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Clearance of once-infected red blood cells in African children with malaria treated with artesunate

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Abreviation

ART-FU	–	artesunate follow-up study
ACT	–	artemisinin-based combination therapy
CSP	–	circumsporozoite protein
DHA	–	dihydroartemisinin
ELISA	–	enzyme-linked immunosorbent assay
FSC	–	forward scatter
GDP	–	gross domestic product
HRP	–	horseradish peroxidase
HRP2	–	histidine rich protein 2
IQR	–	interquartile range
iRBC	–	infected red blood cell(s)
KS-t	–	Kolmogorov-Smirnov-test
LDH	–	lactate dehydrogenase
MSP	–	merozoite surface antigen
oRBC	–	once-infected red blood cell(s)
PADH	–	post-artesunate delayed haemolysis
PfEMP1	–	Plasmodium falciparum erythrocyte membrane protein 1
RBC	–	red blood cell(s)
RDT	–	rapid diagnostic test
RESA	–	ring-infected erythrocyte surface antigen
SSC	–	side scatter
TMB	–	tetramethylbenzidine
VSA	–	variant surface antigen
WHO	–	World Health Organisation

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1 Introduction

1.1 Malaria

Malaria is a vector-borne disease caused by single-celled *Plasmodium* parasites and transmitted by the twilight-active *Anopheles* mosquito. Despite large efforts to reduce parasite prevalence the overall burden of disease remains substantial (1). In 2018 there were still 228 million cases and 405,000 deaths due to malaria infections. The countries suffering most from malaria infections are resource-poor countries of subtropical and tropical regions, with about 93% of the cases in the WHO African region (2). In Ghana, where this study took place, malaria is highly prevalent throughout the population. The highest burden of disease is carried by children, whereas the infection rates in adults decline and reach a stable state of transmission. Thereby, malaria is holoendemic in the area (3). Figure 2 shows regional differences in cases per 1,000 inhabitants. In 2018 there were 4,808,163 confirmed *Plasmodium falciparum* cases in Ghana and 67% of the patients dying from malaria were children under the age of five (2).

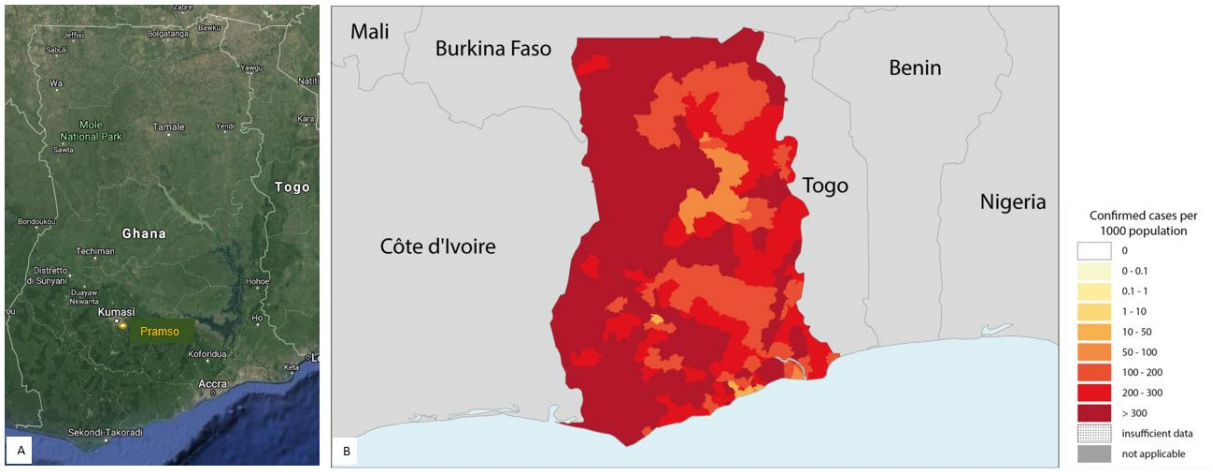

Figure 1 location of study sites(4). Figure 2 malaria cases in Ghana per 1000 inhabitants in 2017 (5)

Five different *Plasmodium* species are known to be pathogenic to humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi.* Their lifecycles are similar, with some characteristics being unique to each species. This work refers mostly to *P. falciparum*, which was responsible for 99.7% of the malaria cases in the WHO African region in 2018 (5). After an infectious mosquito bite *Plasmodium* sporozoites migrate to the liver and infect hepatocytes. After an asymptomatic replication period the infected hepatocytes rupture and release merozoites into the bloodstream. The parasites invade red blood cells and grow until they are mature to release new merozoites. A small number of parasites, however, develop into sexual gametocytes, which are taken up by subsequent mosquito bites (1).

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P. falciparum is the most virulent representative of the *Plasmodium* species. Infections with *P. falciparum* manifest with higher parasite counts and lead to clinical complications more frequently. Adults living in malaria-endemic countries achieve a semi-immunity through repeated infections. Hence, infections are predominantly asymptomatic or result in uncomplicated malaria, which is characterized by general malaise and fever, possibly accompanied by headache, nausea, vomiting or diarrhoea. Non-immune children and travellers, however, are prone to develop severe malaria (1). Severe malaria symptoms, such as severe anaemia, acidosis, hypoglycaemia, organ dysfunction or shock, often coincide with high parasite density in the patient's blood (6). Therefore, besides those symptoms hyperparasitaemia on its own is a criterion defining severe malaria according to the WHO criteria. However, the threshold for hyperparasitaemia is a topic of research, as it has been described that the parasite density coinciding with severe symptoms differs in different areas and has been changing over time. This may be due to the form of data collection, or due to changes in treatment regimens (7). In areas of stable transmission, such as Ghana, the WHO defines hyperparasitaemia as >5% infected erythrocytes or >250,000 parasites/µL (6).

During the infection the parasite is exposed to the host's immune defence. *P. falciparum* has been adapting to its host for millenia and has developed various mechanisms to evade the detection by the human immune system. In consequence the development of a semi-immunity is a complex process that requires various infections and is not yet fully understood (1). Presupposing a situation of persistent exposure, the clinical severity of disease is most fatal during the first years of life and decreases with adulthood resulting in semi-immunity. However, semi-immunity fades in the absence of exposure to infection, as is observed in individuals emigrating to non-endemic regions (1).

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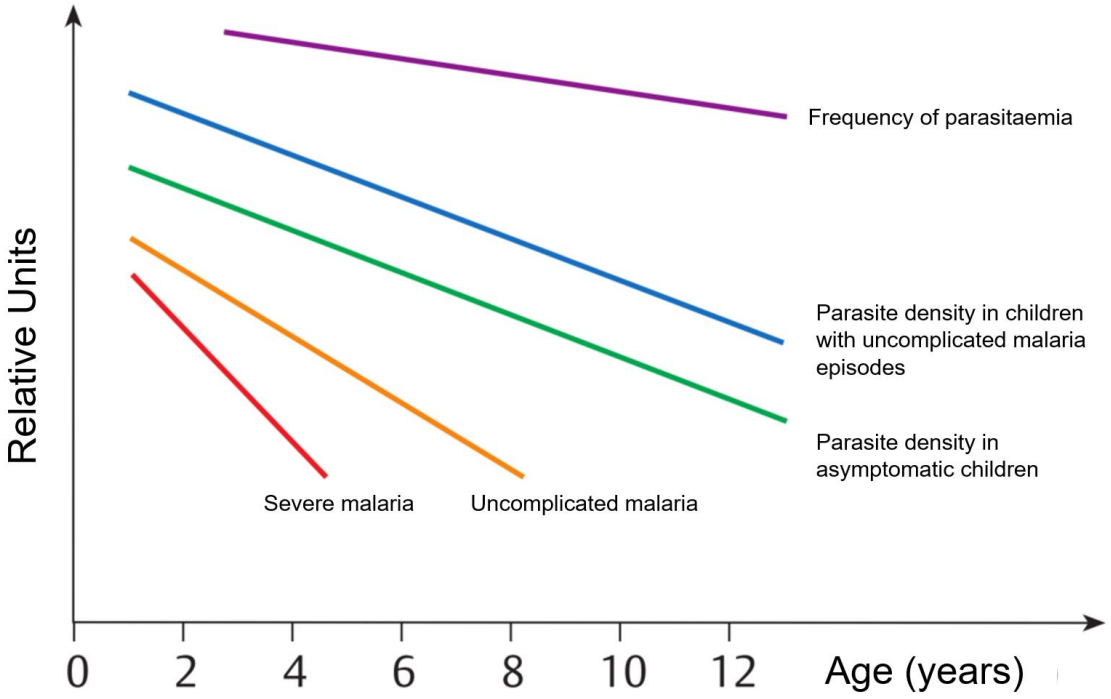

Figure 3 Complexity of naturally acquired semi-immunity. *Illustrated by the development of different markers of infection with increasing age of children from holoendemic areas. Translated into English from chapter 49. Malaria of "Tropenmedizin in Klinik und Praxis" (1).*

1.2 Treatment of malaria with artesunate

Large multi-centre randomized controlled trials have established the efficacy of parenteral artesunate in the treatment of severe malaria. Treatment with artesunate is associated with superior parasite clearance and lower mortality compared to quinine (8–10). Based on these firm results the World Health Organization (WHO) recommends artesunate as the first-line treatment for severe malaria since 2010 (11). Artesunate is a water-soluble derivate of artemisinin, originally called quinghaosu, which was known from Chinese traditional medicine. It was made applicable for worldwide treatment of malaria by a Chinese military project in the 1970s. Chemically it is a 15-carbon sesquiterpene lactone with an endoperoxide bridge (12). Artesunate, or artesunic acid, is a pro-drug that is rapidly activated to dihydroartemisinin (DHA) after uptake. Its mechanism of anti-parasitic activity is not yet fully understood. The most accepted hypothesis is that the haem-dependent cleavage of the endoperoxide bridges causes the generation of reactive oxygen-species, which are harmful to the intraerythrocytic parasites (13). The fast pharmacokinetic clearance of orally administered artemisinin-derivates makes them vulnerable for the development of drug resistance. Therefore, the oral application is only recommended as combination therapy (ACT) with other antimalarial drugs, p.e. lumefantrine, mefloquine or sulfadoxine and pyrimethamine. The longer elimination half-life of the additional

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drug ensures a more continuous and longer drug exposure and thereby reduces the development of resistance (11).

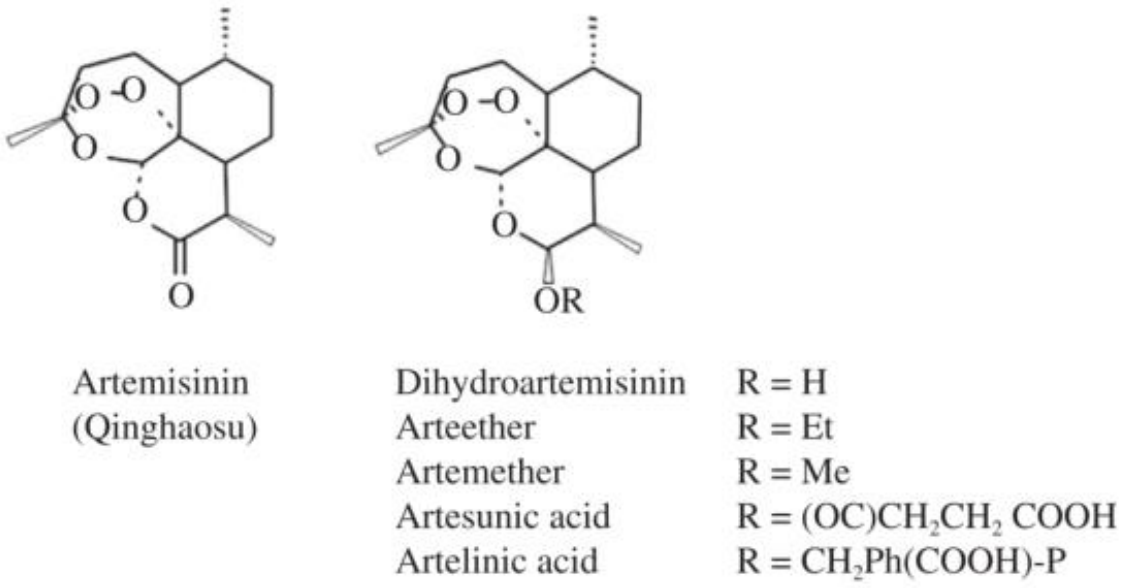

Figure 4 Chemical structure of artemisinin and its derivatives (12).

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1.3 Pitting

During the blood stage of a malaria infection the spleen plays an important role, because the spleen's function is to filter the blood from harmful substances. Red blood cells (RBC) must pass through the spleen's sinuses where stiff intraerythrocytic bodies are retained while the RBC itself is released to the blood stream. This process is called pitting. Changes in deformability of infected red blood cells (iRBC) are detectable already during the ring-stage of the *P. falciparum* lifecycle. Those changes are held accountable for the recognition of the iRBC and subsequent retainment and destruction of the parasite within the spleen, while the RBC returns to the blood stream (14). These pitted or once-infected red blood cells (oRBC) continue to carry plasmodial antigens on their surface.

