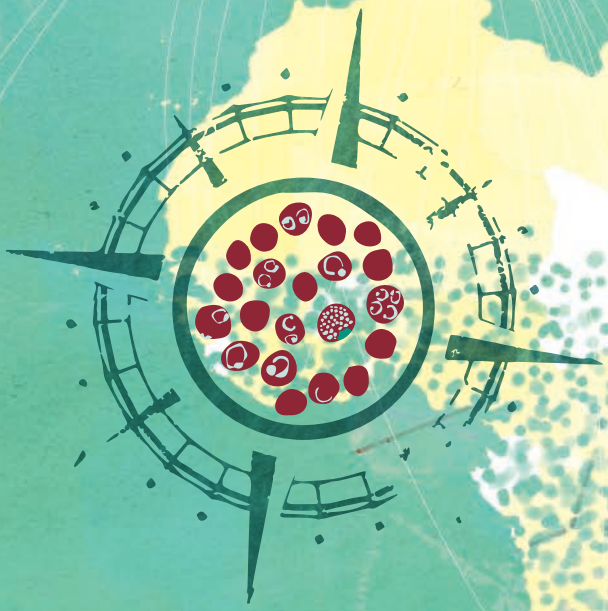
IMPORTED MALARIA

Improving assessment of severity and complications



MARLIES VAN WOLFSWINKEL

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Improving Assessment of Severity and Complications

MARLIES VAN WOLFSWINKEL

COLOFON

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IMPORTED MALARIA

Improving Assessment of Severity and Complications

IMPORTMALARIA

Betere herkenning van ernst en complicaties

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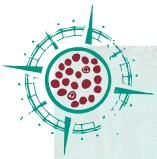

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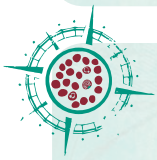

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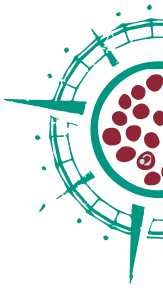
INTRODUCTION

CHAPTER 1

General introduction and outline of the thesis



The global burden of malaria is largely carried by the world's malaria-endemic countries, where the highest death toll is seen in children below five years of age (1). Patients and medical personnel in these areas often have to cope with limited medical facilities and a shortage of beds in intensive care units. In contrast, when patients from high-income countries acquire malaria during international travel to endemic areas, they will usually have access to solid health care systems once they have returned back home.

Imported malaria, however, has its own challenges. Due to the low incidence and the non-specificity of the initial symptoms of the disease, the diagnosis is easily missed. A study in the United Kingdom showed an inverse relationship between the incidence and case fatality of imported malaria (2) and in a London based study in children, the diagnosis was found to be delayed in 53% (3). Diagnosing malaria outside endemic areas is frequently hampered by a lack of sufficiently skilled and experienced laboratory personnel. Hospitals often rely on rapid tests, without possibilities for reliable species differentiation and quantification of the parasite load. Once diagnosis has been made, appropriate treatment forms the next challenge. Few hospitals are experienced in caring for patients with malaria and signs and symptoms indicating a complicated infection might not be promptly recognized. Moreover, appropriate medication is not always readily available. In a survey among all hospitals in the Netherlands in 2008, it was found that 22% did not have intravenous medication for the treatment of severe malaria available and 6.6% did not have any kind of antimalarial treatment stocked (4).

The Harbour Hospital and Institute for Tropical Diseases in Rotterdam, the Netherlands, serves as a national referral center for imported diseases and is one of the country's centers of expertise in malaria. This thesis aims to answer several questions raised while caring for patients with malaria in this institution, and to

improve the care for patients with imported malaria in general. The research questions and outline of the thesis will be formulated at the end of this chapter.

MALARIA

Malaria is a parasitic disease, caused by infection with protozoa of the genus *Plasmodium*. Four species within this genus, *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae,* are strictly human pathogens and cause the vast majority of infections in man. Zoonotic transmission of non-human species was considered rare until 1965, when the first case of a naturally acquired human infection with *Plasmodium knowlesi,* a species primarily found in South East Asian macaques, was reported (5-7). More recently, a naturally acquired infection of another simian *Plasmodium* species, *Plasmodium cynomolgi*, has been described. Although several other simian *Plasmodium* species have been proven to be transmittable to humans in an experimental setting, no naturally acquired infections with these species have been reported to date (8).

Life cycle

Malaria is transmitted by the bite of an infected female mosquito of the genus *Anopheles* (Figure 1). The mosquito carries the sporozoite form of the *Plasmodium* parasite in her salivary glands and, while taking a blood meal, she injects her saliva, containing the sporozoites, into the human skin. The inoculum is usually small, and only a fraction of the injected sporozoites manage to enter the bloodstream and make their way to the liver (9).

After invasion of the hepatocytes, a process of asexual division is started, each sporozoite forming tens of thousands of daughter merozoites over the course of 5 to 8 days. These hepatic schizonts burst and release their merozoites into the blood, where they invade erythrocytes and change into ring-form trophozoites. While feeding on haemoglobin, the parasite starts another phase of asexual division within the red blood cell to form erythrocytic schizonts. This erythocytic cycle takes approximately 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale*, 72 hours for *P. malariae,* and 24 hours for *P. knowlesi*. At the end of the cycle the schizonts rupture to release 8 to 24 merozoites, which are each capable of infecting another erythrocyte to start a new erythrocytic cycle. Simultaneously, some of the blood-stage parasites mature into sexual forms, the male or female gametocytes, which can be picked up by a feeding mosquito. Oocysts are formed in the mosquito gut

after sexual reproduction. Subsequently, the oocysts release sporozoites, which migrate to the salivary glands and inoculate humans during the mosquito's next blood meal (5, 10).

Hepatic-stage parasites of *P. vivax* and *P. ovale*, but not of the other species, may turn into hypnozoites, which can remain in their dormant stage for months to years before resuming replication and thus causing a relapse (5, 10).

Pathogenesis

P. falciparum causes the most severe pathology and causes by far the highest mortality of all *Plasmodium* species. Several factors are responsible for its virulence. *P. falciparum* and *P. knowlesi*, as opposed to *P. vivax*, *P. ovale* and *P. malariae*, do not have a specific affinity for a particular erythrocyte stage and are thus capable of infecting huge numbers of red blood cells, causing very high parasitaemias. The percentage of parasitized erythrocytes in infections with *P. vivax*, *P. ovale*, and *P. malariae* does usually not exceed 1%, while in *P. falciparum* infection percentages of more than 10% are not uncommon and values as high as 80% have been reported in fatal cases (5, 11).

The hallmark of *P. falciparum* however, is its capability to cause trophozoite- and schizont-infected erythrocytes to adhere to the microvascular endothelium. This process, known as sequestration, is mediated by parasite proteins of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) family. Once expressed on the surface of the infected erythrocyte, these proteins bind to adhesion molecules on the endothelium of various organs and tissues, causing obstruction of microcirculatory flow, endothelial activation and pro-inflammatory and pro-coagulatory responses (5, 12, 13). Microcirculation is further compromised by the reduced deformability of both parasitized and unparasitized erythrocytes, which is thought to be caused by oxidative damage to the erythrocyte membrane by haemozoin, a haemoglobin breakdown product formed by the parasite during the erythrocytic cycle and released in large quantities during schizont rupture (14, 15).

Clinical characteristics

During the first days after the *Plasmodium* parasite is transferred into the human host, the infection remains subclinical, reflecting the liver stage of the *Plasmodium* life cycle. Symptoms start to arise when the asexual parasites enter the blood stream and reach a density of about 50 parasites / µL, which is approximately 100 million parasites in total. The initial non-specific symptoms are a direct

consequence of erythrocyte invasion and destruction and the elicited host inflammatory response and commonly include general malaise, fatigue, headache, myalgia and fever. In uncomplicated cases, these symptoms persist and may be accompanied by mild anaemia, jaundice, and sometimes splenomegaly. Fever may start to appear in a regular pattern after about one week of illness, and is thought to be caused by a synchronized formation and rupture of blood schizonts (5, 10).

In severe *P. falciparum* cases, sequestration of erythrocytes in the microvasculature can cause failure of multiple organs. The degree and site of microvascular obstruction correlates with the clinical symptoms. In post-mortem studies of patients with cerebral malaria, a severe and often lethal complication of severe *P. falciparum* infection that is characterized by seizures or decreased consciousness, marked sequestration of the cerebral microvasculature has been found (5, 16, 17). The frequency with which various symptoms of severe malaria occur depends on age. Anaemia and convulsions are seen more often in children, while the incidence of hyperparasitemia, jaundice and renal insufficiency increases with age. Coma and metabolic acidosis are seen at similar rates in all age groups (18). The reported mortality rate of severe malaria is around 23-35% in endemic areas (5) and around 11-25% in imported cases in non-endemic countries (19-22). In imported *P. falciparum* infection, several factors have been found to be associated with an increased risk of severe malaria, including older age, short stays (<21 days), inadequate use of chemoprophylaxis, initial visit to a general practitioner, longer time to diagnosis, and diagnosis during the fall–winter season, which is likely due to more frequent misdiagnosis of the initial symptoms as influenza (23).

Plasmodium knowlesi, although not capable of causing significant sequestration, can cause high parasitaemia and severe disease leading to organ dysfunction and death (24, 25). The other *Plasmodium* species have classically been reported to cause relatively mild infections with very low mortality rates. However, severe manifestations of *P. vivax* infection, including severe anaemia, coma and shock, have been reported and this species in increasingly recognized as a potential cause of severe and even fatal disease (26).

Diagnosis

The gold standard for diagnosis of malaria is the examination of Giemsa-stained thick and thin blood smears by light microscopy. If performed by an experienced laboratory technician, detection of parasite densities as low as 50 parasites / µL

(equivalent to 0.001% parasitized erythrocytes) can be achieved. Microscopic examination is also essential to differentiate between the various *Plasmodium* species and to quantify the parasite load in case of *P. falciparum* infection (27).

Fluorescence microscopy methods have been introduced to further increase the detection sensitivity. The quantitative buffy coat (QBC) technique uses a fluorescent dye with affinity for the parasite nucleus to stain a centrifuged and compressed red cell layer. The sensitivity for detection of low parasite densities (less than 100 parasites/µl or 0.002%) has been found to range from 41 to 93% (27).

Rapid diagnostic tests (RDTs) are mostly based on lateral flow immunochromatography. The majority of the many different commercial RDTs that are currently available are designed to target one ore more of the following three parasite antigens: *Plasmodium falciparum* histidine-rich protein-2 (*Pf*HRP2), *Plasmodium* lactate dehydrogenase (pLDH), and aldolase. *Pf*HRP2 is a *P. falciparum*-specific antigen. *Pf*HRP2-based RDTs can detect low *P. falciparum* parasite densities, but may remain positive up to several weeks after a successfully treated infection. pLDH and aldolase are enzymes produced by all pathogenic *Plasmodium* species, and RDTs targeting these enzymes become negative after parasite clearance. They have a lower sensitivity for *P. falciparum* detection than *Pf*HRP2-based tests, especially at low parasite densities. The sensitivity for non-*falciparum* species of pLDH- and aldolase-based RDTs are comparable (28).

Polymerase chain reaction (PCR) assays are mostly used for research purposes and for species confirmation in clinical cases, which is particularly useful in infections with multiple *Plasmodium* species.

Treatment and prevention

The artemisinin derivatives artesunate and artemether have replaced quinine as the drugs of choice for severe malaria, as they were found to significantly reduce mortality (29-31). While quinine is effective only against the more matured and cytoadhering parasite stages, artemisinin also kills young ring-stage parasites before they can cause the erythrocytes to sequester in the microvasculature. Moreover, treatment with quinine is frequently complicated by severe adverse reactions, and is now only recommended when artemisinin derivates are unavailable (32, 33). The preferred treatment is intravenous or intramuscular artesunate; artemether has a smaller survival benefit in severe cases (34). As the half-life of artesunate is short, treatment should always be followed by a full oral treatment

course (33). The efficacy and safety of exchange transfusion (ET) as adjunctive therapy for severe *falciparum* malaria has always been controversial (35, 36). Shorter parasite clearance times were found when ET was added to treatment with intravenous quinine, but since the introduction of artesunate as the first-choice therapy, the additional effect of ET is lost (37, 38).

Uncomplicated *P. falciparum* infection can be treated orally, with artemisinin combination therapy (ACT, such as artemether-lumefantrine or artenimol-piperaquine) or atovaquone-proguanil as preferred regimens. Uncomplicated non-*falciparum* infections can be treated with oral chloroquine or hydroxychloroquine if acquired in areas without chloroquine resistance, or with ACT or atovaquone-proguanil if resistance is expected. In *P. vivax* and *P. ovale* infections, primaquine is added to target the hepatic hypnozoite forms (33, 39).

Chemoprophylaxis is recommended for travelers to malaria endemic areas. The drugs of choice are atovaquone-proguanil and mefloquine. Doxycyline is also effective but is not registered as prophylaxis in the Netherlands and can only be prescribed off-label. The prophylactic efficacy of chloroquine has become limited by widespread resistance (33, 40).

Despite large amounts of effort, a fully effective malaria vaccine has not yet been developed. One of the most promising vaccine candidates currently being investigated is the RTS,S/AS01 vaccine, which targets an antigen on the surface of the *P. falciparum* sporozoite. Although this vaccine showed an initial efficacy against clinical malaria in African children, this protective effect is of limited duration and is offset by a rebound in later years, when immunity wanes (41).

Acquired immunity against malaria

Repeated infection in holoendemic areas leads to a form of acquired immunity, which protects against the development of high parasitemia and symptoms. This effect is evident by the declining incidence of malaria with increasing age and a reduction in average malaria parasite density with increasing age in holoendemic regions. Immunity declines after leaving an endemic region, but how quickly protection is lost is subject to debate (42).

Epidemiology

One of the Millennium Development Goals set by the World Health Assembly and other global institutions was to have halted and begun to reverse the global

incidence of malaria by 2015. This goal has been met; between 2000 and 2015 the incidence of malaria is estimated to have decreased by 37%. The estimated number of cases fell from 262 million in the year 2000 to 214 million in 2015 and the estimated number of deaths declined from 839 000 to 438 000. The vast majority of malaria fatalities (around 90%) occur in sub-Saharan Africa, where the disease mostly affects children under five (43).

Compared to the huge number of infections that occur in inhabitants of malaria endemic areas, the burden of imported malaria is small. Every year an estimated 25–30 million Europeans travel to areas with malaria transmission, and the number of cases imported to Europe approximates 5000 annually (39, 44).

THE ROTTERDAM MALARIA COHORT

The Harbour Hospital and Institute for Tropical Diseases is a 161-bed general hospital, located in Rotterdam, the Netherlands, which also serves as a national referral centre for travel medicine and provides healthcare to ill sailors in close collaboration with the Port of Rotterdam.

To study the epidemiology and clinical characteristics of patients with imported malaria, the Rotterdam Malaria Cohort (RMC) was started in 1998. Since then, anonymized demographic, clinical and laboratory data of all patients diagnosed with malaria in the Harbour Hospital are routinely collected and stored in an electronic database and form the RMC. As patients are entered into this database regularly, it is a dynamic and gradually expanding cohort.

Patient characteristics

On January 1st, 2016 the cohort comprised 724 patients. Of these patients, 518 had a *Plasmodium falciparum* infection and 200 were infected by a non-*falciparum* species (137 *P. vivax*, 49 *P. ovale*, 13 *P. malariae* and 1 *P. knowlesi*). Of the remaining 6 patients, 5 had a mixed infection with *P. falciparum* and a non-*falciparum* species and in one patient the species could not be determined as only a single trophozoite was seen in the quantitative buffy count (QBC) analysis, without parasites visible in the thick and thin blood slides.
The majority (72%) of the patients was male and the median age was 39 years (interquartile range 30 - 47). The most common reasons for travel were visiting friends or relatives (26%), tourism (22%) and business (16%). Eight percent of

patients were sailors and 6% were Dutch expatriates. The rest of the patients mentioned other reasons for travel (7%) or the reason was not documented (15%). The most commonly presenting complaints were fever (86%), headache (58%) and myalgia or arthralgia (44%).

Most infections (78%) were acquired in Africa, followed by Asia (10%) and South America (3%). The vast majority of the severe *P. falciparum* infections (94%) occurred after travel to an African country. Reversely, malaria occurring after travel to Africa was caused by *P. falciparum* in 85% of cases, while malaria after returning from Asia or South America was mostly caused by non-*falciparum* species (75%) (Figure 2). Figure 3 shows a map with the countries of acquisition of the malaria infections of the patients in the Rotterdam Malaria Cohort.

The majority of patients reported to have not taken antimalarial prophylaxis at all (63%) or to have used it inadequately (17%). Reportedly 14% acquired malaria despite correct use of prophylactic medication. In the patients with severe malaria only 5% reported adequate use of prophylaxis, in 13% the use was inadequate and no prophylaxis was taken in 78%.

Severe malaria

The World Health Organisation (WHO) introduced the malaria severity criteria in 1990, in order to assist clinical and epidemiological studies by creating a case definition of severe malaria (45). These criteria have been revised in the year 2000, in 2010 and most recently in 2014. Due to these changes, different case definitions of severe malaria have been used in this thesis. The 2010 and 2014 WHO severity criteria are shown in Table 1 and Table 2, respectively.

Of the 724 patients, 64 (9%) were classified as severe malaria using the 2014 definitions. The most commonly met criteria, on presentation or later during admission, were jaundice (38 of 64 patients or 59%), hyperparasitaemia (n=26, 41%), acute kidney injury (n=21, 33%) and acidosis (n=15, 23%). None of the patients had prolonged bleeding and hypoglycaemia was observed only in one patient (Table 3).

Treatment and outcome

Oral treatment was given to 82% of patients, most commonly atovaquone / proguanil. In 131 patients initial treatment was given intravenously with either quinine (n=77) or artesunate (n=54). Artesunate became available in the

Netherlands in 2007 as an orphan drug. Although it is still awaiting formal registration at the European Medical Agency (EMA), it gradually took over the place of quinine in the treatment of severe malaria, as evidence of its superiority accumulated (29, 30). In the Rotterdam Malaria Cohort the first patients were treated with artesunate in 2007. The prescription of quinine declined quickly, and it was not prescribed anymore after 2010. Exchange transfusion was performed as an additional treatment to intravenous antimalarials in 46 patients.

Patients stayed in hospital for a median of 3.0 days (interquartile range 1.5-4.5). One-hundred-and-twelve patients (15%) were admitted to the intensive care unit (ICU).

Two patients in the Rotterdam Malaria Cohort died. Both acquired a severe *P. falciparum* infection in sub-Sahara Africa, where they travelled without using chemoprophylaxis. The two deaths yield a mortality rate of 0.3% for all malaria cases, and 3.1% for severe *P. falciparum* infections in this cohort.

Changing incidence of imported malaria

Between 2000 and 2007 there has been a significant decline of the incidence of imported malaria in the Netherlands, which is thought to be due to a decreasing malaria transmission in endemic areas rather than to a change in prophylaxis use (46). This decline is also clear in the Rotterdam Malaria Cohort (Figure 4). In recent years however, an upsurge in the national incidence of malaria has been noted, which is mostly due to an increasing number of immigrants from the Horn of Africa seeking refuge in the Netherlands, and a rise in the incidence of predominantly *P. vivax* infections in this group (47). This recent development shows that the incidence of imported malaria is volatile and is influenced by many factors, including distant wars and conflicts.

OUTLINE OF THE THESIS

The aim of this thesis is to answer several questions raised while caring for patients with imported malaria in Rotterdam.

As described at the beginning of this chapter, the challenge in the care for patients with imported malaria lies in the fact that most hospitals in non-endemic countries have little experience in diagnosing and treating the infection and in recognizing patients at risk for a severe course of the infection. Therefore, in Part I of this thesis, we question which parameters could help to improve the identification of patients at risk for a severe infection. In the first chapters, we focus on the novel host parameters triggering receptor expressed on myeloid cells 1 (TREM-1), neopterin and procalcitonin (Chapter 2) and copeptin (Chapter 3). In Chapter 4 we explore the dynamics of leukocyte count changes in detail in a Controlled Human Malaria Infection trial and we investigate whether the routinely measured differential leukocyte count can be used to predict a severe course of the disease in Chapter 5. In the last two chapters we focus on parasite-derived parameters that can be obtained from routinely performed malaria rapid tests (Chapter 6) or blood slides (Chapter 7).

In Part II we aim to gain insight in two morbidities frequently encountered in the clinical practice of imported malaria. In Chapter 8 we study the frequency of the occurrence of acute kidney injury (AKI) in imported malaria and Chapter 9 describes a pilot study that examines whether the novel marker Neutrophil Gelatinase-Associated Lipocalin (NGAL) could help to early identify malaria patients at risk for AKI. Hereafter we question how commonly hyponatraemia occurs in imported malaria and whether there is an association with severity of the disease (Chapter 10) and we aim to gain insight in its pathophysiology (Chapter 11).

In the Appendix we summarize and discuss our findings and the future perspectives of imported malaria.

