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DISSERTATION

Prävalenz, Risikofaktoren und Medikamentenresistenz der Plasmodieninfektion bei Kindern im Alter von unter fünf Jahren im Hochland des südlichen Ruanda

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Abstract

Fragestellung: Die folgende Arbeit beschäftigt sich mit einer 2010 im Hochland des südlichen Ruanda durchgeführten Querschnittsstudie. Ziel war es, aktuelle Daten zur Prävalenz der Malaria und Plasmodieninfektion sowie zu assoziierten Faktoren bei Vorschulkindern zu erheben, das Ausmaß parasitärer Medikamentenresistenz anhand molekularer Resistenzmarker abzuschätzen und die Bedeutung der Plasmodieninfektion für eine Anämie im Vergleich zum Eisenmangel und einem Eisenstoffwechselpolymorphismus (TMPRSS6 736(V)) einzuordnen.

Methodik: 749 Kinder im Alter unter fünf Jahren wurden in die Studie eingeschlossen: 545 Kinder aus Dörfern des Huye Distrikts, 103 Patienten eines regionalen Gesundheitszentrums und 101 Patienten des Kreiskrankenhaus Butare. Der Nachweis einer Plasmodieninfektion erfolgte mikroskopisch und per PCR. Klinische und sozioökonomische Daten wurden gesammelt und mit einer Plasmodieninfektion assoziierte Faktoren mittels Regressionsanalyse berechnet. Bei den *P. falciparum* positiven Proben erfolgte die Typisierung der Allele der Resistenzmarker *Pfdhfr, Pfdhps, Pfmdr1* und *Pfcrt*. Bestimmt wurden Hämoglobin- und Ferritinwerte, sowie der Eisenstoffwechselpolymorphismus TMPRSS6 736(V), der hier erstmalig bei afrikanischen Kindern getestet wurde.

Ergebnisse: Bei 11,7% bzw. 16,7% der Kinder wurde eine Infektion mit *P. falciparum* mikroskopisch bzw. per PCR nachgewiesen, bei 5,5% lag eine Malaria vor. Als signifikante, mit einer Plasmodieninfektion assoziierte Faktoren erwiesen sich u.a. zunehmendes Alter und Faktoren, die auf einen niedrigen sozioökonomischen Status hinweisen. 69% der *P. falciparum*-Isolate wiesen eine Fünf- oder Sechsfachmutation der *Pfdhfr/Pfdhps*-Gene auf, die mit einem Therapieversagen von Sulfadoxin-Pyrimethamin assoziiert ist. *Pfmdr1* trug in rund 40% das Muster N86-F184-D1246, das mit rekurrenten Infektionen nach Arthemeter-Lumefantrin-Therapie in Zusammenhang gebracht wird. Anämie und Eisenmangel waren bei den Kindern der ländlichen Region häufig (34,4% bzw. 17,5%). Die Plasmodieninfektion erwies sich als stärkster unabhängiger Faktor, der eine Anämie bedingt (Odds-Verhältnis: 10,3); *TMPRSS6* 736(V) hingegen war selten und nicht signifikant mit einer Anämie assoziiert.

Diskussion: Im Studiengebiet des Hochlands von Südruanda wurde eine *P. falciparum* Infektion bei jedem sechsten Kind beobachtet. Der Großteil der Infektionen insbesondere des ländlichen Gebiets ging ohne offenkundige Symptome einher. Diese asymptomatischen Infektionen stellen ein Reservoir für Übertragungen in dieser ausbruchgefährdeten Region dar. Die Risikofaktoren aus dem Bereich des niedrigen sozioökonomischen Status weisen auf den armutsassoziierten Charakter der Malaria in diesem Gebiet hin. Das hohe Ausmaß an Markern mit Sulfadoxin-Pyrimethamin-Resistenz stützt das Aussetzen Sulfadoxin-Pyrimethamin-basierter Interventionen in Ruanda; das *Pfmdr1*-Muster weist dagegen auf einen intensiven Medikamentendruck mit Arthemeter-Lumefantrin hin. Obwohl weitgehend asymptomatisch, ist die *P. falciparum* Infektion neben dem Eisenmangel ein erheblicher Risikofaktor für das Auftreten einer Anämie. Weitere Anstrengungen zur Kontrolle der Malaria und zur Verbesserung der Ernährungssituation bleiben im südlichen Ruanda notwendig.

Abstract

Background: The following paper concerns a cross-sectional study, conducted in southern highlands Rwanda, 2010. The study aimed to gather current data on the prevalence of malaria and *Plasmodium* infection along with associated factors among pre-school children, on evaluating the level of parasitic drug resistance through molecular resistance markers and on comparing the role of *Plasmodium* infection in anaemia, with that of iron deficiency and of a polymorphism of iron-regulation (TMPRSS6 736(V)).

Methods: There were 749 children under the age of five included in the study: 545 from villages of Huye district, 103 from a local health center and 101 from Butare district hospital. *Plasmodium* infection was identified by microscopy and PCR. Clinical and socio-economic data were gathered and factors associated with *Plasmodium* infection were, using regression analysis, worked out. In the case of *P. falciparum* positive samples, we typed resistance marker alleles *pfdhfr, pfdhps, pfmdr1* and *pfcrt*. We investigated haemoglobin and ferritin levels, along with the polymorphism of iron-

regulation TMPRSS6 736(V), which was tested for the first time in African children.

Results: *P. falciparum* infection was detected in 11,7% and 16,7% of children, microscopically or by PCR, malaria was present in 5,5%. As significant factors associated with *P. falciparum* infection, we identified -among others- age and factors indicating a low socio-economic status. 69% of *P. falciparum* isolates exhibited *pfdhfr/pfdhps* quintuple or sextuple mutations associated with sulfadoxine-pyrimethamine treatment failure. Around 40% of *pfmdr1* carried the N86-F184-D1246 pattern known to be selected in infections reappearing following artemether-lumefantrine treatment. Anaemia and iron deficiency were common in children from rural areas (34,4% and 17,6%). *Plasmodium* infection proved to be the strongest independent risk factor in the formation of anaemia (odds ratio:10,3); TMPRSS6 736(V) was uncommon and not significantly associated with anaemia.

Discussion: In southern Rwanda *P. falciparum* infection was observed in one in six children. Most infections, especially in rural areas, were not accompanied by obvious symptoms, seemingly asymptomatic infections, forming a reservoir for transmission in this endemic region. Risk factors arising from low socio-economic status refer to the poverty-associated nature of Malaria in this area. The high level of molecular markers with sulfadoxine-pyrimethamine-resistence supports the suspension of SP-based interventions in Rwanda; the *pfmdr1* pattern shows an intensive medical pressure from artemether-lumefantrine. Though largely asymptomatic, *P. falciparum* infection is, alongside iron deficiency, an important risk factor in the development of anaemia. Further efforts to control malaria and improve nutrition in southern Rwanda remain crucial.

1. Einleitung

Die Malaria gehört weiterhin zu den bedeutendsten Infektionskrankheiten weltweit, schätzungsweise die Hälfte der Todesfälle durch Malaria tritt bei Kindern im Alter unter fünf Jahren auf (1,2). Die Subsahara-Region gehört nach wie vor zu dem Gebiet mit den meisten Malariafällen, jedoch konnten in einigen Ländern dieser Region in den letzten Jahren große Fortschritte in der Bekämpfung der Malaria erreicht werden. Ruanda ist nach Schätzungen der World Health Organisation (WHO) als ein Beispiel für einen besonders starken Rückgang der Fallzahlen und der Mortalität in der Region zu nennen. So konnte das Land nach WHO-Angaben die Zahl der in Gesundheitseinrichtungen registrierten Erkrankungsfälle von 2000 bis 2011 um mehr als 75% senken (1). Inwiefern der Rückgang der Fallzahlen in den Gesundheits-einrichtungen auch auf einen Rückgang in den ländlichen Gebieten schließen lässt, bleibt unklar (3,4).

Ein zentrales Problem in der Bekämpfung der Malaria ist nach wie vor die Resistenzentwicklung von *P. falciparum* gegen gängige Therapeutika. In Ruanda wurden 2006 die Behandlungsempfehlungen aufgrund zunehmender Resistenzen gegen Sulfadoxin-Pyrimethamin (SP) auf die Therapie mit Arthemeter-Lumefantrin (AL) umgestellt. Für zahlreiche Resistenzen von *P. falciparum* konnten molekulare Resistenzmarker im parasitärem Genom identifiziert werden. In mehreren Ländern haben Daten zu Resistenzmarkern die Empfehlungen zur Leitlinientherapie beeinflusst (5-7). Resistenzen von *P. falciparum* gegen SP korrelieren mit Punktmutationen in den Genen der Dihydrofolatreduktase (*Pfdhfr*) und der Dihydropteroatsynthase (*Pfdhps*). Die Dreifachmutation N108-I51-R59 des *Pfdhfr*-Gens kombiniert mit einer Zweifachmutation in G437 und E540 des *Pfdhps*-Gens, auch als *Pfdhfr/Pfdhps*-Fünffachmutation zusammengefasst, ist in Ostafrika mit dem Versagen einer SP-Therapie assoziiert (8,9). Mutationen des Genlokus T76 des *Plasmodium falciparum Chloroquin Resistenz Transporters* (*Pfcrt*) sind ein Indikator für Resistenz gegen Chloroquin (CQ), wohingegen die Variante des *Plasmodium falciparum* multidrug resistance1-Gens (*Pfmdr1*) das Ausmaß der Resistenz beeinflusst (10,11). Auch hinsichtlich der Resistenz gegen AL sind zahlreiche Genorte des *Pfmdr1*-Gens identifiziert worden. So sind die Allele N86, F184 und D1246 des *Pfmdr1*-Gens, aber auch das Wildtypallel K76 des *Pfcrt*-Gens Indikatoren für ein Wiederauftreten von Erregern unter der Therapie mit AL

(12-17). Bisher liegen keine veröffentlichten Daten über die Prävalenz von Mutationen in den *Pfcrt*- und *Pfmdr*-Genen in Ruanda vor. Für *Pfdhfr* und *Pfdhps* hingegen sind Daten aus Ost- und Westruanda verfügbar, wo eine hohe Mutationsprävalenz nachgewiesen wurde (18).

Die Anämie trägt neben der Malaria wesentlich zur Mortalität und Morbidität von Vorschulkindern in der Subsahara-Region bei. Nach Daten der WHO liegt bei zwei von drei Vorschulkindern in der Subsahara-Region eine Anämie vor (19). Malaria kann einen deutlichen Abfall des Hämoglobins verursachen und somit zu einer Anämie beitragen. Weitere häufige Ursachen der Anämie bei Kindern in der Subsahara-Region sind die Eisenmangelanämie, aber auch genetische Faktoren wie die Sichelzellanämie, der G6PD-Mangel oder die Alpha-Thalassämie. Bei der Eisenmangelanämie scheinen neben Ernährungsfaktoren auch genetische Faktoren von Bedeutung zu sein. Die Punktmutation V736A des *Transmembran Serine Protease 6-G*ens (*TMPRSS6)* verursacht bei kaukasischen und asiatischen Menschen hohe Hepcidin-Werte. Hohe Hepcidin-Werte können für einen niedrigen Wert des Serumeisens und einen Abfall des mittleren Zellvolumens und des Hämoglobins verantwortlich sein (20-23). In Ruanda bleibt die Bedeutung des Eisenstoffwechselpolymorphismus TMPRSS6 736(V) für das Risiko einer Anämie bisher unerforscht und soll daher im Rahmen dieser Arbeit untersucht werden.

2. Zielsetzung

Die folgende Arbeit beschäftigt sich mit einer 2010 im südlichen Ruanda durchgeführten Querschnittsstudie. Ziel der Studie war es, aktuelle Daten zur Prävalenz von Plasmodieninfektion und Malaria bei Vorschulkindern in einer Region zu erfassen, wo bisher keine veröffentlichten Daten existieren. Da Schätzungen zu Infektionsprävalenzen überwiegend auf Daten basieren, die in Gesundheitseinrichtungen erhoben werden, wurden malariabezogene Daten von Kindern sowohl in zwei Gesundheitseinrichtungen als auch von Kindern aus Dörfern einer ländlichen Region erfasst. Zudem sollten mit einer Plasmodieninfektion assoziierte Faktoren, wie beispielsweise sozioökonomische Faktoren, erfasst werden. Zur Charakterisierung der *P. falciparum* Infektion wurde die Prävalenz verschiedener molekularer Resistenzmarker

in der Region bestimmt. Erstmalig sollten Daten zu Mutationen in den *Pfcrt*- und *Pfmdr*-Genen in Ruanda generiert werden. Medikamentenspiegel für CQ und Pyrimethamin wurden in Plasmaproben gemessen, um zu erfahren, ob diese Zweitlinienmedikamente weiterhin eingenommen werden. Des weiteren wurden Prävalenzen und Risikofaktoren für die Ausbildung einer Anämie und Eisenmangelanämie untersucht und in Bezug zur Bedeutung der Malaria gesetzt. TMPRSS6 736(V) und dessen Rolle in Bezug auf das Risiko für Eisenmangel und Anämie wurde hier erstmalig bei afrikanischen Kindern getestet.

3. Methoden

3.1. Studienaufbau

Der vorgelegten Arbeit liegt eine zwischen Januar und März 2010 durchgeführte Querschnittstudie zugrunde. Es wurden 749 Kinder im Alter von unter fünf Jahren in der Region Butare, im südlichen Ruanda eingeschlossen (*Details zum Studiengebiet siehe Publikation 1*).