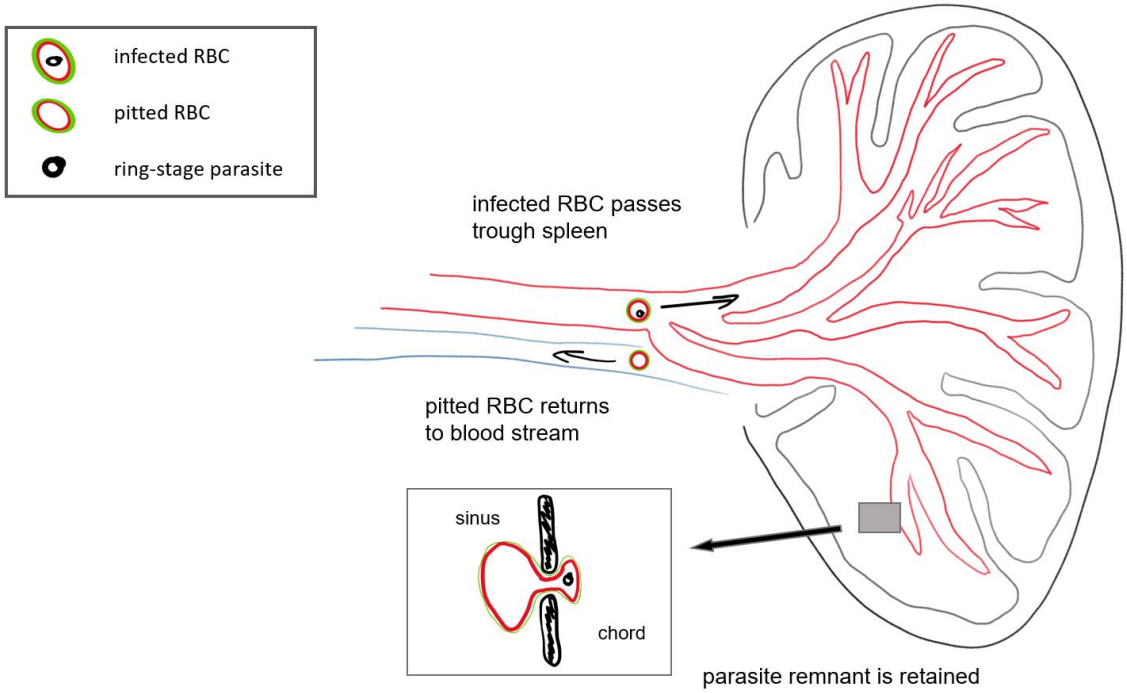

Figure 5 the physiological process of pitting by the spleen. *A parasitized RBC circulates the bloodstream and enters the spleen, where it passes through the slow circuit. Whilst the parasite is retained the now oRBC returns to duty.*

oRBC have mostly been identified by staining for ring-infected surface antigen (RESA/Pf155) and subsequent detection by flow-cytometry or immunofluorescence-microscopy (15). Also, the concentration of HRP2, a plasmodial protein detected in the malaria bedside test (RDT), has been shown to correlate with the number of RESA-carrying cells (16).

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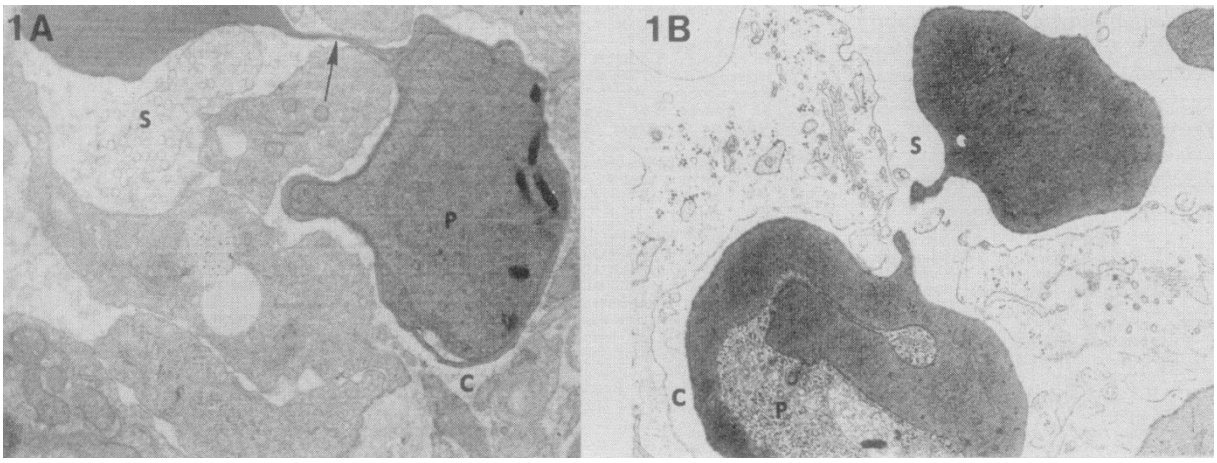

Figure 6 ultrastructural observations of spleen tissue of Plasmodium knowlesi infected monkeys. *1A/B RBC passing from chord (C) to sinus (S), whilst parasitized part of RBC (P) is retained in chord. (17)*

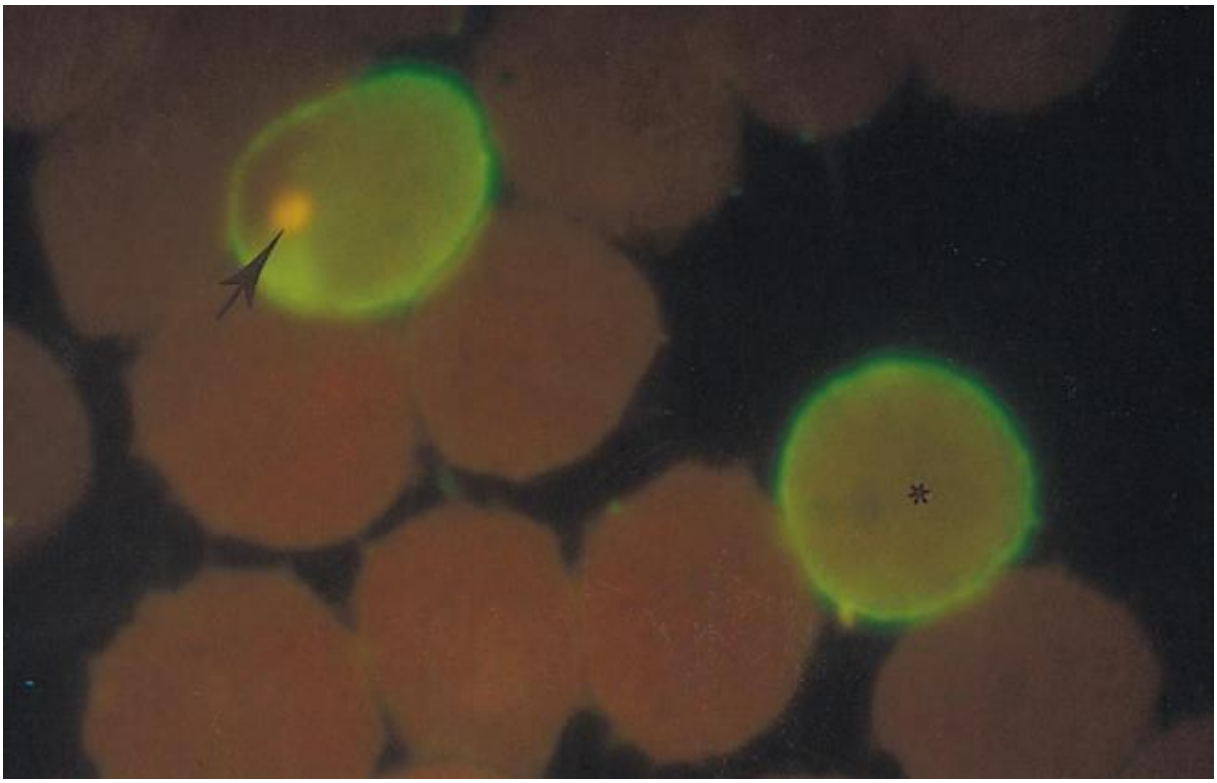

Figure 7 immuno-fluorescence staining of ring-infected erythrocyte surface antigen. *oRBC (*) and iRBC (arrow). (18)*

Pitting is involved in parasite clearance during acute infection (15) and contributes to the clearance of parasitic remnants after treatment. This rescues a large amount of iRBC from destruction, preventing corresponding clinical complications (19). The number of oRBC appears to increase after the initiation of treatment with both artemisinin derivates and quinine (20, 21). However, the increase is much larger after the application of artesunate than after quinine treatment (16, 21, 22).

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1.4 Post-artesunate delayed haemolysis (PADH)

Despite not being licensed in Europe, the WHO recommendation of artesunate as first-line treatment for severe malaria in 2010 (11) resulted in an increased use of the drug in industrialized countries and came along with an extended quantity and quality of follow-up data from artesunate treated patients. The additional clinical observations led to the first reported cases of delayed haemolysis from several European countries (23, 24). The condition has been characterized as a secondary decrease in haemoglobin and the presence of haemolytic activity after successful treatment and complete parasite clearance. Definitions have been slightly different but coincide in a drop of haemoglobin after an initial rise, a low haptoglobin and an increased lactate dehydrogenase (LDH). For this thesis PADH was defined as previously reported by our group, criteria were both any decrease in haemoglobin and any increase in LDH between Days 7 (±2) and 14 (±2) in combination with both an elevated LDH (>350 IU/L) and a low haptoglobin (<0.3 mg/dL) on Day 14 (25).

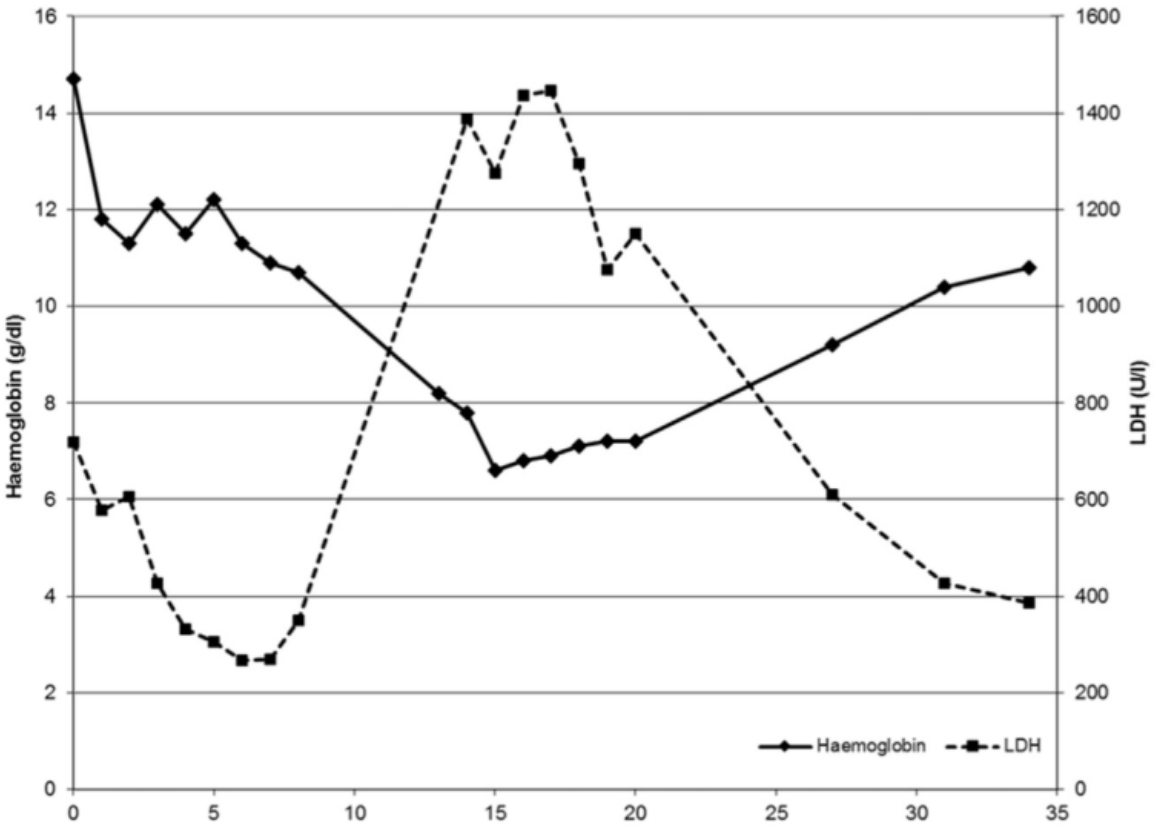

Figure 8 time course of delayed haemolysis. Illustrated by haemoglobin and LDH development adapted from original patient's data (21).

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In 2014 the first two prospective studies on PADH were published. They showed an incidence of 22% in French travellers and an incidence of 7% in children recruited in a double-centre observational study in Ghana and Gabon (25, 26). The latest review on imported severe malaria by Roussel et al. from 2017 analysed data on 624 travellers treated with artesunate with an overall incidence of delayed haemolytic events of 15% (27). Also, cases of delayed haemolysis after the treatment with other artemisinin derivatives were seen. So far, delayed haemolysis has not been reported in patients exclusively treated with quinine (28).

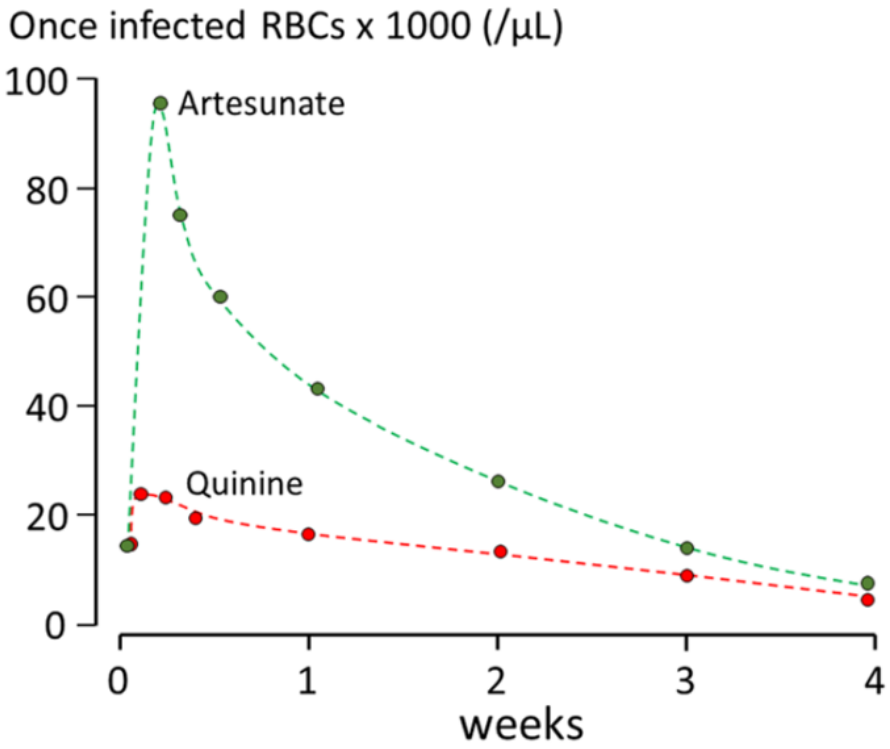

Figure 9 decline model of oRBC.

