Splenic clearance of rigid erythrocytes as an inherited mechanism for splenomegaly and natural resistance to malaria

Benoît Henry,[a,b,c,d,e] Geoffroy Volle,[a,b,c,1] Hilaire Akpovi,[f,1] Laure Gineau,[b,g,1] Camille Roussel,[a,b,c] Papa Alioune Ndour,[a,b,c] Félicien Tossou,[h,i] Felipe Suarez,[b,j,k] Friso Palstra,[b,g] Aurélie Fricot,[a,b,c] Charlotte Chambrion,[a,b,c] Julien Solinc,[a,b,c] Julie Nguyen,[b,g] Mathilde Garé,[f,g] Florentin Aussenac,[f,g] Charles-Henry Cottart,[l] Christine Keyser,[m] Rafiou Adamou,[f] Magali Tichit,[n] David Hardy,[n] Nadine Fievet,[b,f,g] Jérôme Clain,[b,g] André Garcia,[b,f,g] David Courtin,[b,f,g] Olivier Hermine,[b,j,k] Audrey Sabbagh,[b,g,2] and Pierre Buffet[a,b,c,o,2*]

[a]Université Paris Cité, Biologie Intégrée du Globule Rouge, UMR_S1134, BIGR, INSERM, Paris, France
[b]Laboratoire d'Excellence Gr-Ex, Paris, France
[c]Institut National de la Transfusion Sanguine, Paris, France
[d]Service des maladies infectieuses et tropicales, APHP, Hôpital Necker Enfants Malades, Centre d'Infectiologie Necker-Pasteur, Institut Imagine, Paris, France
[e]Service des maladies infectieuses et tropicales, APHP. Université Paris Saclay, Hôpital Bicêtre, Le Kremlin-Bicêtre, France
[f]CERPAGE (Centre d'Etude et de Recherche sur les Pathologies Associées à la Grossesse et à l'Enfance), Cotonou, Bénin
[g]Université Paris Cité, IRD, MERIT, Paris, France
[h]Centre Interfacultaire de Formation et de Recherche en Environnement pour le Développement Durable (CIFRED), Université d'Abomey-Calavi, Cotonou, Bénin
[i]Ministère de la Santé, Cotonou, Bénin
[j]Service d'hématologie adultes, APHP, Hôpital Necker Enfants Malades, Paris, France
[k]Université Paris Cité, INSERM U1163, CNRS ERL 8654, Paris, France
[l]Service de biochimie générale, APHP, Hôpital Necker Enfants Malades, Faculté de pharmacie, Paris, France
[m]Université Paris Cité, CNRS, UMR 8045, Paris, France
[n]Institut Pasteur, Experimental Neuropathology Unit, Paris, France
[o]Institut Pasteur, Paris, France

Summary

Background In malaria-endemic areas, subjects from specific groups like Fulani have a peculiar protection against malaria, with high levels of IgM but also frequent anaemia and splenomegaly. The mechanisms underlying this phenotype remain elusive.

Methods In a cohort study set up in Benin, West Africa, after a careful evaluation of malaria-related phenotypes, we measured the deformability of circulating erythrocytes in genetically distinct groups (including Fulani) living in sympatry, using ektacytometry and microsphiltration, a mimic of how the spleen clears rigid erythrocytes. Heritability of erythrocytes deformability was calculated, followed by a genome-wide association study (GWAS) of the same phenotype.

Findings Compared to non-Fulani, Fulani displayed a higher deformability of circulating erythrocytes, pointing to an enhanced clearance of rigid erythrocytes by the spleen. This phenotype was observed in individuals displaying markers of *Plasmodium falciparum* infection. The heritability of this new trait was high, with a strong multigenic component. Five of the top 10 genes selected by a population structure-adjusted GWAS, expressed in the spleen, are potentially involved in splenic clearance of erythrocytes (*CHERP, MB, PALLD, SPARC, PDE10A*), through control of vascular tone, collagen synthesis and macrophage activity.

Interpretation In specific ethnic groups, genetically-controlled processes likely enhance the innate retention of infected and uninfected erythrocytes in the spleen, explaining splenomegaly, anaemia, cryptic intrasplenic parasite

eBioMedicine 2022;82: 104167
Published online 15 July 2022
https://doi.org/10.1016/j.ebiom.2022.104167

*Corresponding author at: Institut National de la Transfusion Sanguine, UMR_1134, 6, rue Alexandre Cabanel, 75015 Paris, France.
E-mail address: pierre.buffet@pasteur.fr (P. Buffet).

[1] GV, HA and LG equally contributed to this work.

[2] Joint senior authorship.

loads, hyper-IgM, and partial protection against malaria. Beyond malaria-related phenotypes, inherited splenic hyper-filtration of erythrocytes may impact the pathogenesis of other hematologic diseases.

Funding ANR, National Geographic Society, IMEA, IRD, and Région Ile-de-France.

Copyright © 2022 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Keywords: Malaria; Falciparum; Spleen; Erythrocytes; Ethnic groups; Heritability; Splenomegaly; Genome-wide association study

Research in context

Evidence before this study

The genetic background of individuals influences their susceptibility to infectious diseases. Specific human groups, like the Fulani in Africa, react to malaria parasites (named *Plasmodium*) in a specific way. Upon infection, Fulani develop a grossly enlarged spleen, and high levels of anti-*Plasmodium* antibodies in their blood. They also carry smaller numbers of parasites in their blood, and thus are considered partially protected against malaria. The mechanisms underlying this natural protection, different from other natural protective mechanisms such as the sickle cell trait, are not well understood.

Malaria impairs the deformability of red blood cells and the spleen is a key organ to controlling red blood cell quality. We have recently demonstrated that red blood cells containing live malaria parasites accumulate intensely in the spleen of subjects with long term exposure to these parasites. Enhanced retention of infected and uninfected red blood cells in the spleen would explain why the spleen is larger and why lower numbers of parasites are left in circulation. We thus explored whether the retention of infected and uninfected red blood cells could explain why Fulani are partially protected against malaria. As it is unethical to perform spleen puncture or biopsies for research purposes, our explorations were indirect by carefully analyzing the properties of circulating red blood cells in a large number of subjects and by assessing whether observations could be explained by their genetic make-up.

Added value of this study

In more than 500 subjects, we confirmed the high frequency of large spleens in Fulani and, through 2 different methods, we demonstrated an enhanced deformability of their circulating red blood cells, that likely stems from the more efficient removal of the less deformable ones. This enhanced deformability was found to be inheritable based on carefully collected family links and refined analysis of genetic markers.

Implications of all the available evidence

Our findings indicate that genes potentially driving the filtration of red blood cells by the spleen likely influence how subjects in specific groups in Africa and elsewhere react to malaria. While most previous hypotheses pointed to conventional immunological mechanisms as the trigger, we propose that a simple physiological mechanism that controls the quality of red blood cells may drive natural protection from malaria even before the intervention of immunological cells. A better understanding of these processes is of great importance in the context of malaria elimination efforts.

These findings may also have an impact on the understanding of other red blood cell-related disorders, such as inherited red cell diseases, in which splenic filtration of abnormal red blood cells may precipitate splenic complications.

Introduction

Malaria due to *Plasmodium falciparum* is the most frequent acquired red blood cell (RBC) disease worldwide. Natural protection against malaria has been extensively studied, predominantly leading to the identification of RBC polymorphisms, such as sickle cell trait as protective genotypes.[1] Fulani, traditional nomadic pastoralists living in West Africa, display a specific phenotype in relation to malaria. Compared to other ethnic groups living in sympatry, Fulani have a higher prevalence of splenomegaly,[2–7] higher total IgM levels,[8] and a stronger humoral immune response against plasmodial antigens,[9,10] In some but not all studies, malaria-related fever and parasitemia are lower and/or less prevalent in Fulani than in other groups,[4,6,11–15] and their haemoglobin levels are generally lower than in control groups.[16] Not least, the immune response against *P. falciparum* in Fulani displays specificities with regards to cytokines production,[17,18] monocytes activity,[19] B-cell subpopulations,[20] and regulatory T-cell function.[21]

Intra-erythrocytic stages of *P. falciparum* are mostly confined to the blood[22] and splenic circulations.[23,24]

The mechanical properties of *P. falciparum*-infected RBC have been extensively studied over the last 40 years. A decrease in deformability of RBC infected with mature, but also ring-stage parasites, has been observed and contributes to vascular flow obstruction observed in severe malaria.[25,26] This reduced deformability extends to uninfected RBC[27] and is correlated with disease severity.[28] The human spleen plays a key role in the quality control of the circulating RBC. The splenic red pulp senses the surface and mechanical properties of RBC in the specific anatomic structures of the cords and inter-endothelial slits. After observing the mechanical retention of parasitized RBC in human spleens perfused *ex-vivo*,[29] we had proposed that in malaria-infected subjects, the clearance of infected (and possibly uninfected) RBC by the spleen could modulate the size of the parasite biomass as well as where the antigenic stimulus predominates. A stringent retention of RBC in the spleen would confer protection by reducing the parasite biomass in circulation, but would also be pathogenic by precipitating splenomegaly and anaemia.[30] Recent observations of tissue samples from splenectomy in a malaria-endemic area have revealed the intense accumulation of RBC containing live malaria parasites in the human spleen,[23,24] bringing further substance to our hypothesis. To test whether such splenic retention contributes to the distinctive patterns of response to malaria infection among Fulani population, we have quantified the deformability of circulating RBC in a multi-ethnic cohort of Beninese subjects living in sympatry in an area of high malarial transmission, using a filtration method that mimics the mechanical sensing of RBC by the human spleen.[31] The objectives were to deliver a more precise definition of malaria-related phenotypes in different African groups with regards to RBC and spleen, to explore whether Fulani display specific spleen-related red cell characteristics, and to investigate the potential genetic components underlying these phenotypes.

Methods

Study setting and clinical evaluation

This study was part of a program led by the French Research Institute for Development (Institut de Recherche pour le Développement, IRD) and Université Paris Cité, aiming at deciphering the genetic, parasitological, environmental and behavioural determinants of the relative protection of Fulani from malaria (BAOBAB study: Biocultural AdaptatiOn to malaria in Atacora, northern Benin). This study recruited nuclear families and, in some instances, extended families. Participation was voluntary and the inclusion was proposed to subjects from nuclear families with at least 2 children aged 1 to 7 years, and parents (younger than 71) belonging to the same ethnic group. Exclusion criteria was the inability to provide informed written consent.

The study was conducted in 4 rural villages (Goufanrou, Gorgoba, Tamande, Kouboro) located 40 km from the Natitingou city centre, 3-15 km apart, in the Birni district of the Atacora department, a region of north-western Benin with one of the highest prevalence of malaria in the country.[32] Malaria transmission is seasonal and *Plasmodium falciparum* is the predominant parasite species transmitted by *Anopheles gambiae s.s.* (85%) and *Anopheles arabiensis* (15%).[33] Highest infection rates occur during the rainy season, from May to late October, with mean sporozoite inoculation rates reaching two infective bites per person per night (A. Djènontin, personal communication, 2015), and then drop during the dry season from November to April.[34] Most of the field work and experiments were performed at the end of the transmission season in December, 2017. This cross-sectional survey systematically recorded clinical and biological information related to malaria (i.e., temperature, spleen enlargement rate, haemoglobin level, parasite carriage and parasite density). Included subjects underwent a physical examination, including tympanic temperature measurement, spleen palpation, signs of portal hypertension in case of splenomegaly. A rapid haemoglobin determination using the Hemocue© (Radiometer), and a rapid diagnostic test for malaria (RDT; SD BIOLINE Malaria Ag P.f©, Abbott) using immunochromatographic detection of Histidine Rich Protein 2 (HRP2) of *P. falciparum* were also performed. A thick smear was prepared from all subjects, fixed with methanol, stained with Giemsa and read at x1000 magnification. Parasitemia was determined by counting parasites against 500 leucocytes and assuming a total leucocyte count of 8000/mm3. A thick blood smear was declared negative when no parasite was detected in 200 fields. Only *P. falciparum* mono infections were analyzed. Treatment of malaria (artemether-lumefantrine, as recommended by the Beninese National Malaria Control Program) was provided to the study population at no cost.

Individual information (age, gender, ethnic background, symptoms, recent use of antimalarial agents) was collected. Ethnicity was assessed through self-declaration, and, to be eligible for the study, participants must have identified their parents and four grandparents as belonging to the same ethnic group as themselves (Bariba, Otamari, Gando or Fulani). Self-declarations were controlled and confirmed by community health workers and community leaders from each village. Familial relationships were built by questioning each of the household members and were further confirmed by village authorities. In the case of conflicting information, the ambiguous kinship relationships were clarified by genotyping individuals with the GlobalFiler® kit (ThermoFischer Scientific). Reconstruction of family relationships resulted in 116 distinct pedigrees,

including 103 nuclear and 13 extended families. Among the 420 individuals included in the heritability and familial correlation analyses, we identified 337 parent-offspring pairs, 138 sibling pairs, 59 half-sibling pairs, 16 grandparent-grandchild pairs, 47 avuncular pairs, 54 cousin pairs, 16 half-avuncular pairs, 6 great avuncular pairs and 4 half-cousin pairs. A field survey revealed that the 116 studied families lived in 189 distinct households at the time of study. Close members of a same pedigree did not always live in the same house and thus did not share the same household environment. Conversely, there were many cases where unrelated individuals or distant relatives (higher than the first degree) shared the same house.