Es wurden drei Studiengruppen gebildet: 1.) Kinder aus den Dörfern des ländlichen Gebietes des Huye Distrikts; 2.) Kinder, die als Patienten ein lokales Gesundheitszentrum aufsuchten (Sovu Health Center); 3.) pädiatrische Patienten des Kabutare Distrikt Hospital in Butare. Für die Stichprobe der Kinder aus den Kommunen wurden im Huye Subdistrikt (ca. 20.000 Einwohner) in 24 randomisiert gewählten Dörfern je 25 Familien zufällig ausgewählt. Jeweils ein Kind dieser Familien wurde von einem Sozialarbeiter zufällig ausgewählt und die Familie gebeten, das Kind in dem Gesundheitszentrum (Sovu Health Center) vorzustellen. Bei der Rekrutierung wurde ein ausgewogenes Verhältnis der Altersgruppen ≤1,1≤2,2≤3,3≤4 und 4≤5 Jahre angestrebt. Parallel wurden je über 100 Patienten in der Altersgruppe bis einschließlich fünf Jahren, die sich im Sovu Health Center und Kabutare Distrikt Hospital vorstellten, rekrutiert. Die Studienteilnahme erfolgte nach Einholen des Einverständnis der Eltern. Die Studie wurde zuvor durch die nationale Ethikkommission geprüft und bewilligt.

3.2. Untersuchungen im Studiengebiet

Die sozioökonomischen Lage der Familien wurde mittels eines Fragebogens erfasst. Es wurde eine medizinische Anamnese erhoben und eine körperliche Untersuchung durch einen lokalen Arzt durchgeführt. Klinische Parameter wie Größe, Gewicht, Mittlerer Armumfang (MUAC) und die Körpertemperatur wurden gemessen. Es wurde eine venöse Blutprobe, eine Urin- und eine Stuhlprobe gewonnen. Der Hämoglobin-Wert (Hb) wurde mithilfe eines HemoCue Photometers (Angelholm,Schweden) gemessen. Eine Anämie wurde als ein Hb-Wert <11 g/dl definiert. Die Urinprobe wurde mithilfe eines Urin-Stix untersucht (Multistix 10SG, Bayer, Deutschland). Vor Ort wurde ein Giemsa-gefärbter Blutstropfen angefertigt und Malariaparasiten pro 200 Leukozyten ausgezählt.

3.3. Laboranalysen

Weitere Laboranalysen der Proben erfolgten im Tropeninstitut Berlin. Es erfolgte eine erneute mikroskopische Untersuchung der Giemsa-gefärbten Präparate und die definitive Auszählung der Parasiten pro 200 Leukozyten. Die Parasitendichte pro Mikroliter wurde bestimmt auf Grundlage der Annahme einer mittleren Leukozytenkonzentration von 8000 Leukozyten pro Mikroliter. Malaria wurde definiert als mikroskopisch sichtbare Parasitämie mit Fieber oder Fieberanamnese in den letzten 48 Stunden. Genomische DNA wurde aus den Blutproben mithilfe des Qiamp blood kit nach Anleitung des Herstellers (Qiagen, Deutschland) extrahiert. Anwesenheit und Spezies einer Plasmodieninfektion wurden mithilfe einer Semi-nested multiplex PCR bestimmt (24). Zudem erfolgte die Bestimmung der Multiplizität der Infektion (MOI: multiplicity of infection) bei den *P. falciparum* positiven Proben. Hierzu wurden eine getrennte Amplifikation der Genorte des *merozoite surface protein1 (msp1)* Block 2 (K1, Mad20 und Ro33) und *msp2* Block 3 (FC27 und IC) mithilfe der PCR durchgeführt (25). Nach Auftrennung der Amplifikate auf einem 3% Agarose-Gel (Biozym, Deutschland) und Analyse mithilfe von Gene Snap Software (SynGene, Großbritannien) konnten unterschiedliche Plasmodienklone durch Fragment-Längen-Polymorphismen detektiert werden. Die MOI wurde als die maximale Anzahl an Allelvarianten für *msp1* oder *msp2* gewertet. Um eine Vormedikation mit CQ oder SP zu erfassen, wurde mithilfe eines

ELISA die Konzentration von CQ und Pyrimethamin (Indikator für SP) im Plasma bestimmt. Eine Kreuzreaktion von CQ mit Amodiaquin ist vernachlässigbar (26). Die Bestimmung der molekularen Resistenzmarker erfolgte bei den *P. falciparum* positiven Proben durch gezielte Amplifikation der entsprechenden Gene und Detektion von Mutationen anhand von Restriktions-Fragment-Längen-Polymorphismen (RFLP). Mithilfe einer nested PCR, Restriktionsverdau und Auftrennung der Fragmente auf einem Agarose-Gel wurden die *Pfdhfr*-Mutationen A16V, N51I, C59R, S108N und I164L sowie *Pfdhps* S436A, A437G, K540E, A581G und A613S detektiert (27). Die Mutationen *Pfmdr1* N86Y, Y184F, S1034C, N1042C und D1246Y sowie *Pfcrt* K76T wurden mithilfe von PCR und Analyse von Schmelzkurven nach Hybridisierung mit speziellen Primern im Roche LightCycler 480 (Roche Diagnostics, Mannheim, Deutschland) bestimmt (28,29). Die *P. falicparum* Isolate 3D7, HB3 und Dd2 dienten bei allen Untersuchungen als Kontrollen.

Die Blutproben der Studiengruppe der Kinder aus der ländlichen Region wurden auf das Auftreten von *TMPRSS6* rs855791 mithilfe einer Schmelzkurvenanalyse (TIB Mobiol, Berlin, Deutschland) getestet. Mithilfe eines ELISA wurde das C-reaktive Protein und die Ferritinkonzentration bestimmt (Assaypro, St. Charles, USA). Eisenmangel wurde definiert als ein Ferritinwert <12 ng/ml (30) und eine Entzündung als CRP >5 ng/ml (31).

3.4. Statistische Auswertungen

Die statistische Aufarbeitung erfolgte mit der StatView (SAS, Cary, USA). Ein p-Wert <0.05 wurde als signifikant gewertet. Odds-Verhältnisse und Konfidenzintervalle mit dem Konfidenzniveau 95% wurden bestimmt. Eine multivariante Regressionsanalyse wurde durchgeführt, um signifikante Beziehungen mehrerer abhängiger Variablen festzustellen. Einflussfaktoren auf das Risiko für die Infektion mit *P. falciparum,* Malaria, Anämie oder Eisenmangel wurden mithilfe einer logistischen Regressionsanalyse untersucht.

4. Ergebnisse

4.1. Studienpopulation und sozioökonomische Faktoren

Insgesamt wurden 749 Kinder in die Studie eingeschlossen, davon 545 aus den Dörfern der ländlichen Region in Huye (Huye Community), 101 aus dem Kreiskrankenhaus (Kabutare Distrikt Hospital) und 103 aus dem Gesundheitszentrum (Sovu Health Center). Die drei Studiengruppen zeigten in sozioökonomischer Hinsicht deutliche Unterschiede. So verfügten die Familien der Kinder aus den Dörfern im Vergleich zu denen aus dem Gesundheitszentrum oder Krankenhaus durchschnittlich über ein deutlich geringeres Einkommen, einen niedrigeren Bildungsabschluss der Eltern und einen deutlich niedrigeren Anteil an Kindern mit Krankenversicherung.
Die häufigsten klinischen Diagnosen der Patienten im Sovu Health Center und Distrikt Hospital waren ein respiratorischer (34,3% bzw. 26,1%) und ein gastrointestinaler Infekt (25% bzw.19,4%), an dritter bzw. vierter Stelle stand die Malaria (19,3% bzw. 11,9%). Nach Einschätzungen der Ärzte war nur jedes vierte Kind aus den Kommunen gesund, bei 30% wurde ein gastrointestinalen Infekt diagnostiziert. Fieber und anamnestisches Fieber waren bei den Kindern aus den Kommunen selten (3% und 10%), im Gesundheitszentrum (35% und 70%) und im Kreiskrankenhaus (27% und 48%) dagegen häufig (V*ergleich Publikation 1, Tabelle 1*).

4.2. Infektionsprävalenz

In der vorliegenden Studie wurde bei insgesamt 16,7% bzw. 11,7% der Kinder *P. falciparum* per PCR bzw. mikroskopisch nachgewiesen werden, in 5,5% lag eine Malaria vor. Alle mikroskopisch positiven Proben konnten durch die PCR bestätigt werden, u.a. eine Monoinfektion mit *P. malariae* und zwei Fälle von *P. ovale*. Die drei Studiengruppen zeigten keine großen Unterschiede innerhalb der mikroskopischen und PCR-basierten Infektionsprävalenzen (*Details siehe Publikation 1)*. Die Diagnose Malaria (Parasitämie und Fieberanamnese) wurde allerdings bei den Kindern aus den Gesundheitseinrichtungen weitaus häufiger gestellt als bei den Kindern aus den Kommunen. So hatte nur ein Viertel der Kinder mit Parasitämie in den Dörfern eine Malaria, während dies bei allen Kindern aus dem Gesundheitszentrum und bei den meisten aus dem Krankenhaus der Fall war.

4.3. Einflussfaktoren auf die Infektionsprävalenz

Mithilfe einer Regressionsanalyse wurden Faktoren, die Einfluss auf die Prävalenz von Plasmodieninfektion und Malaria nehmen, untersucht. Nach multivarianter Analyse verblieben folgende unabhängige Faktoren, die eine *P. falciparum* Infektion begünstigen: zunehmendes Alter, Vorstellung im Gesundheitszentrum oder Krankenhaus, niedriger mittlerer Armumfang, Nichtbesitz eines Schrankes, Radios oder Fahrrads, Anamnese einer AL-Einnahme und Nachweis von CQ im Plasma. Dieselbe Analyse in Hinsicht auf Malariaerkrankung zeigte ähnliche Faktoren*(siehe Publikation 1)*. Für die Benutzung eines Bettnetzes konnte keine signifikante Assoziation mit einer Infektion mit *P. falciparum* oder mit Malaria festgestellte werden.
Für die Kinder aus der ländlichen Region lag eine klare Zunahme der Infektionsprävalenz (PCR und Mikroskopie) und Malariaprävalenz mit zunehmenden Alter vor (p-Werte: p=0,009, p=0,03 und p=0,02). Gleichzeitig schien der Anteil der Kinder mit einer asymptomatischen Parasitämie mit zunehmendem Alter zu sinken (p=0.08) *(siehe Publikation 1, Abbildung 2).*

4.4. Medikamenteneinnahme und Plasmodieninfektion

Die Einnahme von Artemether-Lumefantrin in den letzten zwei Wochen wurde bei jedem achten Kind berichtet. Eine Einnahme von Chloroquin wurde von keiner der Familien bejaht, allerdings wurde CQ in 3,7% (28/747) der Fälle im Plasma nachgewiesen. In der Gruppe der Kindern mit Plasmodieninfektion lag die Nachweisquote von CQ gar bei 18%. Sowohl die anamnestische Einnahme von AL als auch der Nachweis von CQ im Plasma erwiesen sich als ein signifikanter Risikofaktor für eine Plasmodieninfektion.

4.5. Resistenzmarker

Die Bestimmung der Resistenzmarker war bei dem überwiegenden Teil der *P. falciparum* positiven Proben erfolgreich (83% der 125 Proben). Gemischte Proben mit mutierten und nicht mutierten Allelen wurden als mutiert gewertet. Bezüglich des *Pfdhfr*-Gens waren die Genorte Codon 51 (99%), 59 (75%) und 108 (99%) überwiegend

mutiert. Für das *Pfdhps*-Gen hatten Mutationen im Codon 437 (96%), 540 (94%) und 581 (63%) eine sehr hohe Prävalenz. Die untersuchten Genorte des *Pfmdr1* lagen hauptsächlich als Wildtyp vor (N86: 61%, D1246: 88%), mit Ausnahme des Codons 86 und 184. Im Gen des *Pfcrt* T76 zeigte sich in 74% der Isolate eine Mutation.
Es erfolgte die Untersuchung mutierter *Pfdhfr-* und *Pfdhps*-Gene auf Kombinationen von Mutationen und Zusammenfassung zu Zweifach-, Dreifach-, Vierfach- und Fünffachmutationen. Die Dreifachmutation (N108-I51-R59) des *Pfdhfr*-Gens wurde in 75% der Proben festgestellt. Isolate mit kombinierter *Pfdhfr/Pfdhps*-Fünffach- oder Sechsfachmutationen traten in 69% der Fälle auf. Im *Pfmdr1*-Gen zeigte sich eine Zwei- oder Dreifachmutation mit einer Prävalenz von insgesamt 19,2% nur selten. Es konnten keine signifikanten Häufungen von Mutationen oder Mutationskombinationen in Abhängigkeit von Studiengruppe, Wohnort, Malariaerkrankung, Parasitendichte, submikroskopischer Infektion oder Alter festgestellt werden.

4.6. Anämie und Eisenmangel

Eine Anämie wurde bei 34,4% und ein Eisenmangel bei 17,5% der Kinder aus den Kommunen festgestellt. Ein Viertel der anämischen Kinder hatte einen Eisenmangel. Als Faktoren, die mit erhöhtem Risiko für Anämie einhergingen, erwiesen sich Eisenmangel, Alpha-Thalassämie, Brustfütterung, Entzündung und niedriges Familieneinkommen. Nach multivarianter Analyse erwiesen sich das Alter und eine *P. falciparum* Infektion als die stärksten unabhängigen Faktoren, die eine Anämie bedingen, wobei die Odds Ratio für Anämie bei Infektion mit *P. falciparum* bei 10,3 lag. Das Allel *TMPRSS6* 736(V) war bei 17,8% der Kinder mit einer Frequenz von 0,096 feststellbar und war nicht signifikant mit einer Anämie assoziiert *(Vergleich Publikation 3, Tabelle 1)*.