There are several hypotheses on the pathological mechanism of PADH. It has been described that artesunate inhibits adequate reticulocyte release from the bone marrow from animal models and healthy study participants. A direct erythroblastocidal effect has also been shown (29). To what extend this erythropoietic inhibitory effects contribute to the development of PADH in patients, remains unclear.

Immune-mediated haemolysis has been discussed as another possible explanation. There have been reports of autoantibodies present in patients with prolonged anaemia after malaria infection but immuno-haematological testing in delayed haemolysis has so far not been performed in a standardized manner (30).

A third hypothesis is backed by more evidence: PADH could be caused by the synchronized delayed destruction of oRBC. It is known, that artesunate favours pitting and pitted erythrocytes have a reduced life span compared to never infected erythrocytes. Jauréguiberry et al. could

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establish that a threshold value of 180,000 oRBC/µL during the first week after treatment with artesunate was a valid marker for a higher risk to develop PADH (26). High parasitaemia and young age have been reported as risk factors for PADH in endemic countries (25). As shown in figure 3 both factors are not independent from each other: younger age correlates with higher parasite counts. Furthermore, both factors also correlate with higher oRBC counts (22) and in general correspond to a less developed immune response to malaria (1).

The numbers of oRBC detected after treatment with artesunate are about 3-fold to 5-fold higher than after the treatment with quinine (figure 9) (21, 22, 26). With respect to the superiority of artesunate, it has been hypothesized that delayed haemolysis is not primarily a complication of the treatment with artesunate but rather a consequence of an initially very severe infection. This is in line with the idea of synchronous oRBC clearance being responsible for the development of PADH. As artesunate – in contrast to quinine – affects ring-stage parasites, which are subsequently exposed to pitting through the spleen, it saves RBC during the acute infection. Following that hypothesis, the RBC being lost in PADH would have been destroyed directly, if patients had been treated with quinine instead of artesunate (26). This means that even though artesunate is not able to prevent the haemolysis caused by the acute infection, it delays it. This facilitates the separate treatment of the acute infection and the following anaemia resulting in better chances for both treatments being successful. To obtain a better idea, which mechanisms contribute to the development of PADH and how oRBC might be cleared from the blood stream a closer look into the parasite-host interaction is needed.

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1.5 Parasite-host-interaction

1.5.1 Surface structure and remodelling

P. falciparum possesses over 5000 genes coding for many immunogenic proteins (31). These are exposed on the surface of merozoites, sporozoites, gametocytes or infected host cells. After the invasion of a RBC the parasite starts to restructure its environment by altering the deformability and the surface structure of the hosting cell (32). Many of the proteins exported to the infected erythrocyte's surface are variant surface antigens (VSA), which are encoded by multigene families. Due to epigenetic regulation *P. falciparum* is able to express different versions of those genes during a single infection and thereby impairs the process of efficient antibody generation (33). The most prominent VSA is *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), which binds to various host receptors. The binding of PfEMP1 to host receptors results in the accumulation of infected RBC in terminal vessels of different organs. This process is called sequestration and protects the infected RBC from the recognition by the immune system, namely during passage through the spleen. Clinically sequestration often results in severe complications, because it leads to dysfunction of the respective organs (32). Besides PfEMP1 there are various other VSA families, such as STEVOR or RIFIN, present on the surface of infected red blood cells (32).

1.5.2 Development of effective immune response

The humoral immune response plays a crucial role in preventing symptomatic malaria. In the 1960s the transfer of serum from immune adults to patients with malaria resulted in parasite clearance and cure of symptoms (34). Since then, there has been an abundance of research on antibody dependent protection against malaria, but so far, the translation into clinical medicine remains in its infancy and tested vaccinations have not yet reached the desired efficacy (31). Research for vaccine candidates focuses on conserved antigens, such as circumsporozoite protein (CSP) or merozoite surface protein (MSP). Antibodies to pre-erythrocytic stages such as MSP are present in children of endemic countries at young age, independently of the number of malaria episodes or the age at the first malaria infection (35). Higher levels of maternally-derived antibodies against MSP-1 coincide with a longer period of protection against plasmodial infection (36). In a 2010 meta-analysis on the presence of anti-merozoite antibodies including 33 studies a 54% reduction of malaria incidence for IgG to MSP-3 and an 18% reduction for antibodies to MSP-1 was reported (37).

To better understand naturally acquired immunity, not only conserved proteins have to be considered, but the whole variety of *Plasmodium falciparum*'s proteome must be looked at. In this context the development of high-throughput methods, such as microarrays, opened new possibilities for investigation. For example, Dent et al. could show that the reactivity of children's sera to 110 malarial antigens was lower than the reactivity of adults' sera (38). The

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microarray included 824 proteins of which 163 were reactive across all age groups (figure 10). The authors suggest that a complete protective antibody profile is reached approximately after 15 years of repeated natural malaria exposure. The number of publications reporting similar data is increasing within the past years, however, the results come along with the complexity of the statistical analysis and interpretation of large data sets. Therefore, to fully understand all aspects of the parasite-host interaction much more information is still in need.

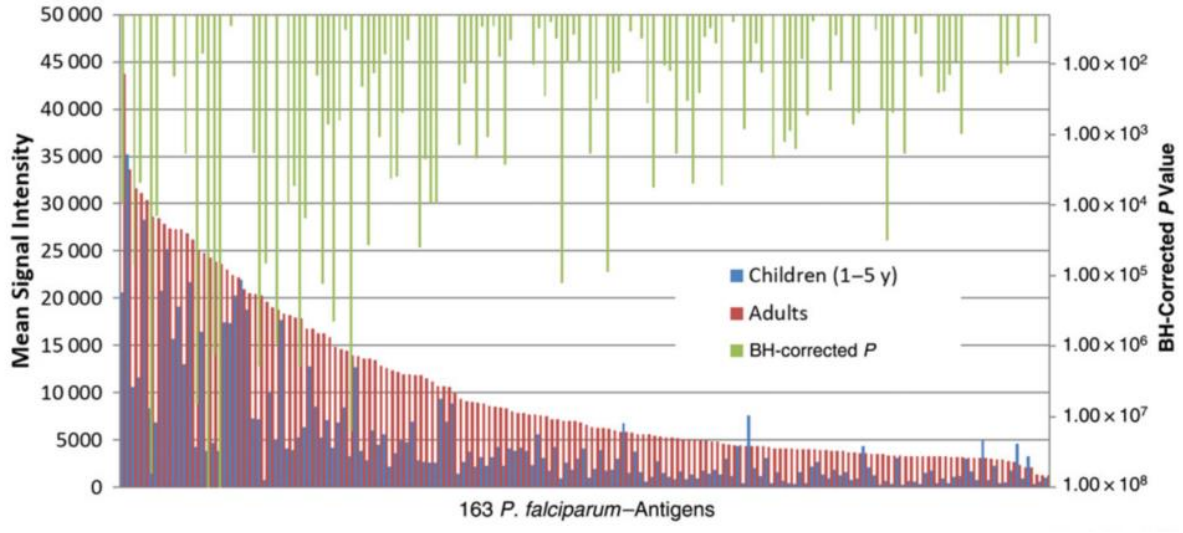

Figure 10 results of microarray analysis from sera of 86 adults (> 18 years) compared with sera from 88 children (<14 years). Of 824 tested plasmodial antigens 163 showed reactivity. For 110 antigens adults had higher reactivity than children. Significant Benjamini-Hochberg (BH)–corrected P values (<.05) for adults versus 1–5-year-old children are shown in green for the comparison (Dent et al. 2015 (34)).

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1.6 Objective

So far, the information on PADH, especially from patients living in malaria endemic countries, is limited. To substantiate the hypothesis that PADH is a relevant complication after the treatment of malaria with parenteral artesunate we conducted a double-center observational study in a malaria endemic area.

To support that the underlining mechanism of the development of PADH is the synchronous clearance of oRBC from the bloodstream, we analyzed the number of circling oRBC over a follow-up period of 28 days via flow cytometry. To further investigate, if the clearance of oRBC depends on the presence of anti-plasmodial antibodies we analyzed serum samples for anti-malarial antibodies with Enzyme-linked immunosorbent assays (ELISA) and by microarray-analysis.

By investigating this topic, we hope to generate a better understanding of PADH and facilitate the identification of additional risk factors for the development of PADH in the future.

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2 Material and methods

2.1 Double centre observational study (ART-FU)

The artesunate Follow-Up study (ART-FU) was conducted from January to July 2015 to investigate the connection between artesunate treatment of severe malaria and subsequent development of delayed haemolysis.

2.2 Study sites

The recruitment for the ART-FU study took place in Ghana. Ghana is a West African country, located on the cost of the Gulf of Guinea. The landscape is mostly plane with moderate elevations not exceeding 1,000 meters. Its fauna is characterized by a costal savanna and a tropical forest zone in the South and a second savanna zone in the North (39).

As classified by the united nations Ghana is a developing country with a median age of the population of 20.7 years in 2015 (40) and an GDP per capita of 2202 US Dollar in 2019 (41). According to Ghana statistical services in 2019 the main economic sector was the service sector (47.2% of GDP) followed the industrial sector (34.2% of GDP) and the agricultural sector (18.5% of GDP) (42).

Our study sites are located in the Ashanti region in the centre of Ghana. With 4,780,380 inhabitants (2010) it is the most populous of Ghana's regions. Over one third of the region's inhabitants (43) live in the urban area of Kumasi, whereas the rest lives in rural areas (42). The climate in the Ashanti region is tropical and daily temperatures reach from 22 to 27°C. There are two wet seasons, one reaches from March to July, and a second one from September to November. (44) Malaria is transmitted perennially with a peak during the wet seasons (45).

Two hospitals participated in the recruitment of patients: the Komfo Anokye Teaching Hospital, Kumasi, and at the Saint Michael's Hospital, Jachie-Pramso. The Komfo Anokye Teaching Hospital is the second largest hospital of Ghana. With 1200 beds it is the largest hospital of the Ashanti Region. As university hospital and a tertiary care center, it has a large catchment area and receives many referrals from smaller hospitals of the surroundings. Saint Michael's Hospital is situated in Jachie-Pramso, a smaller village with approximately 3300 inhabitants about 20 km southeast of Kumasi center. With about 100 beds it is the main referral center in the Bosomtwi district (population 93,910 in 2010 (46)).

2.3 Study protocol

The ART-FU study was designed as an observational study. Patients were all treated with parenteral artesunate, according to hospital guidelines and independent from study participation. Data on the treatment and outcome of severe malaria were collected in a standardized, prospective, observational way. As part of the study a follow-up program was

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implemented. Follow-up visits were implemented weekly for a maximum of 28 days. The patient's parent or legal guardian was contacted by phone one day prior to the scheduled follow-up visit. Inclusion criteria were an age between 6 months and 10 years and the primary diagnosis of *P. falciparum* malaria (> 5000 parasites/µL on a thick blood smear) with symptoms severe enough to require hospitalization, as suggested by the Severe Malaria in African Children (SMAC) network (47, 48). All patients were treated with three weight-adapted doses of parenteral artesunate (2.4 mg/kg body weight) on admission, after 12 and after 24 hrs, followed by a full three-day course of oral weight adapted artemether/lumefantrine (20mg / 120mg). According to their weight (5 - 15kg, 15 - 25kg or >25kg) children received either one, two or three tablets twice per day, respectively. Children with likely other causes for fever, such as pneumonia, otitis media or tonsillitis, or children who had been treated with anti-malarial drugs within the last 48 hrs prior to hospitalization, were excluded.

When a child was considered as eligible for study participation, the legal guardian was informed by trained study personal in local language. If informed consent was provided, the child was recruited for the study. All participants were examined by trained physicians. Data on clinical evaluation, comorbidities and concurrent medication was collected using a standardized case reporting form.

One EDTA blood sample was collected for pitting analysis. One serum sample was collected and stored at -80° and used later for the analysis of LDH, haptoglobin, and for the assessment of anti-plasmodial-antibodies. The described procedure was the same for subsequent follow-up visits, taking place on day 7 (±1), 14 (±2), 21 (±2), 28 (±2).

2.4 Sample size

The sample size for the ART-FU study was calculated with the aim to identify risk factors for developing delayed haemolysis. It was calculated that with a power of 80%, at least 31 patients developing delayed haemolysis were needed to be able to identify risk factors occurring with a proportion of 0.60 in patients with delayed haemolysis and 0.25 in patients without delayed haemolysis at a confidence level of 95%. Based on data from our pilot study at KATH it was anticipated that a minimum of 14% of patients treated with artesunate could be expected to develop delayed haemolysis. Subsequently, at least 221 hyperparasitaemic children had to be included into the analysis to successfully identify such risk factors. With an estimated loss to follow-up of 10% and incomplete data in 5% of the cases, the total sample size was estimated to be 254 patients.

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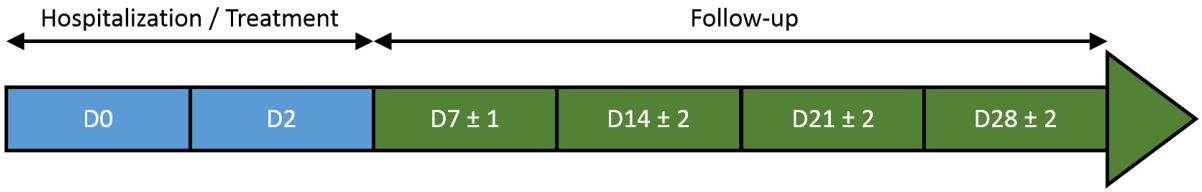

Figure 11 Scheduled study visits. *On all visits clinical evaluation and collection of EDTA- and serum samples were performed.*

2.5 Ethical considerations

This study has been conducted in compliance to the study protocol, according to the principles stated in the Declaration of Helsinki (2008), the guidelines for Good Clinical Practice (GCP) and the laws and regulations of the respective countries. As observational study, the harm for the participants has been minimal and the individuals have been benefitting from a standardized follow-up program. The study protocol has been reviewed and approved by the Committee on Human Research Publication and Ethics of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Children were only included if informed consent was provided by the parent or legal guardian.