Blood samples

Processing of blood samples is shown in Figure 1. Venous peripheral blood (4-6 ml for adults, 1-3 mL for children and infants) was collected on-site on EDTA (BD vacutainer©) and immediately stored at 4-8°C until use. After sedimentation, plasma was collected and cryopreserved at -20°C, then -80°C and shipped to the central laboratory on dry ice. RBC were transferred into SAGM (saline, adenine, glucose, mannitol, Macopharma©) a preservation medium used for transfusion, at 5% haematocrit, and shipped to the central laboratory at 4°C for phenotypic analysis. We had previously shown the stability of measures for up to 3 weeks under these storage conditions (Supplementary Figure 1a and 1b).

Total IgM measurement

Immunoturbidimetric measurement was performed on an Architec C1600 analyzer (Abbott Laboratories Diagnostics Division) according to the manufacturer recommendations (Abbott technical sheet: 1E01-21).

Circulating RBC deformability assays

Ektacytometry[35] was performed on SAGM-preserved RBC, diluted in viscous iso-osmolar polyvinylpyrrolidone (PVP; RR Mechatronics©) at 0·5% haematocrit, temperature 37°C, between 9 and 17 days post sampling. We used a Laser-assisted Optical Rotational Cell Analyzer (RR Mechatronics©), which evaluates the diffraction pattern of RBC through a laser, when elongated between two rotating cylinders at different shear stresses between 0·3 and 30 Pascals (Pa). Results were expressed as an Elongation Index (EI), which is the ratio of the difference between the 2 axes of the ellipsoidal diffraction pattern and the sum of these 2 axes. We collected EI at low (0·3 Pa, 3 Pa), intermediate (1·69 Pa), and high shear stress (30 Pa), the latter being close from what is encountered in the splenic red pulp microcirculation.[36] Ektacytometry was performed on a subset of the population because of a PVP shortage.

Microsphiltration ("microsphere filtration"; MS) assesses the ability of RBC to squeeze through narrow slits between metallic microbeads. This technique reflects the filtration of normal and abnormal RBC in the splenic red pulp, as assessed by direct comparison with retention rates in isolated-perfused human spleens.[31] It has been adapted to 96 and 384-wells microplates, allowing us to process numerous blood samples over a short period of time.[37] Microsphiltration was performed using frozen (-20°C) 96-wells plates thawed just before use. Plates were prepared with a Tecan® automat as described.[37] Control RBC were obtained from an O-negative healthy blood donor (Etablissement Français du Sang, Lille, France), and were carboxyfluorescein diacetate succinyl ester (CFSE)-stained (20 μmol/l; Life Technologies). For each sample tested, the cell mix consisted of 95% control stained RBC, and 5% tested RBC (left unlabelled) from Beninese subjects. The hematocrit of the mix was 1%, and cells were diluted in Krebs-Henseleit buffer (Sigma-Aldrich) modified with 2·1 g/l of sodium bicarbonate and 0·175 g/l of calcium chloride, and supplemented with Albumax© (0·5%; Life Technologies), at a pH of 7·4. Each RBC sample was tested in triplicate. Results were expressed as retention or enrichment rates (RER), using flow cytometry analysis of upstream and downstream samples (BD FacsCantoII©, BD Biosciences). RER was calculated as follows: ([proportion of unlabelled RBC in downstream sample] minus [proportion of unlabelled RBC in upstream sample])/proportion of unlabelled RBC in upstream sample. Positive values, called enrichment, indicate that RBC from the subject are more deformable than control RBC, whereas negative values, called retention, indicate the opposite. Positive controls were glutaraldehyde (0·8%)-treated RBC, which are completely retained; negative controls were unstained control RBC. For each subject, the mean value of RER was calculated after exclusion of outliers, and results were normalized to the negative control value. Experiments were performed 15 to 17 days after RBC collection, after exclusion of unsuitable samples (haemolysis; n = 58). Cytometric data were acquired with FACSDIVA software v6.1.3 (BD Biosciences) and analyzed with FlowJo v10.0 software (FlowJo, LLC).

Nucleic acid extraction and real-time quantitative PCR (qPCR)

Total nucleic acids were extracted from filter papers containing approximately 10 μL of dried whole blood using an optimized Chelex protocol. Final eluate volume was 140 μL. Two species-specific qPCRs were performed. The first qPCR targeted a single-copy gene (human albumin or alb; forward and reverse primers were GCTGTCATCTCTTGTGGGCTG and AAACTCATGGGAGCTGCTGGTT, 140-bp amplicon[38]) and served as an internal extraction control. The second qPCR

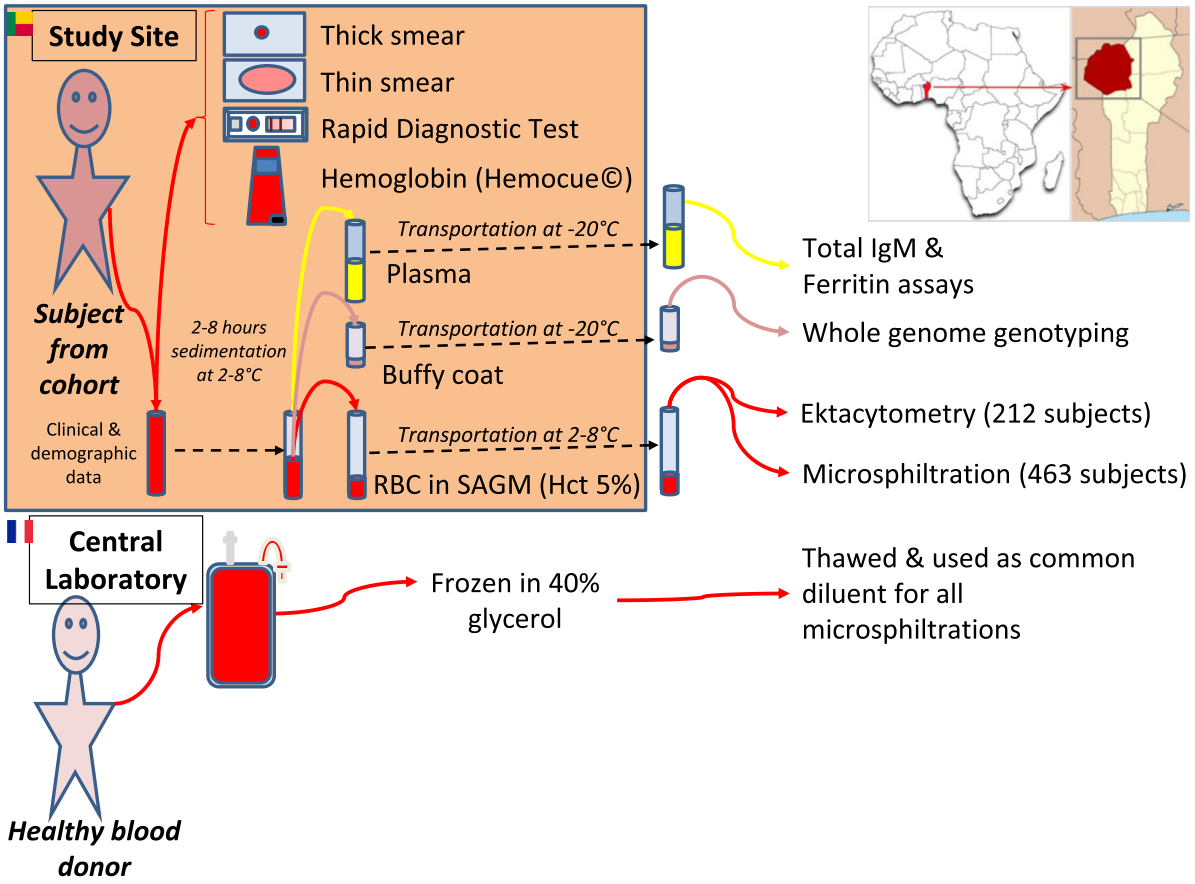


Figure 1. Overview of experimental procedures. Study location is indicated in the upper right corner (Birni district, Atacora department, Benin). SAGM: saline, adenine, glucose, mannitol. Hct: haematocrit. Ektacytometry was performed on 212 samples because of a shortage of polyvinypyrrolidone. PCR was performed on 424 samples because whole blood on filter paper could not be obtained in 97 subjects.

targeted a multiple copy *P. falciparum* gene (cytochrome b or pfcytb, with an average 15-20 copies per parasite; forward and reverse primers were TTGGTGCTAGAGATTATTCTGTTCCT and GGAGCTGTAATCATAATGTGTTCGTC, 188-bp amplicon). qPCRs were carried out in 15 μl in a 384-well plate using SensiFASTTM SYBR Hi-ROX kit (Bioline), 0·5 μM of each forward and reverse primer and 2·5 μl of template DNA. Amplifications were performed in an Applied-Biosystems 7900 HT Fast Real Time PCR machine (Thermo Fisher Scientific): 95°C for 3 min; then 40 cycles of 95°C for 15 sec, and 63°C for 1 min. Several controls were run on each 384-well plate: water, *Plasmodium*-free human DNA, and seven DNA from *Plasmodium*-infected blood isolates (*P. vivax, P. ovale, P. malariae*, and four different *P. falciparum*). For tested DNA samples, the alb and pfcytb qPCRs were performed once and as duplicates, respectively, on each plate. Tested DNA samples were scored as *P. falciparum*-positive when two conditions were met: 1) the alb qPCR gave a threshold value (Ct) < 28 with a melt curve and temperature similar to corresponding positive controls; and 2) the two pfcytb qPCR replicates gave a Ct ≤ 38 with a melt curve and temperature similar to corresponding positive controls. All amplification and melt curves were individually inspected by eye.

Definitions used for phenotypic analysis

Anaemia was defined according to WHO criteria,[39] based on the age-related haemoglobin levels as follows: lower than 11 g/dL in 0-4-year-olds; lower than 11·5 g/dL in 5-11-year-olds; lower than 12 g/dL in 12-14-year-olds; lower than 12 g/dL in in adult women and lower than 13 g/dL in adult men. Fever was defined as a tympanic temperature of 38°C or more. Splenomegaly was defined as any clinically palpable spleen, as assessed by a fully qualified clinician (H.A.).

Heritability analysis

Heritability (h^2) was estimated using the variance-components approach implemented in the SOLAR package, which simultaneously utilizes data on all family relationships.[40] This method applies maximum likelihood estimation to a mixed effects model that incorporates fixed effects for known covariates and variance components for genetic and environmental effects. The genetic component is assumed to be polygenic with no variation attributable to dominance components. A variance component was included in the model to represent the possible shared environmental effect related to the household (environmental exposures shared by individuals living in the same house at the time of study), also referred to as 'household effects' (c^2). Heritability was estimated as the ratio of additive genetic variance to the total phenotypic variance unexplained by covariates. Estimates of the means and variances of components of the models were obtained by maximum likelihood methods and significance was determined by likelihood ratio tests. Intrafamilial correlation coefficients were estimated using the pairwise weighting scheme for all available pairs of relatives in the pedigrees using the FCOR program within the SAGE v6.3 software package (S.A.G.E. [2016], Release 6.4: http://darwin.cwru.edu). Correlations were calculated for the residual trait values obtained from a multivariate linear regression model, including relevant covariates (here RDT positivity and ethnicity). Homogeneity testing of correlations among subtypes (e.g. mother-offspring and father-offspring) within main type (e.g. parent-offspring) was also performed. Under the null hypothesis of homogeneity, the test statistic has an approximate $\chi 2$ distribution with degrees of freedom equal to the number of subtypes minus one.

Genotyping and data quality control

Genotyping was conducted at the Centre National de Recherche en Génomique Humaine (CNRGH, CEA, Evry, France). Before genotyping, a quality control was systematically performed on each DNA sample. All samples were quantified by fluorescence, in duplicate, using the Quant-It kits (Thermo Fischer Scientific). The lowest values systematically underwent a second measurement before any sample was excluded. The quality of material sent was estimated using about 10% of the total samples received (selected randomly throughout the collection) by performing: i) a quality check by migration on a 1% agarose gel to ensure the samples were not degraded, ii) a standard PCR amplification reaction on the samples to ensure that the genomic DNA was free of PCR inhibitors, iii) a PCR test to verify the gender of the individual.[41] All samples with concentrations below 20 ng/µL, or a major quality problem (degradation and/or amplification problems, as well as all samples whose gender verification was discordant with the information provided) were systematically excluded from the study.