5. Diskussion

5.1. Infektionsprävalenz und sozioökonomische Faktoren

Daten zur Prävalenz der Malaria in Ruanda zeigen starke regionale und zeitliche Schwankungen, für das Zentralplateau wird jedoch eine mittleren Prävalenz zwischen 5 und 15% angenommen (32). Allerdings ist davon auszugehen, dass es zu einer deutlichen Abnahme der Prävalenz in den letzten Jahren gekommen ist (1,33,34). Der Wert der mikroskopischen *P. falciparum* Prävalenz für die Studiengruppe der Kinder in den Gesundheitseinrichtungen (12,3%) in dieser Studie deckt sich mit Werten von Vorstudien (35,36). Die Prävalenzen in der Gruppe der Kinder in dem ländlichen Gebieten lagen mit 11,2% mikroskopisch und 16,1% in der PCR allerdings höher als erwartet. Sicherlich spielt die höhere Sensitiviät der PCR im Vergleich zur Mikroskopie und zu Malariaschnelltests eine Rolle (37,38). Obwohl die Daten zur Malariaprävalenz in den Dörfern wahrscheinlich nicht als repräsentativ für das Zentralplateau zu werten sind, bieten sie doch ein aktualisiertes und komplexes Abbild für die Bevölkerung in der Region Butare.

Die Daten zu sozioökonomische Faktoren und der angegebene Nutzung von Bettnetzen decken sich weitgehend mit Daten anderer Studien des Studiengebietes (1,35,36). Unter den häufigsten Diagnosen der Kindern, die in den Gesundheitseinrichtungen vorgestellt wurden, nahm die Malaria neben den Infektionen des Respirationstraktes und der gastrointestinalen Infekte den dritten Platz ein. Das legt nahe, dass trotz des besseren sozioökonomischen Status der Patienten in den Gesundheitseinrichtungen im Vergleich zur Landbevölkerung die Malaria weiterhin zu den häufigsten drei Gründen gehört, eine Gesundheitseinrichtung in diesem Gebiet aufzusuchen.

In der Studiengruppe der Kinder aus dem ländlichen Gebiet fand sich eine sehr hohe Rate an asymptomatischen Infektionen. So lag nur bei ungefähr ein Viertel der Kinder mit Parasitämie Fieber oder eine Fieberanamnese vor. Asymptomatische Infektionen haben häufig einen lang andauernden Verlauf. Studien aus Ghana und dem Sudan zeigen, dass asymptomatische Infektionen ein Jahr oder gar länger anhalten können (39,40). Insgesamt bilden Individuen mit lang andauernden, asymptomatischen Infektionen oder Infektionen mit geringer Parasitenzahl ein wichtiges Reservoir für die Übertragung von *P. falciparum* (38,41,42). Daher wäre zu diskutieren, ob die Behandlung auch von asymptomatisch infizierten Kindern eine sinnvolle Maßnahme zur

Kontrolle der Malaria in der Region darstellt.

5.2. Einflussfaktoren auf die Infektionsprävalenz

Das Alter stellte ein wesentlicher Einflussfaktor auf die Infektionsprävalenz dar. In der Studiengruppe der Kommunen stiegt die Prävalenz der *P. falciparum* Infektion von 10% auf fast 25% von der jüngsten zur ältesten Altersklasse ($p<0.05$).

Neben einem niedrigen durchschnittlichen Einkommen der Familie korrelierte u.a. der Besitz eines Radios oder Fahrrads mit einer geringeren Wahrscheinlichkeit für eine *P. falciparum* Infektion. Die Ergebnisse unterstreichen die Bedeutung der Armutsbekämpfung sowie das Potential von Informationstechnologie und Mobilität für die Malariaprävention. Hier sollten Informationskampagnen und Gesundheits-programme anknüpfen.

Ein weiterer häufig genannter Faktor in Bezug auf die Infektionsrate ist die selbst berichtete Benutzung von Bettnetzen. Die angegebenen Nutzungsquoten in dieser Studie lagen im Referenzbereich anderer Studien (1,35,36). Das festgestellte Ausmaß, in dem Bettnetze vor einer Infektion schützen, war in dieser Studie allerdings nur moderat und in der multivarianten Anlayse nicht signifikant. Obwohl die Verteilung von Bettnetzen eine etablierte Maßnahme zur Bekämpfung der Malaria ist und der Nutzen in Studien erwiesen (1,3,4,33), werfen diese Ergebnisse die Frage nach der Effektivität dieser Maßnahme in der Region auf. Weitere Studien zur Evaluierung dieser Maßnahme in der Region wären daher notwendig.

5.3. Medikamenteneinnahme und Plasmodieninfektion

In der Gruppe der Kinder aus dem Kreiskrankenhaus gaben 38% der Familien eine medikamentöse Vorbehandlung mit einem Malariamedikament an, 6% insgesamt waren mit AL vorbehandelt. In dieser Studie wurden keine Daten zur Dosis und Dauer der Vormedikation gesammelt und Eigenangaben zu Vormedikation sind kritisch zu betrachten (43). Trotzdem bleibt bemerkenswert, dass sich eine kürzlich vorangegangene Einnahme von AL als ein Risikofaktor für eine Malaria erwies. Dies mag ein Hinweis für ein Wiederauftreten von Parasiten unter Therapie sein. Im Jahre 2006 lagen die Heilungsraten unter AL in Ruanda bei 97% (44). Allerdings ist aufgrund

des weiten Einsatzes und der damit einhergehenden deutliche Zunahme des Mutationsdrucks eine aktuelle Evaluierung der Medikamentenwirksamkeit anzustreben.

Auch die Einnahme von Chloroquin stellte in der multivarianten Analyse ein signifikanter Risikofaktor für eine *P. falciparum*-Infektion dar. Die Ergebnisse zeigen nicht nur, dass entgegen der Empfehlungen der WHO Chloroquin weiterhin von der Bevölkerung eingenommen wird, sondern auch, dass es unter der Einnahme zu Persistenz oder Wiederauftreten von Erregern kommen kann. Hier sollten Kampagnen zur Gesundheitsaufklärung verstärkt durchgeführt werden, um die negativen Folgen der nicht leitliniengerechten Behandlung zu verhindern.

5.4. Resistenzmarker

Die Ergebnisse der Untersuchung der molekularen Resistenzmarker hinsichtlich der *Pfdhfr*- und *Pfdhps*-Gene zeigen insgesamt hohe Mutationsraten. Die Rate an Plasmodienisolaten mit Fünf- oder Sechsfachmutationen betrug in der vorliegenden Studie 69,2%. Eine im Jahre 2005-2006 durchgeführte Vergleichsstudie im östlichen bzw. westlichen Ruanda fand diese Mutationskombination mit einer Häufigkeit von 77,8% bzw. 37,5% (18). Der Hochresistenzmarker *Pfdhfr* L164 war allerdings in der vorliegenden Studie gar nicht nachweisbar. Auffallenderweise konnte eine hohe Prävalenz des Codons *Pfdhps* G581 festgestellt werden (63%), das eine Sechsfachmutation in circa der Hälfte der Isolate verursachte. Die G581 Mutation scheint sich in Afrika auszubreiten und neuere Studien aus Tansania vermuten, dass sie für ein frühes Therapieversagen von SP verantwortlich ist (45). Insgesamt wurde ein hohes Maß an Markern mit SP-Resistenz festgestellt, so dass eine Verwendung von SP in der Region weiterhin ungeeignet erscheint.

Erstmalig wurden in der vorliegenden Studie Daten zu *Pfmdr1*-Allelen aus Ruanda veröffentlicht. Die *Pfmdr1*-Mutation F184 trat in über 50% der Isolaten auf, die Kombination N86-F184-D1246 in beinahe 40%. Studien im benachbarten Tansania (13,15) und Uganda (14,46) zeigten geringere Prävalenzen. Das Muster der *Pfmdr1*-Allele spricht für einen hohen Selektionsdruck von AL in der Region. Die Bedeutung dieses Ergebnisses auf die Dauer des wirkungsvollen Einsatzes als

Erstlinienmedikament in der Region bleibt allerdings unklar. Weitere Studien zur Überwachung der molekularen Resistenzmarker in der Region und Korrelation mit Ergebnissen zum Therapieerfolg bleiben zum Therapiemonitoring unabdingbar.

5.5. Anämie und Eisenmangel

Eine Anämie war mit einer Prävalenz von 34,4% der 545 Kinder häufig. Der Wert liegt deutlich niedriger als Vergleichsdaten des Statistikinstitutes Ruanda, wo 2006 eine Prävalenz von 47% im südlichen Ruanda festgestellt wurde (47). Ein Eisenmangel trat bei jedem sechsten Kind auf und war mit einem beträchtlichen Abfall des Hämoglobinwertes um 0,9 g/dl assoziiert. Für die Ausbildung einer Anämie konnten zahlreiche Risikofaktoren festgestellt werden: Infektion mit *P. falciparum*, niedriges Alter des Kindes, Brustfütterung, niedriges Haushaltseinkommen, Alpha-Thalassämie und Entzündung. Mit einem Eisenmangel hingegen waren abgesehen von einem niedrigem Alter keiner der untersuchten Faktoren assoziiert.
Dies galt auch für die Allelvariante TMPRSS6 736(V), für die keine signifikante Häufung von Anämie oder Eisenmangel nachgewiesen werden konnte. Bemerkenswerterweise trat TMPRSS6 736(V) mit einer Allelfrequenz von 0,096 deutlich seltener auf als in Vergleichsgruppen aus Asien ($\geq$0.5) und Europa ($\geq$0.4) (20). Insgesamt ist von einer komplexen Ätiologie des Eisenmangels in der Region auszugehen, möglicherweise überwiegen inflammatorische Faktoren oder andere bisher nicht bekannte andere Einflussfaktoren den Effekt von TMPRSS6 736(V). Weitere Studien mit größeren Fallzahlen und eine umfassende Erfassung von Einflussfaktoren, u.a. Ernährungsfaktoren, wären hier wünschenswert. Trotz allem bleibt die Bedeutung der Anämie und des Eisenmangels für die Kindergesundheit in der Region angesichts der hohen Prävalenzen und der gesundheitlichen Auswirkungen hoch. Eine Infektion mit *P. falciparum* erwies sich mit einer Odds-Ratio von 10,3 als stärkster unabhängiger Risikofaktor für die Ausbildung einer Anämie. Maßnahmen zur Eindämmung der Malaria sind daher weiterhin als wesentliches und beeinflussbares Element zur Verbesserung des hämatologischen Status der Kinder in der Region anzusehen.

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Anteilserklärung der ausgewählten Publikationen

Irene Regina Zeile hatte folgenden Anteil an den ausgewählten Publikationen:

Publikation 1:
Jean-Bosco Gahutu, Christian Steininger, Cyprien Shyirambere, **Irene Zeile**, Neniling Cwinya-Ay, Ina Danquah, Christoph H Larsen, Teunis A Eggelte, Aline Uwimana, Corine Karema, Andre Musemakweri,Gundel Harms, Frank P Mockenhaupt. *Prevalence and risk factors of malaria among children in southern highland Rwanda. Malaria Journal, 2011.* **20 Prozent**
Beitrag im Einzelnen: Diagnostische Plasmodien-PCR und Auswertung

Publikation 2:
Irene Zeile, Jean-Bosco Gahutub, Cyprien Shyirambere, Christian Steininger, Andre Musemakweri, Fidèle Sebahungu, Corine Karema, Gundel Harms, Teunis A. Eggelte, Frank P. Mockenhaupt. Molecular markers of Plasmodium falciparum drug resistance in southern highland Rwanda. Acta Tropica, 2011. **60 Prozent**
Beitrag im Einzelnen: Typisierung der Isolate und Auswertung

Publikation 3:
Ina Danquah, Jean-Bosco Gahutu, **Irene Zeile,** Andre Musemakweri, Frank P. Mockenhaupt. *Anaemia, iron deficiency and a common polymorphism of iron-regulation, TMPRSS6 rs855791, in Rwandan children. Tropical Medicine & International Health, 2014.* **20 Prozent**
Beitrag im Einzelnen: Auswertung und Literaturrecherche

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Prof. Dr. Frank P. Mockenhaupt	Irene Regina Zeile

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Prevalence and risk factors of malaria among children in southern highland Rwanda

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Abstract

Background: Increased control has produced remarkable reductions of malaria in some parts of sub-Saharan Africa, including Rwanda. In the southern highlands, near the district capital of Butare (altitude, 1,768 m), a combined community-and facility-based survey on *Plasmodium* infection was conducted early in 2010.

Methods: A total of 749 children below five years of age were examined including 545 randomly selected from 24 villages, 103 attending the health centre in charge, and 101 at the referral district hospital. Clinical, parasitological, haematological, and socio-economic data were collected.

