-]" of trophozoite stages incubated with PBS-BSA-Hep, instead of plasma).

1026 Data analysis

1027 All flow cytometry data were analyzed using FlowLogic™ version 8.7 (Inivai
1028 Technologies™). Statistical analyses were performed in RStudio software version
1029 2025.09.1+401(©2025 Posit Software; RRID:SCR_000432), with p-values ($p \leq 0.05$)
1030 considered significant. The statistical analysis type was chosen based on the nature of the
1031 variables: proportions based on counts were assessed using binomial and beta-binomial
1032 generalized linear models (GLM) and mixed models (GLMM) with Bonferroni adjustment for
1033 the contrasts in pairwise comparisons. We chose beta-binomial models due to (possible)
1034 violations of the assumptions of the binomial model (overdispersion), this was borderline
1035 acceptable in "*ev*SCA" (GLMM) but not in "*iv*SCA" (GLM) and "IgG/IgM detection" (GLM).
1036 Random factors were used to account for technical replicates in the GLMM for the "*ev*SCA".
1037 To test for the relevance of other factors we also modelled the initial parasitemia ("*ev*SCA" &
1038 "*iv*SCA"), and reticulocyte percentage ("*iv*SCA"). To address the liberal parametric Wald Z
1039 estimates and confidence intervals, we also computed more conservative Bayesian estimates
1040 and credible intervals using a Markov chain Monte Carlo (MCMC) approach when model
1041 specification and convergence allowed. GLM and GLMM analysis results are reported in Table
1042 S1. When the assumptions of the GLM and GLMM beta-binomial models were violated, we
1043 performed Kruskal-Wallis tests as described below.
1044 Continuous variables were first assessed for normality using the Shapiro-Wilk test to guide the
1045 choice between parametric and non-parametric methods. Continuous non-parametric variables
1046 across categorical groups with ≥2 levels were analyzed using the Kruskal-Wallis test, and when

significant, this was followed by Wilcoxon rank sum post-hoc tests for pairwise comparisons with Holm correction. For normally distributed variables, one-way ANOVA was applied when Bartlett's F-test indicated equal variances; when variances were unequal, Welch's ANOVA was used. Bonferroni post-hoc tests were performed for pairwise comparisons when ANOVA results were significant. Associations between categorical variables were evaluated using Pearson's χ^2 test. Continuous non-parametric variables were correlated using Spearman correlation.

In the *ev*SCAs and the *iv*SCAs, risk analyses stratified by HBB genotype were conducted to examine associations with demographic and clinical variables (*e.g.,* SCRs, gender, age, village, ethnicity, …). An additional risk analysis for factors associated with elevated basal SCRs included the same covariates with the addition of HBB genotype (Table S4 for the *ev*SCAs, and Table S5 for the *iv*SCAs).

Ethical approvals

The *InHost* study, a longitudinal cohort in Burkina Faso, was approved by the ethical review board of the Comité d'Éthique pour la Recherche en Santé (CERS: 2018/10/131) in Burkina Faso, and by the IRB-ITM (1261/18) and IRB-UZA (19/06/064) in Belgium. The amendment for our nested study in the *InHost* study was approved by CERS (N°2022000140/MSHP/ MESRI/CERS), the IRB at ITM (1261/18), and the IRB at UZA (3347) in Belgium. Ethical approval for additional sample collection at the Hematology Unit at UZA was granted by the IRBs at ITM (1538/21) and UZA (3215).

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