After quality control, DNA samples were aliquoted in 96-well plates (JANUS liquid handling robot, Perkin Elmer) for genotyping; sample tracking was ensured by a systematic barcode scanning for each sample. Two DNA positive controls were systematically inserted in a random fashion into the plates. Genotyping was performed on Illumina HumanOmni5-4v1 chips, on a high throughput Illumina automated platform, in accordance with the standard automated protocol of Illumina® 'Infinium HD Assay (Illumina®). The PCR amplification of the genomic DNA was performed in a dedicated (« pre-PCR ») laboratory. Fragmentation and hybridization of the DNA on the chips were performed in a dedicated (« post-PCR ») laboratory. Several quality controls were systematically included during the

process, such as visual inspection of the DNA pellets after precipitation, visual inspection of the deposited cocktail of reagents for hybridization, and systematic verification of the temperature of the heating block during the extension and imaging steps. Reading of the chips was performed on iScan+ scanners (Illumina®). Primary analysis of the genotyping results was done using the GenomeStudio software (Illumina®). The analysis of the internal controls provided by Illumina and the randomly distributed positive controls allowed the validation of the technological process.

Standard quality control metrics were then applied to the genome-wide data of the 347 genotyped samples using PLINK v1.90b4.6 software.[42] Single nucleotide polymorphisms (SNPs) with a call-rate < 0·98 (n = 96,834), a minor allele frequency (MAF) < 0·01 (n = 1,740,582) or displaying significant departure from Hardy-Weinberg equilibrium (HWE) ($P < 10^{-4}$) (n = 1,714) were removed from the dataset, as well as duplicated (n = 70,807) and non-autosomal SNPs (n = 109,008). For DNA samples, individuals with discordant sex information (n = 16), an autosomal heterozygosity rate more than three standard deviations on either side of the mean (n = 4), and sample duplicates (n = 4) were excluded. All remaining samples had a call rate >0·98 (mean = 0·998) and were kept for analysis. These quality control checks resulted in a final set of 2,281,899 high-quality SNPs genotyped in 323 individuals. The data sets generated and analyzed during the current study are available from the DataSuds repository (https://doi.org/10.23708/KDRQMQ).

Genotype imputation

Genome-wide imputation was performed using the IMPUTE2 v2.3.2 algorithm.[43] First, genotypes were prephased into best-guess haplotypes with SHAPEIT v2 to increase the computational efficiency of downstream steps.[44–46] We used a window size of 2 Mb, an effective population size of 17,469 (as recommended by the software providers for African populations) and the duoHMM method that allows to incorporate the known pedigree information to improve phasing.[47] Then, IMPUTE2 was run on the prephased haplotypes using the cosmopolitan panel of reference haplotypes from the 1000 Genomes Project Phase 3 (October 2014 release; n = 2,504).[48] The genome was split up into non-overlapping segments of 5 Mb. We used imputation parameter settings of k =500, Ne= 20,000 and an internal buffer region of 250 kb. Imputation was performed in parallel for each segment, and segments were reconstructed into chromosomes once all imputations had finished. Stringent post-imputation quality and accuracy filters were applied to genotypes resulting from the imputation (info ≥ 0·8, certainty ≥ 0·9 and concordance ≥ 0·9 between directly measured and imputed genotypes after masking input genotypes). Further, we applied the same SNP QC criteria as the aforementioned genotyped analysis (removal of variants with call-rate < 0·98, HWE test $P < 10^{-4}$ or MAF < 0·01). Following imputation and quality control, 14,350,106 genotyped or imputed SNPs and indels were available for the genome-wide association analysis.

Genome-wide association study

The genome-wide association analysis was performed in R (version 2.15.1; http://CRAN.R-project.org) using the GRAMMAR−GC (Genome-wide Rapid Association using Mixed Model and Regression-Genomic Control) method[49] implemented in the GenABEL software (version 1.8-0).[50] This method is suitable for the analysis of data in which both ethnic admixture and close family relationships are present while successfully controlling the overall genomic inflation factor to an appropriate level to avoid false-positive results. Phenotypes are regressed on genotypes one SNP at a time in GRAMMAR-GC in three steps. First, phenotypes were corrected by accounting for population stratification and familial dependence among individuals by using the genomic kinship matrix (pairwise kinship coefficients amongst sampled individuals estimated on the basis of genome-wide SNP data). These correlation-free residuals were then used as dependent quantitative traits for association analysis of each SNP using a linear regression model. Finally, genomic control (GC) was applied to correct the test statistic using the genomic inflation factor (λ), which is the regression coefficient of the observed statistic on the expected statistic. We used a genome-wide significance threshold of 5×10^{-8}. Additional loci with a P-value $\leq 10^{-6}$ were listed as suggestive. The quantile-quantile (QQ) plot of the test statistic was visually inspected and the genomic inflation factor λ was calculated to evaluate potential inflation of the test statistic due to sample relatedness and population structure.

Gene mapping and biological prioritization

Loci that showed evidence of association at $P \leq 5 \times 10^{-6}$ with the deformability of circulating RBC (as assessed by microsphiltration, after adjustment for relevant covariates) were mapped to genes using the SNP2GENE tool implemented in FUMA (http://fuma.ctglab.nl/[51]), a web plateform designed for post-processing of GWAS results and prioritizing of genes. FUMA annotates candidate SNPs in genomic risk loci and subsequently maps them to prioritized genes based on (i) physical position mapping on the genome, (ii) expression quantitative trait loci (eQTL) mapping, and (iii) 3D chromatin interactions (chromatin interaction mapping). It incorporates the most recent bioinformatics databases, such as Combined Annotation-Dependent Depletion score (CADD score[52]), regulatory elements in the intergenic

region of the genome (RegulomeDB[53]), Genotype-Tissue Expression (GTEx), and 3D chromatin interactions from Hi−C experiments.[54]

We considered as genomic risk loci those with at least one SNP (lead SNP) with a *P*-value of association $\leq 10^{-6}$. In the initial step, SNPs in LD ($r^2 > 0{\cdot}1$, estimated from the African reference panel of KGP phase 3) in a 250 kb window were considered as belonging to the same risk locus. Thereafter, a set of candidate SNPs were selected, based on LD ($r^2 > 0{\cdot}6$) with the lead SNP (defined as SNP with the lowest *P* value) from either our data set or from the African population data of KGP phase 3. If two independent significant lead SNPs ($r^2 < 0{\cdot}1$) were close to each other (significant loci of interest less than 250 kb apart), the two regions were merged into a single significant region. The major histocompatibility complex (MHC) region was excluded from analysis.

Positional mapping was performed using maximum distance from SNPs to gene of 10 kb and on the basis of annotations obtained from ANNOVAR. Criteria to define deleterious SNPs were a CADD score >12·37 which indicates potential pathogenicity or an RDB score ≤ 4 which indicates that the SNP likely lies in a functional location (categories 1 and 2 of RegulomeDB classification identifed as "likely to affect binding").

EQTL mapping was performed using eQTL data from tissue types considered as relevant for our phenotype of interest. The selection included Alasoo 2018 macrophage; BLUEPRINT monocyte, neutrophil and T-cell; CEDAR B-cell CD19, monocyte CD14, neutrophil CD15, T-cell CD4, T-cell CD8, and platelet; Fairfax 2012 B-cell CD19; Fairfax 2014 IFN24; GENCORD T-cell; Kasella 2017 T-cell CD4 and T-cell CD8; Lepik 2017 blood; Naranbhai 2015 neutrophil CD16; Nedelec 2016 macrophage; Quach 2016 monocyte; TwinsUK blood; van der Wijst scRNA eQTLs (9 tissues); DICE (15 tissues); Westra et al (2013) Blood eQTL Browser; Zhernakova et al (2017) BIOS QTL Browser; eQTLGen (cis and trans eQTLs); GTEx (Genotype-Tissue Expression) v8 blood, blood vessel and spleen tissues. Only significant SNP-gene pairs (with a false discovery rate (FDR) $\leq$ 0.05) were selected.

Chromatin interaction mapping was performed from Hi-C experiments data of three tissues identifed as relevant for our phenotype of interest: aorta, spleen and mesenchymal stem cell (GSE87112), as well as from the FANTOM EP correlations cell type and organ databases. Candidate genes were selected whenever their promoter region (250bp upstream and 500bp downstream of the TSS) belonged to a locus significantly interacting with candidate lead SNPs or SNPs in LD with them in the selected tissues. Additional filtering was performed to restrict the candidate genes (i) to those with a promoter region associated with SNPs overlapping predicted enhancer regions in selected epigenomes and (ii) to those having promoter regions overlapping predicted promoter regions in selected epigenomes. Filtering was restricted to specific epigenomes: blood (23 distinct tissues), spleen and aorta (E065). FDR was used to correct for multiple testing (FDR $< 1.10^{-6}$ for chromatin interaction, the default value in FUMA).

Functional annotation and prediction of SNPs and genes

Annotation of SNPs associated with RBC deformability was conducted using FUMA and the Variant Annotation Integrator tool.[55] The selected lead SNP from each associated locus was annotated using a deleteriousness score (CADD score), a score assessing potential regulatory functions (RegulomeDB score), and the Genomic Evolutionary Rate Profiling (GERP) score.[56] CADD scores of >10 are predicted to be the 10% most deleterious possible substitutions in the human genome, of >20 are predicted to be the 1%, and of >30 are predicted to be the 0·1%. RegulomeDB score is a categorical score from 1a to 7: SNPs showing the strongest evidence of being regulatory (affecting the binding of transcription factor) are given a score of 1, SNPs demonstrating the least evidence of being functional are given a score of 6, and 7 means that those SNPs do not have any annotations. GERP score ranges from −12·3 to 6·17, where higher scores indicate higher evolutionary constraint and a score greater than 2 can be considered constrained.

To further investigate the functional role of genes most associated with RER values, we searched for known functions in the gene database of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/gene/); each gene name, and its potential aliases (according to GeneCards, https://www.genecards.org/), was also entered into a PubMed search through the following algorithm: (GENE) AND ((malaria) OR (Plasmodium) OR (spleen) OR (red blood cell) OR (erythrocyte) OR (smooth muscle) OR (smooth muscular cell) OR (endothelium) OR (endothelial cells) OR (macrophage)) to identify any known association between these genes, malaria, and processes involved in RBC production or clearance, with a focus on cellular and biomechanical control of red blood cell deformability. In case of association between the considered gene and endothelial physiology, search was focused on articles mentioning the spleen. Regarding association with macrophage, we focused on articles mentioning their phagocytic function. We ranked genes according to the number and relevance of potential links between known gene functions and observed phenotypic features (namely, RBC deformability, splenomegaly, and innate processes involved in the quality control of RBC), as follows: no link (red boxes in Supplementary Table 4), potential and indirect link (orange boxes), direct link (green boxes). The relative abundance of each gene transcript in the spleen was estimated through RPKM, retrieved from the NCBI database.

Gene-Based Association Testing

Gene-based association analysis was conducted using MAGMA through FUMA.[51] To identify candidate genes associated with RBC deformability (assessed by RER values adjusted for relevant covariates), the mean association of all SNPs within a gene was calculated, accounting for LD. Gene windows were extended 2kb upstream and 1kb downstream of the annotated gene start and end sites to include regulatory regions.

SPARC splenic immunohistochemistry

Immunohistochemistry was performed on normal splenic tissue retrieved from routine surgery, and performed as described,[57] using a rabbit IgG anti-human SPARC antibody (ab14174, AbCam), that was used as per manufacturer's protocol (RRID : AB_300961).

Statistics

Binary variables were compared between groups using Chi-squared or Fisher's exact test, as appropriate. Continuous variables were compared through Student's t-test or Mann-Whitney test, when comparing 2 groups; and through ANOVA or Kruskal-Wallis test, when comparing more than 2 groups, as appropriate. Normal distribution of continuous variables was assessed through visual inspection of the distribution histogram, along with the D'Agostino-Pearson test. Multivariate analyses were performed using regression models, logistic or linear according to the dependent variable. Variables with *P*-value<0·2 in univariate analysis were included in multivariate models, along with variables with potential strong interaction with the results, based on pathophysiological findings. All tests were 2-sided and a *P*-value lower than 0·05 was considered significant. Analyses were performed with GraphPad Prism 6, R (version 3.4.4), and RStudio (version 1.1.442) softwares.

Ethics

The BAObAB (Biocultural AdaptatiOn to malaria in Atacora, north Benin) research program was reviewed and approved by the ethics committee of the Research Institute of Applied Biomedical Sciences (CER-ISBA/Institut des Sciences Biomédicales Appliquées) in Benin (No 61/CER/ISBA/15). It received support from the Ministry of Health of Benin and has been carried out in close collaboration with the National Malaria Control Programme. All study participants were informed during meetings conducted in villages before the beginning of the study. Individual written informed consent was obtained from adults or from the parents or guardians of children, using a consent form translated in the subject's native language (Ditamari, Bariba or Fulani).