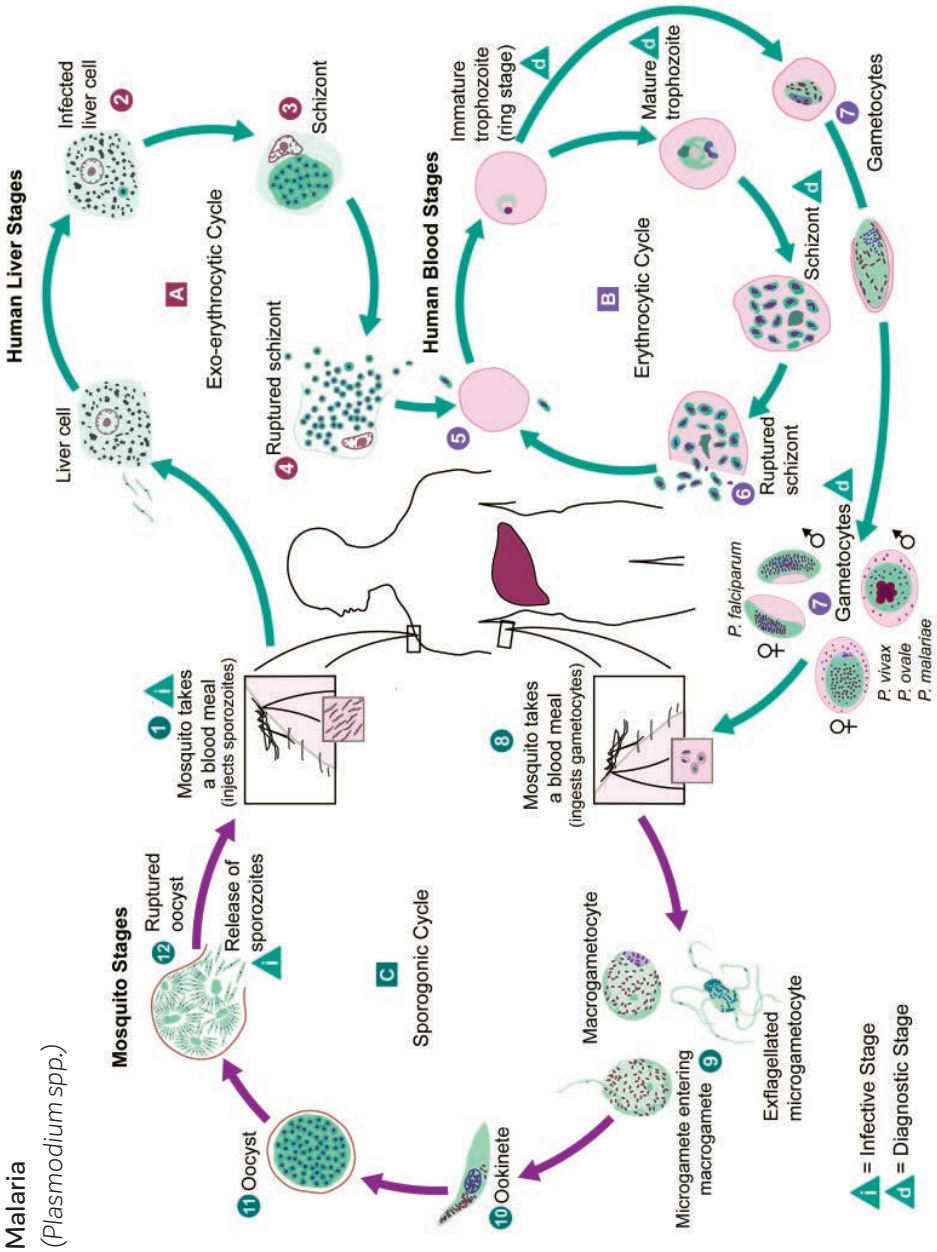


Figure 1. Life cycle of parasites of the genus *Plasmodium* that cause malaria ↻

(courtesy of Centers of Disease Control, Alexander J. da Silva, PhD, Melanie Moser)

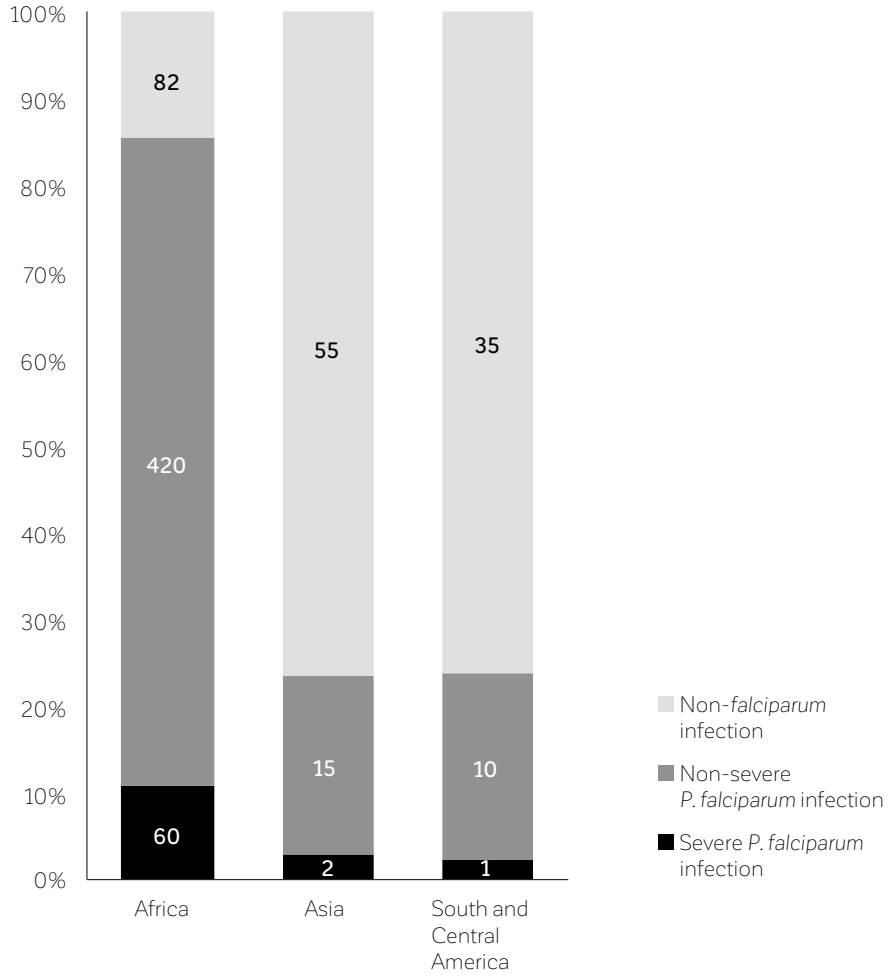
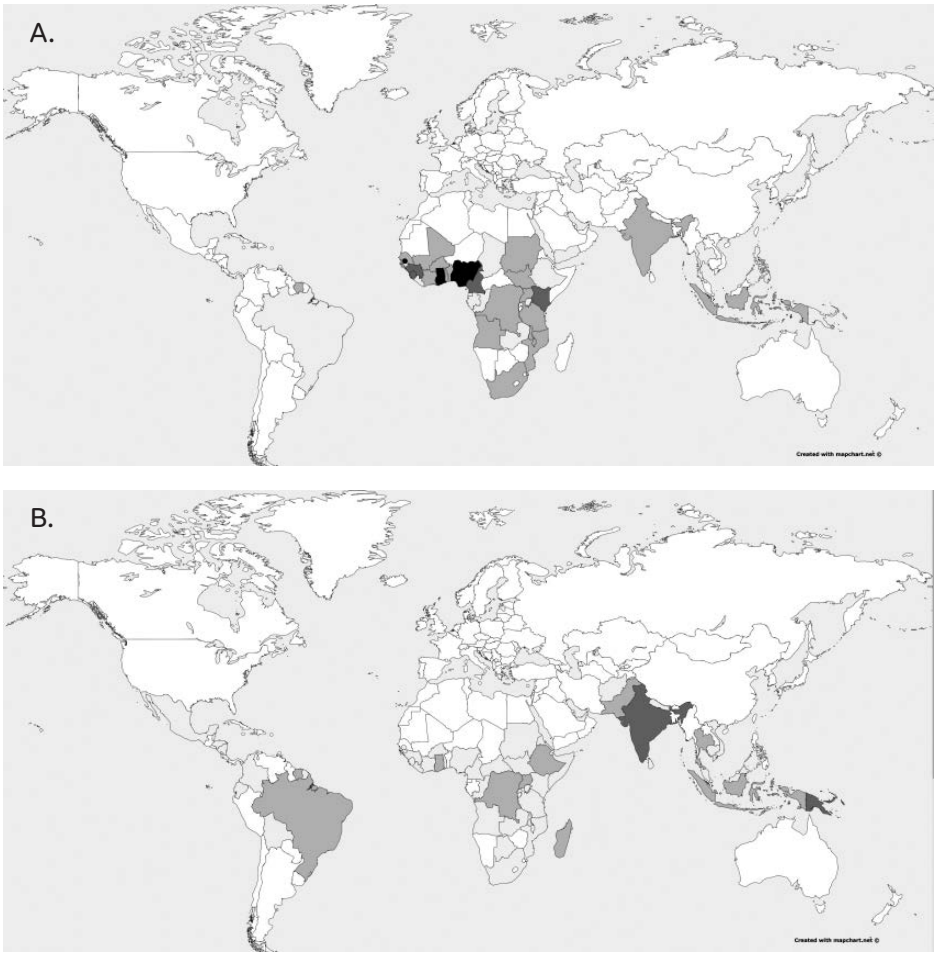


Figure 2. The continent of acquisition of the patients from the Rotterdam Malaria Cohort.

The numbers within the bars indicate the absolute number of cases. The continent of acquisition was unknown in 25 cases (not shown).



Legend

- No cases
- 1 to 4 cases
- 5 to 14 cases
- 15 to 49 cases
- 50 or more cases

Figure 3. World map showing the countries where patients from the Rotterdam Malaria Cohort acquired the infection.

The colour indicates the number of infections acquired in the particular country.

Panel A. shows P. falciparum infections and panel B. non-falciparum infections.

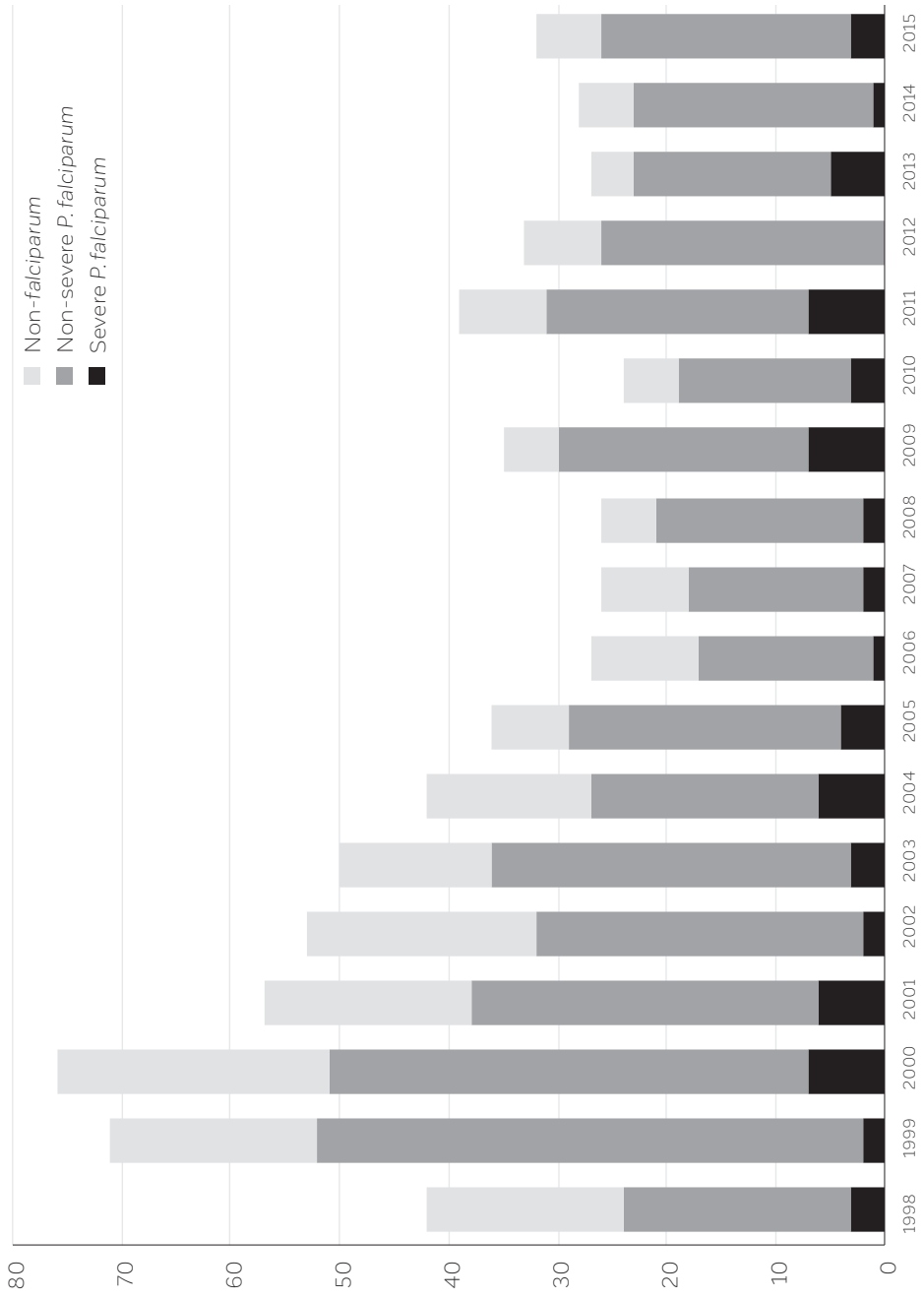


Figure 4. Number of cases per year in the Rotterdam Malaria Cohort. ↻

Table 1. The 2010 World Health Organisation criteria for severe malaria.

2010 WHO malaria severity criteria
Severe malaria is defined, in the absence of other obvious cause, when P. falciparum asexual parasitaemia is accompanied by one or more of the following clinical or laboratory features
Clincal features
Impaired consciousness or unrousable coma
Prostration, i.e. generalised weakness so that the patient is unable to walk or sit up without assistance
Failure to feed
Multiple convulsions - more than two episodes in 24h
Deep breathing, respiratory distress (acidotic breathing)
Circulatory collapse or shock, systoloc blood pressure <70 mmHg in adults and <50 mmHg in children
Clinical jaundice plus evidence of other vital organ disfunction
Haemoglobinuria
Abnormal spontaneous bleeding
Pulmonary oedema (radiological)
Laboratory findings
Hypoglycaemia (blood glucose <2.2 mmol/l)
Metabolic acidosis (plasma bicarbonate <15 mmol/l)
Severe normocytic anaemia (Hb< 5 g/dl, packed cell volume <15%)
Haemoglobinuria
Hyperparasitaemia (>2% or 100 000/µl in low transmisson areas or >5% or 250 000/ µl in high stable malaria transmission areas)
Hyperlactataemia (lactate > 5 mmol/l)
Renal impairment (serum creatinine >265 µmol/l)

Table 2. The 2014 World Health Organisation criteria for severe malaria.

2014 WHO malaria severity criteria	
For epidemiological and research purposes, severe malaria is defined as one of the following, occuring in the absence of an identified alternative cause, and in the presence of P. falciparum asexual parasitaemia	
Impaired consciousness	A Glasgow Coma Score <11 in adults or a Blantyre coma score < 3 in children
Acidosis	A base deficit of >8 meq/l or, if unavailable, a plasma bicarbonate of <15mM or venous plasma lactate >5mM.
Hypoglycaemia	Blood or plasma glucose <2.2 mM
Severe malarial anaemia	A haemoglobin concentration <5 g/dL or a hematocrit of <15% in children <12 years of age (<7 g/dLand <20% respectively in adults) together with a parasite count > 10 000/µl
Renal impairment	Plasma or serum creatinine >265 µM or blood urea >20 mM
Jaundice	Plasma or serum bilirubin > 50 µM together with a parasite count > 10 000/µl
Pulmonary oedema	Radiologically confirmed, or oxygen saturation < 92% on room air with a respiratory rate >30/min, often with chest indrawing and crepitations on auscultation
Significant bleeding	Including recurrent or prolonged bleeding from nose, gums or venepuncture sites, haematemesis or melaena
Shock	Compensated shock is defined as capillary refill >3s or temperature gradient on leg (mid to proximal limb) but no hypotension. Decomensated shock is defined as systolic blood pressure <70 mmHg in children or <80 mmHg in adults with evidence of impaired perfusion (cool peripheries or prolonged capillary refill)
Hyperparasitaemia	*P. falciparum* parasitemia >10%

Table 3. The number of patients that met each of the 2014 WHO criteria for severe malaria.

2014 WHO malaria severity criterion	Frequency
Jaundice	38
Hyperparasitaemia	26
Renal impairment	21
Acidosis	15
Severe malarial anaemia	9
Pulmonary oedema	6
Impaired consciousness	5
Shock	2
Hypoglycaemia	1
Significant bleeding	0

A total of 64 patients were diagnosed with severe malaria after meeting one or more of these criteria

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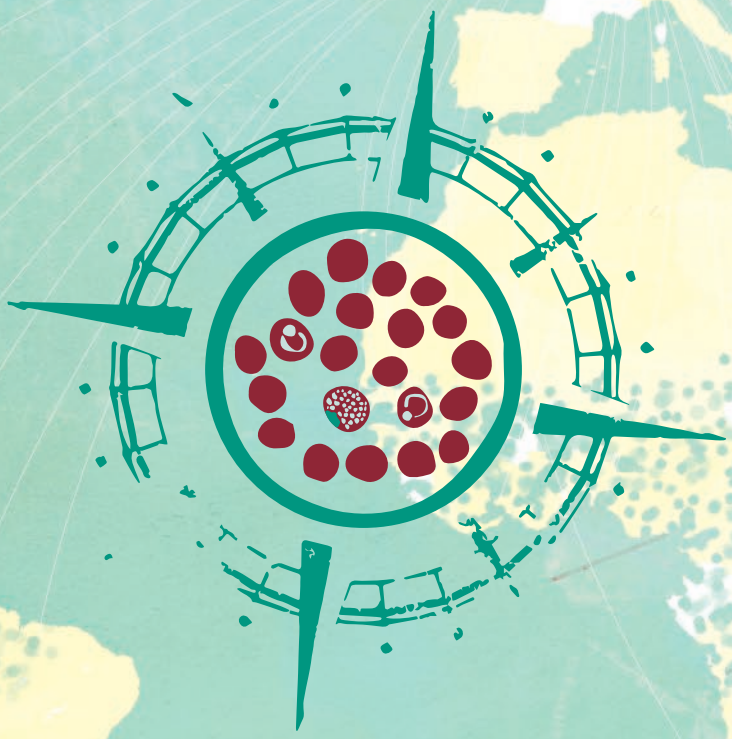
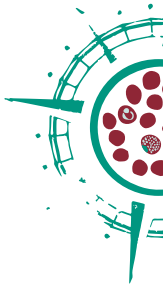
PART I

Assessing and predicting malaria severity



CHAPTER 2

Neopterin and procalcitonin are suitable biomarkers for the exclusion of severe *Plasmodium falciparum* disease in travellers with imported malaria



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Malar J. 2010;9:255

ABSTRACT

Background: Most clinicians in developed, non-malaria endemic countries have limited or no experience in making clinical assessments of malaria disease severity and subsequent decisions regarding the need for parenteral therapy or high-level monitoring in febrile patients with imported malaria. In the present study, the diagnostic accuracy of plasma soluble Triggering Receptor Expressed on Myeloid cells 1 (TREM-1), neopterin and procalcitonin levels as biomarkers for severe *Plasmodium falciparum* disease was evaluated in 104 travellers with imported malaria (26 patients with non-*P. falciparum* malaria, 64 patients with uncomplicated *P. falciparum* malaria and 14 patients with severe *P. falciparum* malaria).

Methods: TREM-1, neopterin and procalcitonin were determined in stored serum samples using commercially available ELISA or EIA tests. The diagnostic performance of these biomarkers for severe disease was compared with the reference marker plasma lactate, a well-validated parameter for disease severity in patients with malaria. Severe malaria was defined according to the modified WHO criteria.

Results: No significant differences in TREM-1 levels were detected between the different patient groups. Patients with severe *P. falciparum* malaria had significantly higher neopterin and procalcitonin levels on admission when compared to patients with uncomplicated *P. falciparum* malaria or non-*P. falciparum* malaria. Receiver Operating Characteristic (ROC) curve analysis showed that neopterin had the highest Area-Under-the-ROC curve (AUROC 0.85) compared with plasma lactate (AUROC 0.80) and procalcitonin (AUROC 0.78). At a cut-off point of 10.0 ng/ml, neopterin had a positive and negative predictive value of 0.38 and 0.98 whereas procalcitonin, at a cut-off point of 0.9 ng/ml, had a positive and negative predictive value of 0.30 and 1.00.

Conclusion: Although the diagnostic value of neopterin and procalcitonin is limited, the high negative predictive value of both neopterin and procalcitonin may be helpful for a rapid exclusion of severe malaria disease on admission. This may be a valuable tool for physicians only occasionally dealing with ill-returned travellers from malaria-endemic regions and who need to decide on subsequent oral anti-malarial treatment or timely referral to a specialized center for high-level monitoring and intensified parenteral treatment.

BACKGROUND

Travellers from industrialized countries and inhabitants of malaria-endemic regions clearly represent two distinct worlds of malaria (1). The global burden of malaria is largely carried by the world's malaria-endemic regions with as many as 500 million cases annually and a death toll of 1 to 3 million children each year. Severe malaria in areas of endemicity is associated with a mortality of 15 to 40% (2, 3). In many malaria-endemic regions, strict triage for admission to ICU facilities must be applied because the ICU capacity is usually limited. Recently, a 5-point Coma Acidosis Malaria (CAM) score based on only acidosis (base deficit) and cerebral malaria (measured with Glasgow Coma Scale) was introduced, which could identify adult patients with severe malaria who were at high risk of death (4).

In striking contrast, in non-endemic industrialized countries malaria is only seen as an occasionally imported disease (5) and is usually associated with a low case-fatality rate (6, 7). Even in the pre-artesunate era, the mortality of severe malaria in non-endemic regions was significantly lower when compared with regions of malaria endemicity (6-8), probably reflecting the availability of adequate supportive care facilities in industrialized countries.