Results: *Plasmodium falciparum* infection (mean multiplicity, 2.08) was identified by microscopy and PCR in 11.7% and 16.7%, respectively; 5.5% of the children had malaria. PCR-based *P. falciparum* prevalence ranged between 0 and 38.5% in the villages, and was 21.4% in the health centre, and 14.9% in the hospital. Independent predictors of infection included increasing age, low mid-upper arm circumference, absence of several household assets, reported recent intake of artemether-lumefantrine, and chloroquine in plasma, measured by ELISA. Self-reported bed net use (58%) reduced infection only in univariate analysis. In the communities, most infections were seemingly asymptomatic but anaemia was observed in 82% and 28% of children with and without parasitaemia, respectively, the effect increasing with parasite density, and significant also for submicroscopic infections.

Conclusions: *Plasmodium falciparum* infection in the highlands surrounding Butare, Rwanda, is seen in one out of six children under five years of age. The abundance of seemingly asymptomatic infections in the community forms a reservoir for transmission in this epidemic-prone area. Risk factors suggestive of low socio-economic status and insufficient effectiveness of self-reported bed net use refer to areas of improvable intervention.

Background

Recent years have seen a substantial increase in malaria control activities. Particularly in East Africa, growing evidence suggests a decline in malaria transmission, morbidity and mortality over the last decade [1-5]. Control measures considered vital to this improvement are the deployment of artemisinin-based combination treatment (ACT), distribution of long-lasting insecticide-treated nets (LLINs), and indoor residual spraying [3,6].

Rwanda is a prime example for the impact malaria control can have. Since 2000, several million insecticide treated nets (ITNs) have been distributed (mostly LLINs) increasing the percentage of the population (10 million) covered by nets to potentially ≥70%. In parallel, ACTs have been dispensed on a large scale. In 2007, 56% of households were considered to own a net and 56% of children to sleep under one [4]. Surveillance and health facility based data indicate that by 2007-2008 these efforts were associated with approximately 50% or higher declines in confirmed outpatient cases, inpatient cases, and deaths due to malaria in children <5 years old [4,7].

While this progress does not appear to be questionable, the extent of the declines as deduced from facility-based data might differ at community level. For instance, community-level case management programmes [8] have been reported to shift primary treatment from health

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centres to villages and thus decrease the health-facility burden [9]. Such a trend, however, does not necessarily reflect the situation in the community [10,11].

One aim of the present study was therefore to provide up-to-date malariologic data at the levels of community, health centre, and district hospital for a highland area in southern Rwanda from where no published material exists so far. In addition, the study aimed at identifying (modifiable) factors associated with *Plasmodium* infection and malaria in this population.

Methods

Study area and sampling

Butare (population approximately 100,000; altitude 1,768 m) is the capital of Huye district, southern province of Rwanda. Located on the central plateau of Rwanda (Figure 1; average altitude, 1,700 m; yearly rainfall, 1,200 mm; mean temperature, 19°C), Butare is surrounded by densely populated farmland hills. Despite two rainy seasons (October-November; March-May), the area is prone to drought. The present study was conducted from January 18 to March 26, 2010 but the rainy season started as early as late January in this year.

Rwanda has a mandatory health insurance system in which the mutual health insurance scheme *(mutuelle de santé*) is the most widespread. At an annual cost of 1,000 Rwandan Francs (1.28 €; February 2010) per capita, treatment of common diseases is basically free of charge including utilization of district and provincial hospitals provided there is adherence to a strict referral system [12]. Governmental health services in Butare area are provided by several primary health centres, Kabutare district hospital and Butare University Teaching Hospital (CHUB, *Centre Hospitalier Universitaire de Butare*).

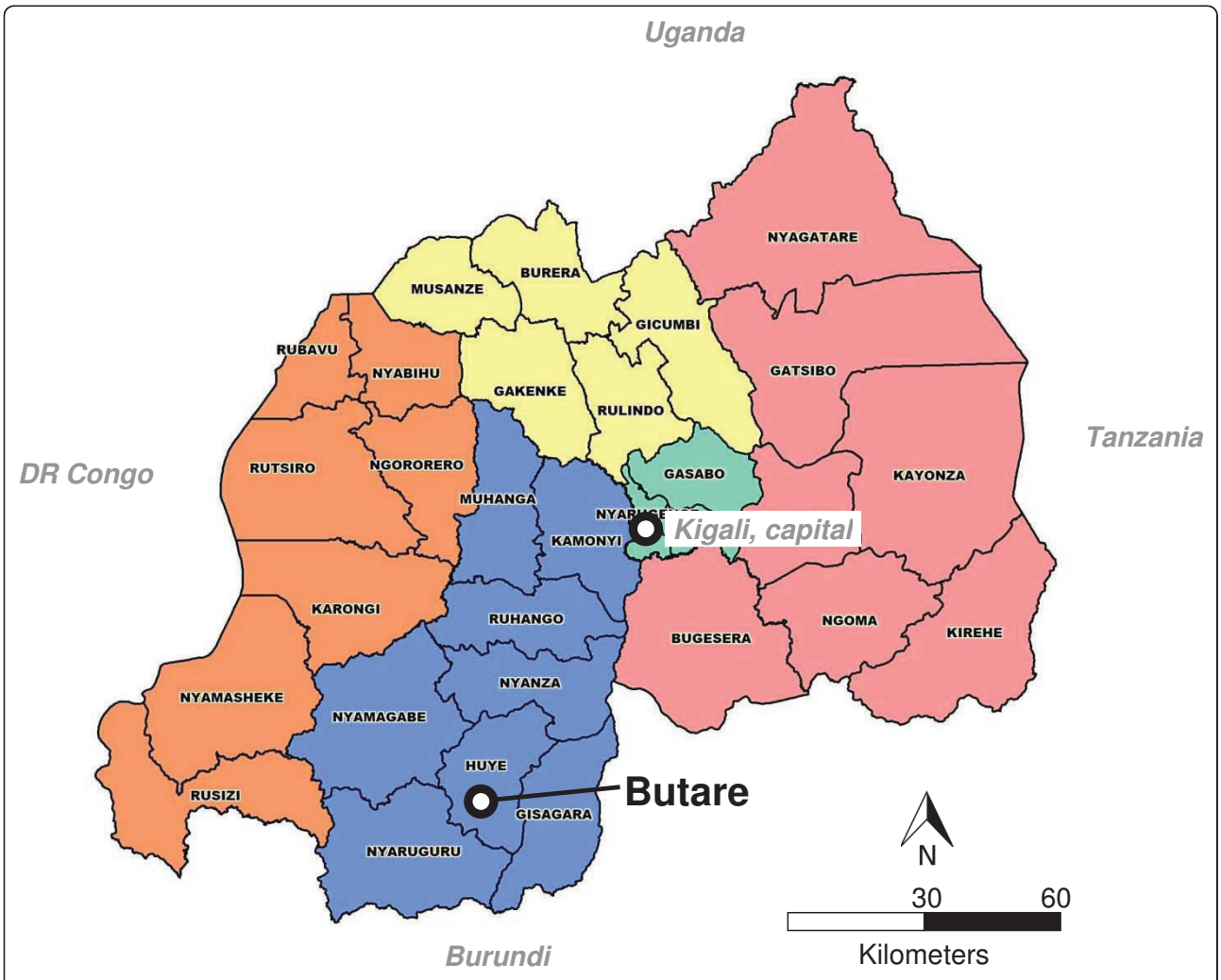

Figure 1 Rwanda administrative map, with Butare location. Map displaying the five provinces of Rwanda (Northern, Eastern, Southern, Western, Kigali) and district boundaries.

The study was designed as a cross-sectional survey to assess the prevalences of malaria, HIV, and soil-transmitted helminths in children under five years of age in the CHUB catchment area, i.e. at the levels of community, health centre, and district hospital. The present report focuses on the malaria situation. For the community level, the neighbouring rural Huye subdistrict (*sector* ; population approximately 20,000) was chosen (Figure 2). Based on most recent census data, each 25 households were randomly chosen in a total of 24 randomly selected villages. Community health workers visited these households, randomly selected one child per family, and asked the child to be presented to the study team located at Sovu health centre (or a non-permanently staffed branch) on a scheduled (usually next) day. Thereby, balanced recruitment into the age strata <1, 1 < 2, 2 < 3, 3 < 4, and 4 < 5 years was aimed at. In parallel, ≥100 paediatric patients aged five years or less and presenting at the primary Sovu health centre and at the referral Kabutare district hospital, i.e. the health facilities serving this population, were successively recruited. All children's parents were thoroughly informed on the purpose and procedures of the study, and recruitment was preceded by HIV pre-counselling and obtaining informed written consent. The study was reviewed and approved by the National Ethics Committee, Republic of Rwanda.

Examinations

Brief questionnaires were filled in on socio-economic aspects of the children's families including household assets; specification of bed nets, e.g. impregnated or not, was omitted. All children were examined by a physician, a medical history obtained, and a venous blood sample collected. Age, sex, weight, height, mid-upper arm circumference (MUAC), and fever (axillary temperature ≥37.5°C) were documented. Haemoglobin (Hb) levels were measured by a HemoCue photometer (Angelholm, Sweden). Anaemia was defined as an Hb level < 11 g/dL. Intestinal parasites were screened for by direct wet

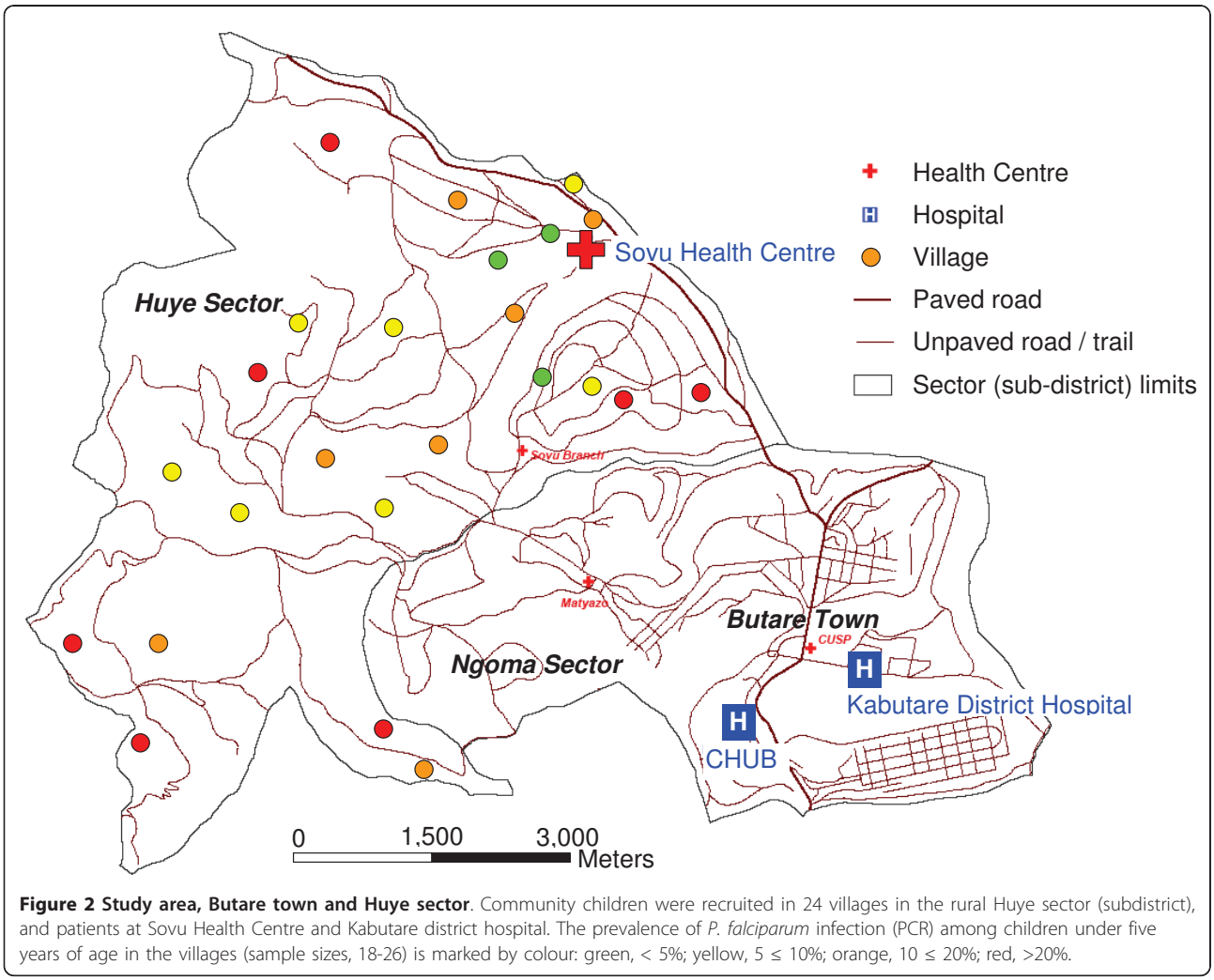

Figure 2 Study area, Butare town and Huye sector. Community children were recruited in 24 villages in the rural Huye sector (subdistrict), and patients at Sovu Health Centre and Kabutare district hospital. The prevalence of *P. falciparum* infection (PCR) among children under five years of age in the villages (sample sizes, 18-26) is marked by colour: green, < 5%; yellow, 5 ≤ 10%; orange, 10 ≤ 20%; red, >20%.

mount microscopical stool examination, and urinary tract infection by dipstick (Multistix 10 SG, Bayer, Germany). Malaria parasites were counted *per* 200 white blood cells (WBCs) on Giemsa-stained thick blood films, while the patient was waiting. Children with malaria parasites were treated with artemether-lumefantrine. Other diseases were treated according to Rwanda health authority guidelines [13]. Following duplicate readings *per* 200 WBCs at the CHUB central laboratory and the Institute of Tropical Medicine & International Health in Berlin, the definite parasite density was calculated on the basis of a putative mean WBC count of 8,000/μL. These data were used for analysis. Malaria was defined as any microscopically visible parasitaemia *plus* fever or a history of fever within the preceding 48 hours. DNA was extracted (Qiamp blood kit; Qiagen, Germany), and *Plasmodium* species and submicroscopic infections were identified by semi-nested multiplex polymerase chain reaction (PCR) assays [14]. For all PCR positive samples, sequences corresponding to the allelic families of the *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) block 2 (K1, Mad20, Ro33) and of *msp2* block 3 (FC27, IC) were amplified in five separate PCR assays [15]. Size variation within the alleles can be used to discriminate different parasite clones by PCR fragment length polymorphism, visualised on 3% GTG® -agarose gels (Biozym, Germany) and analysed using GeneSnap software (SynGene, UK). In case of negative or inconclusive PCR results, assays were repeated maximally twice. Multiplicity of infection (MOI) was calculated as the highest number of fragments for either *msp1* or *msp2*. Residual (pre-treatment) concentrations of chloroquine and pyrimethamine (indicative of sulphadoxine-pyrimethamine) in plasma were determined by ELISA with limits of detection of 5 ng/mL and 10 ng/mL, respectively [16].