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2.6 Laboratory analysis

List of materials used for laboratory analysis	
FASCS analysis	
0.1 – 2.5 µL Eppendorf Research plus pipet	Eppendorf AG
2 – 20 µL Eppendorf Research plus pipet	Eppendorf AG
20 – 200 µL Eppendorf Research plus pipet	Eppendorf AG
100 – 1000 µL Eppendorf Research plus pipet	Eppendorf AG
Eppendorf Tubes® 1.5 mL PCR clean	Eppendorf AG
Mini Vortexer VWR Scientific Products	VWR International
HERAEUS megafuge 16R	Thermo Fisher Scientific Inc.
FACS Calibur	BD Bioscience
CellQuest™Pro software	BD Bioscience
FlowJo_V10 software	FlowJo LCC
FACS Flow	BD Bioscience
FACS Clean	BD Bioscience
FACS Rinse	BD Bioscience
FACS Tubes	BD Bioscience
Dulbecco's Phosphate buffered saline 1x (DPBS)	gibco® life technologies ™
Bovine serum albumin (BSA)	Sigma Aldrich Co.
Glutardialdehyde (25% solution in water)	Merck KGaA
Saponin	Sigma Aldrich Co.
Ring-infected-erythrocyte-surface-antigen-antibody (Stock 1mg/ml)	Walter and Eliza Hall Institute of Medical Research
PerCP conjugated goat $F(ab')_2$ - anti mouse IgG	R&D Systems®
1 mM solution in DMSO Syto®16	Thermo Fisher Scientific Inc.
ELISA analysis	
0.1 – 2.5 µL Eppendorf Research plus pipet	Eppendorf AG
2 – 20 µL Eppendorf Research plus pipet	Eppendorf AG
20 – 200 µL Eppendorf Research plus pipet	Eppendorf AG
100 – 1000 µL Eppendorf Research plus pipet	Eppendorf AG
DYNEX-MRX II Reader	DYNEX Technologies Inc.
Revelation 4.25 Software Copyright © 2003	DYNEX Technologies Inc.
Malaria ELISA kit (EIA-5511)	DRG Instruments GmbH
HRP conjugated goat anti-human IgG (Fc-region) antibody	antibodies-online GmbH

Table 1 list of materials

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2.6.1 Pitting analysis by fluorescence associated cell sorting (FACS)

2.6.1.1 Protocol

To determine the relative amount of oRBC, RBC were analysed by flow cytometry. Therefore, RBC from EDTA blood samples were fixated using 0.05% Glutardialdehyde. Cell membrane was permeabilized with 0.1% Saponin. RBC were incubated with Syto16 (dilution 1:1000) to stain for DNA. To stain for ring-infected surface antigen (RESA), RBC were first incubated with mouse anti-RESA-IgG antibody (dilution 1:1000) and subsequently with a goat anti-mouse IgG antibody, conjugated with the fluorescent agent PerCP (dilution 1:20). For each sample a control staining without mouse anti-RESA-IgG and without Syto16 was performed, to determine the amount of unspecific binding of the PerCP conjugated goat anti-mouse IgG antibody. Acquisition was done accordingly using a FACSCalibur loaded with CellQuest ™ Pro software. Data analysis was done using FloJo_V10 software.

2.6.1.2 Analysis

RBC were identified in the forward scatter vs. side scatter plot. Then Syto16-staining was detected in the FL-1-channel and PerCP-staining was detected in the FL-3-channel. In the FL-1 vs. FL-3 scatter plot PerCP- and Syto16-double-positive RBC were defined as parasite-infected. PerCP-positive, but Syto16-negative RBC were defined as once-infected. Double-negative RBC were defined as never-infected. Syto-16-single-positive cells were also gated, but not evaluated further. The respective number of positive / negative events was expressed as percentage of total number of measured cells. Absolut amounts of pitted erythrocytes were calculated using automated RBC counts [RBCs / µL]. The pitting rate was calculated for each follow-up day as oRBC percentage of initial iRBC count.

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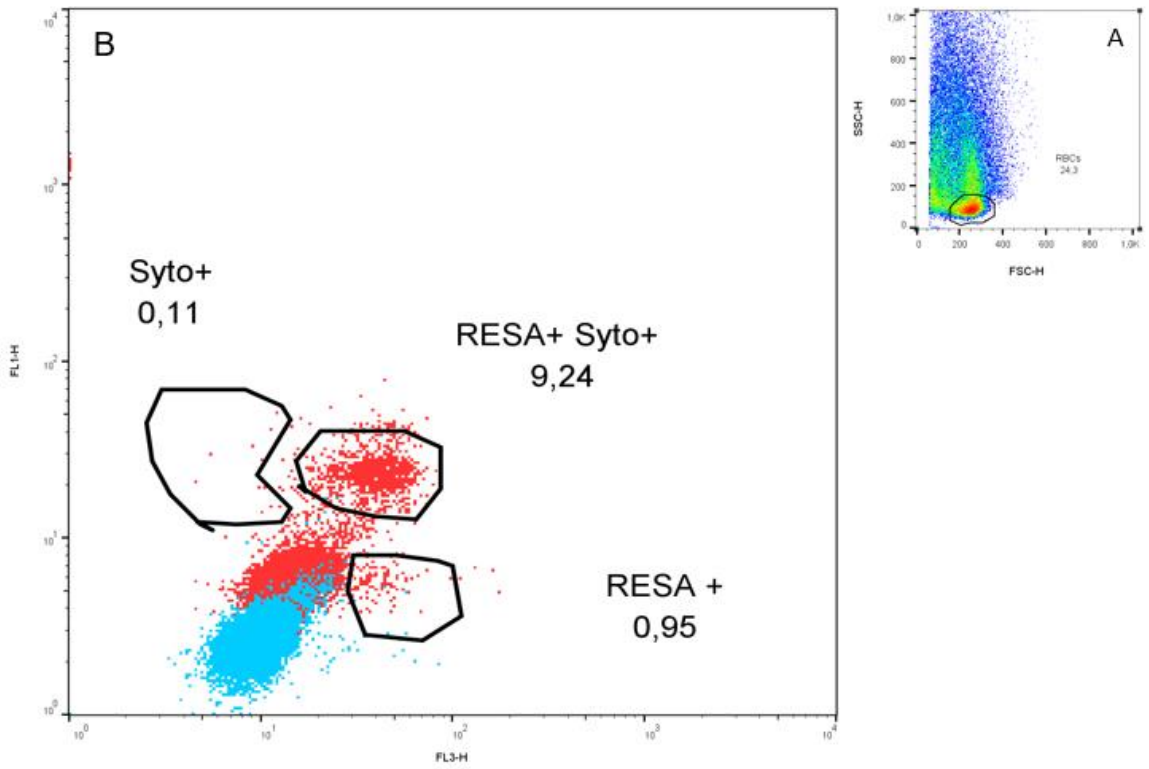

Figure 12 gating strategy for pitting analysis. *(A) shows the whole sample's scatter plot (FSC vs. SSC) with the conservative gating of the main RBC population (percent of all events). (B) the blue population represents the unstained negative control. The red population shows the stained sample. Syto16- and RESA-double positive cells were gated as iRBC (percent of main RBC population), RESA-single-positive cells were gated as oRBC (percent of main RBC population). Syto-16-single-positive cells were gated (percent of main RBC population), but not evaluated further.*

	Double positive	RESA positive	Syto16 positive
Control [pos. ev.]	0.00026	0.0022	0.00016
Staining [pos. ev.]	0.0924	0.0095	0.0011
Staining – Control [pos. ev.] $_{norm}$	0.09224	0.0093	0.00095

Table 2 *flow cytometry: example of normalisation procedure*

Calculation of total number of iRBC or oRBC, respectively:

$$\frac{RESA\ pos.events\ or\ double\ pos.events, respectively}{total\ events} = [pos.ev.]$$

$$STAINING\ [pos.ev.] - CONTROL\ [pos.ev.] = [pos.\ ev.]_{norm}$$

$$[pos.\ ev.]_{norm} * \frac{RBC\ count}{\mu L} = \frac{oRBC\ or\ iRBC, respectively}{\mu L}$$

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2.6.2 ELISA (enzyme-linked immunosorbent assay)

2.6.2.1 Protocol

The used microtiter strip wells were precoated with recombinant MSP I and CSP antigen of *Plasmodium falciparum*. Day 0 serum samples were diluted 1:100 with Sample Diluent, pipetted on the microtiter plates and incubated for one hour at 37° C. After removing all unbound sample material by washing the plates three times, horseradish peroxidase (HRP) labelled goat anti-human IgG (Fc-region) antibody was added, to bind to the captured *Plasmodium*-specific IgG antibodies on the plates.
Tetramethylbenzidine (TMB) substrate then was added and converted into a blue reaction product by the HRP. Then sulphuric acid was added to stop the reaction. Thereby the reaction product turned yellow. The intensity of the developed colour reaction is proportional to the amount of specific anti-*Plasmodium* antibodies in the sample.
All assays were performed in duplicates and each plate was run with a blank, a negative control a positive control and a cut-off control, supplied by the manufacturer.
Absorbance at 450 nm length wave was read using the DYNEX-MRX II ELISA plate reader with the Revelation 4.25 Software Copyright © 2003.

2.6.2.2 Analysis

All assays did fulfil the validation criteria as stated by the manufacturer:

Substrate blank absorbance value < 0.100

Negative control absorbance value < 0.200 and < cut-off absorbance value

Cut-off absorbance value 0.150 – 1.30

Positive control absorbance value > cut-off absorbance value

Absorbance units were calculated according to manufacturer's specifications:

$$\frac{sample\ mean\ absorbance\ value\ x\ 10}{Cut-off\ absorbance\ value} = [Units = DU]$$

Samples were categorized as positive if the absorbance value was 10% over the cut-off absorbance value. Samples were categorized as negative if their absorbance value was less than 10% higher than the cut-off absorbance value.

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2.6.3 Microarray analysis

The microarray analysis was done by Antigen Discovery Irvine (ADI), using the ADI Pf1000, a protein microarray containing about 1,000 recombinant proteins expressed by *Plasmodium falciparum*. According to providers information the microarray slides have been validated and checked for quality by probing with a monoclonal antibody recognizing the N-terminal polyhistidine tag.

At ADI California, microarrays were probed with our samples. IgG antibodies, that bound to proteins, were detected with fluorescent-labelled secondary antibody. Arrays were scanned, raw data was collected, normalized and provided to us for further statistical analysis and interpretation. A reactivity value over 1, which corresponds to a 2-fold increased signal over the negative control spots, was considered positive.

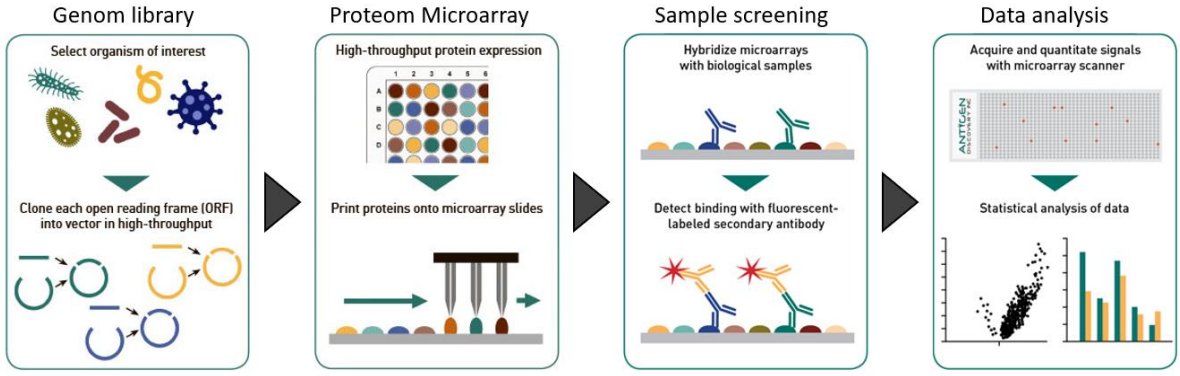

Figure 13 principle of microarray analysis. *Microarrays were prepared and probed by ADI as illustrated. Serum samples, which were collected on admission, were analysed. If antibodies to the respective antigen were present, they were detected by fluorescence-labelled secondary antibody. (source: https://antigendiscovery.com/adi-proteome-microarray-technology/)*

2.7 Statistical analysis

Statistical Analysis was performed using SPSS version 23. Data was analysed for normal distribution by visual inspection of histograms and Q-Q-diagrams, as well as the Kolmogorov-Smirnov-test. Differences between groups were compared using the paired Wilcoxon-rank-sum-test for paired groups and the Mann-Whitney-U-Test for two unpaired groups. Correlation analysis for pitting rate and reactivity against single antigens was performed using R.

2.8 Literature research

Literature search was done using Pubmed. Searching for the term “Malaria AND Pitting” resulted in 7 clinical studies (15, 16, 18, 20–22, 26) presenting data on oRBC. However, there is a wide difference in number and origin of included patients, severity of disease and treatment. Apart from clinical data there are several experimental studies, aiming to give a closer insight to physiological pitting function (14, 17, 49, 50).