Industrialized countries, however, have to face other -more trivial- problems. For instance, the expertise on diagnosis and treatment of malaria is usually focused in some specialized hospitals and institutes but many ill-returning travellers may present to non-specialized hospitals or even general practitioners. Making a proper diagnosis of malaria may be troublesome under these circumstances, for instance, by lack of experience in the examination of malaria thick and thin blood smears and in the assessment of parasite load. These non-specialized centers therefore often rely on rapid diagnostic tests for the diagnosis of malaria (9). Although sensitive in diagnosing *P. falciparum* malaria, these rapid tests do not provide any information about the severity of the infection. Moreover, although artesunate, which is now considered the parenteral drug of choice for treatment of severe *falciparum* malaria, is available as an orphan drug in The Netherlands, it is currently only in stock in some specialized centers but certainly not available in every Dutch hospital. Some of these general hospitals do not even have any drug in stock for the treatment of malaria (10). To prevent unnecessary delay in diagnosis of severe malaria and institution of proper parenteral treatment, a simple, well-validated, laboratory-based biomarker that predicts or excludes severe disease accurately would be of great help for those clinicians occasionally dealing with

febrile travellers returning from malaria endemic regions. These clinicians have to decide on subsequent oral anti-malarial treatment or a timely referral to a specialized center for high-level monitoring and intensified parenteral treatment. In the present study, the diagnostic accuracy of plasma soluble Triggering Receptor Expressed on Myeloid cells 1 (TREM-1), neopterin and procalcitonin were evaluated as potential markers for malaria disease severity in travellers with imported malaria. These bio-substances are all involved in the systemic pro-inflammatory response of the host to invading pathogens. Some of these biomarkers are already in use for the diagnosis and follow-up of sepsis or used in treatment algorithms, resulting in a successful reduction of antibiotic use and duration (11, 12).

METHODS

Study population

The Harbour Hospital is a 161-bed general hospital located in Rotterdam. It also harbours the Institute for Tropical Diseases, which serves as a national reference center. In the period 1999-2008 almost 500 cases of imported malaria were diagnosed (13). For the majority of these cases, demographic, clinical and laboratory data and serum samples were available. For the present study, a representative sample of this cohort was taken and analysed.

Definitions

- Patients were classified as having severe *P. falciparum* malaria if they met one or more of the WHO criteria for severe malaria, as modified by Tran et al (14):
- A score on the Glasgow Coma Scale of less then 11 (indicating cerebral malaria).
- Anaemia (haematocrit < 20%) with parasite counts exceeding 100,000/µl (roughly corresponding to 2% parasitaemia) on a peripheral blood smear.
- Jaundice (serum bilirubin > 50 µmol/l) with parasite counts exceeding 100.000/µl on a peripheral blood smear. Renal impairment (urine output < 400 ml/24 h and serum creatinine > 250 µmol/l).
- Hypoglycaemia (blood glucose < 2.2 mmol/l).
- Hyperparasitaemia (> 10% parasitaemia).
- Systolic blood pressure < 80 mm Hg with cold extremities (indicating shock).

Study design

In previous studies (6, 13, 15) these severity criteria were also used to define severe malaria in non-immune travellers. In the present study the occurrence of severe

malaria was considered a primary end-point. This contrasts with the design of many studies in patients with severe malaria in regions of malaria endemicity where the severity criteria are used as an entry criterion. In the present study, plasma lactate was used as a surrogate parameter for acid-base dysbalance and reference biomarker. It was evaluated in a previous study in non-immune travellers with imported malaria (15). The diagnostic performance of TREM-1, procalcitonin and neopterin for malaria disease severity was compared with that of plasma lactate, which is routinely measured at the Institute for Tropical Diseases in ill-returning travellers.

Procedures

On admission, blood samples were taken for analysis of the red blood cell count, haematocrit, white blood cell count, platelet count, serum electrolytes, total bilirubin, serum creatinine, liver enzymes, and blood glucose. In addition, a serum sample was taken on admission which was stored at -20°C until analysis. For the determination of plasma lactate, a separate blood sample was drawn on admission without congestion and placed on melting ice after which it was immediately analysed after isolation of plasma. Malaria was diagnosed by QBC (Quantitative Buffy Coat) analysis, by a rapid diagnostic antigen test for malaria (Binax NOW® Malaria Test, Binax Inc., Maine, USA) and by conventional microscopy of stained thick and thin blood smears. In case of *P. falciparum* infections, parasite density was determined. When the parasitaemia was less than 0.5% infected erythrocytes, parasites were counted per 100 leucocytes in thick smears. When the parasitaemia was equal or higher than 0.5% infected erythrocytes, infected erythrocytes were counted in thin blood smear and expressed as a percentage of the total erythrocytes. The number of parasites per microliter was subsequently calculated from these data.

TREM-1 and neopterin levels were determined in serum samples using commercially available ELISA tests (R&D Systems, Abingdon, UK; DRG, Marburg, Germany, respectively). Procalcitonin levels in serum samples were determined using a commercially available EIA test (VIDAS BRAHMS Procalcitonin, bioMérieux, Lyon, France). All tests were performed according to manufacturer's instructions. Detection limits were 3.88 pg/ml for TREM-1, 0.2 ng/ml for neopterin and 0.05 ng/ml for procalcitonin, respectively. According to the manufacturers, normal serum values are < 100 pg/ml for TREM-1, < 3 ng/ml for neopterin and < 0.1 ng/ml for procalcitonin.

Statistical methods

For comparison between groups, the Mann-Whitney U-test was used and P-values of < 0.05 were considered statistically significant. The diagnostic performance of each biomarker was reported as sensitivity, specificity, positive and negative predictive value for severe *P. falciparum* malaria and their corresponding 95% confidence intervals. Of each test a Receiver Operating Characteristic (ROC) curve, a graphical plot of sensitivity (true positive rate) versus 1-specificity (false positive rate), was constructed as a summary statistic and the area under the ROC curve (AUROC) and its corresponding 95% confidence intervals were calculated. Youden's index J (J = sensitivity+specificity-1) was used to choose the most appropriate cut-off point for each biomarker. All statistical analyses were performed using SPSS 15.0.

RESULTS

Patient characteristics

In total 104 travellers with imported malaria were included in this study, of which 26 patients were diagnosed with a non-*P. falciparum* infection (*Plasmodium malariae* n = 2; *Plasmodium ovale* n = 5; *Plasmodium vivax* n = 19) and 78 patients were diagnosed with *P. falciparum* infection. The general characteristics of all patients are shown in Table 1.

Characteristics of patients with severe malaria

Thirteen patients fulfilled the criteria for severe malaria at initial presentation. Another patient did not fulfill these criteria on admission, but the clinical course deteriorated shortly hereafter with impaired consciousness and hyperparasitaemia. Procalcitonin and neopterin levels were already increased on admission in this particular patient. Eventually, at admission to the ICU, all 14 patients fulfilled one or more of the severity criteria (GCS < 11, n = 1; anaemia with a parasite count exceeding 100,000 trophozoites per µl, n = 2; icterus with a parasite count exceeding 100,000 trophozoites per µl, n = 8; acute oliguric renal insufficiency, n = 0; hypoglycaemia, n = 0; hyperparasitaemia, n = 5 and shock, n = 1, respectively). Five patients had an impaired conscious level but a GSC above 11; eight patients had a parasitaemia > 5%, respectively. The first arterial blood gas analysis on ICU showed a median bicarbonate level of 22 mmol/l (range 17 to 26 mmol/l) and a median base deficit of 2 (range -3 to 8). Median GCS was 15 (range 9 to 15). One patient needed mechanical ventilation. Eleven patients received exchange

transfusion as an adjunct therapy. No case fatalities were observed. The laboratory results on admission of travellers with imported severe *P. falciparum* malaria were further characterized by significantly lower platelet counts and haemoglobin levels and by significantly higher plasma lactate, bilirubin and C-reactive protein levels and erythrocyte sedimentation rates, respectively (Table 1).

Analysis of biomarkers for severe malaria

TREM-1

No statistically significant differences were observed in TREM-1 levels in serum, between patients with severe *P. falciparum* malaria, uncomplicated *P. falciparum* malaria and non-*P. falciparum* malaria (Figure 1A).

Neopterin

Neopterin levels on admission were significantly higher in travellers with severe *P. falciparum* malaria when compared to travellers with uncomplicated *P. falciparum* malaria ($p<0.0001$) and travellers with non-*P. falciparum* malaria ($p<0.0001$) (Figure 1B). ROC curve analysis showed an AUROC of 0.85 (95% Confidence Interval 0.76-0.94), suggesting a good accuracy (Figure 2). As shown in Table 2, at a cut-off point of 10.0 ng/ml, neopterin had an excellent sensitivity and negative predictive value but a poor specificity and positive predictive value for severe disease.

Procalcitonin

Procalcitonin levels were significantly higher in travellers with severe *P. falciparum* malaria when compared to travellers with uncomplicated *P. falciparum* malaria ($p=0.0022$). However, no significant differences were noted in comparison to travellers with non-*P. falciparum* infections ($p=0.17$) (Figure 1C). ROC curve analysis showed an AUROC of 0.78 (95% CI 0.66-0.91), compatible with a fair accuracy (Figure 2). At a cut-off point of 0.9 ng/ml, procalcitonin had an excellent sensitivity and negative predictive value, whereas specificity and positive predictive value for severe *P. falciparum* malaria was poor (Table 2).

Plasma lactate

Plasma lactate levels were significantly higher in travellers with severe *P. falciparum* malaria when compared to travellers with uncomplicated *P. falciparum* malaria ($p=0.0012$) and travellers with non-*P. falciparum* malaria ($p=0.0040$). ROC curve analysis of plasma lactate levels showed an AUROC of 0.80 (95% CI 0.65-0.96) compatible with a good accuracy (Figure 2). At a cut-off point of 1.8 mmol/l, lactate

had an excellent sensitivity and negative predictive value, but a poor specificity and positive predictive value for severe *P. falciparum* malaria (Table 2), respectively.

Combination of various biomarkers for severe falciparum disease

Analysis of various combinations of newly tested biomarkers and the use of different cut-off levels did not result in better discrimination of patients with severe *P. falciparum* malaria.

DISCUSSION

Severe malaria is disreputable for its high case-fatality rate, but the outcome of severe *P. falciparum* infections has significantly improved since the introduction of artesunate as first line treatment of severe malaria, in particular in developing countries (2). In industrialized countries such as The Netherlands, the case-fatality rate of imported malaria is low and fatal cases are only occasionally reported. In the present study, in which the biomarkers TREM-1, neopterin and procalcitonin were evaluated for their potential to be used as a marker for severe malaria disease upon admission. This contrasts with the design of many studies in regions of malaria endemicity where severe malaria is usually the entry criterion. For reasons of comparability, the same set of criteria for severe malaria was strictly applied for the diagnosis of severe malaria in this study, even though the study population comprised of presumably non-immune travellers and some authors even suggest a threshold of 5% in stead of 10% parasitized erythrocytes to define hyperparasitaemia in non-immune individuals.

The quantification of soluble TREM-1 levels on admission did not result in proper discrimination of severe *P. falciparum* malaria from uncomplicated *P. falciparum* malaria and non-*P. falciparum* malaria. In contrast, travellers with severe *P. falciparum* malaria had significantly higher levels of neopterin and procalcitonin on admission as compared with travellers with uncomplicated *P. falciparum* malaria or non-*P. falciparum* malaria, respectively. These findings correspond with the results of several other studies performed in semi-immune malaria patients living in malaria-endemic regions (16-18). When the ROC curve characteristics of neopterin and procalcitonin were compared to that of plasma lactate, the AUROC of neopterin appeared superior whereas the AUROC of procalcitonin appeared inferior to that of lactate, suggesting that neopterin pro- vided the most accurate diagnostic performance for severe *P. falciparum* malaria in this cohort of travellers.

Unfortunately, the applicability of these tests in the initial clinical assessment of patients with severe *P. falciparum* malaria will probably be limited by the poor positive predictive value of neopterin and procalcitonin indicating that neither test can serve as a valuable tool for the diagnosis of severe *P. falciparum* malaria. For illustration, applying a procalcitonin level > 0.9 ng/ ml or a neopterin level > 10.0 ng/ ml as a guide to intensified monitoring and treatment would result in more than 20 of 64 patients with uncomplicated *P. falciparum* malaria receiving more intensive monitoring and treatment than strictly necessary. On the other hand, the high negative predictive value of both neopterin and procalcitonin suggests that these tests can still be of value by providing a tool for exclusion of severe disease. With either a procalcitonin level of less than 0.9 ng/ml or a neopterin level of less than 7.9 ng/ml in serum on admission as a cut-off point for severe *P. falciparum* malaria, no patient with severe disease would have been denied access to high-level monitoring and intensive treatment. In a previous study, in which a semi-quantitative 'point-of-care' procalcitonin test as a diagnostic tool for severe *P. falciparum* malaria was evaluated prospectively, all 6 patients with severe *P. falciparum* malaria had procalcitonin values classified as either "moderate" or "high" (corresponding to a procalcitonin level ≥ 2 ng/ml), but never as "normal" or "low" (12). This is compatible with the findings of the current retrospective serum sample-based study in which procalcitonin was measured quantitatively.

Although severe or fatal malaria rarely results from infections with the non-sequestering *Plasmodium* species *vivax, ovale* and *malariae*, increased neopterin and procalcitonin serum levels were also observed in the majority of these patients, although levels were lower than compared with severe *P. falciparum* malaria patients. Although speculatively, these observations suggest that the mechanism whereby neopterin and procalcitonin levels increase in malaria, is not specific for severe *P. falciparum* malaria alone. Therefore, it may not accurately reflect the pivotal pathophysiological events in *complicated P. falciparum* malaria, such as the sequestration of infected red blood cells in the microcirculation of vital organs and disturbance of microcirculatory flow. Whereas an increased plasma lactate level conceivably reflects a significant reduction in microcirculatory flow in vital organs, the elevated neopterin and procalcitonin levels are probably the result of activation of a common inflammatory host response evoked by infection with the respective *Plasmodium* parasites. In fact, some reports even suggest that *P. falciparum* malaria per se is not associated with a stronger host response than *P. vivax* or *P. ovale* malaria, but that the parasite burden of the causative *Plasmodium* species may also modulate the extent of the host inflammatory response (19).

CONCLUSION

Although neither neopterin nor procalcitonin can probably serve as a useful single diagnostic tool for severe *P. falciparum* malaria, the high negative predictive value of both neopterin and procalcitonin may be helpful for a rapid exclusion of severe *P. falciparum* malaria on admission. This may be a valuable tool - particularly if available as a rapid diagnostic test - for physicians only occasionally dealing with ill-returned travellers and who need to decide on subsequent oral anti-malarial treatment or a timely referral to a specialized center for high-level monitoring and intensified parenteral treatment.

Table 1. General characteristics and laboratory results on admission of patients with various species of malaria.

	Non- *P. falciparum*	*P. falciparum*	
	(n=26)	Uncomplicated (n=64)	Severe (n=14)
Demographics			
Male/female	20/6	51/13	6/8
Age (years)	40 (17-62)	40 (11-67)	40 (26-57)
Continent of acquisition			
Africa	12 (46%)	60 (94%)	12 (86%)
Asia	9 (35%)	3 (5%)	1 (7%)
South America	5 (19%)	1 (2%)	1 (7%)
Patient immunity			
Non-immune	20 (77%)	35 (55%)	10 (72%)
Partially immune	6 (23%)	28 (44%)	4 (28%)
Semi-immune	0	1 (2%)	0
Vital signs on admission			
Body temperature (Celsius)	38,8 (36,1-41,5)	38,7 (36,1-40,6)	38,8 (36,8-40,6)
Pulse rate (beats per minute)	90 (60-130)	95 (68-120)	108 (78-140)
Systolic blood pressure (mmHg)	123 (100-196)	120 (95-185)	118 (80-160)
Glasgow coma score			
normal (=15)	26 (100%)	63	12 (86%)
mildly impaired (11 - 14)	0	1 (2%)	1 (7%)
severely impared (<11)	0	0	1 (7%)

Table 1. Continued

	Non- *P. falciparum*	*P. falciparum*	
	(n=26)	Uncomplicated (n=64)	Severe (n=14)
Laboratory parameters			
Parasite load (trophozoites/ul)	ND	5502 (1,0-385.000) *	205.600 (80.500-860.000)
Plasma lactate (mmol/l)	1,4 (0,7-3,0) *	1,5 (0,5-4,4) *	2,6 (0,9-5,8)
Haemoglobin (mmol/l)	8,2 (6,1-10,1)	8,7 (5,3-11,1) *	7,6 (3,8-10,2)
Leucocytes (*10e9/l)	5,2 (1,9-9,3)	5,5 (1,8-11,3)	6,6 (3,2-18,5)
Platelets (*10e9/l)	93,0 (10,0-205,0) *	78,5 (16,0-247,0) *	27,0 (3,0-152,0)
C-reactive protein (mg/l)	86,5 (18,0-208,0) *	109,0 (5,0-278,0) *	190,0 (91,0-265,0)
Serum creatinine (umol/l)	94,0 (66,0-149,0)	103,5 (63,0-208,0)	102,5 (70,0-199,0)
Total bilirubin (µmol/l)	24,0 (6,0-84,0) *	25,0 (7,0-164,0) *	54,0 (20,0-269,0)

** p<0.01. P-values as compared with severe P. falciparum malaria. ND = not determined*

Table 2. Descriptive statistics of diagnostic accurary of neopterin, procalcitonin as compared zith lactate for the diagnosis of severe *falciparum* malaria on admission.
The data within brackets are the 95% confidence intervals.

	Neopterin	Procalcitonin	Lactate
Optimal cut-off value	10 ng/ml	0.9 ng/ml	1.8 mmol/l
Youden's index	0.60	0.53	0.58
Sensitivity	0.93 (0.64-1.00)	1.00 (0.70-1.00)	0.92 (0.60-1.00)
Specificity	0.67 (0.54-0.78)	0.53 (0.40-0.66)	0.66 (0.51-0.78)
Positive predictive value	0.38 (0.23-0.56)	0.30 (0.17-0.47)	0.39 (0.22-0.59)
Negative predictive value	0.98 (0.86-1.00)	1.00 (0.87-1.00)	0.97 (0.83-1.00)

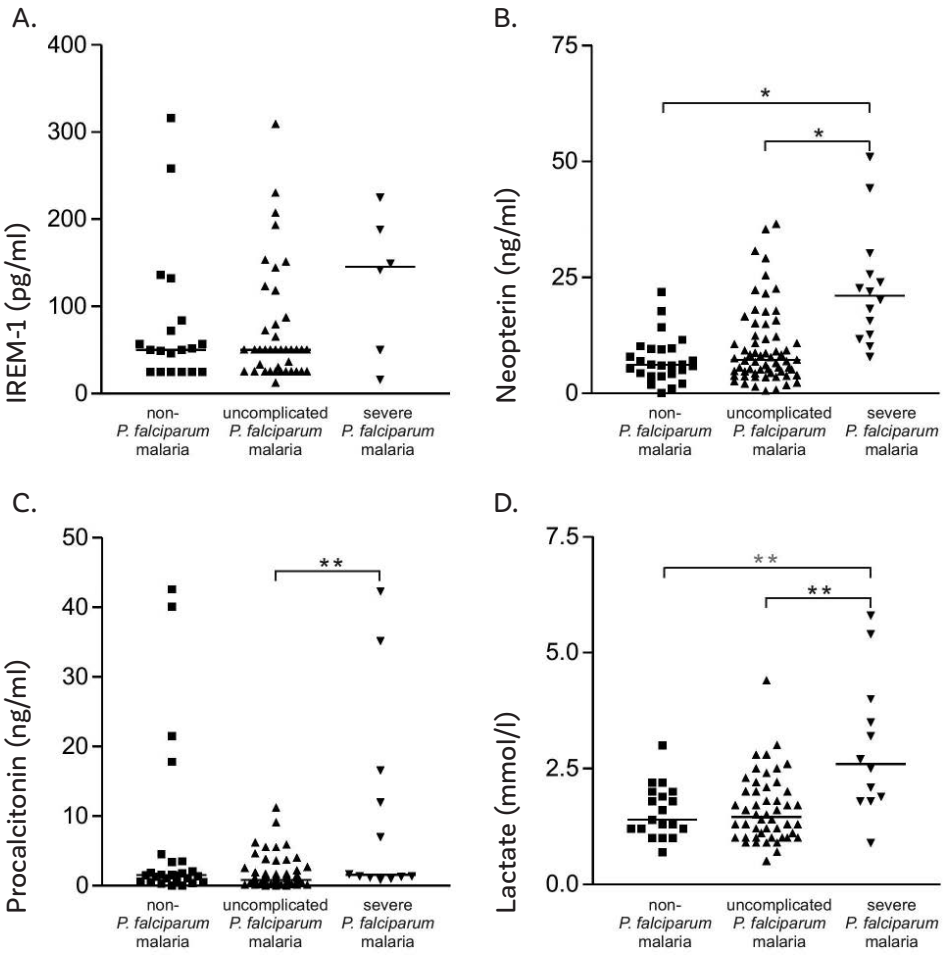

Figure 1 Concentrations of potential biomarkers for disease severity in malaria patients on admission.