Statistical analysis

Data analysis was performed using Statview 5.0 (SAS Institute Inc.). Continuous variables were compared between groups by the non-parametric Mann-Whitney or Kruskal-Wallis tests, and proportions by χ^2 test or Fisher's exact test. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were computed. Non-parametric ordinal regression analysis was performed to assess significantly differential effects of independent factors, e.g. infection, on a dependent variable, e.g. Hb levels, between groups. Despite non-parametric comparisons and for tangibility, parasite densities and Hb concentrations are displayed as geometric mean parasite densities (GMPDs) and means, respectively. Evaluation of determinants of *P. falciparum* infection and malaria was performed by logistic regression analysis. Stepwise backward selection was performed, and final models included those factors that retained statistical significance. A p-value < 0.05 was considered statistically significant.

Results

Study participants

A total of 749 children were examined including 545 from the rural Huye communities, 103 from Sovu health centre, and 101 from Kabutare district hospital (Table 1). As compared to community children, those attending the health centre or the district hospital were slightly younger. In the communities, fever and a history of fever within the preceding 48 hours were rare (3% and 10%) but common in the health centre (35%, 70%) and in the district hospital (27%, 48%). The primary clinical diagnoses differed between the groups (overall, $P < 0.0001$). In community children, the leading one was gastro-intestinal affection including gastroenteritis, amoebiasis and helminthiasis. In health centre and hospital, the leading primary diagnosis was respiratory tract infection (Table 1). Chloroquine in plasma was found in 3.7% (28/747) of all children at a median concentration of 15 ng/mL (range, 8-240). Only one child exhibited pyrimethamine in plasma (50 ng/mL).

The three groups showed large differences in the socio-economic characteristics of the children's families (Table 2). In the communities, most children lived in rural areas, the average monthly family income was low; one third of the parents had no education at all, and almost all worked as farmers or labourers. Accordingly, asset ownership was generally limited. Less than half of the children were covered by any health insurance; for slightly more than half, a bed net was reported to have been used in the preceding night. Compared to that, socio-economic parameters almost consistently indicated better conditions among children attending the health facilities (Table 2). In particular, among health facility attendees, 85% were covered by a health insurance, and the rate of self-reported bed net use was 71%. Many socio-economic parameters were inter-related. Monthly family income, for instance, was higher in those with a health insurance than in those without (medians, 10,000 *vs*. 5,000 Rwandan Francs, $P < 0.0001$) and higher in those using bed nets as compared to non-users (8,000 *vs*. 5,000 Rwandan Francs, $P = 0.0003$).

Parasitological parameters

Overall, 16.7% of all 749 children were found by PCR to harbour *P. falciparum* , 11.7% had microscopically visible parasitaemia, and 5.5% malaria. All microscopically positive samples were also positive by PCR (including one *Plasmodium malariae* and two *Plasmodium ovale* mono-infections). The prevalences of *P. falciparum* infections detected by PCR (range, 16-21%) and of

Table 1 Characteristics of 749 children from southern highland Rwanda

Parameter	Huye communities	Sovu Health Centre	Kabutare District Hospital	*P*
No. (%)	545 (72.8)	103 (13.8)	101 (13.5)	
Age (months)	31.1 (1-60)	28.3 (1-59)	27.2 (1-60)*	0.03
Proportion girls (%)	45.5	50.5	50.5	0.48
Weight (kg)	11.3 (3.5-18.8)	10.9 (3.8-20.0)	11.0 (3.3-19.0)	0.24
Height (cm)[a]	80.0 (41-108)	76.1 (42-112)*	78.5 (52-110)	0.02
MUAC (cm)[b]	13.7 (5.0-18.0)	14.2 (10.5-19.0)*	13.6 (8.0-18.5)†	0.04
Axillary temperature (°C)[c]	36.7 (36.0-40.6)	37.4 (36.0-40.1)*	37.0 (35.8-39.4)†	<0.0001
Fever (%)	3.3 (18/543)	35.0*	26.7*	<0.0001
History of fever, last 2 days (%)	9.8 (49/502)	69.4 (68/98)*	47.5 (47/99)*†	<0.0001
Hb (g/dL)[b]	11.3 (1.7-15.3)	11.4 (1.4-16.6)	11.1 (4.3-16.8)	0.89
Anaemia (Hb < 11 g/dL),%	34.1	35.0	32.0 (32/100)	0.89
Severe Anaemia (Hb<7 g/dl),%	1.8	1.9	7.0 (7/100)*	0.01
Primary diagnosis on examination[d]				
Healthy child	25.4	4.3*	1.5*	<0.0001
Gastro-intestinal tract affection[e]	31.9	25.0	19.4*	0.04
Respiratory tract infection	7.3	34.3*	26.1*	<0.0001
Malaria (suspected)[f]	9.7	19.3*	11.9	0.0001
Severe malnutrition (clinically)	8.2	1.4*	3.7	0.01
Skin infection	4.6	5.7	4.5	0.67
Burns, wounds, accidents, etc.	2.1	2.1	14.9*†	<0.0001
Severe anaemia (clinically)	2.4	0.7	3.0	0.41
Conjunctivitis	1.4	1.4	3.7	0.11
Disability	1.1	0	5.2*†	<0.0001
Oral problems	0.9	1.4	3.7*	0.02
Urinary tract infection	0.6	2.9*	2.2	0.02
Others, missing data	4.4	1.4	0*	0.03

Numerical data are means (range) unless otherwise indicated, and compared by the non-parametric Kruskal Wallis or Mann Whitney U tests. Proportions were compared by χ^2 test or Fisher's exact test. MUAC, mid upper arm circumference; Hb, haemoglobin; GMPD, geometric mean parasite density; Malaria, definition [a], n = 746; [b], n = 748; [c], n = 747; [d], as judged by study physician, several diagnoses per child possible; No. of diagnoses: community, n = 658; health centre, n = 134; hospital, n = 140. [e], includes gastroenteritis, amoebiasis, helminthiasis, and others; [f], based on field-based microscopy and clinical judgement. *, difference to Huye communities, $P < 0.05$; †, difference to Sovu health centre, $P < 0.05$

microscopically visible parasitaemia (range, 10%-17%) did not differ between the groups (Table 3). However, whereas only one quarter of community children with parasitaemia was classified as having malaria, this was the case in all children at the health centre and in most at the district hospital. Likewise, GMPDs were lower in community children as compared to health centre (P = 0.02) or, non-significantly, to district hospital (P = 0.40; Table 3). As for the non-falciparum parasites, *P. malariae* was rare in the community but reached 3% in the health centre (P = 0.01).

In the villages, the prevalence of *P. falciparum* infection (PCR) ranged from 0 to 38.5% (P = 0.0002; Figure 2). The number of children in these communities allowed age-stratified analysis of parasitological parameters (numbers in age-groups: <1 year, 59; 1<2 years, 136; 2<3 years, 136; 3<4 years, 120; 4<5 years, 94). By χ^2 test for trend, the prevalences of *P. falciparum* -infection by PCR (P = 0.009), of microscopically visible parasitaemia (P = 0.03), and of malaria (P = 0.02) increased with age (Figure 3). Likewise, the proportion of asymptomatically infected children (PCR positive but no current or history of fever) among all infected children tended to decline with every year of age (100% (6/6), 88.8% (16/18), 85.0% (17/20), 81.0% (17/21), and 73.9% (17/23), P = 0.08). GMPDs (95% CIs) did not show a clear trend: in the above age groups, they were 308 (143-663), 1,374 (513-3,680), 1,162 (339-3,975), 3,491 (714-17,063), and 2,061 (781-5,437) parasites/μL, respectively (P = 0.29).

Multiplicity of infection (MOI) was successfully typed for 88.8% (111/125) of all *P. falciparum* isolates. MOI ranged from one to five (mean, 2.02; median, 2.0); 65% (72/111) of infections were polyclonal (MOI >1). MOI did not differ between community children and those attending health facilities (Table 3), did not correlate with age (months; Spearman's r = 0.14; P = 0.33; community children only, r = 0.19, P = 0.20) but was increased in microscopically visible parasitaemia as compared to submicroscopic infections (means, 2.15 *vs.* 1.67;

Table 2 Selected socio-economic characteristics in 749 children from southern highland Rwanda

Parameter	Huye communities	Sovu Health Centre	Kabutare District Hospital	*P*
No.	545	103	101	
Rural residence (%)	95.1 (507/533)	73.7 (70/95)*	66.0 (66/100) *	<0.0001
Monthly family income (RwF)[a]	9124 (0-100,000)	31,505 (0-300,000) *	28,916 (500-350,000) *	<0.0001
Mothers education (%)				
None	30.4 (165/543)	20.4 (21/103)	14.9 (15/101)	
Primary	67.0 (364/543)	68.0 (70/103)	69.3 (70/101)	
Secondary or higher	2.6 (14/543)	11.7 (12/103) *	15.8 (16/101) *	<0.0001
Mother's occupation farmer/labourer (%)	98.7 (533/540)	92.2 (95/103) *	78.2 (79/101) *†	<0.0001
Father's education (%)				
None	36.5 (195/534)	19.6 (20/102)	19.8 (20/101)	
Primary	60.1 (321/534)	67.6 (69/102)	60.4 (61/101)	
Secondary/tertiary	3.4 (18/534)	12.7 (13/102) *	19.8 (20/101) *	<0.0001
Father's occupation (%)				
Farmer/labourer	86.9 (472/543)	73.3 (74/101)	65.0 (65/100)	
Else	5.5 (30/543)	25.7 (26/101)	35.0 (35/100)	
Died/left/prisoner	7.6 (41/543)	1.0 (1/101) *	0 *	<0.0001
No. of people/household[a]	5.5 (2-12)	5.2 (3-12)	5.0 (2-12) *	0.03
No. of siblings[a]	2.0 (0-9)	1.8 (0-7)	1.4 (0-6) *	0.002
Household asset present (%)				
Electricity	1.3 (7/542)	11.7 (12/103) *	23.8 (24/101) *†	<0.0001
Piped water	14.3 (77/540)	6.8 (7/103) *	37.6 (38/101) *†	<0.0001
Radio	43.2 (233/539)	67.0 (69/103) *	77.2 (78/101) *	<0.0001
TV	0.7 (4/541)	5.9 (6/102) *	12.9 (13/101) *	<0.0001
Cupboard	8.9 (48/540)	24.3 (25/103) *	37.6 (38/101) *†	<0.0001
Bicycle	9.1 (49/540)	37.9 (39/103) *	27.7 (28/101) *	<0.0001
Motor-bike	0.6 (3/540)	2.9 (3/102)	1.0 (1/101)	0.07
Fridge	0 (0/541)	1.0 (1/102) *	2.0 (2/101) *	0.01
Cattle	13.0 (70/539)	43.7 (45/103) *	20.8 (21/102) *†	<0.0001
Health insurance present (%)[c]	43.0 (234/544)	90.2 (92/102) *	79.3 (80/101) *†	<0.0001
Child received any drug in last 2 weeks	8.3 (45/545)	13.7 (14/102)	38.0 (38/100) *†	<0.0001
Child used bed net last night (%)	52.7 (286/543)	69.6 (71/102) *	73.3 (74/102) *	<0.0001
Chloroquine in plasma (%)	3.7 (20/545)	4.0 (4/101)	4.0 (4/101)	0.98

Numerical data are means (range) unless otherwise indicated, and compared by the non-parametric Kruskal Wallis and Mann Whitney U tests. Proportions were compared by χ^2 test or Fisher's exact test. [a] , n = 748; *, difference to Huye communities, $P < 0.05$; †, difference to Sovu health centre, $P < 0.05$

Table 3 Parasitological parameters in 749 children from southern highland Rwanda

Parameter	Huye communities	Sovu Health Centre	Kabutare District Hospital	*P*
No.	545	103	101	
Parasitaemia (%)	11.2	16.5	9.9	0.25
GMPD (parasites/μL; 95%CI)	1574 (913-2714)	7603 (2127-27185) *	5508 (701-43251)	0.04
MOI (mean, range)	2.05 (1-5)	1.95 (1-4)	1.92 (1-4)	0.92
Malaria (%)	2.9	16.5 *	7.9 *	<0.0001
P. falciparum infection, PCR (%)	16.1	21.4	14.9	0.37
P. ovale infection, PCR (%)	0.9	1.9	3.0	0.22
P. malariae infection, PCR (%)	0.2	2.9 *	2.0	0.006
Proportion of submicroscopic infections (%, n/n)	33.7 (31/92)	22.7 (5/22)	41.2 (7/17)	0.45
Child received artemether-lumefantrine in last 2 weeks	3.1	1.0	5.9	0.13