Role of funders

The funders had no role in the study design, data collection, data analysis, data interpretation. BH, AS and PB had full access to study data, were responsible for writing of the manuscript and for the decision to submit it.

Results

Splenomegaly is more frequent in Fulani

In a cross-sectional study, we examined 521 subjects (mean age, 21·3 +/- 17·6 years; range, 3 months-72 years; 47% female) of whom 157 (30·1%) were Fulani, 85 (16·5%) Gando, 159 (30·5%) Bariba, and 119 (22·8%) Otamari (Table 1). For clarity, Gando, Bariba and Otamari

Characteristic	Fulani	Bariba	Gando	Otamari	Whole cohort	Missing data
Number of subjects	157	159	85	119	520	1 (Ethnicity missing)
Age, mean (SD)	21.3 (17.61)	21.02 (16.86)	20.96 (16.83)	20.79 (16.68)	21.3 (17.61)	55
Gender (% female)	49.22%	47.74%	49.41%	42.86%	47.2%	34
Fever (≥ 38°C)	2 (1.28%)	3 (1.92%)	2 (2.35%)	4 (3.36%)	11 (2.13%)	4
Splenomegaly	59 (37.58%)	38 (23.9%)	13 (15.29%)	32 (26.89%)	142 (27.31%)	0
Recent use of antimalarial	11 (7.48%)	13 (8.5%)	7 (8.54%)	12 (10.17%)	43 (8.58%)	20
Anaemia	62 (60.2%)	77 (53%)	36 (45%)	57 (53%)	232 (53.58%)	88
Malaria RDT positivity	95 (63.76%)	90 (58.4%)	43 (51.19%)	68 (58.12%)	297 (57.56%)	5
Positive thick smear (with *P. falciparum*), n (%)	62 (44.9%)	64 (42.11%)	19 (33.93%)	45 (47.37%)	189 (43.15%)	36
Parasite density (*P. falciparum*), median (IQR)	2500 (630-5200)	3089 (620-6912)	2520 (267.5-11867)	4200 (1200-12382)	2435 (600-5920)	36

***Table 1*: Clinical and biological characteristics of 521 subjects from the BAObAB cohort, enrolled in the December, 2017 cross-sectional study. The four ethnic groups are described separately. Splenomegaly was defined as any palpable spleen. Qualitative data are described as numbers and percentages. Quantitative data are described as mean (standard deviation, SD) or median (interquartile range, IQR), as appropriate. Recent use of antimalarial was defined as receiving any antimalarial within the last month. RDT: rapid diagnostic test.**

were generally pooled in the non-Fulani group. Splenomegaly was more prevalent in Fulani than in non-Fulani (37·6% vs 22·9%; P = 0·0008, Fisher's exact test; Figure 2a). There was a not statistically significant trend towards more prevalent anaemia in Fulani (60·2% vs 51·5%, P = 0·12, Fisher's exact test; Figure 2b). Fever was rare in Fulani and non-Fulani (1·3% vs 2·5%, Fisher's exact test; P = 0·52). Parasite carriage was comparable in Fulani and non-Fulani (63·8% vs 56·6% for rapid diagnostic test (RDT) positivity, P = 0·14 (Chi-squared test), and 44·9% vs 42·1% for thick smear, P = 0·57 (Chi-squared test), Figure 2c-d). Median *P. falciparum* densities were insignificantly lower in Fulani than in non-Fulani (2500 vs 3440/μl, P = 0·28, Mann-Whitney test, Figure 2e 1-3). Antimalarials had been used in the last 30 days by 7·48% and 9·07% of Fulani and non-Fulani subjects, respectively (P = 0·57, Chi-squared test). In a multivariate analysis of factors influencing splenomegaly, we observed a strong association with the Fulani ethnicity (P = 0·0007), the other significantly associated factors being young age, low haemoglobin and RDT positivity (Supplementary Table 1). On a longitudinal analysis of 6 cross-sectional studies performed in the same cohort from November 2015 through December 2017, the proportion of 113 subjects who remained RDT-negative across time was greater in Fulani than in non-Fulani.

Circulating RBC are more deformable in Fulani than in other ethnic groups

Ektacytometry was performed on RBC from 248 randomly selected subjects (108 Fulani, 140 non-Fulani; 48·4% adults). Median elongation indexes of RBC measured at a shear stress of 30 Pascals (Figure 3a) were higher in Fulani than in non-Fulani (0·595 vs 0·589, P = 0·039, Mann-Whitney test). Differences were also significant when comparing the four ethnic groups separately (P = 0·0057, Kruskall-Wallis test; Supplementary Figure 2). Upon univariate analysis, splenomegaly and ethnicity were significantly associated with the Elongation Index of RBC (P = 0·026 and P = 0·013, respectively; Mann-Whitney test; Table 2). Upon multivariate analysis, only ethnicity remained significantly associated with the Elongation Index (P = 0·016; Table 2).

Microsphiltration was performed on blood samples from 463 subjects (149 Fulani, 314 non-Fulani; 47·1% adults). For the 16 microplates used, negative control values were within the expected +/- 20% range, with a mean (IQR) of -7·66% (-13·91%; -6·71%). All positive controls displayed retention rates > 75% with a mean (IQR) value of 96·47% (95·87%; 99·66%). Retention or Enrichment Rates (RER) were significantly and markedly different between ethnic groups. Fulani had higher median RER, indicating enhanced deformability of their circulating RBC, compared to non-Fulani (30·1% vs 11·73%, P < 0·0001, Student's t-test; Figure 3b). RBC deformability was highest in Fulani, lowest in Bariba and intermediate in Gando and Otamari (Supplementary Figure 2). In univariate analysis, RER was influenced by ethnicity, parasite density and RDT positivity ($P = 8{\cdot}1\times10^{-12}$, 0·046 and 0·008, respectively); in the multivariate analysis, factors still significantly affecting deformability were ethnicity and RDT positivity ($P = 8{\cdot}74\times10^{-11}$ and 0·016, respectively) (Table 2). In summary, circulating RBC were more deformable in Fulani than in subjects from other ethnic groups. Two different methods, microsphiltration and ektacytometry, displayed consistent results. In the multivariate analysis, apart from ethnicity, the only factor associated with the deformability of circulating RBC was a positive RDT.

Markers of infection with *P. falciparum* in Fulani are associated with a greater deformability of circulating RBC

As differences were greater with microsphiltration than with ektacytometry we focused further analyses on results obtained with this method, which had also been performed on a larger number of samples. When considering only subjects with a negative RDT, RBC deformability was mildly (and insignificantly) higher in Fulani than in non-Fulani (median RER: 14·04% vs 11·72%, P = 0·46, Student's t-test), meaning that deformability was not demonstrated to be higher or lower in a group than in the other. In subjects with a positive RDT, RBC deformability was markedly and significantly higher in Fulani than in other ethnic groups (median RER: 38·70% vs 11·96%, P < 0·001, Student's t-test). Within Fulani, RBC deformability was markedly greater in RDT-positive than in RDT-negative subjects (38·70% vs 14·04%, P = 0·0007, Student's t-test).

To analyse how markers of infection impact the deformability of circulating RBC, we then compared results from three groups, i.e. subjects in whom RDT and thick smears were both negative (a surrogate for absence of infection), subjects in whom RDT and thick smear were both positive (a surrogate for ongoing infection), and subjects in whom only the RDT was positive (a surrogate for recently resolved infection[58] or of major splenic tropism of parasitized RBC[23,24]). The very few subjects (4 Fulani and 7 non-Fulani) in whom only thick smear was positive were not included in this analysis. In the context of this framework, marked differences were observed between Fulani and other ethnic groups (Figure 3c). In the Fulani group, RBC deformability was lowest in subjects with negative markers of infection, intermediate in subjects with markers both positive (thick smear and RDT), and highest in subjects with a positive RDT and a negative thick smear (median RER: 16·63%, 36·97% and 53·55% for both negative, both positive, and only RDT positive subjects, respectively, P = 0·0022, ANOVA). By contrast, markers of infection

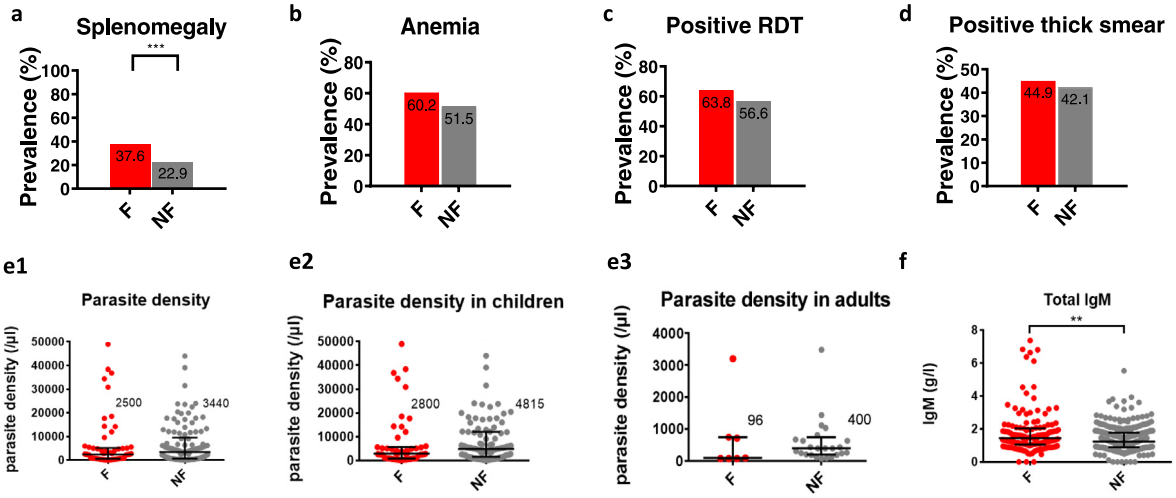


Figure 2. Splenomegaly is more prevalent in Fulani than in subjects from other ethnic groups. (a-d): Prevalence (%) of **(a)** splenomegaly, **(b)** anaemia according to the WHO definition, **(c)** positive rapid diagnostic test for malaria and **(d)** positive thick smears in Fulani (F) and non-Fulani (NF). **(e1)** *Plasmodium falciparum* density (median, IQR) in Fulani and non-Fulani, shown separately in children **(e2)** and adults **(e3)** on thick smears (only positive values are shown). **(f)** Concentration of total IgM (median, IQR) in plasma from Fulani and non-Fulani. *: 0·01<*P* value<0·05; **: 0·001<*P* value<0·01. No symbol is shown if the difference is not significant. Categorical variables were compared through Chi-squared of Fisher's exact test, as appropriate; parasite densities and total IgM values were compared through Mann-Whitney test. For each parameter, the number of individuals on which data were available was: splenomegaly, 521; anaemia, 508; positive rapid diagnostic test, 521; positive thick smear, 521; total IgM measurement, 419.