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3 Results

3.1 Descriptive statistics

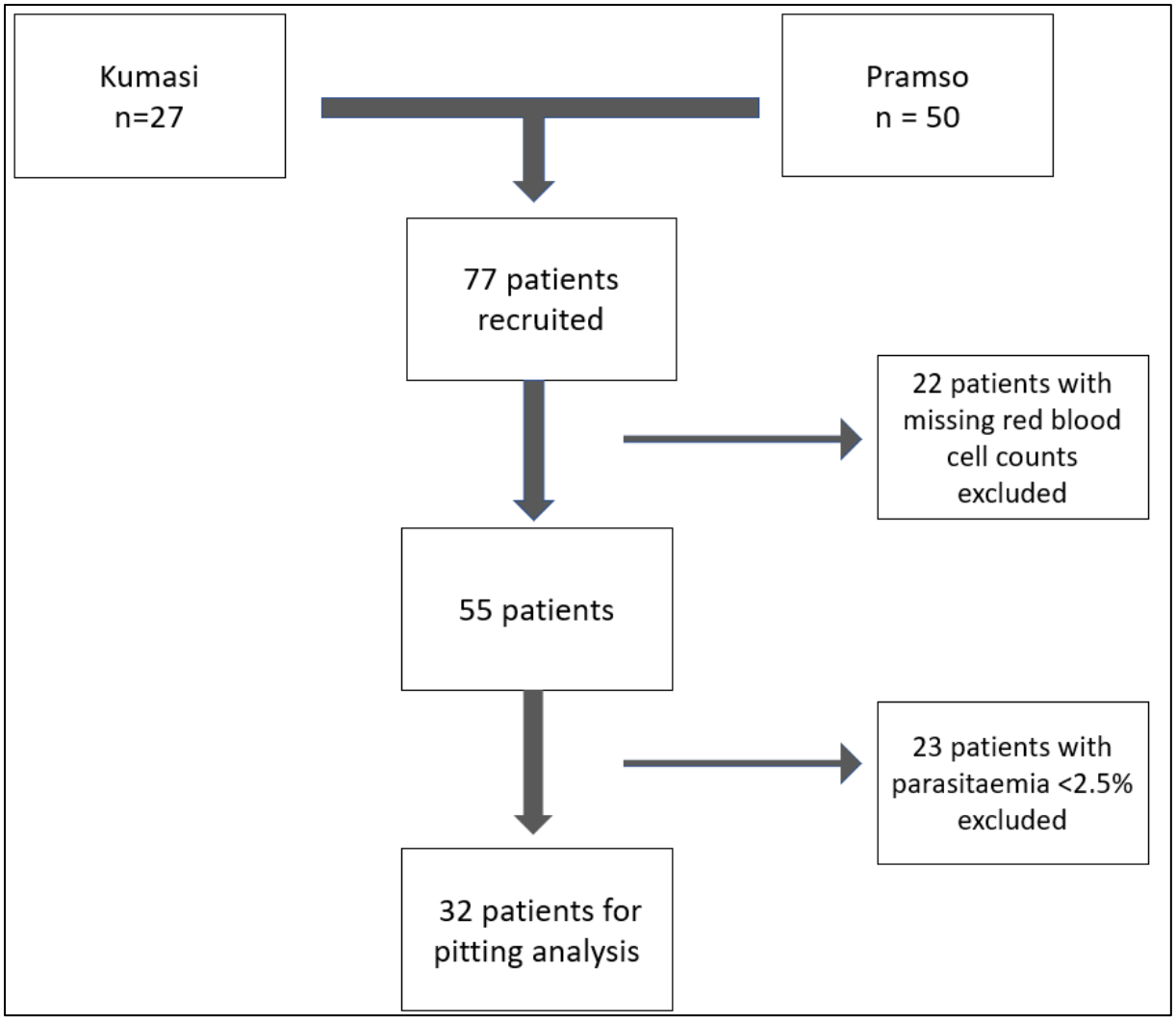

Figure 14 number of patients available for pitting analysis

During the recruitment period from January to July 2015 we recruited 77 patients. Because of missing red blood cell counts 22 patients were excluded from further analysis. The remaining 55 patients had a median number of iRBC of 106,596/µL (IQR: 60,126-152,205) and a median rate of parasitized cells of 2.7% (IQR:1.7-4.0). To reduce the impact of unspecific fluorescence on the pitting analysis, a threshold of 2.5% infected red blood cells was implemented. That resulted in a total number of 32 patients to analyse further (figure 14). Of those 32 patients data was available for 30 patients (94 %) on day 2, for 28 patients (88 %) on day 7, for 25 patients (78 %) on day 14, for 22 patients (69 %) on day 21 and for 23 patients (72 %) on day 28. 13 patients were girls, 19 were boys (table 4). The median age was 48 months. The lower inclusion limit for our study was 6 months, however the youngest patient who was included was 12 months old, because no younger children were eligible during the recruitment period. The oldest patient was ten years old. Age was approximately normally distributed, as assessed

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by the Kolmogorov-Smirnov-test (KS-t p = 0.2) and visual inspection of the histogram and Q-Q-diagram.

The median number of iRBC on day 0 was 147,040/µl (table 3). The Kolmogorov-Smirnov-test (KS-t) and the visual inspection of the histogram and Q-Q-diagram showed discordance with normal distribution for the iRBC count (KS-t p < 0,001). The median number of oRBC on day 2 was 76,340/µl (table 3). oRBC counts were approximately normally distributed, as assessed by the Kolmogorov-Smirnov-test (KS-t p = 0.175) and visual inspection of the histogram and Q-Q-diagram.

Median and IQR were used as measures of location and dispersion for all parameters. The decision to do so was based on the likelihood to miss minor deviations in a small sample size and the fact that normal distribution could not be assumed for all parameter.

The applied method of normalisation ([sample value] – [control value]) resulted in negative results for some samples with low pitting rates. To overcome that limitation, we considered to apply a detection limit of 0 but we decided to maintain the negative values for following analysis, assuming an equal distribution of standard error. In doing so, we sought to avoid the risk of one-sided distortion.

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3.2 oRBC numbers over time

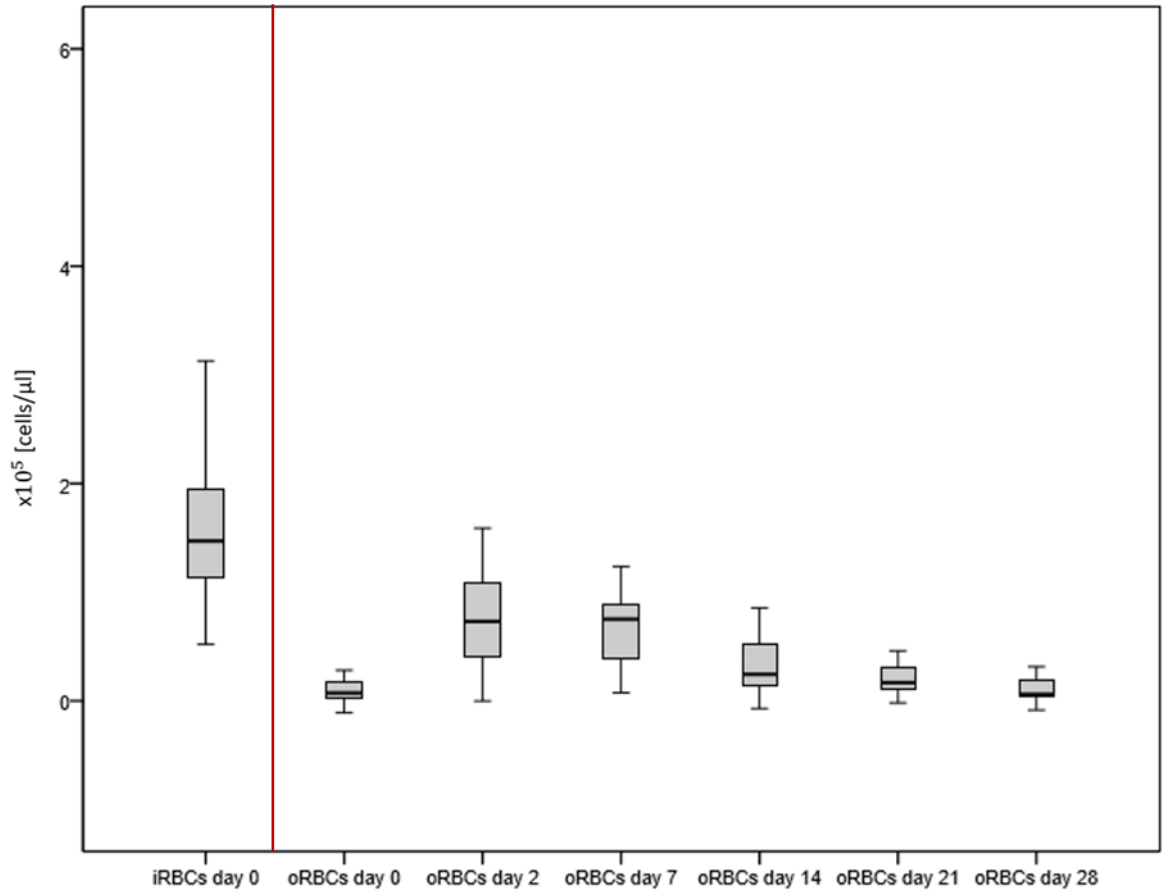

Figure 15 oRBC counts over follow-up period in relation to iRBC count on day 0

	iRBCs day 0 [/µl]	oRBCs day 0 [/µl]	oRBCs day 2 [/µl]	oRBCs day 7 [/µl]	oRBCs day 14 [/µl]	oRBCs day 21 [/µl]	oRBCs day 28 [/µl]
median	147,040	7,286	76,340	75,104	24,339	16,657	6,058
IQR	88,987	39,075	71,161	50,335	42,892	21,283	18,654
25%-percentile	110,291	3,113	40,304	38,337	13,724	9,591	3,810
75%-percentile	199,278	17,794	111,466	88,672	56,616	30,874	22,464
pitting rate [%]		5	52	51	17	11	4

Table 3 iRBC numbers day 0, oRBC numbers day 0 - day 28 and pitting rate day 2 - day 28

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	Day 0	Day 2	Day 7	Day 14	Day 21	Day 28
N	**32**	**30**	**28**	**25**	**22**	**23**
median age in months (IQR)		52 (33-71)	52 (35-72)	53 (36-75)	55 (36-74)	53 (36-52)
gender (male/female)		19/11	16/12	13/12	13/12	13/12

Table 4 number, age and gender of patients included for pitting analysis

To calculate the pitting rate, the median iRBC count of day 0 was used as relative maximum (100%) and the median numbers of oRBC were used for each follow-up day, respectively. The pitting rate on day 2 was 52%, 51% on day 7, 17% on day 14, 11% on day 21 and 4% on day 28.

Looking at absolute oRBC numbers over the follow-up period, our data showed that the median number of oRBC was 76,340/µl on day 2 and 75,105/µl on day 7. Thereby, the median value of oRBC remained approximately equal over the first week of observation. The paired Wilcoxon-rank-sum-test, however, showed a statistically significant difference (W=272.00, negative ranks=18, positive ranks=9, z=1.994, p=0.046, r=0.384) between the two observation timepoints (figure16). The oRBC count dropped from day 7 to day 14 to 24,339/µl (W=302.00, negative ranks=21, positive ranks=4, z=3.754, p=0.001, r=0.751). On day 21 the median oRBC count was 16,657/µl and on day 28 6,058/µl. The paired Wilcoxon-rank-sum-test showed no statistically significant difference comparing the oRBC numbers on day 14 to the oRBC numbers on day 21 or comparing the oRBC numbers between day 21 and day 28. In summary, the strongest drop of oRBC numbers was 50,765/µl (66% of the initial oRBC number) and took place between day 7 and day 14.

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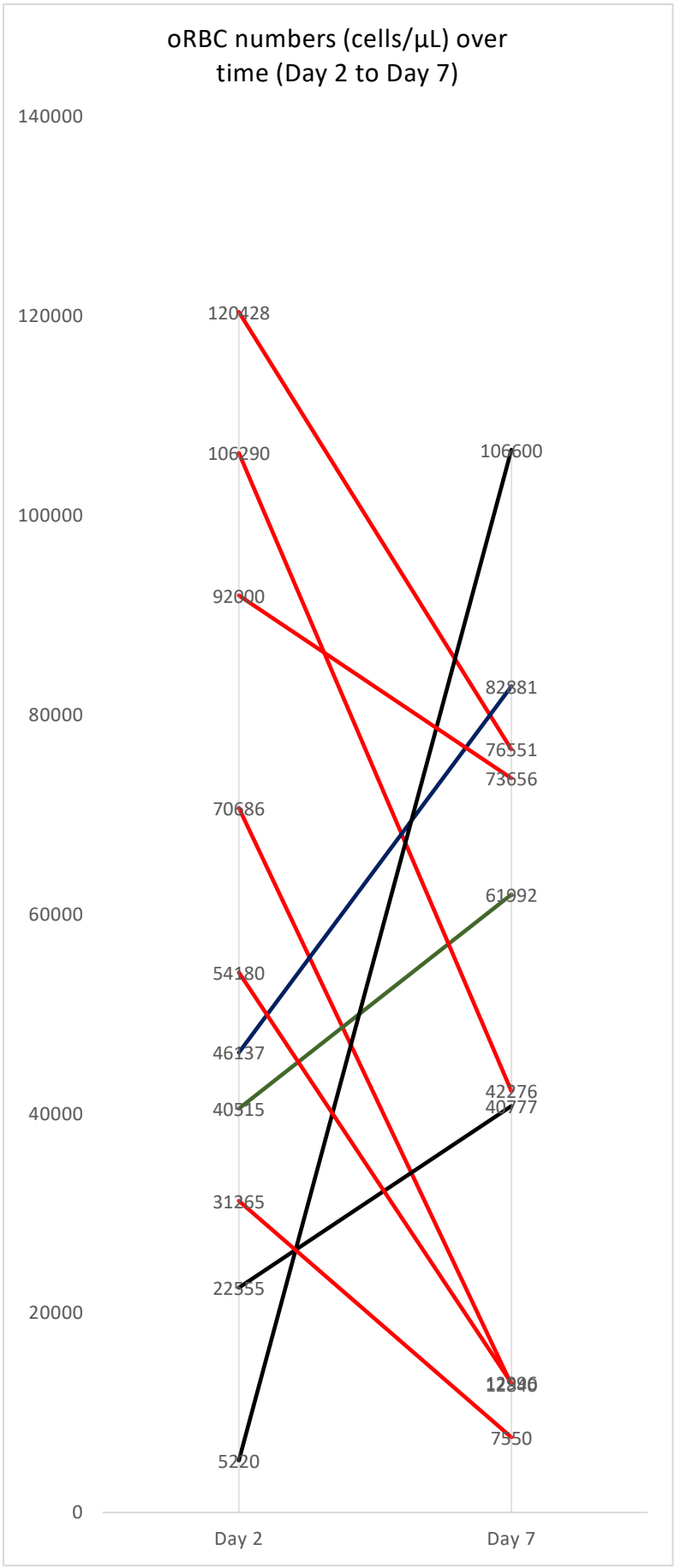

Figure 16 exemplary demonstration of individual changes in oRBC numbers between day 2 and day 7 (selection of representative samples, not all data shown for clarity of presentation).