*Individual data are shown with the median value of each biomarker; TREM-1 (panel A), neopterin (panel B), procalcitonin (panel C) and plasma lactate (panel D). Significant differences in biomarker concentrations between patient groups (black square = non-P. falciparum malaria; black triangle up = uncomplicated P. falciparum malaria; black triangle down = severe P. falciparum malaria) with P-values < 0.0001 and < 0.005 are indicated by * and **, respectively.*

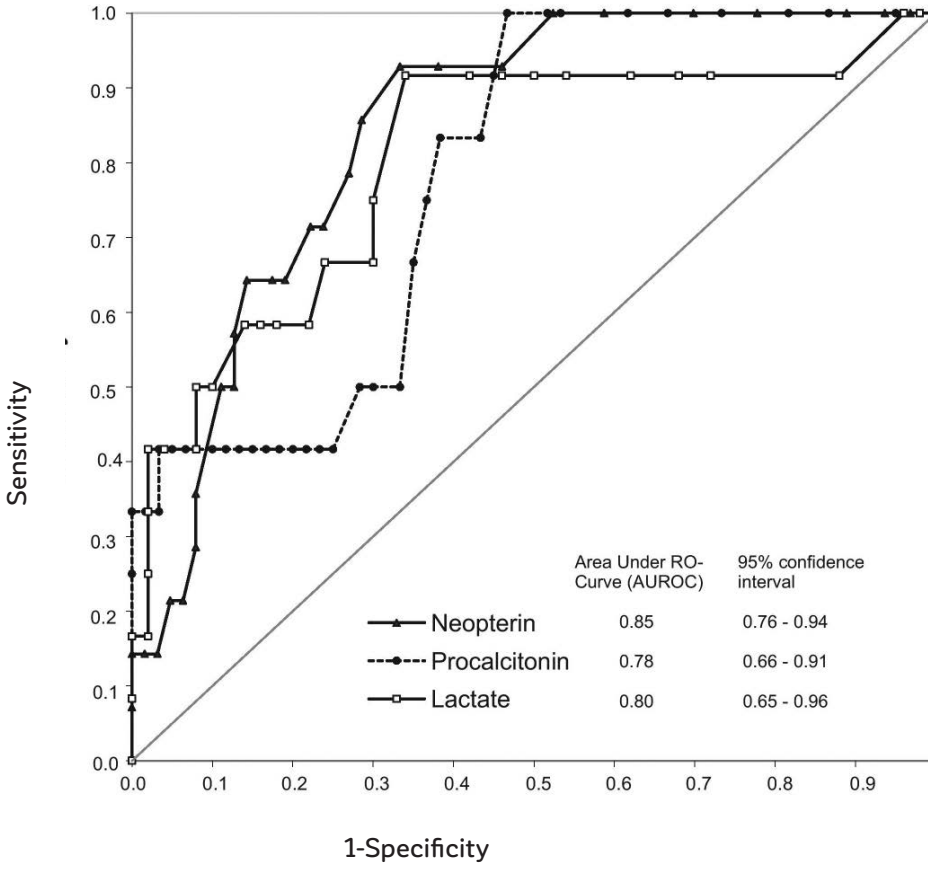

Figure 2. Receiver Operating Curves (ROC) characteristics of the diagnostic performance of neopterin, procalcitonin and lactate for severe *P. falciparum* malaria.

The ROC curve is a graph of sensitivity (true positive fraction) plotted against 1-specificity (false positive fraction). The performance of a diagnostic variable can be quantified by calculating the area under the ROC curve (AUROC). The ideal test would have an AUROC of 1, whereas a random guess would have an AUROC of 0.5.

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CHAPTER 3

Copeptin does not accurately predict disease severity in imported malaria

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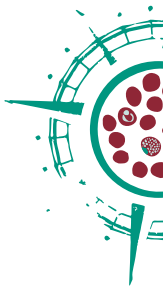
Malar J. 2012;11:6.



ABSTRACT

Background: Copeptin has recently been identified to be a stable surrogate marker for the unstable hormone arginine vasopressin (AVP). Copeptin has been shown to correlate with disease severity in leptospirosis and bacterial sepsis. Hyponatraemia is common in severe imported malaria and dysregulation of AVP release has been hypothesized as an underlying pathophysiological mechanism. The aim of the present study was to evaluate the performance of copeptin as a predictor of disease severity in imported malaria.

Methods: Copeptin was measured in stored serum samples of 204 patients with imported malaria that were admitted to our Institute for Tropical Diseases in Rotterdam in the period 1999-2010. The occurrence of WHO defined severe malaria was the primary end-point. The diagnostic performance of copeptin was compared to that of previously evaluated biomarkers C-reactive protein, procalcitonin, lactate and sodium.

Results: Of the 204 patients (141 *Plasmodium falciparum*, 63 non-*falciparum* infection), 25 had severe malaria. The Area Under the ROC curve of copeptin for severe disease (0.66 [95% confidence interval 0.59-0.72]) was comparable to that of lactate, sodium and procalcitonin. C-reactive protein (0.84 [95% CI 0.79-0.89]) had a significantly better performance as a biomarker for severe malaria than the other biomarkers.

Conclusion: C-reactive protein but not copeptin was found to be an accurate predictor for disease severity in imported malaria. The applicability of copeptin as a marker for severe malaria in clinical practice is limited to exclusion of severe malaria.

BACKGROUND

Arginine vasopressin (AVP), also known as the antidiuretic hormone, is a key hormone in maintaining fluid balance and vascular tone. Despite its clinical relevance, measurement of mature AVP is difficult and subject to (pre)analytical errors. Recently, copeptin, a 39-amino acid glycopeptide that comprises the C-terminal part of the AVP precursor was found to be a stable and sensitive surrogate marker for AVP release (1, 2). In recent studies in patients with bacterial sepsis but also in patients with leptospirosis it was shown that copeptin levels correlate well with disease severity and outcome when compared to other commonly used biomarkers like C-reactive protein (CRP) and more experimental biomarkers like procalcitonin (3, 4). Copeptin levels may even be used as a valuable tool to guide management of critically ill patients (4). Leptospirosis and bacterial sepsis are strikingly similar to severe malaria in terms of case-fatality rates and clinical presentation including the presence of hyponatraemia (5). Hyponatraemia was found to be highly prevalent in severe malaria and suggested to be the result of a dysregulated vasopressin release (6-9).

The present study, therefore, aimed to evaluate copeptin as a pathophysiologic predictor of disease severity in patients with imported malaria. The diagnostic performance of copeptin was compared to that of several previously validated biomarkers to provide the clinician with the most accurate tool for clinical decision making in the acute care setting. A reliable test, easily discriminating severe from non-severe disease in malaria, would be clinically useful, especially in the setting of imported malaria, given the observation that most clinicians in non-endemic areas have little expertise with identifying severe malaria but these patients require a rapid triage for parenteral anti-malarials and intensive monitoring.

METHODS

The Harbour Hospital is a 161-bed general hospital located in Rotterdam, The Netherlands. It also harbours the Institute for Tropical Diseases, which serves as a national reference centre. In the period 1999-2010 more than 500 cases of imported malaria were diagnosed. The characteristics of the Rotterdam Malaria Cohort in the period 1999-2008 were previously described (9). For the majority of these cases, demographic, clinical and laboratory data and serum samples were available. In the present study copeptin and procalcitonin were retrospectively measured in stored serum samples of 204 patients with imported malaria. The

tested serum samples were not from consecutive patients entering this cohort but based on availability. The occurrence of severe malaria was considered a primary end-point given the low case-fatality rate of imported malaria in the Netherlands. Because severe malaria can also occur in non-*falciparum* infections (albeit rare in returning travellers), patients with non-*falciparum* infections were not excluded. This contrasts with the design of many studies in patients with severe malaria in regions of malaria endemicity where the severity criteria are usually used as an entry criterion (10) and focus on *P. falciparum* malaria. In the present study, the previously validated biomarkers plasma lactate (11), procalcitonin (12, 13), sodium and CRP (9) were used as comparative biomarkers for copeptin to evaluate its accuracy for use in clinical practice to differentiate severe from non-severe malaria. For that purpose, the patients with non-severe *P. falciparum* malaria and non-severe non-*falciparum* malaria were grouped as the non-severe malaria patients.

Procedures

On admission, blood samples were taken for analysis of the red blood cell count, haematocrit, white blood cell count, platelet count, CRP, serum electrolytes, total bilirubin, serum creatinin, liver enzymes, and blood glucose. A separate blood tube was drawn on admission for determination of plasma lactate, which was immediately analysed after isolation of plasma. Malaria was diagnosed by QBC (Quantitative Buffy Coat) analysis, by a rapid diagnostic antigen test for malaria (Binax NOW® Malaria Test, Binax Inc. Maine, USA) and by conventional microscopy of stained thick and thin blood smears to identify the causative *Plasmodium* species. In case of *Plasmodium falciparum* infections, parasite density was determined. When the parasitaemia was less than 0.5% infected erythrocytes, parasites were counted per 100 leucocytes in thick smears. When the parasitaemia was equal or higher than 0.5% infected erythrocytes, infected erythrocytes were counted in thin smear and expressed as a percentage of the total erythrocytes. The number of parasites per microliter was subsequently calculated from these data. Since non-*falciparum* infections are naturally limited by their specific preference for certain erythrocytic subpopulations, non-*falciparum* malaria infection were not routinely quantified. Patients were classified as having severe malaria if they met one or more of the WHO criteria for severe malaria, as published (14). Procalcitonin levels were determined in serum samples using a commercially available EIA test (VIDAS BRAHMS Procalcitonin, bioMérieux, Lyon, France). Normal serum values for procalcitonin are <0.1 ng/ml. Copeptin was measured with a commercial sandwich immunoluminometric assay (Brahms Copeptin,

Thermo Fisher Scientific, Hennigsdorf/Berlin, Germany). Normal values for serum copeptin are 1.70-11.25 pmol/L (2).

Statistical methods

Differences between patients with severe malaria, non-severe *P. falciparum* malaria and non-severe non-*falciparum* malaria were analysed with the Kruskall-Wallis test followed by Dunn's post-hoc tests. Two group comparisons were done by Student's unpaired t-test, Student's unpaired t-test with Welch correction or with the non-parametric Mann Whitney test depending on the distribution of the data. The diagnostic performance of each biomarker was reported as sensitivity, specificity, positive and negative predictive value for severe malaria and their corresponding 95% confidence intervals. Youden's index J (J= sensitivity+specificity-1) was used to identify the most appropriate cut-off point for each biomarker. Of each test a Receiver Operating Characteristics (ROC) curve, a graphical plot of sensitivity (true positive rate) versus 1-specificity (false positive rate), was constructed as a summary statistic and the area under the ROC curve (AUROC) and its corresponding 95% confidence intervals were calculated. The AUROC of each of the biomarkers was compared to that of copeptin in a pair-wise comparison with the method of DeLong et al (15).

RESULTS

Patient characteristics

In total, 204 travellers with imported malaria were included in this study; 63 patients were diagnosed with a non-*falciparum* infection (45 with *Plasmodium* vivax, 15 with *Plasmodium* ovale, three with *Plasmodium* malariae) and 141 patients were diagnosed with *P. falciparum* infection. Of the patients with *P. falciparum* infections, 25 (17.7%) patients had severe malaria. There were no cases of severe non-*falciparum* malaria. The infection was most frequently acquired in Africa (n=156), followed by Asia (n=27) and South America (n=14). Thirty (14.7%) patients reported adequate use of malaria chemoprophylaxis, 24 (11.8%) used it inadequately and 143 (70.1%) patients did not use any chemoprophylaxis (data on prophylaxis use were not available in seven patients). The general characteristics of all patients are shown in Table 1.

Characteristics of patients with severe malaria

Twenty-one of 25 patients (84.0%) with severe malaria acquired a *P. falciparum* infection in West-Africa. The others acquired severe malaria in East-Africa (n = 2), South-East Asia (n = 1) and South-America (n = 1). Twenty-four patients with severe malaria did not use any form of malaria chemoprophylaxis and one patient was using it inadequately. At admission to the intensive care unit (ICU), the 25 patients with severe malaria all fulfilled one or more of the severity criteria (Glasgow Coma Score ≤ 11, n = 2; haematocrit < 0.20 L/L n = 5; creatinine > 250 µmol/L n = 2; bilirubin > 50 µmol/L n = 12; lactate > 5.0 mmol/L n = 5; hyperparasitaemia > 5% n = 14; schizontaemia n = 14). The first arterial blood gas analysis on the ICU showed a median bicarbonate level of 21mmol/L (range 9 to 25 mmol/L) and a median base excess of -2 mmol/L (range -18 to 3). The median Glasgow Coma Scale score was 15 (range 11 to 15). Two patients required mechanical ventilation. Two patients were referred to another hospital for renal replacement therapy because of persistent acute oliguric renal insufficiency. Thirteen patients received exchange transfusion as an adjunct therapy to parenteral anti-malarial treatment. One case fatality was observed. The laboratory results of travellers with severe malaria were further characterized by significantly lower platelet counts and haemoglobin levels and by significantly higher plasma lactate, bilirubin and CRP levels compared to patients with non-severe *P. falciparum* malaria (Table 1).

Analysis of biomarkers for severe malaria

In order to evaluate the diagnostic accuracy of copeptin for severe malaria, the performance of copeptin was compared to that of sodium, CRP, lactate and procalcitonin. Assuming that AUROCs are a measure of diagnostic accuracy, the performance of copeptin for severe malaria was comparable to that of sodium, lactate and procalcitonin, as is shown in Figure 1. The AUROC of copeptin did not statistically differ from the AUROC of sodium, lactate and procalcitonin in a pairwise comparison (Table 2). As shown in Figure 1 and Table 2, CRP had a significantly better performance than either copeptin, sodium and lactate for prediction of severe malaria but its usefulness in clinical practice is probably limited. For illustration, when a hypothetical decision rule for severe malaria was created solely based on a CRP cut-off level of ≥ 155 mg/L, 10 of 62 (or 16.1%) evaluable patients with non-*falciparum* disease and 26 of 116 (or 22.4%) evaluable patients with uncomplicated *P. falciparum* would have been falsely diagnosed as having severe malaria. In contrast, the diagnosis severe malaria would not have been considered in 5 of 24 (20.8%) evaluable patients with severe disease. Addition of either lactate, sodium or copeptin as a secondary selection criterion to the prediction rule did

not result in a proper identification of all patients with severe disease on admission. Only after adding procalcitonin (at a cut-off of ≥ 0.9 ng/mL) as a secondary selection criterion to the prediction rule, all evaluable patients with severe malaria would have been correctly identified on admission (See Supplementary Table 1). However, still nine of 29 evaluable patients with non-severe *P. falciparum* malaria and 10 of 15 evaluable patients with non-severe, non-*falciparum* malaria would have been falsely diagnosed with severe malaria, respectively (Supplementary Figure 1).

Analysis of biomarkers in relation to the number of severity criteria in patients with severe disease

When focusing on the 25 patients with severe malaria, 11 patients fulfilled a single criterion for severe malaria on admission, 11 patients fulfilled two malaria severity criteria, 2 patients fulfilled 3 malaria severity criteria and one patient fulfilled 4 malaria severity criteria on admission to the ICU, respectively. As a consequence of this distribution a proper stratification by numbers of malaria severity criteria present on admission was not feasible. When the severe malaria patients fulfilling a single malaria severity criterion on admission were compared to the patients with more than one criterion for severe malaria on admission, only for the biomarker plasma lactate a statistically significant difference was found (1.8±0.6 versus 3.3±1.6 mmol/L, p=0.0042). See Supplementary Table 1 for further details.

DISCUSSION

In the present study, the diagnostic performance of several biomarkers for severe malaria was evaluated. In contrast to studies in patients with severe bacterial sepsis (3) and severe leptospirosis (4), copeptin was not found to be a more accurate marker to predict severe disease in malaria than the previously validated biomarkers procalcitonin, sodium, and lactate. This may suggest that the major clinical manifestations of severe bacterial sepsis and leptospirosis may follow other pathophysiologic pathways than in patients with severe malaria. In the group of malaria patients, CRP had a clearly superior performance as compared to the previously mentioned biomarkers including copeptin. This finding is certainly of clinical relevance since measurement of CRP protein is not only widely available in industrialized countries but also readily available during night shifts in the acute care setting.

In addition, there may be another pitfall with CRP-based decision rules in malaria.

As is known from studies in patients with severe malaria, co-infections may occur frequently in patients with malaria, which in itself may lead to a rise in CRP levels, irrespective of the malaria disease severity. For example, in a large cohort of 400 cases of imported severe malaria, 96 first episodes of co-infection were seen, in particular pneumonia (n=61), bacteraemia (n=18) and urinary tract infections (n=12). Interestingly, these episodes were related to both community-acquired (n=30) as well as nosocomial infections (n=66) (16). In our study blood cultures of patients with severe malaria did not demonstrate presence of co-existing bacteraemia on admission.

These abovementioned observations underline the limited value of these biomarkers for a rapid diagnosis of severe disease in the acute care setting in non-endemic industrialized countries; as for now, a biomarker-based decision rule can certainly not replace the current clinical evaluation. It is noteworthy that all currently studied biomarkers were characterized by high negative predictive values, which may be helpful for exclusion of severe malaria on admission. The combined use of biomarkers may look promising since they allow correct identification of all patients with severe malaria but their use in the current clinical practice is hampered by their poor positive predictive values and lack of prospective validation.

In conclusion, copeptin was not found to be a good biomarker for severe malaria in imported malaria. Instead, CRP appeared to be a more accurate predictor for disease severity than the other investigated biomarkers. The main clinical applicability of the current biomarkers or combination of biomarkers is probably limited to a rapid exclusion of severe disease given their high negative predictive values and low positive predictive values.

Table 1. Characteristics of malaria patients at initial presentation

	Severe malaria [all *P. falciparum*] (n=25)	Non-severe *P. falciparum* (n=116)	Non-severe Non-*falciparum* (n=63)	P-value
Demographics				
Age, years	44 (23 – 70)	41 (11 – 69)	38 (8 – 62)	n.s.
Male, female, n (%)	15 (60), 10 (40)	92 (79), 24 (21)	44 (70), 19 (30)	n.s.
Duration of complaints, n (%)				
Less than 1 week	16 (64)	83 (72)	31 (49)	0.0905
1-2 weeks	8 (32)	20 (17)	15 (24)	
3-4 weeks	0 (0)	8 (7)	5 (8)	
More than 4 weeks	0 (0)	1 (1)	3 (5)	
Vital signs on admission				
Body temperature, °C	38.4 (35.7 – 40.6)	38.6 (35.7 – 41.0)	38.3 (36.0 – 41.2)	n.s.
Pulse rate, beats per minute	108 (75 – 140)[A<0,001; B<0,01]	90 (68 – 130)	90 (58 – 138)	0.0005
Systolic blood pressure, mm Hg	118 (80 – 150)	120 (88 – 185)	120 (95 – 196)	n.s.

Table 1. Continued

	Severe malaria [all *P. falciparum*] (n=25)	Non-severe *P. falciparum* (n=116)	Non-severe Non-*falciparum* (n=63)	P-value
Laboratory data on admission				
Parasite density (parasites/µL)	284,005 (39,600-1,380,600)	22,657 (2-156,600)	n.a.	<0.0001
C-reactive protein, mg/L	186 (71 -407)$^{A<0,001; B<0,001}$	95 (7 – 310)	83 (14 – 348)	<0.0001
Hemoglobin, mmol/L	7.8 (2.5 – 10.2)$^{A<0,05}$	8.5 (5.3 – 11.1)	8.3 (5.6 – 10.7)	0.0281
Haematocrit, L/L	0.37 (0.12 – 0.50)$^{A<0,05}$	0.41 (0.24 – 0.52)	0.39 (0.26 – 0.53)	0.0067
Leucocyte count, x 109/L	6.9 (2.5 – 18.5)$^{A<0,05}$	5.0 (2.2 – 12.6)	5.3 (1.9 – 11.0)	0.0361
Thrombocytes, x 109/L	36 (11 – 164)$^{A<0,001; B<0,001}$	93 (16 – 385)	95 (10 – 292)	0.0004
Serum glucose, mmol/L	6.8 (4.1 – 8.8)	6.8 (4.4 – 26.0)	6.4 (4.2 – 22.1)	n.s.
Serum sodium, mmol/L	131 (124 – 139)$^{A<0.05;B<0,01}$	134 (122 – 141)$^{C<0,05}$	136 (127 – 141)	<0.0001
Serum creatinine, µmol/L	110 (70 – 1081)$^{B<0,001}$	97 (51 – 208)$^{C<0,05}$	91 (46 – 126)	0.0010
Serum urea, mmol/L	7.6 (3.8 – 55.8)$^{A<0,01; B<0,01}$	5.2 (2.2 – 18.7)	5.1 (2.7 – 10.9)	0.0018
Plasma lactate, mmol/L	2,3 (1,0 – 5,8)$^{A<0,001; B<0,05}$	1,4 (0,5 – 5,5)	1.7 (0.7 – 4.0)	0.0014
Procalcitonin, ng/mL	1.9 (0.9-42.3)$^{A<0.01}$	0.6 (0.0 – 11.2)	1.6 (0.0 – 42.6)	0.0057
Copeptin, pmol/L	22.9 (5.1 – 91.5)$^{B<0,05}$	13.9 (1.6 – 67.8)	12.0 (1.9 – 82.9)	0.0249
Duration hospitalisation, days	**7 (3 – 13)**$^{A<0,001; B<0,001}$	**5 (0 – 10)**$^{C<0,001}$	**2 (0 – 9)**	**<0.0001**

Statistical analysis: Kruskall-Wallis (P-values in column) followed by Dunn's post-hoc tests (P-values in superscript); n.s. denotes "not significant"; n.a. denotes "not applicable". A: severe malaria vs non-severe >>

<< P. falciparum malaria; B: severe malaria vs non-severe non-falciparum malaria; C: non-severe P. falciparum malaria vs non-severe non-falciparum malaria. Comparison of parasite density was calculated for P. falciparum infections only (Mann Whitney test).

Table 2. Descriptive statistics of diagnostic accuracy of the various biomarkers for severe malaria.