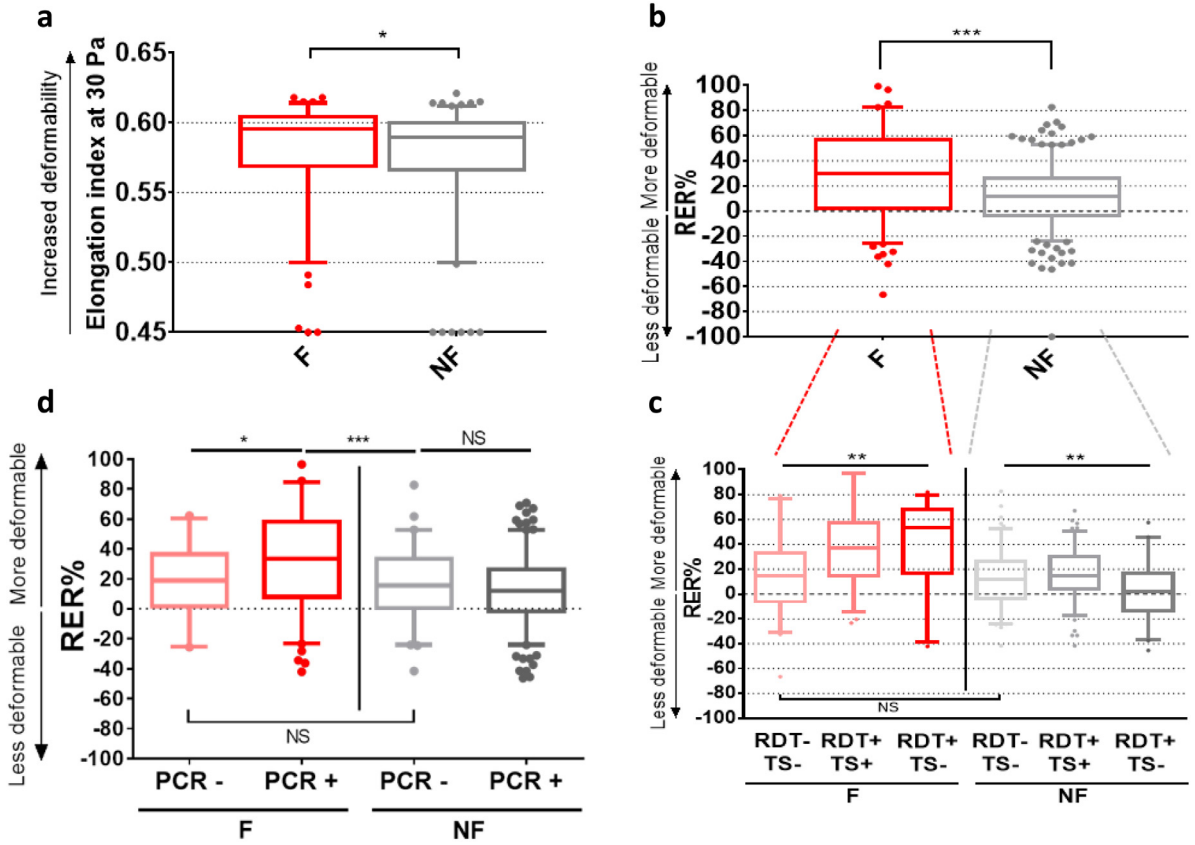


Figure 3. Circulating RBC are more deformable in Fulani than in subjects from other groups, and markers of recent *P. falciparum* infection are associated with enhanced RBC deformability in Fulani. (a) Elongation index (EI) of RBC measured by rotational ektacytometry at 30 Pa in Fulani (F) and non-Fulani (NF) (n = 248). Higher EI values indicate enhanced RBC deformability. **(b)** Retention or enrichment rates (RER%) of circulating RBC from Fulani (F) and non-Fulani (NF) determined by filtration through microsphere layers (microsphiltration) (n = 463). Positive RER (i.e., enrichment in test RBC downstream from filter) indicate enhanced RBC deformability (n = 463). **(c)** RER determined by microsphiltration in Fulani (n = 131) and non-Fulani (n = 282) subjects with both RDT and thick smear negative for malaria parasites (RDT- TS-), or with both tests positive (RDT+ TS+) or with only RDT positive (RDT+ TS-). **(d)** RER values in Fulani (n = 136) and non-Fulani (n = 271) when considering subjects with negative or positive PCR for *P. falciparum*. Box plots indicate median and IQR values; bars indicate 5th and 95th percentiles. NS: no significant difference; *: 0·01<P value<0·05; **: 0·001<P value<0·01; ***: P value<0·001. Ektacytometry data were analyzed through Mann-Whitney test; Microsphiltration data were analyzed through Student's t-test and ANOVA, as appropriate.

A	Ektacytometry (elongation index at 30 Pa)		
Parameter	Univariate analysis		Multivariate analysis
	Correlation or mean comparison	*P*-value	*P*-value
Age	0.055	0.430	
Haemoglobin	0.114	0.075	0.104
Anaemia	0.571 (vs 0.581)	0.575	
Splenomegaly	0.577 (vs 0.575)	0.026	0.640
RDT positivity	0.579 (vs 0.571)	0.369	
Parasite density	0.062	0.348	
Ethnicity		0.013	0.016
Temperature	-0.052	0.422	

B	Microsphiltration (retention or enrichment rate)		
Parameter	Univariate analysis		Multivariate analysis
	Correlation or mean comparison	*P*-value	*P*-value
Age	-0.040	0.409	
Haemoglobin	0.003	0.944	
Anaemia	17.123 (vs 15.042)	0.451	
Splenomegaly	17.471 (vs 16.341)	0.702	
RDT positivity	20.060 (vs 12.885)	0.008	0.016
Parasite density	0.096	0.046	0.149
Ethnicity		8.1×10^{-12}	8.74×10^{-11}
Temperature	-0.018	0.703	

***Table 2*: Univariate and multivariate analysis of factors associated with RBC deformability assessed by ektacytometry (A: upper panel) or microsphiltration (B: lower panel). Variables with a *P*-value of 0.2 or less in univariate analysis were included in the multivariate analysis (multiple linear regression). Variables with a significant association with deformability measures after multivariate analysis are displayed in italic.**

correlated only mildly with RBC deformability in other ethnic groups (Figure 3c). PCR performed on a 407 subjects showed similarly that circulating RBC are more deformable in PCR-positive than in PCR-negative Fulani (RER=33·29% vs 18·91%, $P = 0{\cdot}02$, Student's t-test, Figure 3d). No significant difference was observed when comparing PCR-negative and PCR-positive subjects from non-Fulani groups (RER=11·85% vs 15·55%, $P = 0{\cdot}19$, Student's t-test, Figure 3d).

Serum concentrations of total IgM are higher in Fulani than in non-Fulani

Total plasma IgM values (Figure 2f) were determined in 421 subjects. Median values were 1·41 g/L, and 1·57 g/L in non-Fulani and Fulani, respectively ($P = 0{\cdot}009$, Mann-Whitney test).

A strong genetic component determines RBC deformability in Fulani

In a model incorporating household effects, the global heritability (h^2) of RBC deformability for the whole sample of 420 subjects was 0·36 (likelihood ratio test, $P = 0{\cdot}007$). A stratified analysis by ethnicity revealed striking differences between Fulani and non-Fulani, with a very high and highly significant heritability estimate in the 135 Fulani (h^2=0·71, $P = 0{\cdot}0028$, likelihood ratio test) and a near zero and nonsignificant one in the 285 non-Fulani (h^2=0·01, $P = 0{\cdot}49$, likelihood ratio test) (Figure 4a). The variance component attributable to shared household environmental factors (c^2) was only significant in non-Fulani (c^2=0·30, $P = 0{\cdot}02$, likelihood ratio test). These results are consistent with the patterns of phenotypic correlations among relative pairs observed in Fulani, with correlations being highest between first-degree relatives, with similar correlation values between siblings and parent–offspring pairs (r=0·65, $P < 10^{-5}$ and r=0·51, $P = 0{\cdot}005$, respectively, correlation test), lower but still significant between second-degree relatives (r=0·37; $P = 0{\cdot}015$, correlation test) and even lower and not significant between more distant relatives (r=0·23; $P = 0{\cdot}22$, correlation test) (Supplementary Table 2). This supports the existence of a strong, mainly additive, genetic component determining the deformability of circulating RBC in Fulani.

In the 160 subjects without markers of infection (thick smear and RDT both negative), the heritability of RBC deformability was almost identical to that observed in the whole cohort (h^2=0·38, $P = 0{\cdot}15$, likelihood ratio

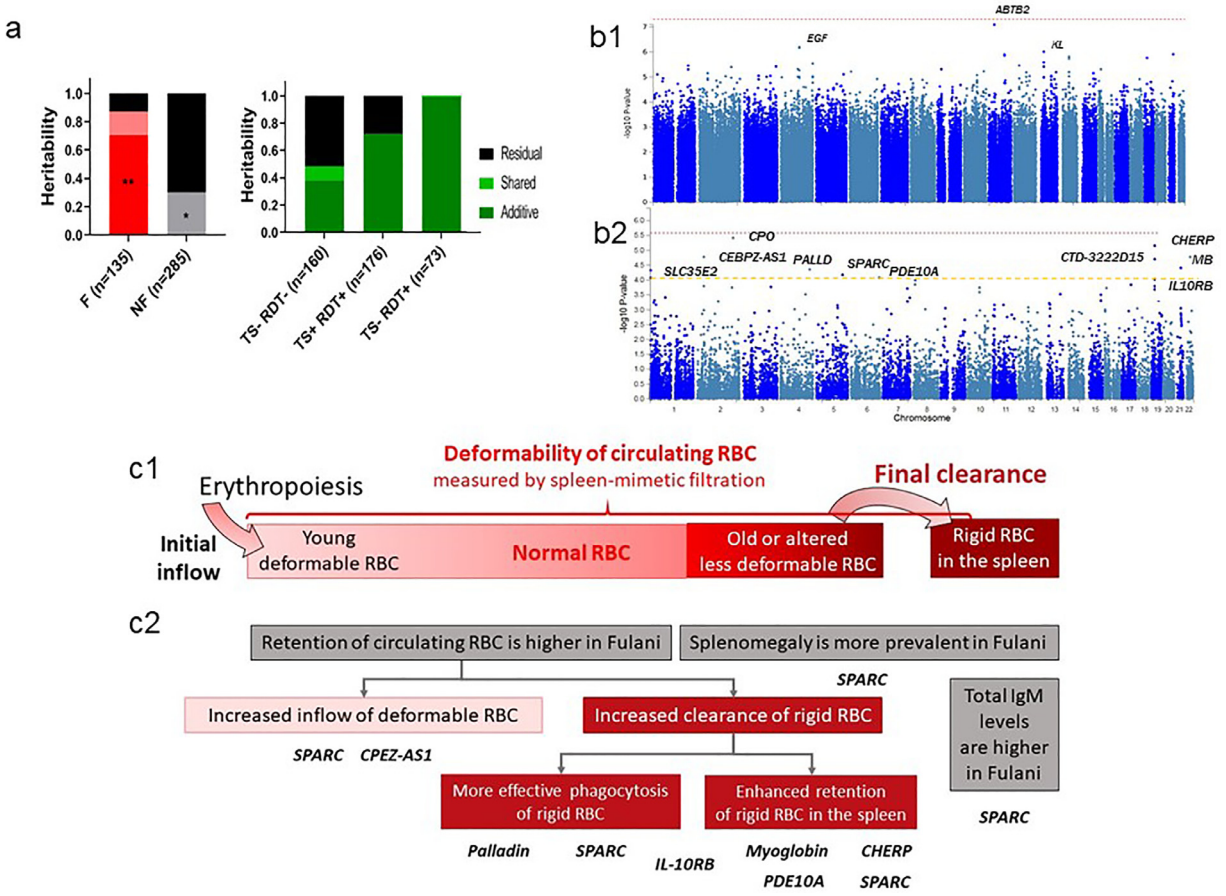


Figure 4. (a) Heritability of splenic RBC filtration. Using a variance component approach, the heritability of the deformability of circulating red blood cells (RBC) was determined either in relation with ethnicity in Fulani (F) or non-Fulani (NF) (left) or in the whole population depending on the status of *P. falciparum* infection (right). Additive heritability is estimated as the ratio of the additive genetic variance component to the total phenotypic variance. *P*-values were estimated using likelihood ratio tests. n: number of subjects; *: 0·01<*P* value<0·05; **: 0·001<*P* value<0·01; ***: *P* value<0·001; TS: thick smear; RDT: malaria rapid diagnostic test. Analysis was performed on 420 subjects. **(b): Genome-wide association analysis for RBC deformability.** (*n* = 323) **(b1)** Each point of the Manhattan plot shows a single-marker variant, with genomic positions across chromosomes on the *X*-axis, and the association level corrected by genomic control on the *Y*-axis. The red dashed line shows the genome-wide significant *P*-value of 5×10^{-8}. **(b2):** Gene-based test as computed by MAGMA. Each point of the Manhattan plot indicates a gene. Input SNPs were mapped to 19185 protein-coding genes. Genome wide significance (red dashed line in the plot) was defined at $P = 0{\cdot}05/19185 = 2{\cdot}606 \times 10^{-6}$. **(c1)** Determinants of retention upon splenic filtration of circulating RBC. **(c2)** Malaria-related phenotypic features observed in Fulani (grey boxes), potential mechanisms underlying the increased retention of circulating RBC in Fulani (red boxes), and genes potentially involved in these mechanisms as per literature analysis, the specific mechanisms being indicated in details in Supplementary Table 4 (Bold letters).

test) but raised to 0·72 ($P = 0{\cdot}0002$, likelihood ratio test) in the 173 subjects with both markers positive, and to 0·99 with wide dispersion ($P = 0{\cdot}07$, likelihood ratio test) in the 73 subjects with a positive RDT and a negative thick smear. This indicates that variations in the deformability of circulating RBC are under a strong influence of genetic determinants in Fulani and in subjects with positive markers of infection. The heritability of RBC deformability in Fulani with a positive RDT reached the maximum value of 1 ($P < 10^{-5}$, likelihood ratio test) with a household effect estimated to be zero.