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3.3 PADH

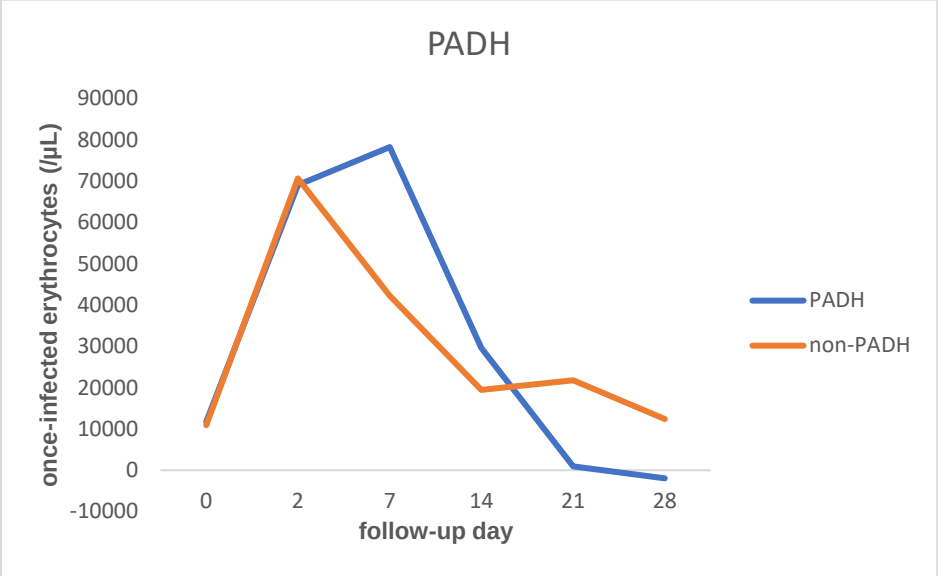

Figure 17 median number of pitted erythrocytes over time. PADH: n=2; non-PADH: n=13

	PADH	Non-PADH
Total number of patients	2	13
Age in months, median (IQR)	57	53 (31-84)
Gender (male/female)	2/0	7/6
Median Hb on day 14 in g/dL	10.0	10.8 (10.1-11.4)
Median LDH on day 14 U/L	455	281 (155-247)
Median haptoglobin on day 14 in g/L	0.26	0.20 (0.20-0.26)
iRBC on day 0 in cells / µL	131,118	132,112 (119,574-147,040)
oRBC on day 2 in cells /µL	78,269	42,276 (27,368 - 70,281)

Table 5 statistics of patients included for PADH pitting analysis

There were 2 cases of PADH. Both patients were only moderately affected with a nadir in haemoglobin of 10 g/dL on day 14. Compared to children without PADH with a similar parasitaemia (median iRBC numbers: 131,118 vs. 131,699/µL) there was a trend towards a higher median number of oRBC in the children with PADH on day 7(78,269 vs. 44,276 /µL, Mann-Whitney-U-test, mean rank = 7.31 vs. 12.50, U=22.000, z=1.529, p=0.171, r=0.395). Numerically there was also a trend for a larger decrease in oRBC numbers between day 7 and day 14 in children with PADH (48,617 vs. 23,419 /µL, Mann-Whitney-U-test, mean rank = 9.50 vs. 6.55, U=16.000, z=0.987, p=0.410, r=0.255). Both results, however, were not statistically significant.

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3.4 Differences in age and serological status

3.4.1 Age

Patients' iRBC and oRBC numbers were analysed stratified by age [1.5 – 4 years] with n = 13 and [> 4 years] with n = 16.

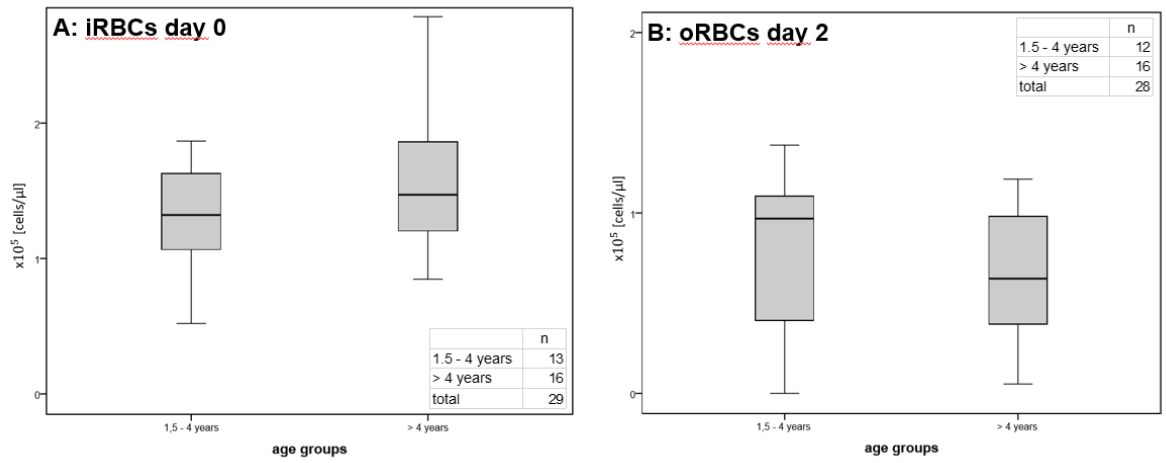

Figure 18 iRBC/µl (A) on day 0 and oRBC/µl (B) on day 2 vs. age

age groups	**1,5 years – 4 years**	**> 4 years**
Total number of patients	13	16
Age in months median (IQR)	35 (25-42)	65 (59-83)
Sex (Male / Female)	8/4	9/7
iRBC on day 0 in cells / µL	136,413 (78,516-180,701)	147,040 (119,393-194,813)
oRBC on day 2 in cells /µL	106,290 (46136-120428)	63,630 (37905-101240)

Table 6 number, age and gender of patients, iRBC and oRBC numbers per age-group

Median iRBC numbers were approximately equal in younger and older children ([1.5 - 4 years] 136,413/µL, vs. [> 4 years] 147,040 /µL, Mann-Whitney-U-test, mean rank 13.77 vs. 16.00, U=270,00, z = 0.702, p=0.503, r = 0.130). Numerically the median oRBC number on day 2 was lower in older children than in younger children, although the difference was not statistically significant ([1.5 - 4 years] 106,290 /µL vs. [> 4 years] 63,630 /µL, Mann-Whitney-U-test, median rank 16.25 vs. 13.19, U=75.00, z = -0.975, p=0.347, r =-0.184).

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3.4.2 Serological status

The serological analysis showed that out of 32 patients on the day of recruitment (day 0) seven patients were negative for anti-MSP1/CSP-IgG and 23 patients were positive for anti-MSP1/CSP-IgG. The serum of 2 patients could not be analysed, because of insufficient sample volume.

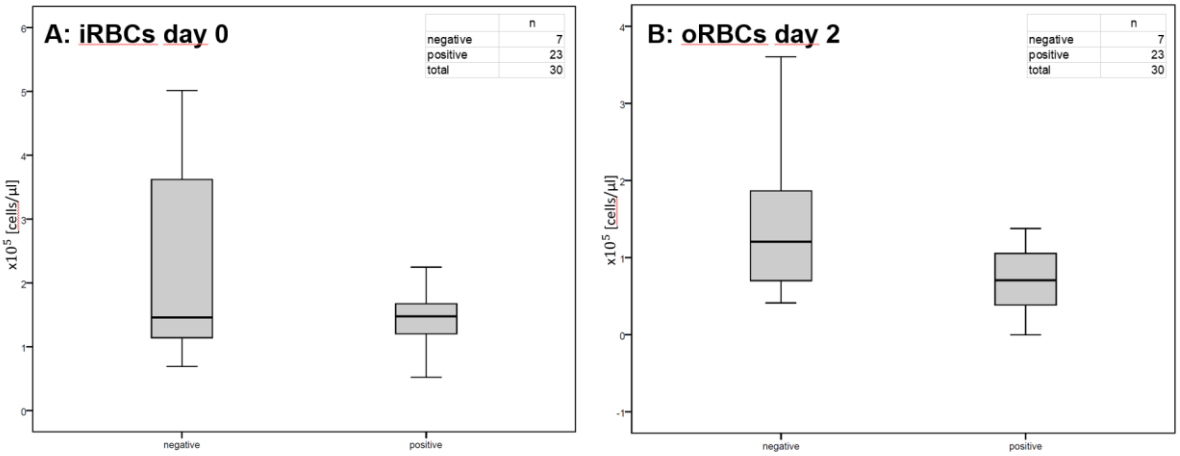

Figure 19A: iRBC/µl on day 0 vs. IgG status. 19B: oRBC/µl on day 2 vs. IgG status

ELISA	positive	negative
Total number of patients	23	7
age in months median (IQR)	53 (34-70)	38 (28-72)
Gender (male/female)	15/8	3/4
iRBC in cells / µL (median+IQR)	147,534 (107,545-445,450)	146,076 (118,528-168,835)
oRBC in cells /µL (median+IQR)	70,686 (37,315-106,289)	120,428 (48,124-213,850)

Table 7 number, age and gender of patients, iRBC and oRBC numbers per group

The median number of iRBC seen on day 0 in patients with a positive ELISA test (147,534/µL) did not differ from the amount of iRBC seen in patients with a negative test (146,076/µL,) (Mann-Whitney-U-Test, median rank 15.04 vs. 17.00 U=70.00, z=-0.515, p=0.631, r=-0.09), as shown in figure 18A.There was, however, a difference in oRBC numbers on day 2 between patients with positive ELISA result (70,686/µL) and patients with negative ELISA result (120,428/µL) (Mann-Whitney-U-Test, median rank 13.61 vs. 21.71, U=37.00, z=-2.133, p=0.033, r=-0.39), as shown in figure 18B.

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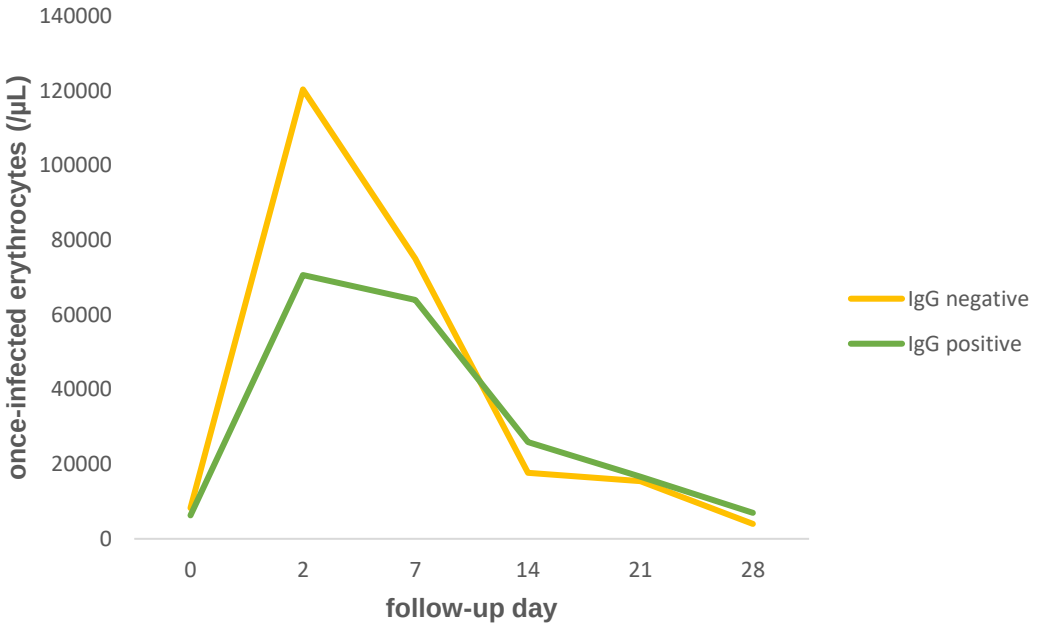

Figure 20 variation of oRBC kinetics depending on antibody status. *oRBC numbers on day 2 differed between ELISA positive and ELISA negative patients (Figure 18B). There was a notable drop in oRBC numbers in IgG-negative patients between day 2 and day 7, what resulted in similar oRBC counts for both groups on day 7. The decay after day 7 appeared to be similar for both groups.*

		Day 0	Day 2	Day 7	Day 14	Day 21	Day 28
Number of patients	**total**	30	30	27	24	21	23
	positive	23	23	21	18	16	17
	negative	7	7	6	6	5	6
Age in months (IQR)	**positive**	53 (28-70)	53 (28-70)	56 (40-75)	58 (45-77)	55 (44-77)	56 (45-75)
	negative	38 (34 – 70)	38 (34 – 70)	37 (29-78)	37 (29-78)	60 (25-85)	37 (29-78)
Gender (male/female)	**positive**	17/8	17/8	15/8	10/8	9/7	10/7
	negative	4/3	4/3	3/3	3/3	4/1	3/3
oRBC numbers median (IQR)	**positive**	6,283 (3,033-18,894)	70,686 (37,315-106,290)	64,010 (27,368 - 87,950)	25,907 (13,692-54,594)	16,657 (8,661-31,523)	6,929 (4,255-25,588)
	negative	8,289 (5,085-16,135)	12,0428 (48,124-213,850)	75,104 (42,085-105,819)	17,728 (13,402-88,569)	15,435 (9,100-27,787)	39,98 (22,37-28,656)

Table 8 number of patients, age, gender and oRBC number for patients stratified by ELISA result

The paired Wilcoxon-rank-sum-test showed a statistically significant difference in oRBC numbers between day 2 and day 7 for on day 0 IgG negative patients (W<0.00, negative

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ranks=6, positive ranks=0, z= -2.201, p=0.028, r=-0.899), but not for IgG positive patients (W=85, positive ranks=9, negative ranks=12, z=-1.060, p=0,289, r=-0.231).

The Mann-Whitney-U-Test showed no difference in oRBC numbers on day 7 between both groups (median rank 16.67 for IgG negative group vs. 13.24 for IgG positive; U=47.00, z=-0.933, p=0.376, r=-0.180). In both groups there was a statistically significant decline in oRBC numbers after day 7, as tested with paired Wilcoxon-rank-sum-test (IgG negative: W<0.00, negative ranks=6, positive ranks=0, z=-2.201, p=0,028, r=-0.899 and IgG positive: W=17.00, negative ranks=14, positive ranks=4, z=-2.983, p=0.003, r=-0.703)

Interestingly, all anti-MSP1-/CSP negative patients showed their peak number of oRBC on day 2. In contrast, within the group of IgG-positive patients only 9 patients showed the peak-number on day 2, while 12 patients peaked on day 7.

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3.5 Microarray analysis

22 samples of patients with representative pitting rates and enough remaining sample volume were selected for further exploratory microarray analysis (table 2). Peak pitting rate, oRBC counts on day 2, mean reactivity and number of reactive antigens were approximately normally distributed, as assessed by the Kolmogorov-Smirnov-test (KS-t p = 0.2) and the visual inspection of the histogram and Q-Q-diagram.