GMPD, geometric mean parasite density, and MOI, multiplicity of infection, are compared by the non-parametric Kruskal Wallis and Mann Whitney U tests. Proportions were compared by χ^2 test or Fisher's exact test. *, difference to Huye communities, $P < 0.05$;

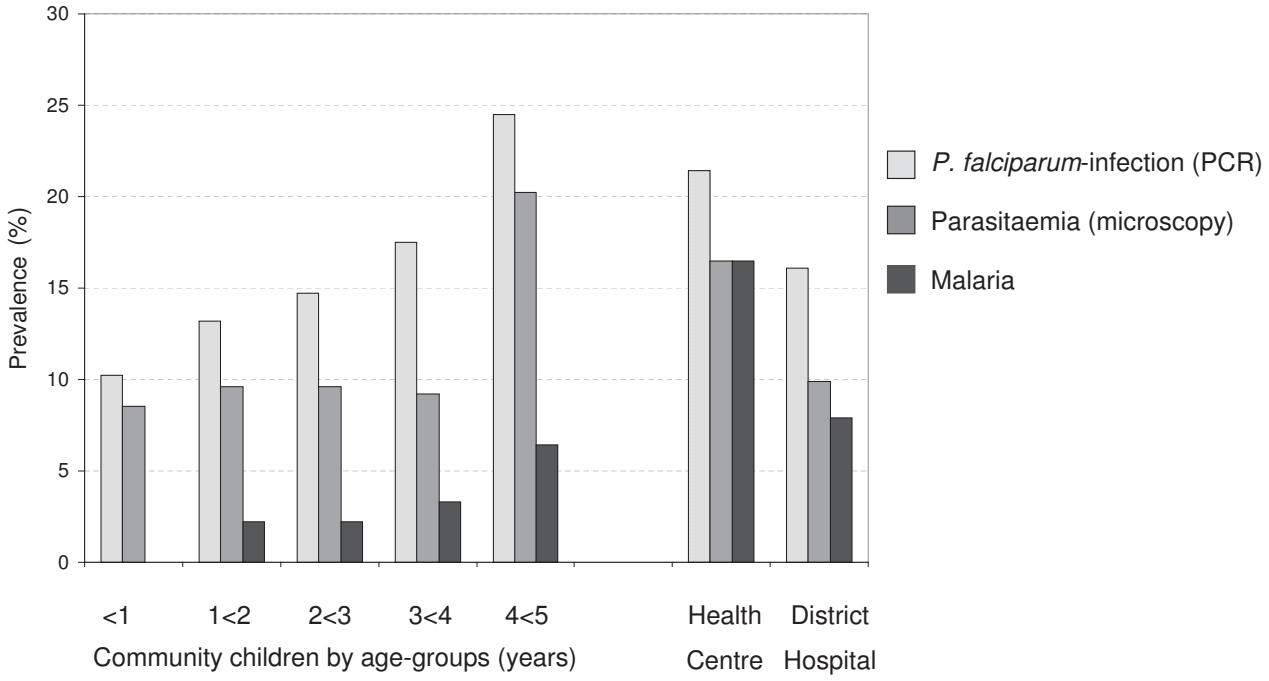

Figure 3 Prevalences of *P. falciparum* infection (PCR), parasitaemia (microscopy) and malaria in 749 children from southern highland Rwanda.

P = 0.03), correlated positively with parasite density (r = 0.28, P = 0.006), and tended to be reduced in former artemether-lumefantrine (AL) recipients as compared to children without such reported intake (means, 1.54 *vs*. 2.08; P = 0.06).

Factors associated with *P. falciparum* infection and malaria

Beyond age, a number of factors influenced the presence of infection or malaria. In explorative univariate analysis, socio-economic parameters (Table 2), MUAC, self-reported bed net use, previous AL treatment, and chloroquine in plasma were tested for association with *P. falciparum* infection. The odds of *P. falciparum* was found to increase with increasing age, decreasing MUAC, low educational level, absent father, absence of several household assets, a low family income, lacking use of a bed net, intake of AL within the preceding two weeks (median 7 days before; range, 1-14), and the presence of chloroquine in plasma. For multivariate analysis, univariately associated factors, adjusted for study sub-groups, were entered into a logistic regression model, and subjected to stepwise backward removal (Table 4). Independent predictors of *P. falciparum* infection included increasing age, health centre attendance, low MUAC, absence of cupboard, radio and bicycle, recent AL intake, and presence of chloroquine in plasma.

For malaria, the same analysis produced the following independently associated factors (aOR (95% CI)): age (months, 1.02 (1.0-1.05), P = 0.04), absent father (6.53 (2.15-19.83), P = 0.0009), presence of radio (0.30 (0.13-0.65), P = 0.003), recent intake of AL (4.40 (1.24-15.57), P = 0.02), and chloroquine in plasma (4.87 (1.43-16.57), P = 0.01), adjusted for attendance at health centre (15.93 (6.47-39.26), P < 0.0001) or district hospital (9.16 (3.16-26.52), P < 0.0001). The role of village among community children lost significance in multivariate analysis (all, P > 0.05). In the above final models, self-reported bed net use showed no association with *P. falciparum* infection (aOR, 0.88 (0.56-1.38), P = 0.58) or malaria (aOR, 0.99 (0.47-2.10), P = 0.98).

Limiting multivariate analysis to community children produced basically the same results. However, household possessions of cupboard or bicycle lost significant association with *P. falciparum* infection. In multivariate analysis of current malaria in community children, chloroquine in plasma (prevalence, 3.7%) lost significant association, and that of age (months) became borderline significant (aOR, 1.03; 95%CI, 1.0-1.07; P = 0.07).

Clinical manifestations

Because ordinal regression analyses revealed that *P. falciparum* infection (PCR) in the three groups had significantly differing effects on Hb (r = 0.6; standard error (SE) = 0.25; P = 0.02) and body temperature (r = 0.83;

Table 4 Univariate and multivariate analysis of factors associated with *P. falciparum* infection (PCR)

Parameter	No.	Proportion infected (%)	Univariate analysis			Multivariate analysis		
			OR	95%CI	*P*	aOR	95%CI	*P*
Group								
Huye communities	545	16.1	1			1		
Health Centre	101	21.4	1.41	0.81-2.45	0.20	2.74	1.44-5.19	0.002
District Hospital	103	14.9	0.91	0.48-1.70	0.74	1.72	0.86-3.44	0.12
Age (months)	749	n.a.	1.02	1.01-1.03	0.001	1.03	1.01-1.04	<0.0001
MUAC (cm)	748	n.a.	0.88	0.79-0.99	0.03	0.83	072-0.95	0.008
Mother's education								
None	201	20.9	1					
Primary	504	16.1	0.72	0.47-1.12	0.13			
Secondary/tertiary	42	4.8	0.19	0.02-0.79	0.01			
Father's education								
None	235	23.0	1					
Primary	451	14.2	0.55	0.36-0.85	0.004			
Secondary/tertiary	51	7.8	0.29	0.07-0.84	0.01			
Father's occupation								
Farmer/labourer	611	16.9	1					
Else	91	8.8	0.48	0.21-1.05	0.05			
Died/left/prisoner	42	31.0	2.21	1.05-4.60	0.02			
Pipe-born water								
No	622	18.3	1					
Yes	122	7.4	0.35	0.16-0.75	0.003			
Cupboard								
No	633	18.3	1			1		
Yes	111	6.3	0.30	0.11-0.66	0.002	0.37	0.15-0.92	0.03
Radio								
No	363	23.7	1			1		
Yes	380	9.5	0.34	0.22-0.52	<0.0001	0.40	0.24-0.66	0.0003
Bicycle								
No	628	18.5	1			1		
Yes	116	6.0	0.28	0.11-0.63	0.0009	0.38	0.15-1.0	0.049
Household income								
>= 5000 RwF (median)	498	14.5	1					
< 5000 RwF	250	21.2	1.59	1.05-2.40	0.02			
Use of bed net								
No	315	21.0	1					
Yes	431	13.7	0.60	0.40-0.90	0.009			
Intake of AL, preceding 2 weeks								
No	725	15.4	1			1		
Yes	24	54.4	6.47	2.64-15.9	<0.0001	6.93	2.70-17.78	<0.0001
Chloroquine in plasma								
No	719	14.5	1			1		
Yes	28	67.9	12.48	5.19-32.1	<0.0001	17.18	6.84-43.16	<0.0001

n.a., not applicable; MUAC, mid upper arm circumference; RwF, Rwandan Francs; AL, artemether-lumefantrine; OR, odds ratio; aOR, adjusted OR derived from logistic regression including all parameters listed here and following stepwise backward removal of factors not associated in multivariate analysis.

SE = 0.25; P = 0.001), the main analysis was performed for community children (Table 5). In these, anaemia (Hb<11 g/dL) was observed in 34% and fever in 3%. Parasitaemia was associated with a reduction in mean Hb of-2.2 g/dL, and, age-adjusted, eighteen-fold and five-fold increased odds of anaemia and fever, respectively. These effects were pronounced at increasing parasite density. However, even in submicroscopic infections (all afebrile), mean Hb was significantly reduced by-1.4 g/dL (Table 5).

Table 5 Manifestation of malaria in rural Huye subdistrict

		Fever			Anaemia (Hb < 11 g/dl)			Hb (g/dL)	
	No.	%	*P*	aOR (95%CI)	%	*P*	aOR (95%CI)	Mean	*P*
Parasitaemia									
Absent	484	2.3 (11/482)			28.1			11.5	
Present	61	11.5	0.002	4.8 (1.8-13.2)	82.0	<0.001	17.5 (8.4-36.4)	9.3	<0.0001
Parasite density									
None	484	2.3 (11/482)			28.1			11.5	
< 1000	26	7.7	0.1	3.4 (0.7-16.5)	65.4	0.0002	6.0 (2.5-14.4)	9.9	<0.0001
1000 < 10000	25	12.0	0.01	4.8 (1.2-18.9)	96.0	<0.0001	105.7 (13.8-812.5)	9.1	<0.0001
> = 10000	10	20.0	0.005	8.6 (1.6-46.9)	90.0	0.003	40.5 (4.9-336.7)	8.3	<0.0001
P. falciparum infection, PCR									
Absent	457	2.4 (11/455)			26.7			11.6	
Present	88	8.0	0.008	3.0 (1.1-8.1)	72.7	<0.0001	10.8 (6.1-18.9)	9.6	<0.0001
Staged infection									
None	453	2.4 (11/451)			26.5			11.6	
Submicroscopic	31	0	1	0 (0-∞)	51.6	0.004	4.0 (1.9-8.8)	10.2	0.0009
Microscopic	61	11.5	0.004	4.4 (1.6-12.2)	82.0	<0.0001	20.3 (9.6-42.6)	9.3	<0.0001

aOR, age-adjusted odds ratio. 95%CI, 95% confidence interval. Hb, haemoglobin concentration. Hb levels are compared by the non-parametric Mann Whitney U test.

In children attending the health centre, there were trends only of an association between anaemia and *P. falciparum* infection by PCR (age-adjusted OR (95% CI), 2.77 (0.90-8.55), $P = 0.08$) or microscopically visible parasitaemia (2.69 (0.80-9.0), $P = 0.11$). In contrast, at the district hospital, these figures yielded statistical significance (PCR, 4.62 (1.34-15.95), $P = 0.02$; microscopy, 10.3 (1.89-55.88), $P = 0.007$). For fever, the opposite was seen: Parasitaemia, e.g. detected by microscopy, increased the risk of fever nine times in the health centre (age-adjusted OR (95% CI), 8.88 (2.54-31.04), $P = 0.0006$) but showed no association in the hospital (1.27 (0.30-5.39), $P = 0.74$).

Discussion

Malaria transmission in Rwanda varies widely. Traditionally, the central plateau (altitude 1,500 to 1,800 m) is considered as one of four distinct ecological zones with overall *P. falciparum* prevalence rates of 5% to 15% [17]. While transmission in Rwanda is regarded to be stable with seasonal peaks in the valleys and unstable (and potentially epidemic-prone) at higher altitude [18], a linear correlation between altitude and transmission would be oversimplified: in a recent tabulation of the years 2001-2007, endemicity at 1,600-2,000 m above sea level ranged from hypo-to holoendemic and annual malaria incidences (presumed and confirmed) from 2.4 to 20.4 per 1000 capita [17]. Likely, these figures have declined in recent years [4,7,8]. Data from the 2007-2008 Demographic and Health Survey (DHS) [19] indicate that in >4,600 children <5 years of age and sampled across Rwanda, 2.6% had malaria infection based on rapid *Plasmodium* lactate dehydrogenase tests. In the present study from the vicinity of Butare (altitude, 1,768 m), 11% and 16% of community children were infected with *P. falciparum* based on microscopy and PCR, respectively. Data from the 2010 Rwanda Health Management Information System indicate that 13.4% of patients attending health facilities in the Huye district had microscopically confirmed malaria. In the present study, this figure was 12.3%.