Genome-wide association study of red blood cell deformability

Association of RBC deformability (through RER, adjusted for relevant covariates) with 2,281,899 high-quality SNPs, genotyped in 323 individuals, was evaluated genome-wide through a linear mixed model implemented in GenABEL.[50] The λ genomic inflation factor (ratio of the median of the observed distribution of the test statistic to the expected median) was 0·99997, consistent with a reliable adjustment for sample relatedness and population structure (Supplementary Figure 3). No variant reached the genome-wide significance threshold

of 5×10^{-8} (Figure 4b1). However, three independent genomic loci on chromosomes 4, 11 and 13 showed suggestive association with RBC deformability (*P*-value $\leq10^{-6}$), one of them being very close to the genome-wide significance threshold (SNP rs149514849 located at 11p13, close to gene *ABTB2*, *P*-value=$8{\cdot}08\times10^{-8}$; Table 3). None of the three lead SNPs in the three genomic risk loci were annotated as having predicted functional effects according to the three functional scores considered (CADD, RegulomeDB and GERP). However, according to ENSEMBL, the lead SNP rs149514849 is located on the binding site of a transcription factor, and has been associated with variations of the expression of CD44 in macrophages. Additionally, the lead SNP rs76559378 at 13q13 is in strong LD (r^2 = 0·897) with the exonic *KL* SNP rs201936594 (same GWAS, $P = 9{\cdot}74\times10^{-7}$) which displays a CADD score of 13·94, suggesting a likely deleterious effect. No other variant in the three associated genomic loci was annotated as functional. No significant association between our phenotype of interest and *ABTB2, EGF* and *KL* genes was found in the literature.

To link the variants associated at $P < 5\times10^{-6}$ to genes, we applied three gene-mapping strategies implemented in FUMA.[51] These three methods identified a total of 25 genes (12, 7 and 11 by positional mapping, eQTL mapping and chromatin interaction mapping, respectively), three of which were identified by two mapping methods (*CHERP, CTD-3222D19* and *ABCC1*) and only one (*SLC35E1*) by the three (Supplementary Table 3). A thorough literature review did not find any relevant association between any of these 4 genes and mechanisms potentially underlying the newly defined phenotype. Regarding the remaining 21 genes, few associations were found in the available literature, all for genes identified through chromatin interaction mapping (Supplementary Table 4).

Gene-based association testing was then performed with MAGMA,[59] which uses the mean association signal from all SNPs within each gene while accounting for LD. A total of 19,185 genes were analyzed. A multiple testing-corrected *P* value threshold of $2{\cdot}61\times10^{-6}$ was applied to identify genes significantly associated with RBC deformability. No gene score was above this significance threshold (Figure 4b2; see the corresponding quantile-quantile plot in Supplementary Figure 3b). Nevertheless, we analysed the top ten genes that displayed *P*-values between $3{\cdot}84\times10^{-6}$ and $8{\cdot}25\times10^{-5}$ (Figure 4b2 and Supplementary Table 4). A thorough search and analysis of available information was performed to identify potential associations between these 10 genes and malaria, erythrocytes biology, spleen physiology, smooth muscle, endothelium, and macrophages (Supplementary Table 4). According to the Gene database of the National Center for Biotechnology Information, 5 of the top 10 genes (*CHERP, CEBPZ-AS1, IL10RB, PALLD, SPARC*) displayed a significant splenic

Genomic risk loci	Position (start-end in hg19)	No. GWAS SNPs	Lead SNP (rsID)	Non-effect allele	Effect allele	MAF	GWAS *P*-value	β	SE	Location	Nearest gene	CADD score	Regulome DB score	GERP score
11p13	34385832	1	rs149514849	C	T	0.0211	8.08×10^{-8}	-2.168	0.441	Intergenic	*ABTB2*	1.853	4	-2.5
4q25	110854595-110907748	6	rs528481745	G	A	0.0477	6.35×10^{-7}	-1.653	0.362	Intronic	*EGF*	2.394	6	-0.35
13q13	33567899-33590718	12	rs76559378	G	T	0.0446	9.74×10^{-7}	1.560	0.347	Promoter flanking region	*KL*	0.729	7	-2.34

Table 3: **Independent genomic risk loci suggestively associated (*P*-value $\leq 10^{-6}$) with RBC deformability (RER values adjusted for relevant covariates) in genome-wide association analysis. MAF, minor allele frequency; β is the estimate of the SNP effect under an additive model; CADD, Combined Annotation-Dependent Depletion score; GERP, Genomic Evolutionary Rate Profiling.**

expression (RPKM values ranging from 10·66 to 123·8). Immunohistochemistry performed on a sample of human spleen showed the presence of SPARC within the red pulp, especially at the sinus wall (Supplementary Figure 4) and in smooth muscular cells of arteries.

Discussion

Malaria is an archetypical example of how an infectious disease has shaped the human genome, and haemoglobin S mutation is the first genetic trait robustly shown to innately protect against a microbial agent.[1] Recently, polymorphisms in the Dantu blood group were related to protection against severe malaria[60,61] through a reduction of RBC invasion linked to increased membrane tension.[62] Natural resistance to malaria however may extend well beyond RBC polymorphisms and we show here that a peculiar malaria-related phenotype observed in African subgroups likely results from spleen-related mechanisms. Splenomegaly, anaemia and hyper-IgM are indeed prevalent in Fulani and we found circulating RBC to be more deformable in this group. These original results were obtained by filtering RBC in a spleen-mimetic way and no known RBC-related trait was associated with this new cell-based, highly inheritable phenotypic trait. Our preliminary genome-wide association study points to several genes expressed in the spleen and potentially involved in the clearance of RBC, one of which, *SPARC*, has previously been linked to splenomegaly and IgM production. Taken together, these results suggest that the mechanisms underlying the peculiar, "malaria-hyperreactive" phenotype of Fulani, shared by other groups worldwide,[63] may stem from innate processes driving more altered and infected RBC to the spleen, thereby increasing the antigenic stimulus to this immunologically reactive organ. Recent major observations[23,24] in Indonesia have indeed shown that in asymptomatic carriers of malaria parasites, far more intact infected RBC are in the spleen than in the peripheral blood. The original reactivity to malaria in some subgroups may therefore be only partially immunological in essence, and also depend on microvascular and interstitial processes that regulate the filtration of RBC. Hyper-stringent filtration of RBC by the human spleen parsimoniously explains the original splenomegaly-anaemia-Hyper-IgM syndrome of Fulani.

Assuming that mature RBC cannot autonomously modify their deformability, an increase in the deformability of circulating RBC can result either from an inflow of more deformable cells or from the clearance of the less deformable cells (Figure 4c1-2). Acute symptomatic malaria induces an inflow of infected and modified uninfected RBC in the circulation[22,26] which are less deformable than normal RBC,[25–27,29,31,64,65] but the situation is different in our study where almost all subject were asymptomatic with negative or low parasitaemia. Therefore, in subjects from our cohort, an inflow of new RBC would rather come from erythropoiesis, and is expected to be small. The maximal previously observed inflow is a peak 7% reticulocytaemia in subjects recovering from an acute, anemic malaria attack.[66–68] When comparing parasite-positive with parasite-negative Fulani, the difference in the proportion of circulating rigid RBC was greater than 20%. Therefore, the inflow of new RBC from erythropoiesis is unlikely to explain the malaria-related enhancement of RBC deformability in Fulani. The measured hyperdeformability of circulating RBC observed in Fulani thus likely increases essentially in reaction to an enhanced clearance of the least deformable subset of these circulating RBC. The major organ that controls RBC deformability is the spleen. Several RBC labelling studies in humans[67,69–71] have shown that the clearance of RBC with either mechanical or surface alterations is enhanced in *P. falciparum*-infected subjects, very strongly so when splenomegaly is present.[69] The "hungry" post-malarial spleen even displays an increased clearance of normal RBC.[68,71] Taken together, these observations suggest that, in Fulani, splenic filtration of RBC is enhanced, with strong association with the presence of parasite antigens in circulation. Interestingly, when malaria parasites intensely accumulate in the spleen, as recently uncovered,[23,24] very few parasites circulates but parasite antigens are released in plasma and captured by RDT.

If Fulani display indeed a particularly stringent splenic filtration of RBC, this not only explains the higher deformability of their circulating RBC, but also partial antimalarial protection, and the more prevalent anaemia and splenomegaly, three hallmarks of the malaria-related 'Fulani phenotype'.[5] This new 'stringent splenic filtration' phenotypic trait is expected to be partially protective by clearing part of the circulating parasite load[29] and pathogenic by precipitating splenomegaly and anaemia.[22,30] The deformability of ring-infected and that of uninfected RBC overlap,[25] so that protective retention of a high proportion of infected RBC would also precipitate anaemia, caused by an enhanced retention of uninfected RBC in the spleen. Enhanced retention of RBC also cause congestive splenomegaly observed indeed in a recent splenectomy study in a malaria-endemic area (Kho *et al.*, in preparation). This accumulation of infected RBC in the spleen of Fulani perfectly matches the frequent discrepancy between a positive RDT and negative thick smear displayed by subjects who had the most deformable RBC in circulation, reflecting the most stringent splenic retention. Splenic parasites release indeed antigens visualized by RDT but do not circulate hence the negative smear. Not least, by dispatching more parasites to the responsive splenic environment,[72] malaria-enhanced splenic filtration of infected RBC would also explain the strong immunological stimulation observed

in Fulani.[4,6,9,18,19] Higher circulating IgM levels observed previously[8] and confirmed in this study, fit a predominantly splenic stimulation, as many IgM-producing cells are in the spleen.[73] Interestingly, antibody responses against *Helicobacter pylori* or rubella virus are not greater in Fulani than in other groups[8] suggesting that the enhanced stimulation in this group is specific to *Plasmodium*. The mechanism of this microbe-specific response had not been identified yet and is explained by our model. Along these lines, the proposed 'stringent splenic filtration' trait is reminiscent of hyper-reactive malarial splenomegaly (HMS),[3] a syndrome defined by gross splenomegaly, elevated antibody concentrations to *P. falciparum*, low parasitemia, and elevated total IgM levels.[74] Hyper-reactive malarial splenomegaly is more frequent in Fulani than in other groups,[3,5,7] and may be the chronic complication of repeated episodes of hyper-reactive splenic filtration in infected subjects. Chronic antigenic stimulation of the spleen is a convincing explanation for the link between hyper-reactive splenomegaly and marginal zone splenic lymphoma.[75] Our phenotypic observations in Fulani are however milder than HMS, as only very few subjects had a full-blown HMS with gross splenomegaly and very high IgM levels.

This study is not devoid of weaknesses and some of our assumptions would benefit from more direct demonstrations. We provide no direct experimental evidence that the malaria-enhanced clearance of less deformable RBC is operated by the spleen. We nevertheless favour a spleen related process because ektacytometry results were significant only at 30 Pa, a surrogate for shear stress in the splenic red pulp,[36] and because ethnicity- and infection-related differences in RBC deformability were the most pronounced with microsphiltration, a mimic of splenic retention of RBC.[31] This assumption is also based on reports of experimental transfusion of malaria patients with labelled RBC showing increased RBC retention during or after malaria attacks.[69–71] Not least, that several genes selected by our preliminary GWAS approach are expressed in the spleen, which strengthens the suspicion of a spleen-related process. The mechanisms underlying the putative malaria-enhanced splenic hyperfiltration in Fulani are currently unknown and difficult to explore directly. Contrast-enhanced ultrasonography can quantify the proportion of splenic blood flow to the slow, filtering compartment[29] but can hardly be performed in asymptomatic naturally infected subjects in remote areas. Direct analysis of human spleen samples following splenectomy or autopsy in West Africa would bring relevant information but requires considerable resources. Currently available mouse models of malaria are not adapted to these explorations. Mechanical processes affecting *Plasmodium*-infected RBC display indeed marked differences between human and rodent parasite species, and humanized mice infected with *P. falciparum* have a defective splenic filtration, as illustrated by the presence of stiff mature asexual stages and immature gametocytes in circulation.[76,77] Regulation of the width of inter-endothelial slits likely exists in humans[78,79] but exploring it directly is beyond the scope of current experimental methods. Recently, natural selection on genetic variants in the *BDKRB2* and *PDE10A* genes has been shown to affect the human ability to withstand diving-related hypoxia likely through pooling of RBC in the spleen.[80] This strongly supports the existence of genetically-driven mechanisms that regulate splenic filtration of RBC. Of interest, *PDE10A* is among the top-10 genes revealed by gene-based testing in our preliminary GWAS. Future replication studies in other areas of Benin and in other West African countries should soon extend and refine the findings reported here.

Due to recruitment constraints (for safety reasons, North Benin has become incompatible with field studies since 2018), our GWAS did not reach a fully accurate power. In keeping with previous reports in Fulani,[81] none of the usual genetic polymorphisms associated with protection from malaria, such as haemoglobinopathies or blood groups, had high scores. Nevertheless, several genes warranting further investigations were identified. Our lead SNP (rs149514849) may influence the expression of CD44, a pleiotropic glycoprotein involved in cell-cell interactions, cell adhesion and migration, and has a role in phagocytosis by macrophages.[82,83] Through our mapping strategy, we also identified CD59, whose expression is decreased on *Plasmodium*-infected erythrocytes, which may promote erythrophagocytosis.[84] LMO2 enhances erythropoiesis,[85] and IL1RAP upregulates the expression of the "don't eat me" signal CD47 to inhibit macrophage phagocytosis.[86] Of the 10 genes the most significantly associated with RBC deformability in the gene-based tests, 5 (*CHERP, CEBPZ-AS1, IL10RB, PALLD, SPARC*) are highly transcribed in the spleen (detailed in Supplementary Table 4). Four selected genes (*CHERP, MB, PALLD, PDE10A*) play a role in smooth muscular contraction and thus may regulate the dispatching of splenic blood flow to the slow filtering circulation of the spleen red pulp, as well as the opening of splenic inter-endothelial slits where altered and parasitized RBC are mechanically trapped.[29,78,87] SPARC has been involved in vascular smooth muscle cells proliferation,[88] is expressed on splenic sinus cells (Supplementary Figure 4 and[89]) and impacts the production of collagen IV,[90] a major component of filaments surrounding splenic sinuses.[91] Like other selected genes, it may therefore impact the regulation of RBC filtration by the spleen, but SPARC is also linked to the recruitment of macrophages, that eliminate altered RBC, and SPARC-null mice have an altered splenic marginal zone and B-cell response,[89] including abnormal levels of IgM.