	Copeptin (pmol/L)	Sodium (mmol/L)	Lactate (mmol/L)	C-reactive protein (mg/L)	Procalcitonin (ng/mL)
Cut-off value	≥ 21	≤ 132	≥ 1.6	≥ 155	≥ 0.9
Youden's index	0.33	0.44	0.41	0.59	0.51
Sensitivity	60 (39-78)	72 (50-87)	86 (64-96)	79 (57-92)	100 (68-100)
Specificity	73 (66-79)	72 (64-78)	56 (45-65)	80 (73-85)	51 (38-64)
Positive Predictive Value	31 (25-38)	26 (0.17-0.38)	29 (19-42)	35 (23-49)	28 (15-44)
Negative Predictive Value	93 (87-96)	95 (89-98)	95 (85-99)	97 (92-99)	100 (86-100)
Area Under the ROC curve	0.66 (0.59-0.72)	0.72 (0.66-0.78)	0.74 (0.66-0.81)	0.84 (0.79-0.89)	0.76 (0.65-0.86)
P-value*		P=0.4289	P=0.6546	P=0.0237	P=0.6479

*Data are given as mean (95% confidence interval). *P-values of pair-wise comparison of ROC curves (with copeptin ROC curve as comparator)*

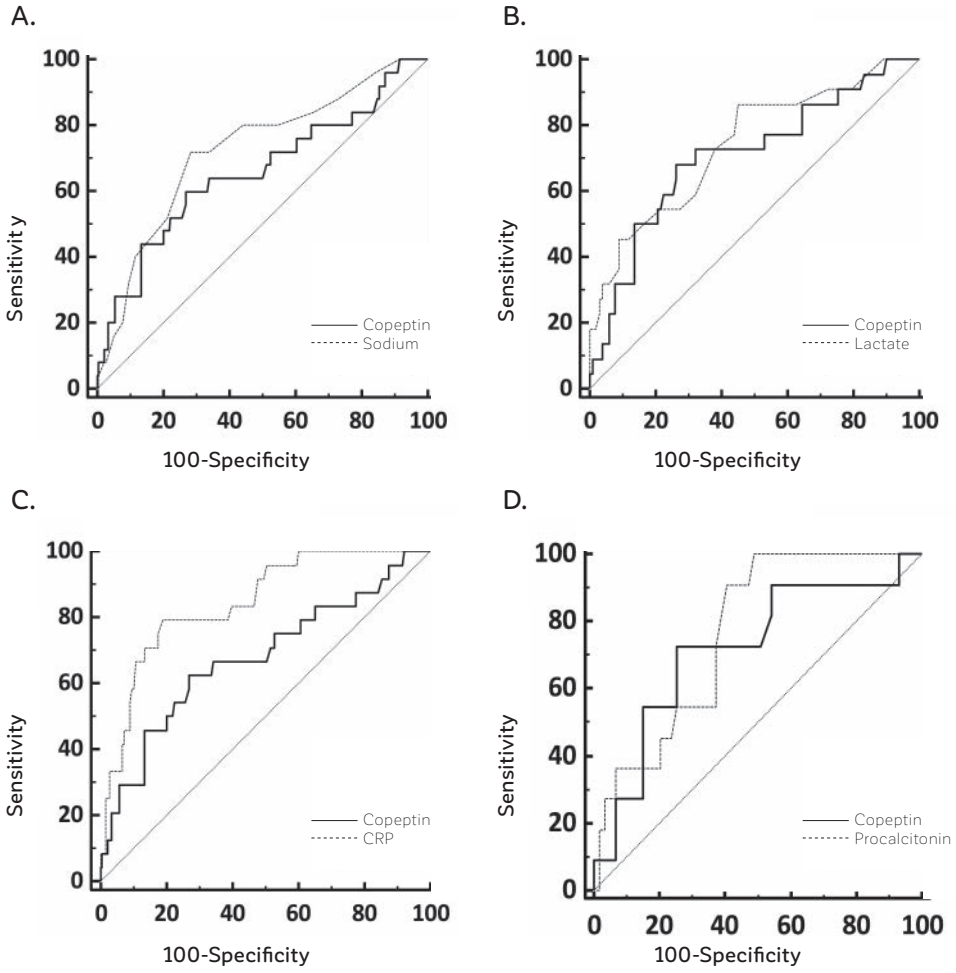
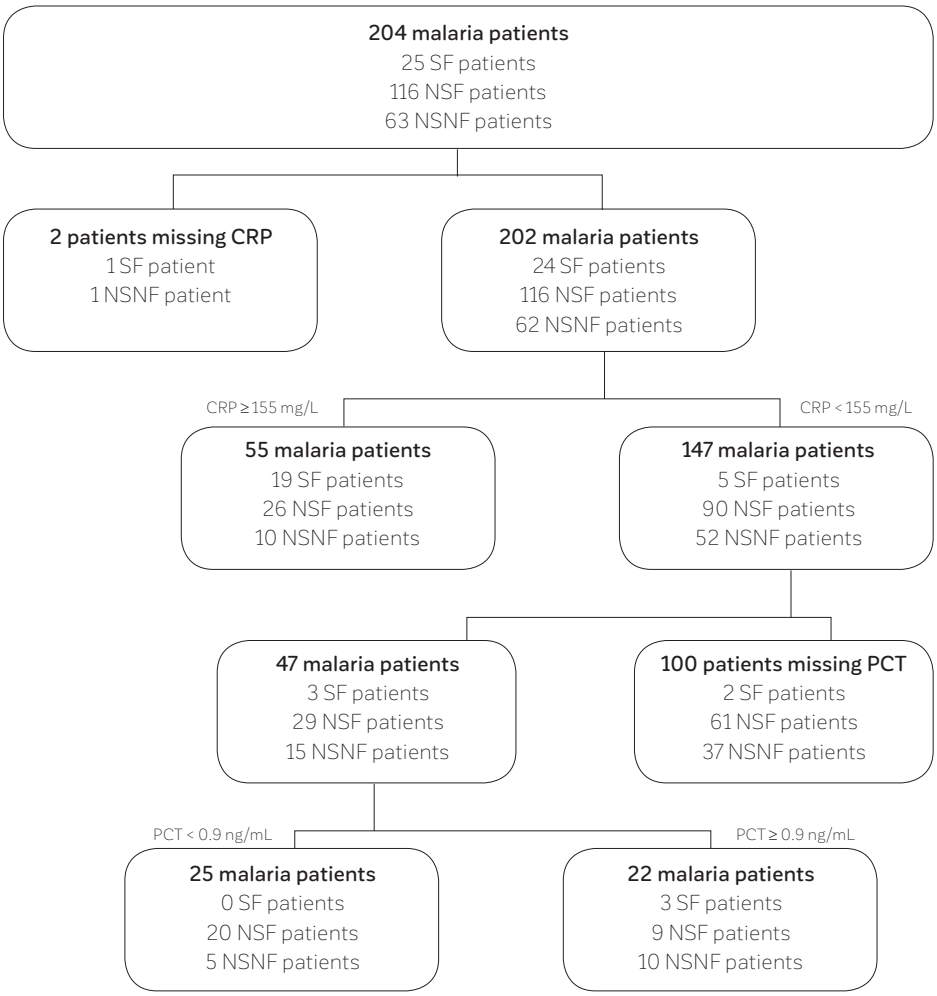


Figure 1. Receiver Operating Curves (ROC) characteristics of the diagnostic performance of copeptin for severe malaria, compared to that of sodium, lactate, CRP and procalcitonin.

The ROC curve is a graph of sensitivity (true positive fraction) plotted against 1-specificity (false positive fraction). The performance of a diagnostic variable can be quantified by calculating the area under the ROC curve (AUROC). The ideal test would have an AUROC of 1, whereas a random guess would have an AUROC of 0.5. The results of the pair-wise comparison of ROC curves are shown (with copeptin ROC curve as comparator). Figure 1A: copeptin vs sodium (n=204 pairs, P-value not significant); Figure 1B: copeptin vs lactate (n=124 pairs, P-value not significant); Figure 1C: copeptin vs CRP (n=202 pairs, p=0.02); Figure 1D: copeptin vs procalcitonin (n=70 pairs, P-value not significant). The copeptin graphs are not all identical due to missing values in pair-wise comparisons.



Supplementary Figure 1.

A two-step decision rule involving the combined use of C-reactive protein and Procalcitonin to identify all patients with severe malaria on admission.

Legend

SF *Severe P. Falciparum malaria*

NSF *Non-severe P. Falciparum malaria*

NSNF *Non-Severe, Non-Falciparum malaria*

CRP *C-Reactive Protein*

PCT *Procalcitonin*

Supplementary Table 1.
Impact of the number of severity criteria on the level of the biomarker on admission in malaria patients with severe disease

Parameter	Patients with a single criterion for severe disease (N=11)	Patients with more than one criterion for severe disease (N=14)	P-value
C-reactive protein (mg/L)	161 (111-210)	225 (177-272)	P=0.058
Sodium (mmol/L)	133 (130-135)	131 (128-133)	P=0.349
Lactate (mmol/L)	1.8 (1.0-2.7)	3.3 (2.4-4.3)	P=0.004
Copeptin (pmol/L)	25.4 (13.1-37.7)	30.5 (15.7-45.2)	P=0.687
Procalcitonin (ng/mL)	4.1 (-2.3-10.6)	17.4 (-7.1-42.0)	P=0.360

Data are given as mean (95% confidence interval)

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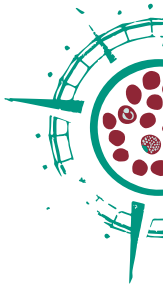
CHAPTER 4

Total and differential leukocyte count changes in a Controlled Human Malaria Infection in malaria-naïve Dutch volunteers



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ABSTRACT

Background: Both in endemic countries and in patients with imported malaria, changes in total and differential leukocyte count during *Plasmodium falciparum* infection have been described. To study the exact dynamics of the changes of the differential leukocyte counts and their ratios, they were monitored in a group of healthy non-immune volunteers who were exposed to a Controlled Human Malaria Infection (CHMI) with *P. falciparum*.

Methods: The study was performed as a substudy of two CHMI trials in which 15 and 24 healthy malaria-naive volunteers, respectively, were exposed to bites of infected mosquitos, using .the *Plasmodium falciparum* research strain NF54 and the novel clones NF135.C10 and NF166.C8. After mosquito bite exposure, all volunteers were followed up clinically and twice-daily blood draws were taken to detect the development of parasitemia in peripheral blood,and to monitor the total and differential leukocyte counts. All subjects received a course of atovaquone / proguanil when meeting the treatment criteria.

Results: A rise in total leukocyte, lymphocyte and monocyte count was observed during the liver phase of the malaria infection. Hereafter a leukocytopenia (nadir median 3.3 x 109 leukocytes/L), lymphocytopenia (nadir median 0.7 x 109 lymphocytes/L) and a borderline neutropenia (nadir median 1.5 x 109 neutrophils/L) developed in the parasitemic subjects. The neutrophil to lymphocyte count ratio (NLCR) reached a maximum of 4.0. Significant correlations were found between parasitemia and absolute lymphocyte count and between parasitemia and NLCR. All parameters normalized after parasite clearance. No changes in differential cell counts or their ratios were observed in the four non-parasitemic subjects.

Conclusion: A not previously described rise in leukocyte count, lymphocyte and monocyte count was observed during the liver phase of a CHMI. Subsequently a marked lymphocytopenia and rise in the NLCR was observed, both correlating with the parasite load.

BACKGROUND

Changes in total and differential leukocyte count during *Plasmodium falciparum* infection have been described previously, both in endemic countries and in patients with imported malaria (1-4). In patients diagnosed with malaria, the most pronounced change is the decrease of peripheral lymphocytes. Lymphocytopenia has been observed in 45% to 63% of patients with an imported *P. falciparum* infection, but was less prominent in patients with some degree of antimalarial immunity (5, 6). In a recent large cross-sectional study on leukocyte count changes in returning travellers, malaria was the second most common cause of absolute lymphocytopenia (7).

Recently, several studies have focused on the value of differential leukocyte count ratios as parameters in critical illness. The neutrophil to lymphocyte count ratio (NLCR) was introduced by Zahorec *et al.* as a parameter of systemic inflammation in critically ill surgical and medical patients (8). The NLCR outperformed traditional infection markers including C-reactive protein (CRP) in predicting bacteremia and the severity and outcome of community-acquired pneumonia (9). In patients with imported malaria, the NLCR correlated with parasitemia and normalized after parasite clearance (5, 6). One study on imported malaria showed correlations between parasitemia and both the monocyte to lymphocyte count ratio (MLCR) and neutrophil to monocyte count ratio (NMCR) (5). The exact dynamics and underlying pathophysiological mechanism of leukocyte count changes in malaria are not fully understood yet.

Controlled Human Malaria Infection (CHMI) is a well-established clinical model that was developed for the evaluation of candidate malaria vaccines (10). In this model, healthy volunteers are infected by malaria sporozoites via exposure to the bites of infected mosquitos. CMHI studies have traditionally been performed using the *P. falciparum* strain NF54, which is thought to originate from Africa (11). To extend the genetic diversity of strains available for laboratory use, the clones NF135.C10, and NF166.C8, originating from *P. falciparum* strains from Cambodia and Guinea, respectively, have been acquired in recent years. (12, 13).

In the present study the changes of differential leukocyte counts and their ratios were monitored during the liver phase and during the development of detectable blood parasitaemia in a group of healthy non-immune volunteers in the CHMI model using the NF54, NF166.C8 and NF135.C10 *Plasmodium falciparum* strains.

METHODS

Study design

The present study was performed as a substudy of the Controlled Human Malaria Infection study CHMI-b, which is one of two CHMI studies that were performed using the well-established *Plasmodium falciparum* research strain NF54 and the novel clones NF135.C10 and NF166.C8. The primary objective of CHMI-a was to compare parasite dynamics of these isolates during infection and that of CHMI-b was to determine the proportion of subjects in each group who developed patent parasitaemia in relation to the number of infected mosquito bites. Secondary objectives of both studies included clinical parameters and further measures of parasite kinetics (14).

Healthy malaria-naïve adult Dutch volunteers were recruited at the Harbour Hospital, Rotterdam, after signing informed consent. The screening procedures have been described previously (15). In CHMI-a, fifteen subjects were randomly allocated to three groups of n=5, to be infected by bites of five mosquitoes per subject carrying either the NF54 strain of *P. falciparum*, the NF135.C10 clone or the novel NF166.C8 clone. In CHMI-b, 24 subjects were randomly allocated to six groups of n=4, to bites by one, two or five mosquitoes carrying either NF135.C10 or NF166.C8. Sporozoite-carrying mosquitoes were allowed to feed for ten minutes on the forearms of the volunteers. If the predetermined number of bites was not achieved after this exposure, the subjects were exposed to additional mosquitoes. From day 5 after exposure, subjects were seen in clinic twice daily for registration of vital parameters and adverse events and for venous blood draws for thick blood smear, qPCR, and a wide range of laboratory parameters including differential leukocyte counts. *Plasmodium falciparum* parasitaemia was quantified by qPCR as described before (16). In CHMI-a, subjects were treated with atovaquone-proguanil 1000/400mg daily for three days upon their first positive thick blood smear, defined as ≥2 parasites per 225 high-powered fields (equivalent to 0.5 µl blood). In CHMI-b, subjects were treated with the same regimen as soon as 2 consecutive blood samples were positive by qPCR, defined as >500 parasites/ml. This change in treatment initiation between the two studies was implemented to minimize safety risks following a cardiac serious adverse event in a previous study (15) (17). Daily thick blood smears and qPCR were continued after treatment until complete clearance of asexual parasites. These studies were approved by the Netherlands' Central Committee on Human Research (CCMO NL41004.078.12 and NL48704.000.14, respectively) and are registered with ClinicalTrials.gov (registration numbers NCT01627951 and NCT02149550, respectively).

Data selection for the substudy

For the present substudy the data of CHMI-b were used, as this study had included a larger number of participants. The described differences in methodology regarding the initiation of treatment did not permit combination of blood phase CHMI-a and CHMI-b data. However, data from the liver phase of CHMI-a were used to confirm findings during the liver phase of CHMI-b subjects.

Statistical analysis

To determine whether the parameters of interest changed over time a Friedman test was performed. Next, a more detailed analysis of the timing of these changes was done using a linear model. Correlations were tested with the Spearman's rank-order correlation. Data were not Normally distributed and given as median (interquartile range) unless stated otherwise.

Definitions

- Leukocytopenia: Leukocyte count < 4.0 × 109/L;
- Lymphocytopenia: Lymphocyte count < 1.0 × 109/L;
- Neutropenia: Neutrophil count < 1.5 × 109/L
- Day of treatment (DT): The day the subject started a 3-day course of atovaquone / proguanil, directly upon meeting the treatment criteria. The days from two days before until three days after this day are named DT-2 to DT+3.
- Liver phase: The subjects who eventually developed parasitemia were regarded as being in the liver phase on day 5 and 6 of the study protocol, when the peripheral blood samples of all subjects were still qPCR negative
- Detectable blood parasitaemia: Detectable blood parasitaemia is defined as qPCR positive, a method with a detection limit of 100 parasites per ml.

RESULTS

Development of parasitemia and phases of the infection

Of the 24 volunteers in CHMI-b, 13 (54%) were female and the median age was 22 (range 19-34). Twenty of the twenty-four volunteers (83%) developed parasitemia. The median time between exposure to mosquitoes and the development of blood parasitemia detected by qPCR was 7 days (range 7 - 9 days) and the median time between exposure and the day the treatment criteria were met (hereafter called day of treatment [DT]) was 8 days (range 7 – 11 days). All 20 subjects who developed detectable parasitemia were still qPCR negative at day 5 and 6 of

the study protocol, and the infection was therefore regarded as being in the liver phase (18).

The data from two days before DT (DT-2) until three days after DT (DT+3) were synchronized on DT (Figure 1). On DT-2, four subjects had a positive qPCR, all with a low parasitemia (385 to 713 parasites/ml) and did not meet the treatment criteria at that moment. The number of parasitemic subjects increased to nine subjects on DT-1. On DT all subjects met the treatment criteria. The median parasite load on DT was 6,265 parasites per ml. Hereafter the parasite load declined to 1,099 on DT+1 and 100 parasites per ml on DT+2.

Changes in differential leukocyte counts and count ratios during the liver phase

During the liver phase of the infection (from baseline until day 6 after mosquito bite exposure), an increase of the lymphocyte count and the monocyte count was observed in the 20 subjects who developed parasitemia later on in CHMI-b. Changes in total leukocyte count, neutrophil count and the differential cell count ratios were not significant. No significant changes were observed in the four subjects who remained qPCR negative. As these changes during the liver phase of infected subjects were an unexpected finding, we reviewed the data of the CHMI-a study to see if this finding could be confirmed. In CHMI-a, all 15 subjects developed parasitemia, but, like in CHMI-b, all were still qPCR negative at day 5 and 6 of the study protocol, and regarded as being in the liver phase during these days. The combined data of both CHMI studies also showed a significant increase of the total leukocyte count and the differential lymphocyte and monocyte counts (Table 1).

Changes in differential leukocyte counts and count ratios after the liver phase

In the group of twenty subjects who developed parasitemia in CHMI-b, highly significant changes in the total leukocyte count and differential cell counts of neutrophils, lymphocytes and monocytes were observed in the days before DT and during treatment (Table 2 and Figure 1). These changes were not seen in non-parasitemic individuals (Supplementary Table 1 and Supplementary Figure 1). Of interest, both parasitemic and non-parasitemic individuals received antimalarial treatment, thus excluding an effect of atovaquone/proguanil treatment on the observed changes.

Linear model analysis showed that in the individuals who develop parasitemia, the leukocyte count remained stable until DT-1 and then showed a fall, which continued until it reached its nadir on DT+2, with a median of 3.3 x 105 leukocytes/L. On day DT+2, a leukocytopenia was observed in 70% (14 / 20) of the subjects. The absolute neutrophil count started to decrease on DT-1 to a median value of 1.5 x 105 neutrophils/L on DT+3. Neutropenia was found in 40% (8 / 20) of the subjects on this day. A steady fall in absolute lymphocyte count was observed from DT-2 until DT+2, when the median lymphocyte count was 0.7 x 105 lymphocytes/L. The percentage of subjects with an absolute lymphocytopenia increased from 30% (6 / 20) on DT to 70% (14 / 20) two days later. The median absolute monocyte counts remained within the normal range, but showed a mild drop on DT+2. On day 35 of the study protocol, all parameters had returned to baseline values. The evaluated parameters did not differ significantly between subjects infected with NF166.C8 and NF135.C10 (not shown).

The differential cell count ratios were also found to change significantly over time using the Friedman test (Table 1 and Figure 2). The linear model analysis showed a gradual rise of the NLCR, reaching a maximum of 4.0 on DT+1. After DT+1 a rapid decrease was observed. The MLCR showed a rise from DT-2 to DT+1 after which it decreased. The NMCR seemed to show a rise followed by a fall but these changes were not significant in the linear model analysis.

In the four subjects that remained qPCR negative throughout the study period, the differential leukocyte counts remained within normal values and no significant changes in cell counts or cell count ratios were observed. All four received a full course of atovaquone / proguanil at day 13 of the study protocol (Supplementary Table 1 and Supplementary Figure 2).

Correlations

Significant correlations were found between parasite load and absolute lymphocyte count ($p < 0.001$, correlation coefficient - 0.46) and between parasite load and NLCR ($p < 0.001$, correlation coefficient 0.50). Correlations between parasite load and total leukocyte count, MLCR and NMCR were significant, but weak ($p = 0.004$, Spearman's rho (rs) -0.23, $p < 0.001$, rs 0.36 and $p = 0.003$, rs 0.23, respectively). There was no significant correlation between parasite load and absolute neutrophil count or absolute monocyte count.

DISCUSSION

The present study shows an increase in total leukocyte, lymphocyte and monocyte count during the liver phase of infected subjects. After the liver phase, a decrease of total leukocyte , neutrophil, lymphocyte and monocyte count is observed; these changes clearly relate to the occurrence of blood parasitemia and are not caused by treatment with atovaquone / proguanil, as shown by the control group of four subjects who did not develop parasitemia.