Given the scarcity of published community-based data, the reasons for the discrepancy in the prevalence of infection reported in the DHS and observed in the present study are difficult to appraise. Higher sensitivity of PCR as compared to microscopy or rapid test devices [20,21] may partially be involved. Selection bias during recruitment at home, e.g., due to preferential presentation by the parents of children with (a recent history of) sickness cannot completely be excluded. However, recruitment teams were instructed to select children from households randomly and into pre-defined age strata. Also, most infections in the communities were asymptomatic. One limitation of the present study is its cross-sectional nature by which e.g., seasonal fluctuations are not

reproduced. Geographical variation of infection between villages was evident (Figure 2) but attempts to relate this to e.g. altitude or proximity to a water stream, failed. Likely involved, most villages comprise homesteads scattered in the hills rather than agglomerated settlements [22]. Other parameters in the present study, e.g. self-reported bed net use, socio-economic factors, anaemia, were largely in the reported range [4,19,22]. Thus, the present data are not representative for the central plateau, let alone Rwanda, but rather provide a detailed and up-to-date picture of *P. falciparum* infection in southern highland communities and in health facilities serving this population. In contrast, routine health facility based surveillance has clear limitations in providing complete or representative data on e.g., malaria in the community, also because patients lacking access or choosing alternatives are not registered [23]. A low health insurance coverage (43%) in the communities may have deterred parents from seeking formal health care. Such, in turn, could have lead to an over-estimation of disease burden at the community level as compared to the end of the year when more have paid their fees. Nevertheless, only 20% of African children with suspicion of malaria are considered to come to the attention of any formal health system [24], a figure that might have improved in recent years [4]. Community-based surveys, despite their local limitations, thus provide essential information, also for control campaign monitoring [10,11].

In the communities, infection prevalence increased from 10% to almost 25% at four years of age, which was not accompanied by a decline in parasite density with age or increase in MOI. The additional age-dependent increase in malaria and trend for declining asymptomatic infections indicate that semi-immunity did not develop to the extent observed in highly endemic areas [25-27]. In line with this, *P. falciparum* infection including submicroscopic ones had an impact on Hb levels, which exceeds the one commonly seen in children in high-endemicity areas [27-29]. On the other hand, only a quarter of parasitaemic children had malaria, suggesting a majority of asymptomatic infections. Irrespective of the erratic nature of fever in the definition of malaria, the presence of (usually undetected) asymptomatic *P. falciparum* infections has important implications for malaria control in highland areas. In two sites in highland Kenya, both, high and low levels of asymptomatic *P. falciparum* infections have been observed among children and adults [30,31]. Studies from Ghana and Sudan indicate that asymptomatic infections can persist for a year or longer [32,33]; in highland Kenya the median duration in children aged 5-9 years was five months [31]. Gametocyte carriage was not consistently assessed in the present study but appeared to be low. Nevertheless, individuals with low level, long-lasting, and asymptomatic infections form a major reservoir for transmission [21,34,35]. In situations of increased rainfall, higher temperatures, or changed land use such asymptomatic infections may give rise to epidemics which have increased in frequency and intensity in East Africa during the last two decades [36-39]. Targeted antimalarial treatment even of asymptomatic children may thus be a justifiable part of malaria control in highland areas. However, the differing findings on the level of asymptomatic parasite carriage in the present and the two Kenyan studies [30,31] illustrate that results may not be readily extrapolated.

At the health facilities, roughly half of the children had respiratory tract infections or gastrointestinal problems. Every sixth child at the health centre had malaria and every fifth was *P. falciparum* infected. Irrespective of the better socio-economic status of the patients' families as compared to the communities (Table 2) this indicates that malaria is among the top three reasons to seek primary health care in this area. At eight percent prevalence, malaria was of lesser importance at the district hospital which receives referrals from several health centres and patients bypassing the referral system by self-paying. There, 38% of the patients were reportedly pre-treated including 6% with AL. The validity of (malaria) treatment histories frequently is questionable [40], and no data on the dose and duration of treatment were collected in the present study. Nevertheless, the finding that recent AL treatment was positively associated with current malaria is remarkable. This is suggestive of recurrence of parasitaemia following treatment. In fact, drug resistance markers associated with reappearing parasitaemia following AL treatment tended to be increased in these infections (Zeile *et al* , unpublished observations). Latest cure rates of AL in Rwanda from 2006 have been reported as 97% [41]. Nevertheless, against the background of intense AL drug pressure in Rwanda in recent years, this finding underlines the necessity of the upcoming re-evaluation of the drug's efficacy in this country.

Intake of chloroquine was stated by none of the respondents but the drug was present in plasma in 1.4% and 15.4% of non-infected and infected children, respectively. With the assay applied, chloroquine intake can be detected for several weeks, depending on the dose; cross-reactivity with amodiaquine is negligible [16]. Likely, the finding of an increased infection prevalence in chloroquine positive children reflects the combination of previous home-treatment and persisting or recrudescent parasites due to intense chloroquine resistance which is prevalent in Rwanda [42].

Among the age-adjusted risk factors for *P. falciparum* infection was a decreasing MUAC. This crude proxy parameter for malnutrition was, however, not associated with malaria itself. Chronic malnutrition affects every second child in Rwanda [19] and compromises anti-

pathogen immunity [43]. Lacking effect on malaria morbidity as observed in the present study corresponds with previous findings [29,44] but contrasts with others [28,45,46]. Possibly, the differential effect on infection and malaria depends on the specific yet unknown type of malnutrition in the study area. Considering the modifiable nature of this risk factor and Rwanda's recently renewed commitment to fight malnutrition, more research into this field is needed.

At variable statistical significance, several parameters reflecting low socio-economic status were associated with increased risks of infection. Remarkably, possession of a radio or a bicycle were independently associated predictors of reduced *P. falciparum* prevalence. This may reflect increased access to malaria-related information, improved awareness and increased usage of curative services. Given the latter is true, this points to accomplishable ways of reducing *P. falciparum* infection in the area, i.e. health communication and education.

Lastly, although self-reported bed net usage was in the previously observed range [4,19,22], the detectable impact was modest and non-significant in multivariate analysis. This finding points to deficits in an established mean of malaria prevention the efficacy of which has been confirmed in many studies [4,7,10,11]. The reasons may be diverse and rather involve caregivers' beliefs about causation and vulnerability as well as obstacles in translating knowledge into behaviour than insecticide resistance [47-49]. Nevertheless, these actual reasons need to be assessed at the community-level and subsequent campaigns should address potential obstacles to promote consistent and correct use.

Conclusions

In this community and facility based survey on malariologic parameters in southern highland Rwanda, *P. falciparum* infection was observed in one out of six children under five years of age, without much variation between community and health facilities. While facility-based, most infections were symptomatic, the opposite was seen in the communities. These seemingly asymptomatic infections greatly contributed to anaemia and form an unrecognized source of transmission in the epidemic-prone highland area. Improved nutrition, identification and elimination of causes of low bed net effectiveness, and reinforced health education are promising and tangible measures to further reduce *P. falciparum* in this area of Rwanda. In parallel, community-based surveillance of malaria should be included to monitor the progress of malaria control.

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Authors' contributions

JBG, FPM, AM, and GH designed the study. JBG, CSt, CSh, NCA, CHL, and CK were responsible for patient recruitment, clinical and laboratory examinations, and logistics. IZ did the PCR analyses, TEA the ELISA assays, and ID, JBG, AU and FPM the statistical analyses. JBG and FPM wrote the paper with major contributions of the other authors. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Short Communication

Anaemia, iron deficiency and a common polymorphism of iron-regulation, *TMPRSS6* rs855791, in Rwandan children

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Abstract Anaemia in children living in sub-Saharan Africa is common, but its causes are diverse. In 545 children below 5 years of age from rural southern Rwanda, we assessed the role of iron deficiency (ID) and of the *TMPRSS6* 736(V) (rs855791) allele, known to reduce iron status and haemoglobin (Hb) levels, in anaemia and Hb concentrations. Anaemia (Hb <11 g/dl) was present in 34.4% of the children and ID (ferritin <12 ng/ml) in 17.6%. The *TMPRSS6* 736(V) allele was uncommon (allele frequency, 0.096) and not associated with ID. In multivariate analysis, ID was positively associated with anaemia (adjusted odds ratio, 1.67) to an extent comparable with α^+-thalassaemia, breastfeeding, inflammation and low household income, but the odds were substantially higher in *Plasmodium falciparum* infection (adjusted odds ratio, 10.3). These findings were verified in a multivariate analysis of Hb concentrations. The *TMPRSS6* 736(V) allele only tended to be associated with low Hb levels. *TMPRSS6* 736(V) is comparatively rare among Rwandan children and may only slightly contribute to low Hb concentrations. Preventable causes of anaemia, notably ID and *P. falciparum* infection, largely outweigh its impact and need to be addressed to improve the haematological status of children in the study area.

keywords anaemia, *TMPRSS6*, iron deficiency, Rwanda

Introduction

Anaemia is present in two of three pre-school children in sub-Saharan Africa (SSA) (World Health Organization 2008). Iron deficiency (ID) – largely due to low intake, poor absorption or increased requirements (e. g., pregnancy, growth) – accounts for roughly half of anaemia worldwide, but its relative contribution varies with, for example, age and region. Severe anaemia is a major cause of childhood mortality in SSA, whereas milder and often chronic anaemia, notably ID anaemia, substantially impairs cognitive and physical development (World Health Organization 2001, 2008).

In Rwanda, a national survey in 2005 revealed 56% of pre-school children to be anaemic (haemoglobin (Hb) <11 g/dl) with a slightly lower prevalence (47%) in the South province (Institut National de la Statistique du Rwanda, ORC Macro 2006). In a rural area of that province in 2010, we found anaemia in 34% of 545 pre-school children, and associations with *Plasmodium falciparum* infection and α^+-thalassaemia (Gahutu *et al.* 2011, 2012). Here, we re-assessed risk factors for anaemia focusing on ID. Specifically in this respect, we examined the role of a common mutation (rs855791, valin (V) to alanin (A) change at codon 736, V736A) in the transmembrane serine protease 6 gene (*TMPRSS6*). The product of this gene, matriptase-2, influences the transcriptional regulation of hepcidin, the key regulator of iron homoeostasis. High levels of hepcidin cause ID (Hentze *et al.* 2010). The *TMPRSS6* 736(V) allele is associated with comparatively high hepcidin serum levels (Nai *et al.* 2011) and, in Caucasians and Asians, with low values for serum iron, transferrin saturation, mean cell volume (Benyamin *et al.* 2009) and Hb (average difference between homozygotes, 0.2 g/dl) (Chambers *et al.* 2009). However, recent work suggests other, yet unknown, serum hepcidin independent mechanisms, which play a role in the association of *TMPRSS6* variants with serum iron parameters (Galesloot *et al.* 2013). In SSA, common infections and (secondary) inflammatory

processes complicate the assessment of ID and of its contribution to anaemia (Mockenhaupt *et al.* 1999). Against this background, we explored whether *TMPRSS6* 736(V) is associated with anaemia and ID among African children.

Subjects and methods

We conducted a cluster-sampled survey on common diseases in children under 5 years of age in Butare and its rural surroundings in 2010. The study procedures and details on malaria and erythrocyte variants have been published elsewhere (Gahutu *et al.* 2011, 2012). The present report focuses on 545 children from the rural but densely populated farmland hills of Huye subdistrict (1700–1800 m asl). Briefly, 25 households each were randomly chosen in 24 randomly selected villages, and one child was randomly selected per household. Informed written consent was obtained from the children's parents, and the study was approved by the National Ethics Committee, Republic of Rwanda.

All children were clinically examined, medical and socio-demographic data documented, and a venous blood sample was collected. Fever (axillary temperature ≥37.5 °C), underweight (weight-for-age *z*-score <−2) and anaemia (Hb <11 g/dl; HemoCue, Angelholm, Sweden) were documented. Malaria parasite density was estimated microscopically (Gahutu *et al.* 2011), and the species of *Plasmodium* as well as submicroscopic infections (i.e. below the detection threshold of microscopy) were identified by polymerase chain reaction (PCR) assays (Rubio *et al.* 2002) after DNA extraction (QIAamp; Qiagen, Germany). *TMPRSS6* rs855791 was typed by melting curve analysis using commercially available primers and probes (TIB Mobiol, Berlin, Germany). Erythrocyte variants were identified as previously described (Gahutu *et al.* 2012). Plasma concentrations of ferritin and C-reactive protein (CRP) were measured by ELISA (Assaypro, St. Charles, MO, USA). ID was defined as ferritin <12 ng/ml (Cook & Skikne 1989), and inflammation as CRP >5 ng/ml (Erhardt *et al.* 2004). Children were treated according to Rwandan health authority guidelines.

Data were analysed using the survey data analysis module in Stata 9.0 (Stata Corporation, College Station, TX, USA). Due to the cluster sampling and the non-normal distribution of continuous variables, weighted non-parametric tests for survey data were used to compare means (95% confidence intervals [CIs]) and proportions (95% CIs). As for factors associated with anaemia, odds ratios (ORs) and 95% CIs were calculated applying logistic regression, weighting for the population size of each sampling cluster cell, that is, residence. Therefore, prevalences (%) do not necessarily correspond to absolute numbers. Stepwise backward removal of exposure variables was performed to identify independently associated variables. Weighted linear regression was used to estimate factors associated with normalised Hb concentrations (to the three transformation). A *P*-value ≤0.05 was considered statistically significant.

Results

Selected characteristics of the children are shown in Table 1. Most parents were farmers, many had no formal education, and income and asset ownership were low. One in four children was underweight. *P. falciparum* infection (predominately asymptomatic) and inflammation were each seen in 1 in 6 children, and anaemia in one-third of the children. The *TMPRSS6* 736(V) allele was observed in 17.8% (allele frequency 0.096).