Hyper-reactive filtration of RBC by the spleen is the most parsimonious explanation for a new phenotypic

trait in Fulani where, in addition to well-known over-prevalence of splenomegaly and anaemia, we reveal that circulating RBC are more deformable, almost exclusively in subjects with markers of ongoing low-grade (likely intrasplenic) accumulation of parasites,[23,24] or recent parasite carriage. Beyond the understanding of the malaria-related phenotype in Fulani, infection-related splenic hyper-filtration of RBC may contribute to the pathogenesis of other RBC diseases. For example, splenic sequestration crises in sickle cell disease - the acute trapping of RBC in the spleen - often follow acute episodes of fever or infections.[92] The potential impact of RBC filtration on antigen dispatching in the spleen also deserves consideration as it may display a major impact on the understanding of immunization by intravascular pathogens.

Contributors

A.S., A.G., P.B. and B.H. designed the study; B.H., G.V., C.R., P.-A.N., A.F., C.C., J.S., J.N., C.-H.C., F.A., E.C., R. A., N.F., J.C., A.S. and P.B. performed the experiments; H.A., A.S., A.G., M.G., F.T., D.C., J.C., P.B., and B.H. collected clinical data; F.P., L.G., C.K. and A.S. analyzed genetic data; M.T. and D.H. performed splenic immunohistochemistry; F.S., M.M. and O.H. provided scientific insights to experimental design; B.H. and P.B. wrote the initial draft of the manuscript. B.H., A.S. and P.B. had full access to study data and verified the data. All authors have critically reviewed and approved the final version of the manuscript.

Data sharing statement

The data sets generated and analyzed during the current study are available from the DataSuds repository (https://doi.org/10.23708/KDRQMQ). The data set is not publicly available due to patient confidentiality but are available for researchers who meet the criteria for access to confidential data. All other relevant data are available within the manuscript and its Online Resource files.

Declaration of interests

Dr Clain reports receiving a grant from the French national research agency (Grant ANR-17-CE15-0013-03) to conduct research (materials, equipment, post-doc salary) on artemisinin resistance in malaria parasites. All the other authors have declared no conflict of interest.

Acknowledgements

We thank all the subjects who participated in the study; Michaël Dussiot, Mickaël Marin and Nathalie Chartrel for outstanding technical support; our research team in Benin for excellent technical and logistical achievements: Séverin Vignigbe, Pépin Kounou, Sabi N'Dah Kouagou, Wilson Houssou; Jehane Fadlallah for her help in the recruitment of control patients; Nicolas Bréchot for his logistical help; Geneviève Milon, Adrian Luty for giving scientific guidance and participating in discussions during the course of this research. This research was supported by a grant from Labex GR-Ex and from the National Geographic Society Committee for Research and Exploration (Grant 9804-15). The labex GR-Ex, reference ANR-11-LABX-0051 is funded by the program Investissements d'avenir of the French National Research Agency, reference ANR-11-IDEX-0005-02. It also received funding from IMEA (Institut de Médecine et d'Epidémiologie Appliquée) and IRD (Institut de Recherche pour le Développement); BH was supported by a grant from Région Ile de France (DIM Malinf; grant dim150030).

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104167.

References

1 Mangano VD, Modiano D. An evolutionary perspective of how infection drives human genome diversity: the case of malaria. *Curr Opin Immunol.* 2014;30:39–47. https://doi.org/10.1016/j.coi.2014.06.004.
2 Bereczky S, Dolo A, Maiga B, et al. Spleen enlargement and genetic diversity of Plasmodium falciparum infection in two ethnic groups with different malaria susceptibility in Mali, West Africa. *Trans R Soc Trop Med Hyg.* 2006;100:248–257. https://doi.org/10.1016/j.trstmh.2005.03.011.
3 Bryceson AD, Fleming AF, Edington GM. Splenomegaly in Northern Nigeria. *Acta Trop.* 1976;33:185–214.
4 Dolo A, Modiano D, Maiga B, et al. Difference in susceptibility to malaria between two sympatric ethnic groups in Mali. *Am J Trop Med Hyg.* 2005;72:243–248.
5 Greenwood BM, Groenendaal F, Bradley AK, et al. Ethnic differences in the prevalence of splenomegaly and malaria in The Gambia. *Ann Trop Med Parasitol.* 1987;81:345–354.
6 Modiano D, Petrarca V, Sirima BS, et al. Different response to Plasmodium falciparum malaria in west African sympatric ethnic groups. *Proc Natl Acad Sci USA.* 1996;93:13206–13211.
7 Oomen JM, Meuwissen JH, Gemert W. Differences in blood status of three ethnic groups inhabiting the same locality in Northern Nigeria. Anaemia, splenomegaly and associated causes. *Trop Geogr Med.* 1979;31:587–606.
8 Bolad A, Farouk SE, Israelsson E, et al. Distinct interethnic differences in immunoglobulin G class/subclass and immunoglobulin M antibody responses to malaria antigens but not in immunoglobulin G responses to nonmalarial antigens in sympatric tribes living in West Africa. *Scand J Immunol.* 2005;61:380–386. https://doi.org/10.1111/j.1365-3083.2005.01587.x.
9 Arama C, Skinner J, Doumtabe D, et al. Genetic resistance to malaria is associated with greater enhancement of immunoglobulin (Ig)M than IgG responses to a broad array of plasmodium falciparum antigens. *Open Forum Infect Dis.* 2015;2:ofv118. https://doi.org/10.1093/ofid/ofv118.
10 Arama C, Maiga B, Dolo A, et al. Ethnic differences in susceptibility to malaria: what have we learned from immuno-epidemiological studies in West Africa? *Acta Trop.* 2015;146:152–156. https://doi.org/10.1016/j.actatropica.2015.03.023.
11 Arama C, Giusti P, Boström S, et al. Interethnic differences in antigen-presenting cell activation and TLR responses in Malian children during Plasmodium falciparum malaria. *PLoS One.* 2011;6:e18319. https://doi.org/10.1371/journal.pone.0018319.

12 Cherif M, Amoako-Sakyi D, Dolo A, et al. Distribution of FcγR gene polymorphisms among two sympatric populations in Mali: differing allele frequencies, associations with malariometric indices and implications for genetic susceptibility to malaria. *Malar J*. 2016;15:29. https://doi.org/10.1186/s12936-015-1082-8.
13 Creasey A, Giha H, Hamad AA, El Hassan IM, Theander TG, Arnot DE. Eleven years of malaria surveillance in a Sudanese village highlights unexpected variation in individual disease susceptibility and outbreak severity. *Parasitology*. 2004;129:263–271.
14 Dolo A, Maÿga B, Dara V, et al. [Place of malaria among febrile illnesses in two ethnic tribes living in sympatry in Mali from 1998 to 2008]. *Bull Soc Pathol Exot 1990*. 2012;105:377–383. https://doi.org/10.1007/s13149-012-0249-6.
15 Maiga B, Dolo A, Campino S, et al. Glucose-6-phosphate dehydrogenase polymorphisms and susceptibility to mild malaria in Dogon and Fulani, Mali. *Malar J*. 2014;13:270. https://doi.org/10.1186/1475-2875-13-270.
16 Dolo A, Maiga B, Tolo Y, et al. [Relationship between malaria and anemia in two ethnic groups living in sympatry in Mali]. *Bull Soc Pathol Exot 1990*. 2012;105:370–376. https://doi.org/10.1007/s13149-012-0265-6.
17 Boström S, Giusti P, Arama C, et al. Changes in the levels of cytokines, chemokines and malaria-specific antibodies in response to Plasmodium falciparum infection in children living in sympatry in Mali. *Malar J*. 2012;11:109. https://doi.org/10.1186/1475-2875-11-109.
18 McCall MBB, Hopman J, Daou M, et al. Early interferon-gamma response against Plasmodium falciparum correlates with interethnic differences in susceptibility to parasitemia between sympatric Fulani and Dogon in Mali. *J Infect Dis*. 2010;201:142–152. https://doi.org/10.1086/648596.
19 Quin JE, Bujila I, Chérif M, et al. Major transcriptional changes observed in the Fulani, an ethnic group less susceptible to malaria. *ELife*. 2017;6. https://doi.org/10.7554/eLife.29156.
20 Portugal S, Doumtabe D, Traore B, et al. B cell analysis of ethnic groups in Mali with differential susceptibility to malaria. *Malar J*. 2012;11:162. https://doi.org/10.1186/1475-2875-11-162.
21 Torcia MG, Santarlasci V, Cosmi L, et al. Functional deficit of T regulatory cells in Fulani, an ethnic group with low susceptibility to Plasmodium falciparum malaria. *Proc Natl Acad Sci USA*. 2008;105:646–651. https://doi.org/10.1073/pnas.0709969105.
22 Buffet PA, Safeukui I, Deplaine G, et al. The pathogenesis of Plasmodium falciparum malaria in humans: insights from splenic physiology. *Blood*. 2011;117:381–392. https://doi.org/10.1182/blood-2010-04-202911.
23 Kho S, Qotrunnada L, Leonardo L, et al. Hidden biomass of intact malaria parasites in the human spleen. *N Engl J Med*. 2021;384:2067–2069. https://doi.org/10.1056/NEJMc2023884.
24 Kho S, Qotrunnada L, Leonardo L, et al. Evaluation of splenic accumulation and colocalization of immature reticulocytes and Plasmodium vivax in asymptomatic malaria: A prospective human splenectomy study. *PLoS Med*. 2021;18:e1003632. https://doi.org/10.1371/journal.pmed.1003632.
25 Cranston HA, Boylan CW, Carroll GL, et al. Plasmodium falciparum maturation abolishes physiologic red cell deformability. *Science*. 1984;223:400–403.
26 Miller LH, Usami S, Chien S. Alteration in the rheologic properties of Plasmodium knowlesi–infected red cells. A possible mechanism for capillary obstruction. *J Clin Invest*. 1971;50:1451–1455. https://doi.org/10.1172/JCI106629.
27 Dondorp AM, Nyanoti M, Kager PA, Mithwani S, Vreeken J, Marsh K. The role of reduced red cell deformability in the pathogenesis of severe falciparum malaria and its restoration by blood transfusion. *Trans R Soc Trop Med Hyg*. 2002;96:282–286.
28 Dondorp AM, Angus BJ, Hardeman MR, et al. Prognostic significance of reduced red blood cell deformability in severe falciparum malaria. *Am J Trop Med Hyg*. 1997;57:507–511.
29 Safeukui I, Correas J-M, Brousse V, et al. Retention of Plasmodium falciparum ring-infected erythrocytes in the slow, open microcirculation of the human spleen. *Blood*. 2008;112:2520–2528. https://doi.org/10.1182/blood-2008-03-146779.
30 Buffet PA, Safeukui I, Milon G, Mercereau-Puijalon O, David PH. Retention of erythrocytes in the spleen: a double-edged process in human malaria. *Curr Opin Hematol*. 2009;16:157–164. https://doi.org/10.1097/MOH.0b013e32832a1d4b.
31 Deplaine G, Safeukui I, Jeddi F, et al. The sensing of poorly deformable red blood cells by the human spleen can be mimicked in vitro. *Blood*. 2011;117:e88–e95. https://doi.org/10.1182/blood-2010-10-312801.
32 Institut National de la Statistique et de l'Analyse Economique (INSAE), ICF International. *Enquête Démographique et de Santé du Bénin, 2017-2018*. Cotonou, Benin and Rockville, Maryland, USA: INSAE and ICF; 2019.
33 Yadouléton A, N'Guessan R, Allagbé H, et al. The impact of the expansion of urban vegetable farming on malaria transmission in major cities of Benin. *Parasit Vectors*. 2010;3:118. https://doi.org/10.1186/1756-3305-3-118.
34 Gnanguenon V, Govoetchan R, Agossa FR, et al. Transmission patterns of plasmodium falciparum by anopheles gambiae in Benin. *Malar J*. 2014;13:444. https://doi.org/10.1186/1475-2875-13-444.
35 Bessis M, Mohandas N, Feo C. Automated ektacytometry: a new method of measuring red cell deformability and red cell indices. *Blood Cells*. 1980;6:315–327.
36 Chien S. Physiological and pathophysiological significance of hemorheology. *Clin Hemorheol Dev Cardiovasc Med*. 1987;74:125–164. Springer.
37 Duez J, Carucci M, Garcia-Barbazan I, et al. High-throughput microsphiltration to assess red blood cell deformability and screen for malaria transmission-blocking drugs. *Nat Protoc*. 2018;13:1362–1376. https://doi.org/10.1038/nprot.2018.035.
38 Sabbagh A, Pasmant E, Imbard A, et al. NF1 molecular characterization and neurofibromatosis type I genotype-phenotype correlation: the French experience. *Hum Mutat*. 2013;34:1510–1518. https://doi.org/10.1002/humu.22392.
39 World Health Organization. *The Global Prevalence of Anemia in 2011*. Geneva: World Health Organization; 2015.
40 Almasy L, Blangero J. Multipoint quantitative-trait linkage analysis in general pedigrees. *Am J Hum Genet*. 1998;62:1198–1211. https://doi.org/10.1086/301844.
41 Wilson JF, Erlandsson R. Sexing of human and other primate DNA. *Biol Chem*. 1998;379:1287–1288.
42 Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet*. 2007;81:559–575. https://doi.org/10.1086/519795.
43 Howie B, Marchini J, Stephens M. Genotype imputation with thousands of genomes. *G3 Bethesda Md*. 2011;1:457–470. https://doi.org/10.1534/g3.111.001198.
44 Howie B, Fuchsberger C, Stephens M, Marchini J, Abecasis GR. Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. *Nat Genet*. 2012;44:955–959. https://doi.org/10.1038/ng.2354.
45 Delaneau O, Marchini J, Zagury J-F. A linear complexity phasing method for thousands of genomes. *Nat Methods*. 2011;9:179–181. https://doi.org/10.1038/nmeth.1785.
46 Delaneau O, Zagury J-F, Marchini J. Improved whole-chromosome phasing for disease and population genetic studies. *Nat Methods*. 2013;10:5–6. https://doi.org/10.1038/nmeth.2307.
47 O'Connell J, Gurdasani D, Delaneau O, et al. A general approach for haplotype phasing across the full spectrum of relatedness. *PLoS Genet*. 2014;10:e1004234. https://doi.org/10.1371/journal.pgen.1004234.
48 1000 Genomes Project ConsortiumAbecasis GR, Altshuler D, et al. A map of human genome variation from population-scale sequencing. *Nature*. 2010;467:1061–1073. https://doi.org/10.1038/nature09534.
49 Amin N, van Duijn CM, Aulchenko YS. A genomic background based method for association analysis in related individuals. *PLoS One*. 2007;2:e1274. https://doi.org/10.1371/journal.pone.0001274.
50 Aulchenko YS, Ripke S, Isaacs A, van Duijn CM. GenABEL: an R library for genome-wide association analysis. *Bioinforma Oxf Engl*. 2007;23:1294–1296. https://doi.org/10.1093/bioinformatics/btm108.
51 Watanabe K, Taskesen E, van Bochoven A, Posthuma D. Functional mapping and annotation of genetic associations with FUMA. *Nat Commun*. 2017;8:1826. https://doi.org/10.1038/s41467-017-01261-5.
52 Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic Acids Res*. 2019;47:D886–D894. https://doi.org/10.1093/nar/gky1016.
53 Boyle AP, Hong EL, Hariharan M, et al. Annotation of functional variation in personal genomes using RegulomeDB. *Genome Res*. 2012;22:1790–1797. https://doi.org/10.1101/gr.137323.112.