Microarray array analysis	
Total number of patients	22
Age in months median (IQR)	58 (33-77)
Gender (male/female)	13/9
iRBC in cells / µL (median+IQR)	147,040 (121,645-173,301)
oRBC in cells /µL (median+IQR)	86,337 (41,011-111,466)
Pitting rate in % of iRBC day 0	55 (33 – 73)

Table 9 age, gender, iRBC *and oRBC numbers and pitting rate of patients selected for microarray analysis*

ID	peak pitting rate [%]	oRBC [/µL]	reactive antigens	median reactivity
PR029	21	31265	882	2.09
PR035	28	41177	588	1.45
PR014	31	22555	806	3.03
PR011	36	158968	628	1.69
PR010	38	39672	836	2.54
PR003	40	73080	523	1.06
PR042	40	109322	767	2.27
PR001	47	5220	749	2.41
PR026	47	40515	650	1.67
PR006	53	70686	769	2.28
PR039	60	96861	655	1.88
PR028	64	54180	618	1.44
PR022	66	108171	771	2.49
PR041	66	80674	828	2.81
PR013	67	79600	484	0.80
PR017	71	104320	578	1.33
PR046	73	118736	528	1.14
PR021	80	117895	600	1.49
PR009	86	92000	564	1.29
PR019	90	137632	677	1.65
PR048	99	106290	679	1.81
PR024	100	120428	691	2.06

Table 10 overview of samples used for microarray analysis.

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The reactivity against 71 proteins correlated significantly with a lower peak pitting rate ($p<0.05$) (Table 11). 22 (31%) of these proteins belong to the RIFIN-antigen family, nine (13%) belong to the family of Pfemp1-proteins, four did not belong to either of both families but have been detected on the erythrocyte's surface by mass spectrometry. 20 proteins have not been detected on the erythrocyte's surface by mass spectrometry. The location of the remaining 16 antigens has not been described closer in literature.

protein	description	R	p
PF3D7_1146000	nucleolar preribosomal assembly protein, putative	-0,630	0,002
PF3D7_1014300	conserved protein, unknown function	-0,603	0,003
PF3D7_1361700	cytochrome c oxidase subunit 2, putative	-0,588	0,004
PF3D7_0806800	vacuolar proton translocating ATPase subunit A, putative	-0,576	0,005
PF3D7_1146800	conserved Plasmodium protein, unknown function	-0,574	0,005
PF3D7_0827000	ATP-dependent RNA helicase DBP10, putative	-0,556	0,007
PF3D7_1124500	pyruvate dehydrogenase E1 alpha subunit (pdhA)	-0,555	0,007
PF3D7_1405500	conserved Plasmodium protein, unknown function	-0,545	0,009
PF3D7_0513200	conserved Plasmodium protein, unknown function	-0,540	0,009
PF3D7_0317500	kinesin-like protein, putative	-0,540	0,010
PF3D7_1040400	rifin	-0,538	0,010
PF3D7_0937300	rifin	-0,538	0,010
PF3D7_1040000	rifin	-0,530	0,011
PF3D7_0909100	conserved Plasmodium membrane protein, unknown function	-0,529	0,011
PF3D7_0113100	surface-associated interspersed protein 1.1 (SURFIN 1.1)	-0,527	0,012
PF3D7_1349300	serine/threonine protein kinase	-0,525	0,012
PF3D7_0525800	membrane skeletal protein IMC1-related	-0,520	0,013
PF3D7_1021700	conserved Plasmodium membrane protein, unknown function	-0,514	0,014
PF3D7_1000300	rifin	-0,511	0,015
PF3D7_0115300	rifin	-0,511	0,015
PF3D7_0812700	RNA-binding protein (U1 snRNP-like), putative	-0,505	0,016
PF3D7_0814600	conserved Plasmodium protein, unknown function	-0,502	0,017
PF3D7_0831100	surface-associated interspersed protein 8.1 (SURFIN 8.1)	-0,500	0,018
PF3D7_0217700	E2F-associated phosphoprotein, putative	-0,493	0,020
PF3D7_0424100	reticulocyte binding protein homologue 5 (RH5)	-0,490	0,021
PF3D7_0600200	erythrocyte membrane protein 1, PfEMP1	-0,482	0,023
PF3D7_1001600	alpha/beta hydrolase, putative	-0,481	0,023
PF3D7_0808200	plasmepsin X	-0,480	0,024
PF3D7_0223200	rifin	-0,479	0,024
PF3D7_1479600	rifin	-0,478	0,025
PF3D7_0700300	rifin	-0,477	0,025
PF3D7_1254200	rifin	-0,477	0,025
PF3D7_1136200	conserved Plasmodium protein, unknown function	-0,475	0,025
PF3D7_0809100	erythrocyte membrane protein 1, PfEMP1	-0,475	0,026
PF3D7_1373300	rifin	-0,470	0,027
PF3D7_0113800	DBL containing protein, unknown function	-0,465	0,029

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PF3D7_1240300	erythrocyte membrane protein 1, PfEMP1	-0,461	0,031
PF3D7_0400100	erythrocyte membrane protein 1, PfEMP1	-0,460	0,031
PF3D7_0830600	Plasmodium exported protein (PHISTc), unknown function	-0,459	0,032
PF3D7_1103700	casein kinase II beta chain (CK2beta1)	-0,455	0,033
PF3D7_0300500	rifin	-0,455	0,033
PF3D7_0632500	erythrocyte membrane protein 1, PfEMP1	-0,453	0,034
PF3D7_1253700	rifin	-0,451	0,035
PF3D7_1200300	rifin (RIF)	-0,450	0,036
PF3D7_0207300	serine repeat antigen 8	-0,449	0,036
PF3D7_0421300	erythrocyte membrane protein 1, PfEMP1	0,449	0,036
PF3D7_0500800	mature parasite-infected erythrocyte surface antigen,erythrocyte membrane protein 2 (MESA)	-0,447	0,037
PF3D7_0115200	rifin	-0,446	0,037
PF3D7_0401200	rifin	-0,445	0,038
PF3D7_0808900	rifin	-0,443	0,039
PF3D7_0808800	rifin	-0,443	0,039
PF3D7_1254000	rifin	-0,441	0,040
PF3D7_1249800	conserved Plasmodium protein, unknown function	-0,439	0,041
PF3D7_0603600	conserved Plasmodium protein, unknown function	0,438	0,041
PF3D7_0412700	erythrocyte membrane protein 1, PfEMP1	0,437	0,042
PF3D7_0815500	conserved Plasmodium protein, unknown function	-0,436	0,042
PF3D7_1334400	MSP7-like protein (MSRP4)	-0,435	0,043
PF3D7_0322100	RNA triphosphatase (Prt1)	-0,433	0,044
PF3D7_0732900	rifin	-0,431	0,045
PF3D7_0115000	surface-associated interspersed protein 1.3 (SURFIN 1.3) (SURF1.3)	-0,430	0,046
PF3D7_0100800	rifin	-0,429	0,046
PF3D7_0900100	erythrocyte membrane protein 1, PfEMP1	-0,429	0,047
PF3D7_1216500	male development gene 1 (MDV1)	-0,428	0,047
PF3D7_1212100	peripheral plastid protein 1, putative;with=UniProt:B6KNA0 (PPP1)	-0,428	0,047
PF3D7_0900700	rifin	-0,427	0,047
PF3D7_1101100	rifin	-0,427	0,047
PF3D7_0900400	rifin	-0,426	0,048
PF3D7_1100100	erythrocyte membrane protein 1, PfEMP1	-0,426	0,048
PF3D7_0833000	rifin	-0,426	0,048
PF3D7_0711700	erythrocyte membrane protein 1, PfEMP1 (VAR)	-0,425	0,048
PF3D7_0207700	serine repeat antigen 4 (SERA4)	-0,425	0,049

Table 11 surface antigens associated with low peak pitting rates

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4 Discussion

The aim of this thesis was to provide more information about post-artesunate delayed haemolysis (PADH) in malaria endemic countries and to elucidate its pathophysiological background. The work was carried out in the framework of a prospective double-centre observational study in the area of Kumasi, Ghana. Overall, we recruited 77 children with *Plasmodium falciparum* infection who were treated with parenteral artesunate. The primary hypothesis of this thesis is that the clearance of once-infected red blood cells (oRBC) is mediated by anti-plasmodial antibodies.

In this study, we identified two patients with signs of PADH but with only mild anaemia. Because of the small sample size, the validity of the results concerning the pathophysiology of PADH is limited. However, details about the kinetics of oRBC numbers and the serological data that was obtained allow us to draw some conclusions about the pathophysiological mechanisms involved in the clearance of oRBC. The data supports earlier research, which reported a difference in oRBC numbers between patients with and without PADH (26). However, our results in this context were not statistically significant. This can be explained by the rather mild clinical symptom severity of the patients with PADH and the large variation of oRBC numbers within the whole study population. Nevertheless, the overall major decrease in oRBC numbers coincided with the time reported for PADH.

The serological analysis substantiated the hypothesis that oRBC are recognized and cleared from the blood stream by the adaptive immune system. In line with this hypothesis, patients with detectable anti-plasmodial antibodies had lower oRBC counts on day 2 than patients with a negative ELISA test. Finally, a microarray method was used to analyse the serological response to 1000 plasmodium falciparum antigens and identified the family of RIFIN antigens as possible target involved in the clearance of once-infected red blood cells.

4.1 PADH frequency

In the ART-FU study, we saw a PADH frequency of 3.6%, which appears to be lower than the frequency seen in a former study by our workgroup conducted in Ghana and Gabon, where the reported overall incidence was 7% (25). Again, considering the small sample size the difference between both results lies within the expected error and therefore could be due to chance. Nevertheless, following the recommendations of the Council for International Organizations of Medical Science (CIOMS) with a frequency of 3.6% PADH belongs to the category of a “common” side effect (51) and must be considered relevant.

If the difference between both studies is not just a coincidence, a lower incidence of PADH in the current study can also be explained by overall lower parasite counts, as compared to the former project (106,596 iRBC/µL vs. 211,103 parasites/µL, respectively). A Congolese study

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from 2017 reported a median parasitaemia of 179,608/µL and 10 patients with a relevant drop in haemoglobin at least seven days after artesunate treatment in uncomplicated malaria (n =109) (21). This corresponds to an incidence of 10.9%. Yet, presumably, not all ten patients completely fulfilled the defined criteria for PADH. Firstly, haptoglobin and LDH levels were not reported separately for the subpopulation in which the reduction in haemoglobin was seen. Secondly, this conclusion can be drawn from the fact that in the ART-FU study, some of the patients with a reduction in haemoglobin did not show the corresponding increase in LDH or decrease in haptoglobin. A reduction in haemoglobin without a corresponding increase in LDH and / or decrease in haptoglobin can be explained by several circumstances. Firstly, it can be related to a mild haemolysis with haptoglobin and LDH levels changing only within the range of the standard values. Secondly, another mechanism for RBC elimination besides haemolysis could explain this, such as depletion by the spleen. Thirdly, it could be due to a reduced reproduction rate of erythrocytes. Another Congolese study from 2014 reported a haemoglobin reduction between 2 and 5 g/dL in 11.4% of the patients occurring during the follow-up period of 28 days (52). Unfortunately, the exact time for the minimum or drop was not described for all patients. For five patients with life-threatening anaemia, however, the time of haemoglobin nadir was after day 7. In this study, no LDH or haptoglobin values were measured.

Current empirical evidence suggests that the occurrence of PADH is much more frequent in returning travellers than in African children. It should be emphasized that all returning travellers but one who were reported with PADH until 2015 initially presented with hyperparasitaemia (23, 24). Alongside the overall lower frequency, the clinical presentation of PADH seems to be less severe in African children than in returning travellers. Both patients who developed PADH in our recent study had only mild anaemia with a nadir in haemoglobin of 10 g/dL. In the Congolese study from 2017 (21), only one patient (< 1%) and in the study from 2014 (52), only five patients (1.4%) showed symptoms of severe PADH. Considering that the subpopulation of returning travellers constitutes a small percentage of the patients at risk for PADH, the overall prevalence of PADH should be estimated based on the data available from endemic countries. The available data, though remaining scarce, indicate an overall frequency of 5% for PADH and about 1% for severe cases of PADH with haemoglobin levels lower than 5 g/dL. Based on these findings, PADH is a frequent and relevant complication of artesunate treatment. With estimated 24 million children infected with *Plasmodium falciparium* in 2018 (2), 1.2 million of PADH cases per year can be estimated, which implies 240 000 patients at risk of developing severe symptoms. If it is considered that the studies conducted in endemic settings reported only a low percentage of patients with hyperparasitaemia, a higher prevalence in patients with higher parasite counts is likely, suggesting that the number of patients at risk for severe PADH could be even higher. If the treatment with artesunate delays

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the haemolysis caused by a severe infection, prolonged clinical observation is urgently needed to treat patients adequately when the haemolysis occurs and to identify reliable risk factors.

4.2 oRBC as predictive marker for PADH

Previous studies have shown that the early concentration of oRBC discriminated patients at risk to develop delayed haemolysis from other patients with similar initial parasitaemia (16, 26). In the ART-FU study we did see a trend for a difference in oRBC numbers between patients with and without PADH. This finding was, however, not statistically significant. Since only two patients in the study cohort developed PADH and since the total number of patients included for the FACS analysis was also small (n= 32), the validity of those findings is limited. We assume that oRBC numbers were not sensitive enough to allow statistically significant discrimination of the two patients with PADH from the rest of the cohort for two reasons. Firstly, with a nadir in haemoglobin of 10 g/dL, the clinical affection of the two patients with PADH was only moderate. Secondly, the pitting rate as well as the total number of oRBC within the study population varied widely.

In our study, both patients with PADH presented with oRBC numbers lower than the threshold of 180,000 oRBC/µL, assumed to be a marker to identify patients at risk of PADH (26). Our findings do not question the validity of oRBC numbers as marker for the identification of patients at risk of severe PADH with the need for treatment. The two patients with PADH in the ART-FU study presented with mild anaemia, which improved without clinical intervention. Therefore, our data does not allow to judge the reliability of oRBC numbers for the identification of patients with a future need for clinical attendance.

Besides the trend towards lower oRBC numbers in the two patients with PADH, the moment of the overall largest reduction in oRBC numbers coincided with the time reported for PADH. Median oRBCs numbers raised from day 0 to day 2 and remained approximately equal till day 7. Thereafter, on day 14, they dropped to a significantly lower number, which correlates with the time of two to three weeks after treatment reported for the occurrence of PADH.