The increase of the total leukocyte count, lymphocytes and monocytes during the liver phase has not been described in other CHMI studies, which generally focus on the blood stage of the infection. During the liver phase of malaria, only a relatively low number of hepatocytes become infected and the host immune responses to this stage of the parasite are poorly understood and the liver phase is often referred to as immunologically silent (19). The finding that the total leukocyte count, lymphocyte count and monocyte count show an increase during the liver phase is remarkable and further studies are needed to confirm this finding and to elucidate its underlying pathophysiological mechanism.

The most pronounced change is a fall in lymphocyte count that starts on DT-2 and reaches its nadir at 0.7 x 105 lymphocytes/L on DT+2, when 65% of subjects are lymphocytopenic. The neutrophil count only starts to show a significant fall after DT. These changes are reflected in the NLCR, which shows a steep rise to 4.0 on DT+2. In comparison, in a previous study on patients with imported malaria the median NLCR on admission of all patients was 3.2, and 3.5 in patients with a severe *P. falciparum* infection (6).

Lymphocytopenia has been described in several large cohorts of patients with malaria (3, 20-22) and is highly prevalent in symptomatic travelers with imported malaria (6). The correlations between parasite and lymphocyte count and between parasite load and NLCR were also demonstrated in a study by Berens-Riha et al (5), in which patients were stratified according to their immune status. Both the MLCR and the NLCR were found to be lower in semi-immune patients as compared to non-immune patients. In the present study, all subjects were non-immune. White blood cell count changes have also been described in previous CHMI studies. In a study on clinical manifestations in CHMI with *P. falciparum,* Church et al described a decrease in total white blood cell count and in neutrophil count, but not in lymphocyte count, with the nadir occurring two days after therapy initiation (23).

A decrease in lymphocyte count was observed in several studies involving sporozoite infection after mosquito bite exposure (24-27) and in a study using experimental inoculation with a low number of parasitized erythrocytes (28). These observations are in line with the findings described here. As no measurements are available between DT+3 and the end of the study protocol at day 35, we are not able to monitor when the changes observed, started to normalize after treatment. This is an important limitation of the study.

Several mechanisms have been proposed as an explanation for malaria induced lymphocytopenia. A temporary reallocation of lymphocytes has been suggested, which is supported by the observation that lymphocytes rapidly re-emerge in the peripheral circulation after treatment is initiated (29). The destination of the lymphocytes, however, is unknown. Others suggest that the soluble Fas ligand induces apoptosis of lymphocytes, which has been observed both *in vitro* and in healthy donors from endemic areas (30-34), and a previous CHMI study found an increased production of Granzyme B, which is also know to induce apoptosis, in exposed individuals, (35). Both mechanisms though might be partly responsible for the observed fall in lymphocyte cell count.

CONCLUSION

In a setting of controlled human malaria infection in 24 healthy non-immune volunteers, an initial increase of peripheral total leukocyte count and differential lymphocytes and monocytes was observed during the liver phase of the infection which has never has been described before. This rise seems to indicate the presence of a malaria infection and is followed 2-3 days later by the appearance of parasites in the peripheral blood, which is accompanied by a marked decrease in total leukocyte count, lymphocyte count and the neutrophil count and a rise of the NLCR. Both the lymphocyte count and the NLCR correlated with blood parasitemia, and all parameters had normalized 3 to 4 weeks after parasite clearance. The non-parasitemic control group in this study excludes a treatment-related effect.

Table 1. The course of differential leukocyte counts and their ratios during the liver phase in the subjects who developed malaria.

	Leuko-cytes $X10^9/L$	Neutro-phils $X10^9/L$	Lympho-cytes $X10^9/L$	Mono-cytes $X10^9/L$	NLCR	MLCR	NMCR
CHMI-b (n=20)							
Baseline	**5.1 (4.9-6.1)**	**2.8 (2.5-3.1)**	**1.6 (1.5-2.0)**	**0.48 (0.35-0.60)**	**1.8 (1.1-2.0)**	**0.3 (0.2-0.3)**	**5.8 (4.7-8.3)**
Day 5	5.7 (5.4-7.2)	3.1 (2.2-3.6)	2.0 (1.7-2.5)	0.49 (0.43-0.60)	1.3 (1.0-2.1)	0.2 (0.2-0.3)	5.9 (4.8-7.2)
Day 6	6.0 (5.3-6.9)	3.0 (2.4-3.8)	2.2 (1.9-2.4)	0.53 (0.46-0.65)	1.4 (1.1-1.8)	0.2 (0.2-0.3)	5.3 (4.6-7.1)
P-value	0.051	0.195	0.004	0.012	0.165	0.196	0.848
CHMI-a (n=15)							
Baseline	**5.8 (5.3-6.5)**	**3.1 (2.9-3.3)**	**1.9 (1.9-2.3)**	**0.51 (0.48-0.58)**	**1.6 (1.4-1.7)**	**0.3 (0.2-0.3)**	**6.2 (5.0-7.9)**
Day 5	6.8 (6.1-7.3)	3.6 (2.9-4.0)	2.3 (2.0-2.5)	0.55 (0.46-0.63)	1.6 (1.4-1.8)	0.2 (0.2-0.3)	6.9 (4.9-8.6)
Day 6	6.9 (5.8-7.6)	3.2 (2.7-4.1)	2.4 (1.9-2.7)	0.55 (0.49-0.58)	1.3 (1.1-1.9)	0.2 (0.2-0.3)	6.0 (5.5-7.2)
P-value	0.105	0.721	0.140	0.208	0.623	0.983	0.633
Combined (n=35)							
Baseline	**5.5 (5.0-6.2)**	**2.9 (2.6-3.2)**	**1.9 (1.6-2.2)**	**0.50 (0.38-0.60)**	**1.7 (1.3-2.0)**	**0.3 (0.2-0.3)**	**6.1 (4.8-8.4)**
Day 5	6.1 (5.5-7.2)	3.3 (2.5-3.7)	2.2 (1.8-2.5)	0.50 (0.43-0.54)	1.5 (1.1-1.9)	0.2 (0.2-0.3)	6.1 (4.9-8.1)
Day 6	6.1 (5.5-7.0)	3.0 (2.6-3.9)	2.2 (1.9-2.6)	0.54 (0.47-0.62)	1.4 (1.1-1.8)	0.2 (0.2-0.3)	5.7 (4.8-7.2)
P-value	0.005	0.413	0.001	0.038	0.121	0.368	0.971

Data in this table are not Normally distributed and shown as median (interquartile range). P-values in this table were derived from Friedman tests

Table 2. The course of differential leukocyte counts and their ratios in the subjects who developed malaria in CHMI-b (n=20)

	Leuko-cytes $X10^9/L$	Neutro-phils $X10^9/L$	Lympho-cytes $X10^9/L$	Mono-cytes $X10^9/L$	NLCR	MLCR	NMCR	Parasite load Parasites/ml
Baseline								
	5.1 (4.9-6.1)	**2.8 (2.5-3.1)**	**1.6 (1.5-2.0)**	**0.48 (0.35-0.60)**	**1.8 (1.1-2.0)**	**0.3 (0.2-0.3)**	**5.8 (4.7-8.3)**	**N/A**
DT-2	5.8 (5.4-6.8)	3.0 (2.3-3.7)	2.1 (1.9-2.4)	0.49 (0.43-0.61)	1.4 (1.0-1.7)	0.2 (0.2-0.3)	5.6 (4.3-6.8)	0 (0-0)
DT-1	5.8 (5.3-6.6)	2.9 (2.6-3.5)	2.0 (1.5-2.3)	0.55 (0.46-0.65)	1.4 (1.1-2.1)	0.3 (0.2-0.3)	5.2 (4.5-6.7)	0 (0-374)
DT	5.6 (4.8-6.1)	3.1 (2.8-3.6)	1.6 (0.9-2.0)	0.53 (0.40-0.58)	2.2 (1.4-3.8)	0.4 (0.4-0.5)	6.4 (5.0-8.0)	6,265 (1,306-12,152)
DT+1	4.4 (3.9-4.7)	3.0 (2.5-3.2)	0.8 (0.6-1.1)	0.44 (0.30-0.60)	4.0 (2.2-5.3)	0.5 (0.5-0.6)	6.9 (5.4-9.5)	1,099 (159-19,494)
DT+2	3.3 (2.9-4.0)	1.9 (1.7-2.5)	0.7 (0.6-1.2)	0.32 (0.24-0.49)	2.6 (1.7-3.7)	0.5 (0.3-0.6)	6.2 (4.1-8.0)	100 (1-20,337)
DT+3	3.4 (2.7-3.7)	1.5 (1.3-1.9)	1.0 (0.8-1.3)	0.36 (0.28-0.60)	1.6 (1.1-2.2)	0.5 (0.3-0.6)	3.6 (2.8-5.4)	1 (0-305)
Day 35	6.2 (5.2-7.2)	2.8 (2.3-3.3)	2.2 (1.9-3.0)	0.53 (0.44-0.71)	1.2 (0.9-1.3)	0.2 (0.2-0.2)	5.6 (4.1-6.4)	0 (0-0)
P-value	<0.0001	<0.0001	<0.0001	0.002	<0.0001	<0.0001	<0.0001	<0.0001

Data in this table are not Normally distributed and shown as median (interquartile range). P-values in this table were derived from Friedman tests

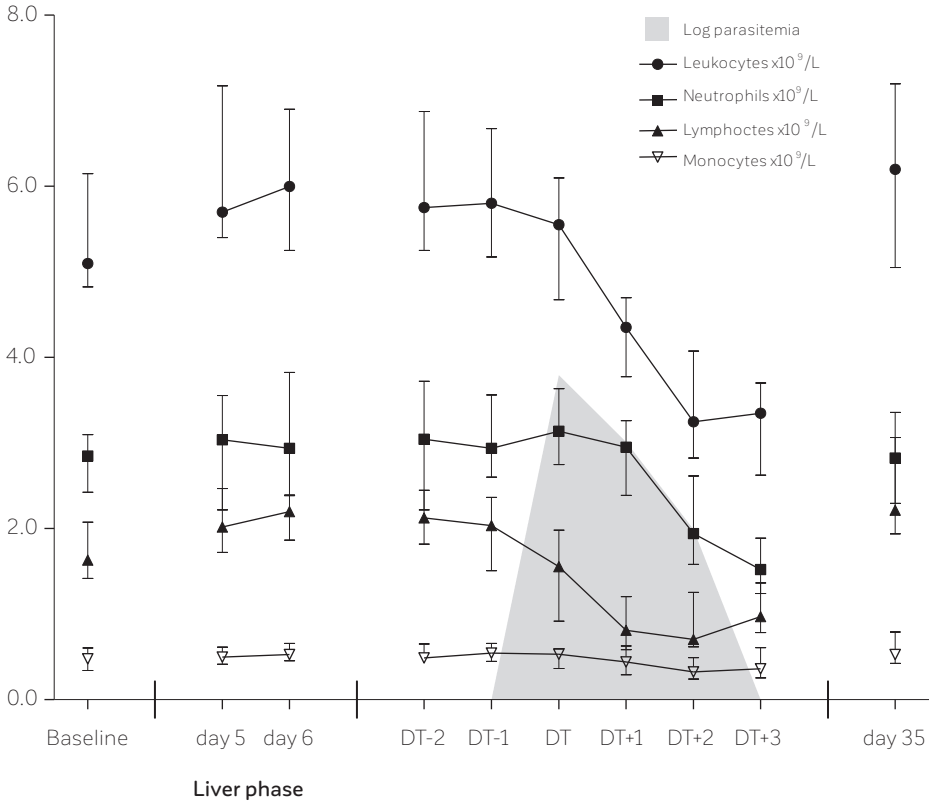


Figure 1. Changes in total and differential leukocyte counts in the volunteers who developed malaria in CHMI-b (n=20).

The data are show as medians (dots) and interquartile ranges (whiskers). The data from DT-2 until DT+3 were synchronized on DT.

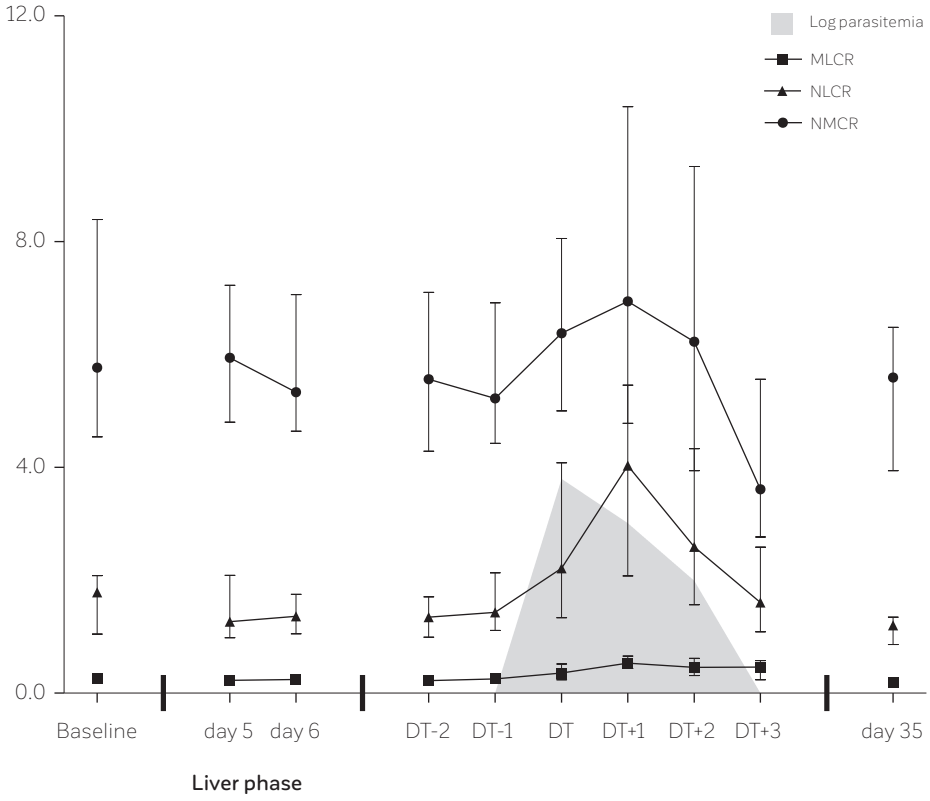
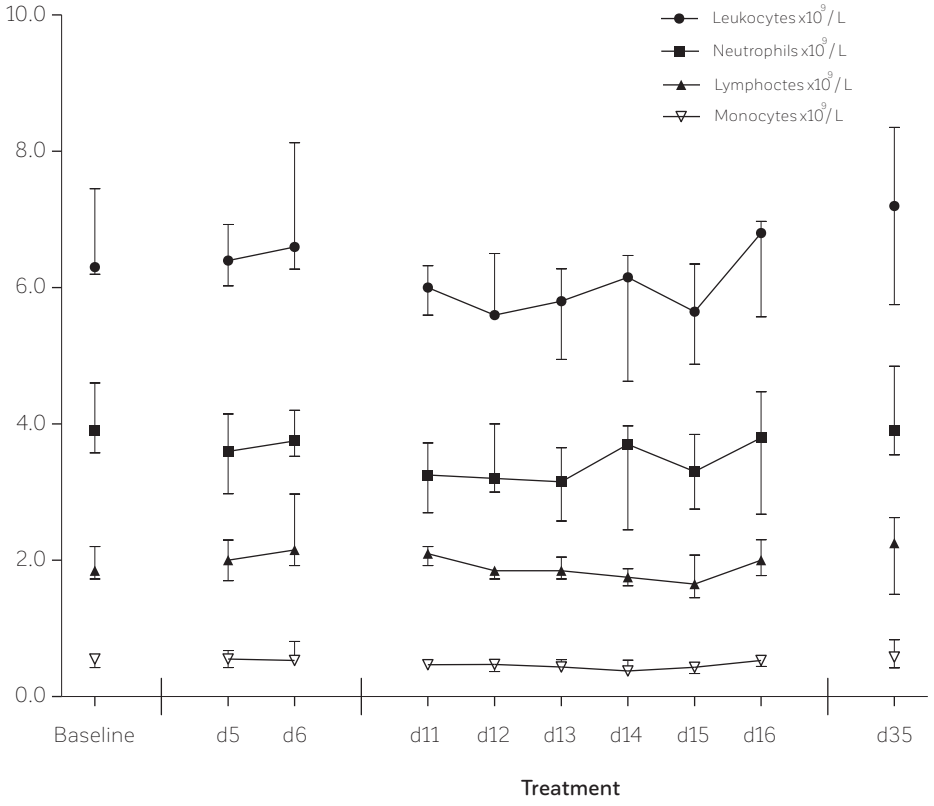


Figure 2. Changes in differential cell count ratios in the volunteers who developed malaria in CHMI-b (n=20).

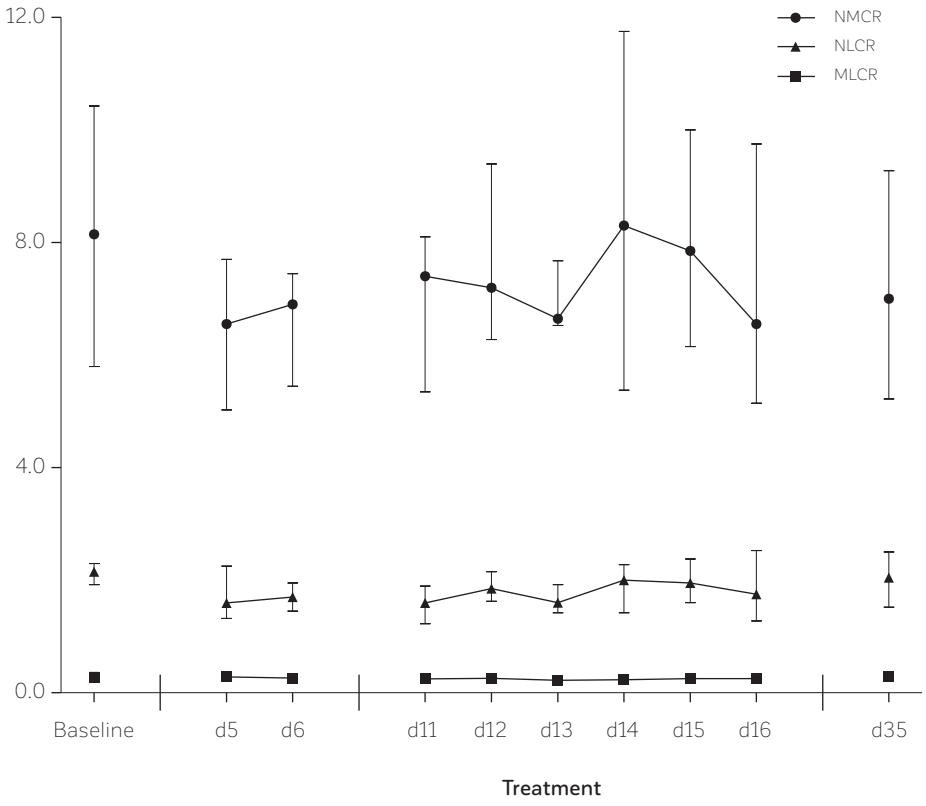
The data are show as medians (dots) and interquartile ranges (whiskers). The data from DT-2 until DT+3 were synchronized on DT.



Supplementary Figure 1.

Changes in total and differential leukocyte counts in the volunteers that did not develop malaria.

The data are show as medians (dots) and interquartile ranges (whiskers). None of the changes in this group were statistically significant.



Supplementary Figure 2.

Changes in differential cell count ratios in the volunteers that did not develop malaria.

The data are show as medians (dots) and interquartile ranges (whiskers). None of the changes in this group were statistically significant.

Supplementary Table 1.

Differential leukocyte counts and their ratios in the non-parasitemic subjects.

	Leuko-cytes $X10^9/L$	Neutro-phils $X10^9/L$	Lympho-cytes $X10^9/L$	Mono-cytes $X10^9/L$	NLCR	MLCR	NMCR
Baseline	**6.3 (6.2-6.8)**	**3.9 (3.7-4.2)**	**1.8 (1.8-2.0)**	**0.54 (0.45-0.62)**	**2.1 (2.0-2.3)**	**0.3 (0.2-0.3)**	**8.1 (6.0-10.3)**
Day 5	6.4 (6.1-6.8)	3.6 (3.1-4.0)	2.2 (2.1-2.3)	0.57 (0.51-0.63)	1.6 (1.4-2.0)	0.3 (0.2-0.3)	6.6 (5.5-7.5)
Day 6	6.6 (6.4-7.2)	3.8 (3.6-4.0)	2.1 (1.9-2.5)	0.53 (0.51-0.62)	1.7 (1.5-1.9)	0.3 (0.3-0.3)	6.9 (6.3-7.1)
Day 7	6.1 (5.9-7.1)	3.5 (3.2-4.5)	1.9 (1.8-2.1)	0.57 (0.52-0.59)	2.0 (1.7-2.4)	0.3 (0.2-0.3)	7.4 (6.4-8.9)
Day 8	6.6 (6.4-6.8)	3.5 (3.4-3.8)	2.1 (2.0-2.2)	0.56 (0.53-0.60)	1.6 (1.4-1.8)	0.3 (0.2-0.3)	6.6 (6.0-7.0)
Day 9	6.0 (5.1-7.2)	3.5 (2.9-4.4)	1.9 (1.7-2.1)	0.50 (0.46-0.51)	2.0 (1.8-2.2)	0.2 (0.2-0.3)	8.0 (6.8-9.1)
Day 10	6.4 (5.8-7.3)	3.7 (3.3-4.2)	2.2 (1.9-2.4)	0.57 (0.49-0.66)	1.7 (1.6-1.9)	0.3 (0.3-0.3)	6.5 (6.4-6.7)
Day 11	6.0 (5.8-6.2)	3.3 (2.9-3.6)	2.1 (2.0-2.2)	0.47 (0.42-0.52)	1.6 (1.3-1.9)	0.2 (0.2-0.3)	7.4 (6.6-7.7)
Day 12	5.6 (5.6-5.9)	3.2 (3.0-3.6)	1.8 (1.7-1.9)	0.47 (0.42-0.51)	1.9 (1.7-2.0)	0.3 (0.2-0.3)	7.2 (6.6-8.2)
Day 13^	5.8 (5.5-6.0)	3.2 (3.0-3.3)	1.9 (1.8-1.9)	0.44 (0.39-0.50)	1.6 (1.5-1.8)	0.2 (0.2-0.2)	6.7 (6.6-7.0)
Day 14	6.2 (5.5-6.4)	3.7 (3.1-3.9)	1.8 (1.7-1.8)	0.39 (0.35-0.46)	2.0 (1.7-2.2)	0.2 (0.2-0.3)	8.3 (6.3-10.5)
Day 15	5.7 (5.0-6.3)	3.3 (3.0-3.5)	1.6 (1.5-1.8)	0.43 (0.40-0.45)	2.0 (1.6-2.3)	0.2 (0.2-0.3)	7.9 (6.5-9.4)
Day 16	6.8 (6.3-6.9)	3.8 (3.2-4.2)	2.0 (1.9-2.1)	0.53 (0.50-0.56)	1.8 (1.4-2.2)	0.3 (0.2-0.3)	6.5 (6.0-7.6)
End	7.2 (6.7-7.7)	3.9 (3.6-4.4)	2.2 (1.9-2.4)	0.58 (0.43-0.77)	2.1 (1.8-2.3)	0.3 (0.3-0.3)	7.0 (5.6-8.5)
P-value	0.21	0.66	0.10	0.08	0.28	0.74	0.65

Data are shown as median (interquartile range). P-values in this table were derived from Friedman tests.