54 Schmitt AD, Hu M, Jung I, et al. A compendium of chromatin contact maps reveals spatially active regions in the human genome. *Cell Rep*. 2016;17:2042–2059. https://doi.org/10.1016/j.celrep.2016.10.061.

55 Kent WJ, Sugnet CW, Furey TS, et al. The human genome browser at UCSC. *Genome Res*. 2002;12:996–1006. https://doi.org/10.1101/gr.229102.

56 Cooper GM, Stone EA, Asimenos G, et al. Distribution and intensity of constraint in mammalian genomic sequence. *Genome Res*. 2005;15:901–913. https://doi.org/10.1101/gr.3577405.

57 Buffet PA, Milon G, Brousse V, et al. Ex vivo perfusion of human spleens maintains clearing and processing functions. *Blood*. 2006;107:3745–3752. https://doi.org/10.1182/blood-2005-10-4094.

58 Bell DR, Wilson DW, Martin LB. False-positive results of a Plasmodium falciparum histidine-rich protein 2-detecting malaria rapid diagnostic test due to high sensitivity in a community with fluctuating low parasite density. *Am J Trop Med Hyg*. 2005;73:199–203.

59 de Leeuw CA, Mooij JM, Heskes T, Posthuma D. MAGMA: generalized gene-set analysis of GWAS data. *PLoS Comput Biol*. 2015;11:e1004219. https://doi.org/10.1371/journal.pcbi.1004219.

60 Malaria Genomic Epidemiology Network, Band G, Rockett KA, Spencer CCA, Kwiatkowski DP. A novel locus of resistance to severe malaria in a region of ancient balancing selection. *Nature*. 2015;526:253–257. https://doi.org/10.1038/nature15390.

61 Leffler EM, Band G, Busby GBJ, et al. Resistance to malaria through structural variation of red blood cell invasion receptors. *Science*. 2017;356. https://doi.org/10.1126/science.aam6393.

62 Kariuki SN, Marin-Menendez A, Introini V, et al. Red blood cell tension protects against severe malaria in the Dantu blood group. *Nature*. 2020;585:579–583. https://doi.org/10.1038/s41586-020-2726-6.

63 Crane GG, Wells JV, Hudson P. Tropical splenomegaly syndrome in New Guinea. I. Natural history. *Trans R Soc Trop Med Hyg*. 1972;66:724–732.

64 Ishioka H, Ghose A, Charunwatthana P, et al. Sequestration and red cell deformability as determinants of hyperlactatemia in falciparum malaria. *J Infect Dis*. 2016;213:788–793. https://doi.org/10.1093/infdis/jiv502.

65 Safeukui I, Buffet PA, Perrot S, et al. Surface area loss and increased sphericity account for the splenic entrapment of subpopulations of Plasmodium falciparum ring-infected erythrocytes. *PLoS One*. 2013;8:e60150. https://doi.org/10.1371/journal.pone.0060150.

66 Jauréguiberry S, Ndour PA, Roussel C, et al. Postartesunate delayed hemolysis is a predictable event related to the lifesaving effect of artemisinins. *Blood*. 2014;124:167–175. https://doi.org/10.1182/blood-2014-02-555953.

67 Looareesuwan S, Merry AH, Phillips RE, et al. Reduced erythrocyte survival following clearance of malarial parasitaemia in Thai patients. *Br J Haematol*. 1987;67:473–478.

68 Woodruff AW, Ansdell VE, Pettitt LE. Cause of anaemia in malaria. *Lancet Lond Engl*. 1979;1:1055–1057.

69 Looareesuwan S, Ho M, Wattanagoon Y, et al. Dynamic alteration in splenic function during acute falciparum malaria. *N Engl J Med*. 1987;317:675–679. https://doi.org/10.1056/NEJM198709103171105.

70 Looareesuwan S, Davis TM, Pukrittayakamee S, et al. Erythrocyte survival in severe falciparum malaria. *Acta Trop*. 1991;48:263–270.

71 Lee SH, Looareesuwan S, Wattanagoon Y, et al. Antibody-dependent red cell removal during P. falciparum malaria: the clearance of red cells sensitized with an IgG anti-D. *Br J Haematol*. 1989;73:396–402.

72 Fendel R, Brandts C, Rudat A, et al. Hemolysis is associated with low reticulocyte production index and predicts blood transfusion in severe malarial anemia. *PLoS One*. 2010;5:e10038. https://doi.org/10.1371/journal.pone.0010038.

73 Kruetzmann S, Rosado MM, Weber H, et al. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. *J Exp Med*. 2003;197:939–945. https://doi.org/10.1084/jem.20022020.

74 Bates I, Bedu-Addo G. Review of diagnostic criteria of hyper-reactive malarial splenomegaly. *Lancet*. 1997;349:1178. https://doi.org/10.1016/S0140-6736(05)63061-9.

75 Bates I, Bedu-Addo G. Chronic malaria and splenic lymphoma: clues to understanding lymphoma evolution. *Leukemia*. 1997;11:2162–2167.

76 Angulo-Barturen I, Jiménez-Díaz MB, Mulet T, et al. A murine model of falciparum-malaria by in vivo selection of competent strains in non-myelodepleted mice engrafted with human erythrocytes. *PLoS One*. 2008;3:e2252. https://doi.org/10.1371/journal.pone.0002252.

77 Duffier Y, Lorthiois A, Cisteró P, et al. A humanized mouse model for sequestration of Plasmodium falciparum sexual stages and in vivo evaluation of gametocytidal drugs. *Sci Rep*. 2016;6:35025. https://doi.org/10.1038/srep35025.

78 Groom AC, Schmidt EE, MacDonald IC. Microcirculatory pathways and blood flow in spleen: new insights from washout kinetics, corrosion casts, and quantitative intravital videomicroscopy. *Scanning Microsc*. 1991;5:159–173. discussion 173-174.

79 Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol*. 2005;5:606–616. https://doi.org/10.1038/nri1669.

80 Ilardo MA, Moltke I, Korneliussen TS, et al. Physiological and genetic adaptations to diving in sea nomads. *Cell*. 2018;173. https://doi.org/10.1016/j.cell.2018.03.054. 569-580.e15.

81 Modiano D, Luoni G, Sirima BS, et al. The lower susceptibility to Plasmodium falciparum malaria of Fulani of Burkina Faso (west Africa) is associated with low frequencies of classic malaria-resistance genes. *Trans R Soc Trop Med Hyg*. 2001;95:149–152.

82 Norris PAA, Kaur G, Khan R, Zhu G, Ni H, Lazarus AH. Anti-inflammatory activity of CD44 antibodies in murine immune thrombocytopenia is mediated by Fcγ receptor inhibition. *Blood*. 2021;137:2114–2124. https://doi.org/10.1182/blood.2020009497.

83 Bousoik E, Qadri M, Elsaid KA. CD44 Receptor mediates urate crystal phagocytosis by macrophages and regulates inflammation in A Murine Peritoneal Model of acute gout. *Sci Rep*. 2020;10:5748. https://doi.org/10.1038/s41598-020-62727-z.

84 Oyong DA, Loughland JR, SheelaNair A, et al. Loss of complement regulatory proteins on red blood cells in mild malarial anaemia and in Plasmodium falciparum induced blood-stage infection. *Malar J*. 2019;18:312. https://doi.org/10.1186/s12936-019-2962-0.

85 Warren AJ, Colledge WH, Carlton MB, Evans MJ, Smith AJ, Rabbitts TH. The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell*. 1994;78:45–57. https://doi.org/10.1016/0092-8674(94)90571-1.

86 Liu F, Dai M, Xu Q, et al. SRSF10-mediated IL1RAP alternative splicing regulates cervical cancer oncogenesis via mIL1RAP-NF-κB-CD47 axis. *Oncogene*. 2018;37:2394–2409. https://doi.org/10.1038/s41388-017-0119-6.

87 Pivkin IV, Peng Z, Karniadakis GE, Buffet PA, Dao M, Suresh S. Biomechanics of red blood cells in human spleen and consequences for physiology and disease. *Proc Natl Acad Sci USA*. 2016;113:7804–7809. https://doi.org/10.1073/pnas.1606751113.

88 Motamed K, Funk SE, Koyama H, Ross R, Raines EW, Sage EH. Inhibition of PDGF-stimulated and matrix-mediated proliferation of human vascular smooth muscle cells by SPARC is independent of changes in cell shape or cyclin-dependent kinase inhibitors. *J Cell Biochem*. 2002;84:759–771. https://doi.org/10.1002/jcb.10095.

89 Rempel SA, Hawley RC, Gutiérrez JA, et al. Splenic and immune alterations of the Sparc-null mouse accompany a lack of immune response. *Genes Immun*. 2007;8:262–274. https://doi.org/10.1038/sj.gene.6364388.

90 Chioran A, Duncan S, Catalano A, Brown TJ, Ringuette MJ. Collagen IV trafficking: the inside-out and beyond story. *Dev Biol*. 2017;431:124–133. https://doi.org/10.1016/j.ydbio.2017.09.037.

91 Chen LT, Weiss L. Electron microscopy of the red pulp of human spleen. *Am J Anat*. 1972;134:425–457. https://doi.org/10.1002/aja.1001340404.

92 Brousse V, Buffet P, Rees D. The spleen and sickle cell disease: the sick(led) spleen. *Br J Haematol*. 2014;166:165–176. https://doi.org/10.1111/bjh.12950.