Iron deficiency was present in 17.5%. This figure was similar when accounting for the role of inflammation in the definition of ID (Table 1; 15.1% (95% CI, 11.6–18.7%) of children with ferritin ≥12 ng/ml showed inflammation). Ferritin levels were comparatively low in children ≤2 years of age (mean, 67.1; 95% CI, 56.4–77.8 ng/ml) and higher thereafter (mean, 87.2; 95% CI, 77.6–96.7 ng/ml, $P = 0.006$). Correspondingly, ID was more common in children ≤2 years of age (22.6%) than in the older children (13.6%, $P = 0.001$). ID was not associated with sex, residence, CRP levels, underweight or socio-economic indicators (data not shown). Nor was the *TMPRSS6* 736 variant associated with ID: in children with AA, VA and VV genotypes, ID was observed in 16.5% (95% CI, 12.9–20.0%; 74/450), 17.5% (95% CI, 9.3–25.6%; 16/85) and 23.7% (95% CI, −5.3 to 52.7%; 2/10), respectively ($P = 0.68$). In these groups, mean (95% CI) plasma ferritin concentrations were 79.0 (71.2–86.8), 88.8 (67.3–110.3) and 58.8 (27.6–90.0) ng/mlL, respectively ($P = 0.57$).

Table 2 displays factors associated with anaemia. Anaemia was observed in 51.5% of children with ID and in 31.2% of children without (OR, 2.3). 25.0% (95% CI, 18.5–31.4%; 47/186) of anaemic children had ID, the proportion being virtually identical in children ≤2 years of age (25.3%; 95% CI, 16.1–34.4%; 24/94) and in older children (24.7%; 95% CI, 15.5–33.8; 23/92). In comparison, 34.6% (95% CI, 27.5–41.8%; 64/186) of anaemic children were *P. falciparum* infected, with a lower proportion in younger (23.6%; 95% CI, 14.6–32.7%; 22/94) than older children (45.2%; 95% CI, 34.6–55.9%; 42/92; $P = 0.004$). The slight overrepresentation of anaemia in children carrying the *TMPRSS6* 736(V) allele did not reach statistical

Table 1 Characteristics of 545 children from Huye subdistrict, South Province, Rwanda

Parameter	Per cent (95% CI)	*n*/*N*
N	545	
Proportion girls	44.6 (39.8–49.3)	247/544
Age (months), *n* = 545 [mean (95% CI)]	31.4 (30.1–32.7)	
Proportion breastfed	51.5 (46.8–56.3)	285/544
Monthly household income (<5000 RwF)	37.3 (32.8–41.8)	218/544
Lacking formal education, mother	30.1 (25.7–34.4)	165/543
Mother's occupation: farmer/laborer	98.7 (97.6–99.8)	533/540
Lacking formal education, father	39.1 (34.4–43.8)	195/534
Father's occupation: farmer/laborer	88.4 (85.2–91.7)	472/543
Absence of assessed household assets	52.1 (47.3–56.9)	185/540
No. of people/household, *n* = 544 [mean (95% CI)]	5.5 (5.4–5.7)	
No. of siblings, *n* = 544 [mean (95% CI)]	2.1 (1.9–2.2)	
Underweight (weight-for-age *z*-score <−2)	24.4 (20.3–28.5)	133/543
Fever (>37.4 °C axillary)	3.1 (1.3–4.9)	18/543
History of fever (last 48 h)	9.4 (6.7–12.2)	49/502
Plasmodium falciparum infection (PCR)	16.2 (12.7–19.7)	88/545
Malaria parasites on blood film	11.7 (8.8–14.6)	61/545
Malaria*	2.5 (1.0–4.0)	16/545
Proportion anaemic (Hb <11 g/dl)	34.4 (29.8–38.9)	88/545
Proportion moderately severe anaemia (Hb <7 g/dl)	1.9 (0.7–3.1)	10/545
Hb (g/dl), *n* = 545 [mean (95% CI)]	11.2 (11.1–11.4)	
Iron deficiency (Ferritin <12 ng/ml)†	16.8 (13.5–20.0)	92/545
Inflammation (CRP >5 mg/l)	16.3 (12.9–19.6)	82/545
α^+-thalassaemia		
None	86.0 (82.9–89.0)	467/545
Heterozygous	13.4 (10.4–16.3)	75/545
Homozygous	0.7 (0.0–1.5)	3/545
Glucose-6-phosphate dehydrogenase deficiency		
None	90.4 (87.8–92.9)	489/544
Heterozygous	5.0 (3.2–6.8)	30/544
Homo-/hemizygous	4.6 (2.7–6.5)	25/544
TMPRSS6 rs855791		
AA	82.2 (78.8–85.6)	450/545
AV	15.6 (12.4–18.8)	85/545
VV	2.2 (0.8–3.6)	10/545

Values for metric variables are presented as means (95% CIs) and for categorical variables as per cent (95% CIs).
Values were calculated using nonparametric tests for survey data weighted for the population size of the sampling cluster.
*Defined as microscopically visible *P. falciparum* infection *plus* fever (>37.4 °C) or history of fever (last 48 h).
†Using alternative definitions, the prevalences were 16.9% (95% CI, 13.6–20.2; *n* = 93) (ferritin <12 ng/ml, or <30 ng/ml if CRP >5 mg/dl) and 21.1% (95% CI, 17.5–24.6; *n* = 118) (ferritin <30 ng/ml).

significance, irrespective of age. In multivariate analysis, age and *P. falciparum* infection were the strongest independent predictors of anaemia. Moreover, the odds were increased by 67% in iron deficient children. Similar estimates were seen for α-thalassaemia, breastfeeding, inflammation and low household income. In this model, *TMPRSS6* 736(V) only tended to be associated with anaemia (Table 2).

Lastly, factors influencing (normalised) Hb concentrations *per se* were analysed. Mean (95% CI) Hb concentrations in children with and without ID were 10.5 (95% CI, 10.0–10.9) and 11.4 (95% CI, 11.3–11.6) g/dl, respectively ($P < 0.0001$). In contrast, the *TMPRSS6* variant was not significantly associated with Hb concentrations (means [95% CIs]; AA, 11.3 [11.1–11.4]; AV, 11.1 [10.7–11.4]; VV, 11.0 [9.3–12.7]; $P = 0.37$). In a multiple linear regression model replicating the above logistic regression model (all univariately associated parameters *plus* the *TMPRSS6* polymorphism), *P. falciparum* infection was the strongest factor independently associated with low Hb concentrations (regression coefficient $\beta = -8.66$ [standard error = 3.87]; $P < 0.0001$)

Table 2 Univariate and multivariate analyses of factors associated with anaemia

Factor	Anaemia (%)	OR (95% CI)	*P*	aOR (95% CI)*	*P*
Age (years)					
0 < 1	64.8	1			
1 < 2	40.8	0.37 (0.19–0.73)	0.004	0.26 (0.13–0.54)	<0.0001
2 < 3	30.0	0.23 (0.12–0.46)	<0.0001	0.12 (0.06–0.27)	<0.0001
3 < 4	26.0	0.19 (0.09–0.39)	<0.0001	0.07 (0.03–0.18)	<0.0001
4 < 5	24.7	0.18 (0.08–0.38)	<0.0001	0.05 (0.02–0.13)	<0.0001
Breastfed					
No	30.6	1		1	
Yes	38.5	1.42 (0.98–2.07)	0.066	2.03 (1.09–3.77)	0.026
History of fever (last 48 h)					
No	32.5	1			
Yes	53.4	2.38 (1.28–4.42)	0.006		
Monthly household income					
≥5000 RwF	30.3	1			
<5000 RwF	41.2	1.62 (1.11–2.35)	0.013	1.62 (1.04–2.52)	0.032
Household assets					
Some	27.2	1			
None	43.1	2.03 (1.39–2.96)	<0.0001		
α-thalassaemia					
Absent	32.6	1			
Present	44.0	1.90 (1.20–3.02)	0.007	2.02 (1.13–3.61)	0.017
P. falciparum infection					
Absent	27.0	1			
Present	73.7	7.57 (4.42–12.95)	<0.0001	10.29 (5.77–18.36)	<0.0001
CRP >5 mg/dl					
No	31.5	1			
Yes	50.2	2.19 (1.33–3.60)	0.002	1.87 (1.08–3.26)	0.026
Ferritin <12 ng/ml					
No	31.2	1			
Yes	51.5	2.34 (1.46–3.76)	<0.0001	1.67 (1.00–2.79)	0.050
Underweight†					
Absent	30.6	1			
Present	45.9	1.92 (1.27–2.92)	0.002		
TMPRSS6 rs855791					
AA	33.7	1			
AV	37.0	1.15 (0.70–1.91)	0.58	‡	
VV	49.0	1.89 (0.50–7.09)	0.34		

*A logistic regression model with weighted estimates for residence by population size of the sample cluster was adjusted for sex, and the other variables remaining significant after stepwise backward removal.
†<−2 weight-for-age *z*-score.
‡If remaining in this model, AV, aOR, 1.45 (95% CI, 0.78–2.70), $P = 0.24$; VV, aOR, 2.90 (95% CI, 0.86–9.80), $P = 0.086$; AV + VV, aOR, 1.57 (95% CI, 0.88–2.80), $P = 0.125$.

followed by α^+-thalassaemia (no/yes, $\beta = -5.63$ [3.63], $P < 0.0001$), ID ($\beta = -5.20$ [3.87], $P = 0.016$), inflammation ($\beta = -5.05$ [3.78], $P = 0.017$), breastfeeding ($\beta = -5.02$ [3.80], $P = 0.02$) and a household income below the median ($\beta = -4.78$, [3.43], $P = 0.007$). The *TMPRSS6* 736(V) allele was nominally associated with low Hb concentrations (no/yes, $\beta = -4.48$ [3.72], $P = 0.08$). Age was the only parameter positively associated with Hb levels in this model (years, $\beta = 5.64$ [2.79], $P < 0.0001$).

Discussion

Contrasting its significance, data on anaemia and ID in children in SSA are remarkably scarce. Here, we show that in the southern highlands of Rwanda, every fourth child with anaemia had ID, and the Hb difference between children with and without iron deficiency was in the range of 1 g/dl. ID was more prevalent in young children but not associated with underweight or socio-economic parameters, suggesting that the actual risk factors

for ID, for example, nutrients intake or micronutrient deficiencies, were not assessed in the present study. In addition to ID, anaemia, present in 34%, was found to be associated with low age, breastfeeding, low household income, α^+-thalassaemia, inflammation and, most strongly, *P. falciparum* infection. This illustrates the complex aetiology of anaemia in African children (World Health Organization 2008). Also, in the present study, these factors clearly outweighed the influence of the *TMPRSS6* polymorphism which, in addition, was not significantly associated with anaemia. Although a larger sample size and a more focused study design would have been desirable, our findings are biologically plausible and the Hb difference between homozygotes resembled the one previously reported (Chambers *et al.* 2009). Ferritin concentrations also did not correlate with the *TMPRSS6* genotypes. Apart from sample size reasons, abundant infectious and inflammatory processes affecting children in SSA may on the one hand influence the validity of ferritin as a marker of iron status (Mockenhaupt *et al.* 1999). On the other hand, the influence of these processes likely overrides the impact of the *TMPRSS6* polymorphism on hepcidin concentrations and other iron-regulatory mechanisms which secondarily influence macrophage iron release, iron absorption and thus plasma iron levels and anaemia (Hentze *et al.* 2010). Notable, however, is the low allele frequency of *TMPRSS6* 736(V) of 0.096 in the present study population. While this accords with genetic data from other African populations, the frequency is considerably higher in European (≥0.4) and Asian (≥0.50) populations (Nai *et al.* 2011). Whether the unfavourable *TMPRSS6* 736 (V) allele is subject to evolutionary selection still needs to be explored.

In conclusion, anaemia among pre-school children in the southern highlands of Rwanda is frequent. One in four anaemic children has iron deficiency. The *TMPRSS6* 736(V) allele previously reported to be associated with ID and anaemia is less common in Rwanda than in non-African populations and seems to contribute only slightly to low haemoglobin levels. Preventable conditions, notably *P. falciparum* infection but also poverty, are common factors strongly associated with anaemia and need to be addressed to improve child health in this area.

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Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Komplette Publikationsliste

1.

Jean-Bosco Gahutu, Christian Steininger, Cyprien Shyirambere, **Irene Zeile**, Neniling Cwinya-Ay, Ina Danquah, Christoph H Larsen, Teunis A Eggelte, Aline Uwimana, Corine Karema, Andre Musemakweri,Gundel Harms, Frank P Mockenhaupt.
Prevalence and risk factors of malaria among children in southern highland Rwanda. Malaria Journal, 2011.
Impact Factor des Journals (2013) : 3,489

2.

Irene Zeile, Jean-Bosco Gahutub, Cyprien Shyirambere, Christian Steininger, Andre Musemakweri, Fidèle Sebahungu, Corine Karema, Gundel Harms, Teunis A. Eggelte, Frank P. Mockenhaupt.
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3.

Ina Danquah, Jean-Bosco Gahutu, **Irene Zeile,** Andre Musemakweri, Frank P. Mockenhaupt.
Anaemia, iron deficiency and a common polymorphism of iron-regulation, TMPRSS6 rs855791, in Rwandan children. Tropical Medicine & International Health, 2014.
Impact Factor des Journals (2013) : 2,302

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Berlin den 16. Dezember 2014 _____

Irene Regina Zeile

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