^Non-parasitemic subjects received a full 3-day course of atovaquone / proguanil, starting on day 13.

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CHAPTER 5

Predictive value of lymphocytopenia and the neutrophil-lymphocyte count ratio for severe imported malaria

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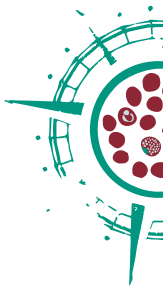
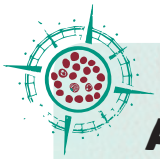
Malar J. 2013;12:101





ABSTRACT

Background: Lymphocytopenia has frequently been described in patients with malaria, but studies on its association with disease severity have yielded conflicting results. The neutrophil/lymphocyte count ratio (NLCR) has been introduced as a parameter for systemic inflammation in critically ill patients and was found, together with lymphocytopenia, to be a better predictor of bacteraemia than routine parameters like C-reactive protein and total leukocyte count. In the present study, the predictive value of the NLCR and lymphocytopenia for severe disease was evaluated in patients with imported malaria.

Methods: All patients diagnosed with malaria at the Harbour Hospital between January 1st 1999 and January 1st 2012 with differential white cell counts determined within the first 24 hours after admission were included in this retrospective study. Severe malaria was defined according to the WHO criteria. The performance of the NLCR and lymphocytopenia as a marker of severe malarial disease was compared back-to-back with that of C-reactive protein as a reference biomarker.

Results: A total of 440 patients (severe *falciparum* malaria n = 61, non-severe *falciparum* malaria n = 259, non-*falciparum* malaria n=120) were included in the study. Lymphocytopenia was present in 52% of all patients and the median NLCR of all patients was 3.2. Total lymphocyte counts and NLCR did not differ significantly between groups. A significant correlation of total leukocyte count and NLCR, but not lymphocyte count, with parasitaemia was found. ROC analysis revealed a good negative predictive value but a poor positive predictive value of both lymphocytopenia and NLCR and performance was inferior to that of C-reactive protein. After complete parasite clearance a significant rise in total leukocyte count and lymphocyte count and a significant decrease in NLCR was observed.

Conclusion: The NLCR was found to correlate with parasitaemia, but both lymphocytopenia and the NLCR were inferior to C-reactive protein as markers for severe disease in patients with imported malaria.

BACKGROUND

Changes in blood cell counts are a well-known feature of malaria. Alterations in leukocyte counts are often less pronounced than in the other blood cell lineages, but in general total leukocyte counts have been found to be low to normal in malaria (1, 2). Lymphocytopenia has frequently been described in malaria patients in endemic areas (3-6), and was found to be present in 63% of patients with imported *Plasmodium falciparum* infection (7). Studies on the correlation between lymphocyte count and malaria severity yielded conflicting results, as both lymphocytopenia (1, 3, 4) and lymphocytosis (8) have been reported to be associated with adverse outcome.

Lymphocytopenia accompanied by a rise in neutrophil count is commonly seen in various infectious and non-infectious causes of systemic inflammation and stress (9-13). Zahorec *et al* introduced the ratio of neutrophil to lymphocyte count as a parameter of systemic inflammation and stress in critically ill surgical and medical patients (14). The predictive value of both lymphocytopenia and the neutrophil-lymphocyte count ratio (NLCR) for bacteraemia was confirmed in a study in an emergency care setting, in which these parameters were found to be better predictors of bacteraemia than routine parameters like C-reactive protein (CRP) level, total leukocyte count or neutrophil count. Recently, another study evaluated this parameter in patients with a community-acquired pneumonia (CAP) (15) and it was found to predict severity and outcome of CAP with a higher prognostic accuracy as compared with traditional infection markers.
Although the NLCR is currently not routinely used as a clinical parameter, the above mentioned studies have demonstrated its value as an infection marker in critically ill patients. Unequivocal data concerning the predictive value of lymphocytopenia and the NLCR in malaria are not yet available.

In non-endemic countries, where malaria is only seen as an imported disease, non-specialized hospitals often rely on rapid diagnostic tests for the diagnosis of malaria and generally lack experience in the examination of thick and thin blood smears to asses the parasite load. There is, therefore, still a need for simple and readily available parameters for the early identification of patients at risk of severe or complicated disease. The present study evaluated lymphocytopenia and the NLCR as predictive markers of severe disease in a large cohort of patients with imported malaria.

METHODS

Patients

The Harbour Hospital is a 161-bed general hospital located in Rotterdam, The Netherlands. It also comprises the Institute of Tropical Diseases, which serves as a national referral centre. All patients diagnosed with malaria at our centre are included in the Rotterdam Malaria Cohort study. Demographic, clinical and laboratory data of all these patients are collected using a standardized form and stored in an electronic database. For the present observational cohort study, patients diagnosed with malaria between January 1st 1999 and January 1st 2012 and with differential white cell counts determined within the first 24 hours after admission were included.

Laboratory investigations

Total and differential leukocyte counts were measured using an automatic cell counters. During the study period three distinct cell counters were subsequently used after careful calibration (Sysmex NE 8000 [in the period January 1st 1999 -July 31st 2002], Beckman Coulter HMX [July 31st 2002- July 31st 2010] and Sysmex XE 2100 [July 31st 2010 - January 1st 2012], respectively). Absolute numbers of lymphocytes and neutrophil subsets were obtained by multiplication of the absolute leukocyte counts with their respective differential leukocyte counts. Manual confirmation of automatic cell count results was performed when immature or aberrant leukocytes, erythrocytes or platelet clumps were detected and when cell count results differed substantially from normal values. Other available laboratory examinations included red blood cell counts, haematocrit, platelet counts, C-reactive protein levels, serum electrolytes, total bilirubin, serum creatinine and urea, sodium, potassium, liver enzymes, blood glucose and venous plasma lactate.

Detection of *Plasmodium* parasites

The standard procedure to diagnose malaria comprised a Quantitative Buffy Coat (QBC) analysis, a rapid diagnostic test (RDT) for malaria antigens (Binax NOW® Malaria Test Binax, Inc. Maine, USA), and thick and thin blood smears using freshly collected blood specimens from finger pricks. The RDT and the QBC analysis were performed according to the manufacturer's instructions. QBC capillaries were examined independently by two technicians by microscopic analysis of two complete rows of the region between the bottom of the capillary and the polynuclear leukocyte layer using an Olympus BX-60 fluorescence microscope equipped with UV-filter, 50x objective and 12.5x oculars (total magnification 625x). These

two lanes represent about 100 microscopic fields (at 625 x magnification) and take an average examination time of 5 min. Subsequently, the polynuclear and mononuclear cell layer was screened for schizonts, gametocytes, malaria pigment and elderly trophozoites of *Plasmodium vivax* and *Plasmodium ovale*.

Thick blood smears were stained with Field's stain (Brunschwig Chemie, Amsterdam, the Netherlands) and thin smears with Diff Quick stain (Medion Diagnostics, Düdingen, Switzerland). Both staining procedures have been optimized for optimal staining of *Plasmodium* parasites as well as Schüffner's dots and Maurer's clefts in infected erythrocytes. Thick and thin smears were examined with regular light microscopes at a total magnification of 1250x.

The same *Plasmodium* detection methods were used throughout the whole study period.

Definitions

Severe malaria

Patients were classified as having severe malaria if they met one or more of the WHO criteria for severe malaria, as published (16), either on presentation or later during hospital admission.

Leukocyte counts and NLCR

Leukocytopenia was defined as a leukocyte count of less than 4.0×10^9/L and leukocytosis was defined as a leukocyte count exceeding 10.0×10^9/L. Lymphocytopenia was defined as a lymphocyte count of less than 1.0×10^9/L and lymphocytosis as a lymphocyte count more than 4.0×10^9/L. Neutropenia was defined as a neutrophil count of less than 1.5×10^9/L and neutrophilia as a neutrophil count of more than 7.0×10^9/L. The neutrophil-lymphocyte count ratio was defined as the ratio of the absolute neutrophil count to the absolute lymphocyte count.

Estimation of immunity to *P. falciparum*

The degree of immunity to malaria was estimated as previously described (17). Adult immigrants from a malaria-endemic country living in the Netherlands were considered partially immune, because they had likely been exposed to *P. falciparum* during childhood. Patients who had been living in a malaria-endemic area for at least 2 years at the time of diagnosis were presumed semi-immune. Tourists from non-endemic regions who travelled to endemic areas were considered non-immune.

Statistical analysis

Data were not normally distributed (Kolmogorov-Smirnov test) and are therefore presented as medians and range. Univariate comparisons were performed using the Chi-Square test and the Kruskall-Wallis test with Dunn's post-hoc tests (three groups), or the Mann-Whitney test and Fisher's Exact test (two groups). Correlations with parasitaemia were calculated using Spearman's rank correlation. For the comparisons of differential leukocyte counts before and after treatment, Wilcoxon's matched pairs test was used.

For clinical reference, the diagnostic performance of NLCR and lymphocytopenia for severe disease was compared to that of the classic biomarker serum CRP using receiver operating characteristic (ROC) analysis. The optimal cut-off point was identified using Youden's index. The areas under the ROC curve (AUROCs) were compared to that of CRP in a pair-wise comparison by the method of Hanley and McNeil (18).

RESULTS

Patient characteristics

Between January 1st 1999 and January 1st 2012 a total of 562 cases of imported malaria were seen. Differential leukocyte counts were available for 440 (78%) of the patients. Of these cases, 120 cases were caused by non-*falciparum Plasmodium* species: 88 by *P. vivax*, 27 by *P. ovale*, four by *Plasmodium malariae* and one by *Plasmodium knowlesi*. The majority of infections (320 or 72%) was caused by *P. falciparum*, including three patients who had a mixed infection; two with *P. falciparum* and *P. ovale*, one with *P. falciparum* and *P. vivax*. Sixty-one patients were classified as having severe malaria. All these patients met the severity criteria upon admission to the hospital, and no patients were re-classified as having severe malaria because of progression of disease during admission. Concomitant infection was present in 29 (7%) of patients and was more common in patients with severe malaria. None of the patients had positive blood cultures. The general characteristics of these patients are shown in Table 1.

Leukocyte counts and NLCR

Leukocytopenia was present in 23% (100/440) of all patients but in only 11% (7/61) of patients with severe malaria. Leukocytosis was seen in 4% (19/440) of all patients and was more common in patients with severe disease (13% or 8/61) compared to those with non-severe *falciparum* malaria (2% or 6/259) and

non-*falciparum* malaria (4% or 5/120). Likewise, leukocyte counts were higher in patients with severe malaria compared to those with non-severe *P. falciparum* malaria and non-*falciparum* malaria (Figure 1 and Table 1).

Lymphocytopenia was present in 52% (227/440) of all patients and was more frequently seen in patients with non-severe *falciparum* malaria (143/259 or 55%) than in patients with severe malaria (28/61 or 46%), and non-*falciparum* malaria (54/120 or 45%). Absolute lymphocyte counts did not differ significantly between the three groups.

Neutropenia was found in 8% (36/440) of all patients and neutrophilia in 4% (17/440), but was more common in patients with severe malaria (7% or 4/61) compared to those with non-severe *falciparum* malaria and non-*falciparum* malaria (3% or 7/259 and 5% or 6/120, respectively). Neutrophil counts were higher in patients with severe malaria compared to those with non-severe *P. falciparum* malaria and non-*falciparum* malaria (Figure 1 and Table 1).

The median NLCR of all patients was 3.2. Although there was a trend towards higher values in patients with severe malaria (3.5) compared to those with non-severe *P. falciparum* (3.3) and non-*falciparum* (2.8) malaria, these differences were not significant.

Correlation with parasitaemia

Significant correlations were found between parasitaemia and total leukocyte count (r_s 0.304, p<0.0001)and parasitaemia and NLCR (r_s 0.165, p=0.03).
Correlations between parasitaemia and absolute lymphocyte or neutrophil counts were not significant.

Predictive value and ROC analysis

Analysis of the diagnostic performance of lymphocytopenia and the NLCR for the detection of severe malaria revealed good negative predictive value (0.87 and 0.92 respectively) but poor positive predictive value (Table 2). The AUROCs of total leukocyte count, neutrophil count, lymphocyte count and NLCR were all significantly inferior to that of CRP (Table 2).

Leukocyte count changes during malaria treatment

Follow-up differential leukocyte counts were available for 40 (66%) patients with severe malaria and 114 patients with non-severe *P. falciparum* infection. By

the time of complete parasite clearance (confirmed by a negative QBC and thick blood smear) a significant rise in total leukocyte count and lymphocyte count and a significant decrease in NLCR was observed. A significant decrease in neutrophil counts after treatment was only seen in non-severe malaria patients, but not in patients with severe malaria (Table 3).

DISCUSSION

In contrast to studies in bacterial sepsis, where lymphocytopenia and NLCR were found to outperform CRP in predicting the presence of bacteraemia, lymphocyte counts and NLCR did not allow for an accurate discrimination between malaria patients with severe disease and those without. Even though lymphocytopenia and NLCR had good negative predictive values, CRP was found to be a superior marker in back-to-back analyses.

This lack of diagnostic power might partly be due to the fact that neutrophilia, while often marked in patients with bacterial sepsis, is not commonly seen in malaria. Some studies even report neutropenia (1). In the present study, neutrophil counts were in the lower range of normal. This is reflected in NLCR values that are much lower than those found in patients with bacteraemia (19) and might be an explanation for the dissimilar performance of the NLCR in bacterial sepsis and malaria. Bacterial co-infection was present in 8% of patients with severe malaria, which is consistent with the findings of a large cohort study on patients with severe malaria admitted to the ICU (20). However, none of the patients in the present study had a positive blood culture. A major confounding effect of concomitant bacterial infections is, therefore, unlikely. Moreover, regarding the design of the study on NLCR in bacterial sepsis by de Jager *et al* (19), an important difference with the present study has to be taken into account. In the former study, lymphocytopenia and the NLCR were evaluated as predictors of the presence of bacteraemia in patients with suspected community-acquired bacteraemia, while in the present study these biomarkers were evaluated in a patient group with confirmed malaria. Considering this, the correlation of NLCR with peripheral parasite count and the fact that lymphocyte count increases and NLCR decreases after complete parasite clearance are interesting findings; although NLCR lacks diagnostic power to accurately identify patients with severe malaria, a high parasite load does seem to result in relative lymphocytopenia.
Surprisingly for such a common phenomenon (3-7, 21) the mechanism behind

malaria-associated lymphopaenia has still not been satisfactorily elucidated and remains the subject of debate (22). The rapid re-emergence of lymphocytes in the peripheral circulation following initiation of treatment has led some authors to suggest transient sequestration during malaria to be responsible (4, 5). The relatively large drop in peripheral lymphocyte numbers would suggest this to be a non-specific effect, *e.g.* pooling in the enlarged spleens of patients (5) rather than a response by malaria-specific lymphocytes only. Others have pointed to the increased propensity of lymphocytes from malaria patients to undergo spontaneous apoptosis *in vitro* (23, 24), possibly induced by soluble Fas ligand (sFasL)-Fas interaction (25). Interestingly, increased apoptosis is also seen in healthy donors from endemic areas, be it to a lesser extent (23, 24, 26), suggesting chronic stimulation of lymphocytes by environmental micro-organisms may be contributing through activation-induced cell death. Presumably both mechanisms are at work in tandem, with activated lymphocytes sequestering during malaria and rates of apoptosis, whether spontaneous or activation-induced, rising due to infection and peaking following treatment in order to restore homeostasis (27).

CONCLUSION

The NLCR was found to correlate with parasitaemia, but both lymphocytopenia and the NLCR were inferior to CRP as markers of severe disease in patients with imported malaria in direct back-to-back comparisons. Although these parameters may have proven their usefulness in predicting bacteriaemia, they are apparently not useful as predictive markers of severe disease in imported malaria in the acute care setting.

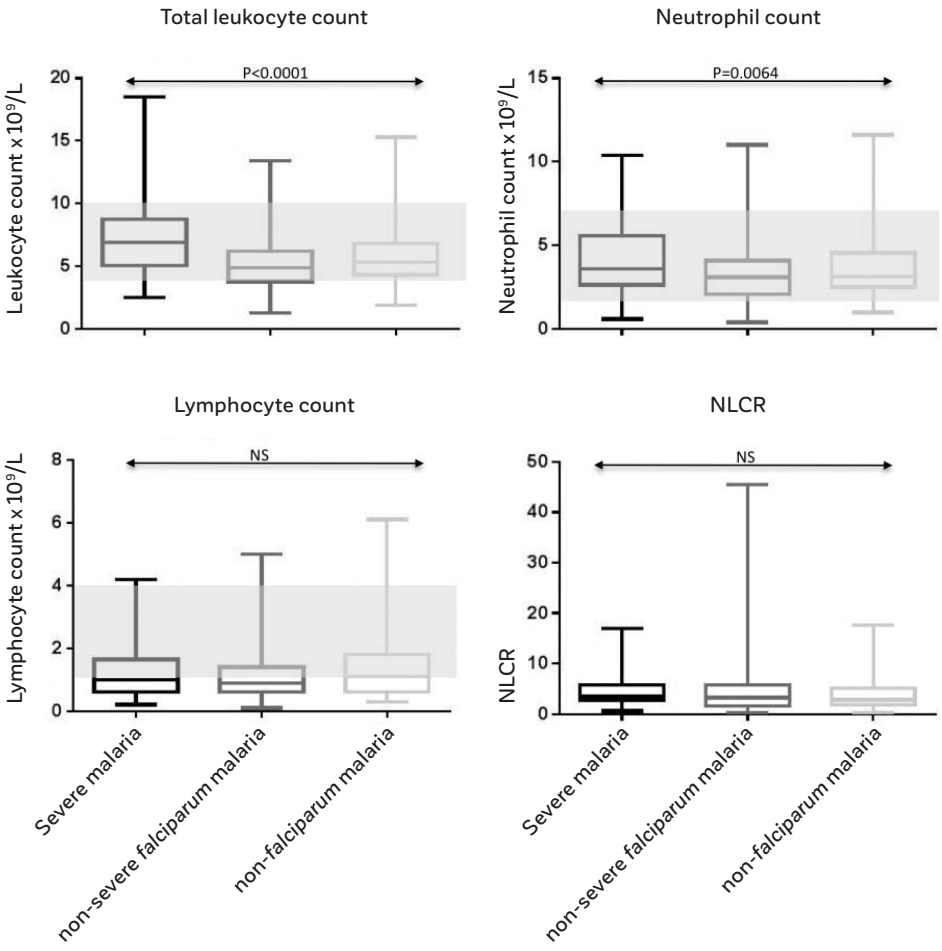


Figure 1. Distribution of leukocyte counts and the NLCR in patients with malaria grouped by severity and causative *Plasmodium* species

The gray areas depict the normal ranges

Table 1. General characteristics

	Severe *P. falciparum* n=61	Non-severe *P. falciparum* n=259	Non-*falciparum* n=120	P-value
Demographics				
Age, years	44 (4-70)	39 (11-78)	36 (15-77)	0.0005*
Male / female, n (%)	39 (64%) / 22 (36%)	191 (74%) / 68 (26%)	83 (69%)/37 (31%)	NS#
Immunity to *P. falciparum*				**0.0029#**
Non-immune	42 (69%)	123 (47%)	N/A	
Partially immune	19 (31%)	118 (46%)	N/A	
Semi-immune	0 (0%)	3 (1%)	N/A	
Unknown	0 (0%)	16 (6%)		
Continent of acquisition				**NS#**
Africa, n (%)	56 (92%)	240 (93%)	42 (35%)	
Asia, n (%)	3 (5%)	11 (4%)	50 (42%)	
South & Central America, n (%)	1 (2%)	5 (2%)	26 (22%)	
Unknown, n (%)	1 (2%)	3 (1%)	2 (2%)	
Duration of signs/symptoms				**0.007#**
<8 days, n (%)	38 (62%)	177 (68%)	58 (48%)	
8-14 days, n (%)	17 (28%)	45 (17%)	24 (20%)	

Table 1. Continued

	Severe *P. falciparum* n=61	Non-severe *P. falciparum* n=259	Non-*falciparum* n=120	P-value
15-28 days, n (%)	2 (3%)	20 (8%)	11 (9%)	
>28 days, n (%)	0 (0%)	5 (2%)	8 (7%)	
Unknown, n (%)	4 (7%)	12 (5%)	19 (16%)	
Use of malaria chemoprophylaxis				**<0.0001#**
No chemoprophylaxis, n (%)	49 (80%)	182 (70%)	51 (43%)	
Inadequate use, n (%)	8 (13%)	46 (18%)	15 (13%)	
Adequate use, n (%)	2 (3%)	20 (8%)	42 (35%)	
Unknown, n (%)	2 (3%)	11 (4%)	12 (10%)	
Vital signs on admission				
Body temperature, °C	38.4 (35.7-41.2)	38.5 (35.5-41.0)	38.9 (36.0-41.2)	NS*
Pulse rate, beats per minute	103 (50-150)	90 (45-140)	90 (58-138)	0.0005*
Systolic blood pressure, mm Hg	117 (80-160)	120 (73-185)	120 (90-196)	N.T.
Impaired consciousness (GCS<15), n (%)	8 (13%)	1 (0%)	0 (0%)	N.T.
Coma (GCS≤11), n (%)	3 (5%)	0 (0%)	0 (0%)	N.T.