Numerically the median oRBC numbers did barely change from day 2 to day 7, however, there was a significant difference between those measurements. This can be explained by the statistical method. To elaborate, the paired Wilcoxon-rank-sum-test is a nonparametric test for matched samples. The test shows whether a single value within a tested population maintains its rank from one test time to another. In our study cohort, it showed a statistically significant difference between day 2 and day 7, because the time to until the peak in oRBC numbers was reached was not the same for all patients. 16 patients' peak value was found on day 2, with a resulting drop of the numbers thereafter. 12 patients peaked on day 7, which implies that in those patients oRBC numbers still increased from day 2 to day 7. We suppose that the

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described phenomenon reflects the known variability of the lag-phase in time to numerical parasite reduction after the initiation of treatment. It is assumed that the duration of this lag-phase depends on the maturity of the parasites at the time of first administration of treatment (54). If the parasite population is at a late stage of the growth cycle, and schizonts are about to rupture, parasite numbers are still increasing, or at least not as rapidly decreasing, with the next parasite count. It is highly probable that this does not only prolong the time until the parasite clearance becomes visible, but also prolongs the process until the peak of circulating oRBC in the blood stream is reached.

As described above, the phenomenon of delayed haemolysis after a successful artesunate treatment appears to be a rather frequent complication in children suffering from malaria. Due to predisposing conditions such as iron deficiency (55) or chronic infections like schistosomiasis many of the children at risk for PADH already suffer from anaemia and the additional development of delayed haemolysis is likely to result in a life-threatening condition for them. Therefore, a reliable marker to identify patients at risk is needed – preferably early during the course of antimalarial treatment.

Besides initial parasitaemia, the number of circulating oRBC on day 7 has proven to be a suitable parameter to identify patients at risk (26). Nevertheless, detecting oRBC by FACS analysis is not a standardised diagnostical method and is too expensive for routine implementation. To overcome that obstacle, NDour et al. have shown that the number of circulating oRBC correlated with the amount of HRP-2 detectable after parasite clearance (16). HRP-2 is a plasmodial protein that can be detected using the commonly available rapid diagnostic test (RDT). A semiquantitative application of the RDT three days after the initiation of treatment allowed the prediction of PADH with a similar sensitivity as described for the FACS analysis. Yet, only limited data about patients with severe PADH in endemic countries is available. To evaluate the correlation of parasitaemia, oRBC numbers and the amount of HRP-2 with clinical data and their usefulness as a screening parameter, further research is warranted.

4.3 oRBC and antibody status

Clinical and demographical data suggest that recognition by the adaptive immune system and subsequent clearance is an important mechanism which leads to the elimination of oRBC from the blood stream by the end of the follow-up period of 28 days. As already indicated, empirical evidence has suggested that pitting rates, and accordingly the number of oRBC, are highest in non-immune travellers (22). Additionally, they are higher in severe than in uncomplicated malaria (20) and finally, in endemic settings the peak pitting rates (= highest percentage of oRBC of initially measured iRBC) is higher in non-immune young children compared to older children (22) with a rising immune response. All described factors and characteristics

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correlating with lower oRBC counts are known to correlate with a maturated immune response. Moreover, there is evidence that lower pitting rates correlate with the binding of antibodies to the surface of the infected erythrocytes (22). Also, the clearance of IgG-labelled red blood cells is increased after a malaria infection for several weeks compared to uninfected control subjects (56). There was a trend towards lower oRBC numbers in older children as compared to younger children but our results were not statistically significant., This might be explained by a selection bias resulting from the implemented threshold of 2.5% parasitaemia for the FACS analysis and by the small sample size. To further examine the influence of antibodies on oRBC numbers, we investigated the presence of anti-plasmodial antibodies in the recruited patients, using a commercially available ELISA-kit precoated with CSP- and MSP1-antigens. Anti-CSP-antibodies are known to be detectable in patients who have suffered several malaria episodes. Their presence is commonly interpreted as a marker for a further developed immune response (57). In contrast, anti-MSP1-antibodies are already detectable after the first infection (35). Therefore, we assume that patients in the ART-FU study with a negative ELISA test experienced a comparably low number of malaria episodes beforehand. For positive ELISA results, the method does not allow to differentiate between patients positive for MSP1 only and patient positive for both MSP1 and CSP. Subsequently, assumptions about the maturity of the patient's immune response are limited. Given the holoendemic prevalence of malaria in the study region, the relatively low number of patients who tested negative in the ELISA analysis (7 vs. 23 children) seems plausible. There was a lower number of oRBC in children with a positive ELISA test. Since neither MSP1 nor CSP are present on the surface of infected red blood cells, it is unlikely that they are directly responsible for the recognition of oRBC. However, a positive test result can be interpreted as a surrogate marker. We suppose that the presence of anti-plasmodial antibodies increases the probability for the presence of antibodies directed specifically to the surface of oRBC.

Interestingly, ELISA negative patients presented not only with overall higher oRBC numbers but all of them showed the corresponding peak in oRBC numbers on day 2, while in over half of the positive patients oRBC numbers peaked on day 7. A correlation between detectable anti-CSP antibodies and a lag-phase in parasite clearance after the start of treatment has been described in literature (57). Again, CSP antigens are not expressed in blood stage parasites. Therefore, it is unlikely that CSP antibodies are directly involved in the process of oRBC clearance and further investigation are needed to elucidate the corresponding mechanisms.

As the results from the ELISA analysis pointed to a difference in the amount of circulating oRBC, depending on the presence of detectable anti-malarial antibodies, we aimed at characterizing the immuno-profiles of our patients more in detail. To do so, 22 samples with a representative oRBC number and enough remaining sample volume were selected and their

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reactivity against 1000 antigens of *Plasmodium falciparum* in a microarray analysis was tested. The reactivity against 71 of those antigens showed a statistically significant correlation ($p<0.05$) with lower pitting rates. Due to multiple testing the relevance of this is unclear. But as 22 antigens belonged to the RIFIN-family there may be a pathophysiologically relevant association. RIFIN-proteins belong to the variable surface antigens, known to be expresses on the surface of infected red blood cells (58). Therefore, it is plausible that antibodies against RIFIN-antigens play a role in the recognition and opsonisation of oRBC.

4.4 Study limitations

4.4.1 Study population

The validity of our results is limited owing to the small sample size. In a prior study of our workgroup from April to September 2012, there were 53 patients recruited at the KATH. In contrast, in the ART-FU study only 31 patients were recruited there in a similar timeframe. The malaria incidence during the time of recruitment was as high as estimated before (59). However, during the former study, the timeframe of recruitment was slightly more suitable as it partly took place during two wet seasons.

We did not reach the calculated sample size of 254 patients due to various reasons. The initially planned recruitment period was from August 2014 to July 2015. Due to a delay in the ethics approval the recruitment start was delayed and the wet season in September and November 2014 was missed. Furthermore, a preliminary data analysis showed a notably lower frequency of PADH in our study cohort (3,6%) than the frequency that was estimated for the initial sample size calculation (14 %). Recalculating the sample size with this information resulted in an even higher sample size necessary to generate statistically significant results. To meet those numbers funding was not sufficient, what finally led to an early abortion of further recruitment.

Next to the overall low recruitment numbers, the parasitaemia of the recruited patients was lower than expected. Furthermore, 22 patients had to be excluded due to missing red blood cell counts. This led to only a small number of patients suitable for the analysis of pitting data. In this context it must also be considered that the selection bias introduced by the threshold of 2,5% parasitaemia for the pitting analysis very likely resulted in a distortion of the age distribution of the remaining data set. As it is known, that both higher parasitaemia and higher numbers of oRBC are correlated with a younger age.

Despite the described limitations, our results do contribute to a better understanding of PADH and of influential factors. As one of the few prospective studies with children living in malaria endemic countries – who are the patients, at greatest risk to develop PADH – our results are in line with the assumption that PADH is a relevant complication of artesunate treatment, but

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the overall incidence is lower than estimated from returning travellers. Furthermore, our data indicates that more studies in children with higher parasitaemia are needed.

4.4.2 number of iRBC

Because of poor staining quality, the microscopically determined parasitaemia was not available for all patients. In order to ensure methodological consistency, for this thesis iRBC numbers determined with flow-cytometry were used for all analysis. For the corresponding paper (60) with a more clinical focus, however, the number of infected red blood cells was calculated as the mean between the FACS results and the microscopical results (as far as they were available). Because microscopy is the gold standard for the determination of parasitaemia. Both calculations led to comparable results for subsequent analyses and can be viewed equally suitable.

4.4.3 Microarray analysis

It should be noted that high-throughput methods, such as the applied microarray analysis, harbour an increased risk to generate random correlations due to the large amount of data originating from a small sample size. Yet, considering the pre-existing evidence of a connection between IgG-reactivity and the number of circulating oRBC, provided by our ELISA results and the publication by NDour et al. (22), and the mechanistical conclusiveness of RIFIN-antigens as a target, we assume that the reported results are valuable for further investigation. Nevertheless, the described results should be viewed as exploratory and should be backed with data from larger study cohorts in the future.

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5 Conclusion

In this work we presented data from an observational prospective study in a malaria-endemic setting. Our findings are in line with the hypothesis that the synchronous clearance of once-infected red blood cells is one mechanism which contributes to the development of delayed haemolysis after artesunate treatment. We observed a lower incidence and severity of PADH in children living in endemic countries than described in former reports on adult returning travellers. Even though there was only a trend for a difference in circulating oRBC between patients with and without PADH, the time of the overall largest reduction in oRBC numbers coincided with the time reported for PADH. Furthermore, we established a difference in the amount of circulating oRBC, depending on the antibody status of the patients. This suggests specific antibodies to be responsible for the recognition and clearance of oRBC. Additionally, the results of our exploratory microarray analysis suggest RIFIN-antigens to be a target for such antibodies.

It should be noted that to date almost all evidence concerning the pathophysiological mechanism of PADH is derived from returning travellers with severe malaria infection and subsequent severe PADH. The data available from patients living in endemic countries, especially on circulating oRBC, is mostly from patients with pre-existing anti-plasmodial antibodies who, if any, showed only mild signs of delayed haemolysis.

It is plausible that findings from adult returning travellers are to some extent generalisable to children living in endemic countries with comparable parasitaemia and comparable oRBC counts. Nevertheless, this has not been empirically confirmed yet and should thus be considered with due caution. Particularly as it is known that the age when an infection is first experienced influences the type of clinical manifestation in many infectious diseases (61). As only a very small part of the vast number of factors influencing the parasite-host-interaction has been established, it can be assumed that there are numerous mechanisms involved, which have not been explored so far.

Despite many similarities in the clinical presentation between children living in endemic countries and adult returning travellers in *falciparum* malaria, there are differences. Therefore, clinical studies in the relevant subpopulation – children living in endemic countries with hyperparasitaemia – are urgently needed.

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6. Zusammenfassung

In dieser Arbeit wurden die Ergebnisse eine prospektiven Beobachtungsstudie aus einem Malaria Endemiegebiet dargestellt. Die vorgestellten Daten unterstützen die Hypothese, dass der synchrone Verlust von ehemals mit Malaria Parasiten infizierten Erythrozyten (engl. „once-infected red blood cells“ = oRBC) zur Entwicklung einer verzögerten Haemolyse nach Artesunat-Therapie (engl. „post-artesunate delayed haemolysis“ = PADH) beiträgt.

Die Daten zeigen, dass diese verzögerte Hämolyse bei Kindern, die in einem Endemiegebiet leben, seltener auftritt als zuvor von erwachsenen Reisenden berichtet wurde.

Zwar ließ sich in unseren Daten lediglich ein tendenzieller Unterschied in der Anzahl zirkulierender oRBC zwischen Erkrankten, die eine PADH entwickelten und Erkrankten, die keine PADH entwickelten zeigen. Jedoch deckt sich der Zeitpunkt, zu dem die Anzahl zirkulierender Erythrozyten signifikant abnimmt mit dem Zeitpunkt, der in der Literatur für das Auftreten der PADH berichtet wird.

Des Weiteren konnten wir abhängig vom malariaspezifischen Antikörper-Status der erkrankten Kinder, einen Unterschied in der Anzahl der zirkulierenden oRBC zeigen. Diese Feststellung legt nahe, dass die Entdeckung und anschließende Entfernung von oRBC aus dem Blutstrom durch spezifische Antikörper vermittelt wird. Zusätzlich weisen explorative Mikroarray Analysen darauf hin, dass Antikörper gegen RIFIN-Antigene hierbei involviert sein könnten.

Es muss berücksichtigt werden, dass bis zum jetzigen Zeitpunkt nahezu alle Daten bezüglich des pathophysiologischen Hintergrundes der PADH von europäischen Reisenden stammen. Diese litten zumeist an schweren Malariainfektionen und anschließend an schwerer PADH. Daten von Erkrankten aus Endemiegebieten stammen vorwiegend von Individuen mit einer vorbestehenden Immunantwort gegen Malariaparasiten, die meiste weniger schwere PADH Symptome zeigten.

Es scheint schlüssig, dass Daten von erwachsenen Reisenden bis zu einem gewissen Grad auf Kinder, die in Endemiegebieten leben, mit vergleichbaren Parasitämien und Anzahlen an oRBC, übertragen werden können. Jedoch wurde dies bisher nicht empirisch gezeigt und sollte dementsprechend nicht uneingeschränkt angenommen werden. So ist zum Beispiel bekannt, dass bei vielen Infektionserkrankungen das Erkrankungsalter einen entscheidenden Einfluss auf die Schwere des Verlaufes hat (61). Da bisher nur ein kleiner Anteil der Faktoren bekannt ist, die die Interaktion zwischen Parasit und Wirt beeinflussen, kann angenommen werden, dass eine Großzahl der involvierten Mechanismen bisher noch nicht untersucht wurde. Trotz vieler Ähnlichkeiten in der klinischen Manifestation einer Malariainfektion bei Kindern, die in Endemiegebieten leben und erwachsenen Reisenden, können diese nicht

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gleichgesetzt werden. Daher sind weitere klinische Studien in der relevanten Subpopulation - Kinder mit Hyperparasitämie, die in einem Endemiegebiet leben - dringend notwendig.

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8 Curriculum Vitae

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt

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9 Eidesstattliche Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

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