Table 1. Continued

	Severe *P. falciparum* n=61	Non-severe *P. falciparum* n=259	Non-*falciparum* n=120	P-value
Laboratory data on admission				
Haemoglobin, mmol/L	7.8 (2.5-10.9)	8.4 (4.0-11.1)	8.2 (4.6-11.2)	N.T.
Thrombocytes, x 10^9/L	39 (3-188)	101 (18-293)	95 (10-292)	<0.0001*
C-reactive protein, mg/L	182 (65-476)	85 (5-320)	71 (14-348)	<0.0001*
Serum creatinine, µmol/L	114 (39-1081)	93 (47-238)	90 (53-255)	N.T.
Serum sodium, mmol/L	131 (115-146)	135 (119-145)	135 (124-148)	<0.0001*
Lactate dehydrogenase, U/L	485 (139-2038)	268 (118-947)	246 (127-775)	<0.0001*
Total bilirubin, µmol/L	53 (13-416)	22 (4-164)	22 (3-99)	N.T.
Plasma lactate, mmol/L	2.3 (0.6-6.2)	1.4 (0.5-4.6)	1.3 (0.6-4.0)	<0.0001*
Leukocyte counts				
Total leukocyte count, x 10^9/L	6.9 (2.5-18.5)	4.9 (1.3-13.4)	5.3 (1.9-15.3)	<0.0001*
Neutrophil count, x 10^9/L	3.6 (0.6-10.4)	3.1 (0.4-11.0)	3.2 (1.0-11.6)	0.0064*
Neutrophil count %	60 (19-85)	64 (13-91)	64 (20-88)	NS*
Lymphocyte count, x 10^9/L	1.0 (0.2-4.2)	0.9 (0.1-5.0)	1.1 (0.3-6.1)	NS*
Lymphocyte count %	15 (4-45)	19 (2-74)	21 (5-74)	0.0036*

Table 1. Continued

	Severe *P. falciparum* n=61	Non-severe *P. falciparum* n=259	Non-*falciparum* n=120	P-value
NLCR	3.5 (0.7-17.0)	3.3 (0.2-46)	2.8 (0.3-17.6)	NS*
Parasite count				
P.falciparum load (asexual parasites/µL)	230,000 (520-1,380,600)	4,288 (2-208,000)	N/A	N.T.
Presence of *P. falciparum* schizonts (%)	33 (54%)	11 (4%)	N/A	<0.0001^
Concomitant infection				
All concomitant infections	8 (13%)	11 (4%)	10 (8%)	<0.0001#
Concomitant bacterial infection	5 (8%)	3 (1%)	2 (2%)	0.004#

** Comparison of 3 groups using the Kruskal Wallis test, # Comparison of 3 groups using the Chi-Square Test. ^ Comparison of 2 groups using Fisher's Exact test. Parameters that are included in the modified WHO severity criteria for severe falciparum malaria were not tested (N.T.).*

Table 2. Descriptive statistics of diagnostic performance for severe malaria

Parameter	Cut-off value	Sensitivity	Specificity	PPV	NPV	Youden's	AUROC
Total leukocyte count	≥6.5 x 10^9/L	0.59 (0.46-0.71)	0.75 (0.71-0.80)	0.28 (0.21-0.37)	0.92 (0.88-0.95)	0.34	0.70 (0.63-0.78)
Neutrophil count	≥3.4 x 10^9/L	0.64 (0.51-0.76)	0.56 (0.51-0.61)	0.19 (0.14-0.25)	0.91 (0.86-0.94)	0.2	0.61 (0.53-0.69)
Lymphocyte count	< 0.7 x 10^9/L	0.33 (0.22-0.46)	0.72 (0.67-0.76)	0.16 (0.10-0.24)	0.87 (0.83-0.90)	0.05	0.51 (0.43-0.59)
NLCR	≥2.8	0.77 (0.64-0.86)	0.44 (0.39-0.49)	0.18 (0.14-0.24)	0.92 (0.87-0.96)	0.21	0.57 (0.50-0.64)
CRP	>141 mg/L	0.80 (0.67-0.89)	0.76 (0.71-0.80)	0.33 (0.25-0.42)	0.96 (0.93-0.98)	0.56	0.84 (0.79-0.89)

Table 3. Leukocyte count changes during malaria treatment

	On admission	Upon clearance of parasitaemia	n	P-value
Severe *P. falciparum*				
CRP, mg/L	184 (108-373)	49 (1-206)	29	<0.0001
Total leukocyte count, x 109/L	6.8 (2.5-18.5)	7.8 (3.1-16.4)	40	0.0045
Neutrophil count, x 109/L	3.5 (1.2-10.4)	4.0 (1.4-10.7)	40	NS
Lymphocyte count, x 109/L	0.9 (0.2-4.2)	2.1 (0.2-5.8)	40	<0.0001
NLCR	4.0 (0.9-17.0)	2.1 (0.5-20.3)	40	0.0011
Non-severe *P. falciparum*				
CRP, mg/L	96 (5-284)	14 (1-250)	116	<0.0001
Total leukocyte count, x 109/L	4.8 (1.3-13.4)	5.6 (2.2-16.9)	161	<0.0001
Neutrophil count, x 109/L	3.0 (0.6-8.8)	2.5 (0.3-11.9)	114	0.0075
Lymphocyte count, x 109/L	0.9 (0.2-3.1)	2.0 (0.3-4.5)	114	<0.0001
NLCR	3.3 (0.5-19.0)	1.3 (0.3-11.0)	114	<0.0001

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CHAPTER 6

A simple and fast method to exclude high *Plasmodium falciparum* parasitaemia in travellers with imported malaria

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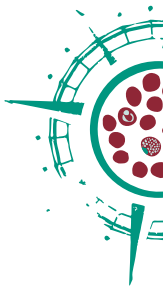


ABSTRACT

Background: Counts of malaria parasites in peripheral blood are important to assess severity of *Plasmodium falciparum* malaria. Thin and thick smears are routinely used for this purpose.

Methods: In this study the Binax NOW® Malaria Test, an easy-to-perform rapid diagnostic test, with Histidine Rich Protein-2 (HRP-2) and aldolase as diagnostic markers, was used for semi-quantitative assessment of parasitaemia of *P. faciparum*.

Results: In 257 patients with imported *P. falciparum* malaria, reactivity of aldolase increased with higher parasitaemia. In all patients with a parasitaemia above 50,000 asexual parasites/ µl (> 1%) co-reactivity of HRP-2 and aldolase was observed. Absence of aldolase reactivity in the presence of HRP-2 was a reliable predictive marker to exclude high (> 1%) parasitaemia in *P. falciparum* malaria.

Conclusion: Assessment of HRP-2 and aldolase co-reactivity can be of help in clinical decision making in the acute care setting of returning travellers suspected of having malaria.

BACKGROUND

The global burden of malaria is largely carried by the world's malaria-endemic regions with as many as 225 million cases and a death toll of more than 750,000 individuals in 2009 (1). In striking contrast, in non-endemic industrialized countries malaria is seen as an occasionally imported disease in non-immune travellers, but it still represents a potentially fatal disease (2, 3). Without prompt and proper treatment malaria may rapidly progress to complications and even death. Hence, all patients must be assessed for signs or symptoms suggestive of an increased risk for complications. Due to unfamiliarity with the disease in non-endemic countries, ill-returning travellers frequently present to physicians who have no tropical medicine expertise and to primary health care facilities that lack expert diagnostic capabilities. As a result, diagnosis of malaria may be delayed or even missed, resulting in more severe disease or even fatalities (4, 5).

Recent studies in non-endemic industrialized countries showed that rapid diagnostic tests (RDTs) for malaria provide an excellent tool for diagnosis of malaria as compared to peripheral blood smears (6). Although highly sensitive in diagnosing *Plasmodium falciparum* malaria, RDTs are not thought to provide sufficient information about parasitaemia, one of the major determinants of disease severity (1). In the present multi-centre operational laboratory study it is shown that the FDA approved three-band immunochromographic RDT Binax NOW® Malaria Test allows a semi-quantitative assessment of parasitaemia and rapid exclusion of high *P. falciparum* parasitaemia, which may facilitate clinical decision making in the acute care setting.

METHODS

In order to assess the utility of this RDT as a semi-quantitative measure of *P. falciparum* load in routine clinical practice, an operational laboratory study was conducted at two hospital-based laboratories with expertise in malaria diagnosis in The Netherlands (Academic Medical Center, Amsterdam, The Netherlands; Harbour Hospital, Rotterdam, The Netherlands). Of all patients detailed demographic, clinical and laboratory data were available, as well as the outcome measures severe malaria and death. Severe malaria was diagnosed according to predefined WHO criteria in travellers (7). In both Dutch centres, parasitaemia was examined using the same protocol. Thick and thin smears were stained with

Giemsa (Giemsa improved R66 Gurr, BDH, diluted 1:10, PH 7,2, 30 min.) For an initial estimate of the parasite load, malaria thin smears were examined by light microscopy (100x objective and 12,5 ocular lens). If the parasitaemia was assumed to be ≤0.5% infected red blood cells, the exact parasite load was determined by counting the number of asexual parasites per 100 leukocytes in a thick smear. In case the initial parasitaemia was assumed to be 0.5-2.0%, the number of infected red blood cells was counted in 10 visual fields of a thin smear. The number of red cells per microscopic field in a thin smear was pre-calculated for the different microscopes in use. In case the initial parasitaemia was assumed to be >2.0%, the number of infected red blood cells was determined using a special ocular lens with a visual field area reduced to approximately 25%. Within this limited field of view both the total number of red blood cells and the number of infected red blood cells were counted in at least 10 visual fields. All counts were performed in duplicate and the final count was given as the average. In case of a discrepancy of >15% between the duplicate counts, a third count was performed. The number of asexual parasites/µl was finally calculated using the actual number of erythrocytes or leukocytes in a blood sample.

The RDTs were performed on fresh blood samples, simultaneously with microscopy of the blood slides. Every RDT and blood slide was read by two independent, experienced laboratory technicians. The Binax NOW® Malaria Test was used as RDT and performed according to the manufacturer's instructions. The Binax NOW® Malaria Test uses monoclonal antibodies that target the histidine-rich protein 2 (HRP-2) antigen specific to *P. falciparum* (the 'T1' line) and the pan-malarial antigen aldolase (the 'T2' line), common to all five *Plasmodium* species that can be detected in humans (*P. falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*) (8). Aldolase co-reactivity was defined as both a reactive HRP-2 line (T1 line) as well as a reactive aldolase line (T2 line). Absence of aldolase co-reactivity was defined as a non-reactive aldolase (T2) but a reactive HRP-2 (T1) line (Figure 1).

For external validation of the reproducibility of the RDT findings in the Dutch centers, a reference center for malaria diagnosis participated (Institute of Tropical Medicine (ITM), Antwerp, Belgium). In this setting in thick and thin blood films, stained with Giemsa (Merck 1.09204.0500, diluted to 3.5% in pH 8.0, 20 min.), parasite densities were determined by counting asexual parasites against 200 white blood cells (WBC) in thick blood films and converting this number to parasites/µl using the actual white blood cell count (9). Parasite densities were next

converted to % infected red blood cells using the red blood cell count. For the purpose of this study, ITM provided RDT findings in relation to the parasite loads of 73 consecutive patients, but did not contribute to the clinical data.

RESULTS

A total of 257 patients with *P. falciparum* malaria were included. All patients had HRP-2 reactivity in the Binax NOW® Malaria Test. The general characteristics and laboratory findings of 184 evaluable patients from Amsterdam (Academic Medical Center) and Rotterdam (Harbour Hospital) are provided in Table 1. The 23 patients with severe malaria presented with impaired consciousness in seven (three of them had a Glasgow Coma Score score < 11), severe anaemia in two, hyperbilirubinaemia in 12 and renal failure in three cases. Eleven patients had a parasitaemia > 5% (4 of them had a parasitaemia > 10%). Five patients received haemodialysis and one patient died. Of the 73 Belgian patients no clinical data were available.

Co-reactivity of HRP-2 and aldolase was observed in blood specimens with both low and high parasitaemia, ranging from 56 to 558,000 (Amsterdam), 23 to 1,380,600 (Rotterdam) and 26 to 400,000 asexual parasites/µl (Antwerp), respectively. The proportion of aldolase co-reactivity substantially increased with increasing parasitaemia (Figure 2). Aldolase co-reactivity was always present when parasitaemia was above 50,000 asexual parasites/µl (corresponding to approximately 1 % parasitized erythrocytes). This observation was valid not only in the centers in Rotterdam and Amsterdam, but also in the laboratory setting of Antwerp. All patients with severe malaria (n=23) invariably showed HRP-2 and aldolase co-reactivity. Hence, aldolase co-reactivity with HRP-2 had a sensitivity of 100% (95% confidence interval 85 –100%) for severe malaria, but a poor positive predictive value (PPV) of only 21% (95% CI 11-25%), since aldolase co-reactivity was also present in 109 patients with uncomplicated malaria. In contrast, absence of aldolase reactivity (n=52) had a negative predictive value (NPV) of 100% (95% CI 93 – 100%) for severe malaria. The implications of the RDT test outcomes for clinical decision- making are shown in Figure 1.

DISCUSSION

The results of the present study indicate that the Binax NOW® Malaria Test allows a semi-quantitative assessment of parasitaemia in travellers returning with *P. falciparum* malaria.
The combination of HRP-2 reactivity (T1) in absence of aldolase reactivity (T2), proved a reliable predictive marker for a low (< 1%) *P. falciparum* parasitaemia. In the current study population HRP-2 reactivity without aldolase reactivity was applicable to 30% of returning travellers with *P. falciparum.* When clinical findings and routine laboratory results in these patients also are not indicative of severe disease, the patient most likely has uncomplicated malaria. This knowledge is important for further clinical decision-making.

Co-reactivity of aldolase and HRP-2 was present in all patients with a parasitaemia above 50,000 asexual parasites/µl (corresponding to approximately 1% infected red blood cells). Others reported co-reactivity with Binax NOW® in 80% of patients with *P. falciparum* parasitaemia > 40,000 parasites/µl (9). The authors suggested co-reactivity of HRP-2 and aldolase possibly could function as a semi-quantitative marker of high *P. falciparum* parasitaemia (10). In the present study this relation proved, however, not straightforward with co- reactivity of HRP-2 and aldolase also being present in patients with low parasitaemia (i.e. < 0.5%). As such co-reactivity of HRP-2 and aldolase is less reliable as marker for high parasitaemia..

The data of the present study suggest that aldolase and HRP-2 co-reactivity is present in all patients with severe malaria. Apparently HRP-2 and aldolase reactivity, which depends on the load of these antigens in the blood specimen, is preserved because these antigens are derived not only from circulating viable and non-viable malaria parasites, but also from sequestered parasites that are abundantly present in severe malaria. As a consequence of this sequestration, microscopic determination of peripheral blood smears might underestimate the total parasitaemia.
The relation between aldolase reactivity and *P. falciparum* parasitaemia, as observed with Binax NOW® in this study, could be dependent of the process of manufacturing. Other RDTs with a similar three-band configuration, therefore, should be studied in detail, to assess about the precise relationship between *P. falciparum* parasitaemia and aldolase reactivity.
HRP-2 and aldolase co-reactivity may also be a feature of a mixed *Plasmodium*

infection. In a recent study of 2,847 cases of imported malaria in the Netherlands (11), 75% of the infections were solely caused by *P. falciparum* whereas the remainder was caused by *P. vivax* (15%), *P. ovale* (7%), and *P. malariae* (3%), respectively. Only 0,7% of all infections was attributable to mixtures of species, mostly involving *P. falciparum*. Thus, in the Dutch setting, HRP-2 and aldolase co-reactivity is far more likely to reflect a mono-parasitic *P. falciparum* infection rather than a mixed infection.

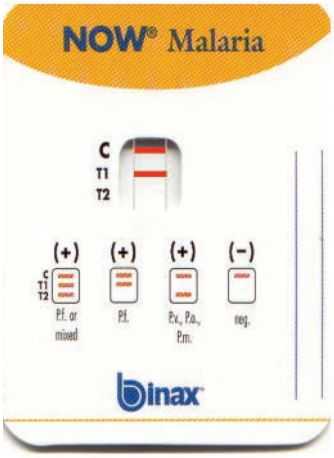
Results of RDT's may facilitate clinical decision making in patients suspected of having malaria. There are however also some drawbacks to consider. First, these tests cannot replace clinical assessment of the ill-returning patient and results of RDT tests should always be confirmed by thin or thick blood smears, including parasite counts in case of *P. falciparum* malaria (6, 12). Second, the diagnostic power of this RDT test is dependent on the epidemiological setting, in particular the prevalence of the disease. The current findings may not simply be extrapolated to regions of malaria endemicity where low-grade malaria infections are far more prevalent and empirical anti-malarial treatment is common use, which may lead to false-negative and false-positive RDT findings, respectively. In addition, the majority of the travellers in this study contracted *P. falciparum* infection in Africa; other malaria-endemic continents like South-East Asia and South-America were underrepresented. Caution is warranted with extrapolating the applicability of the current findings to imported malaria acquired outside Africa. False negative results have been suggested for certain genetic polymorphisms of HRP-2 geographically confined to the Asia-Pacific region (13) and for *P. falciparum* isolates from South America lacking HRP-2 (14). In addition, false negative test results may occur at high parasitaemia due to a so-called prozone effect, defined as false-negative or false-low results in immunological reactions due to an excess of either antigens or antibodies. The prozone effect was observed for HRP-2 in 16 of 17 RDTs (including the Binax NOW® Malaria Test), resulting in a false low HRP-2 signal, whereas aldolase reactivity was not affected (15). Finally, the clinician must also consider the possibility of a *P. knowlesi* infection, which may give rise to severe disease and fatal complications as well (8). Even though early reports suggested that RDTs may not detect *P. knowlesi* infections, later studies demonstrated that *P. knowlesi* was reactive with the aldolase band in the Binax NOW® Malaria Test, but not with HRP-2 and that aldolase reactivity depended on the *P. knowlesi* parasitaemia (8).

CONCLUSION

In conclusion, the RDT Binax NOW® Malaria Test allows a rapid semi-quantitative assessment of *P. falciparum* load in travellers with malaria returning from the tropics, especially for exclusion of high (>1%) parasitaemia in the acute care setting. This may facilitate clinical decision making for subsequent oral antimalarial treatment or timely referral to a specialized centre for high-level monitoring and intensified parenteral treatment.

Table 1. General characteristics and laboratory findings at initial clinical assessment of 184 patients with *P. falciparum* malaria

Parameter	Amsterdam (AMC)	Rotterdam (HZH)
Number of patients, n	77	107
Male/female	52/25	78/29
Age, median (range,) yrs	37 (0 – 61)	40 (16 – 70)
Purpose of travel		
Immigrant, n (%)	4 (5.2)	0 (0.0)
Visiting friends and relatives, n (%)	48 (62.3)	36 (33.6)
Tourist, n (%)	6 (7.8)	20 (18.7)
Business, n (%)	10(13.0)	27 (25.2)
Expatriate, n (%)	0 (0.0)	4 (3.7)
Sailor, n (%)	0 (0.0)	4 (3.7)
Unknown, n (%)	9 (11.7)	11 (10.3)
Continent of acquisition		
Africa, n (%) [West-Africa, n (%)]	73 (94.8) [63 (81.8)]	101 (94.9) [71 (66.4)]
South-America, n (%)	2 (2.6)	1 (0.9)
South East Asia, n (%)	2 (2.6)	7 (6.5)
Clinical assessment		
Severe malaria, n (%)	7 (9.1)	16 (15.0)
Non-severe malaria, n (%)	70 (90.9)	91 (85.0)
Laboratory findings		
Parasite load, median range, trophozoites/µL	4648 (50 – 558000)	10664 (2 – 1380600)
Malaria Now	77 (100)	107 (100)
HRP-2 and aldolase co-reactivity, n (%)	54 (70.1)	78 (72.9)
HRP-2 reactivity, n (%)	23 (29.9)	29 (27.1)



HRP-2 (T1 line) reactivity

- No aldolase (T2 line) reactivity
- *P. falciparum* parasitaemia
  < 1% or < 50.000 asexual parasites / µL

Aldolase co-reactivity (T1 + T2 line)

- *P. falciparum* parasitaemia variable
- Generally present in severe disease

Figure 1. Interpretation of the Binax NOW® Malaria test results in patients with *P. falciparum* infection, related to microscopic parasitaemia.

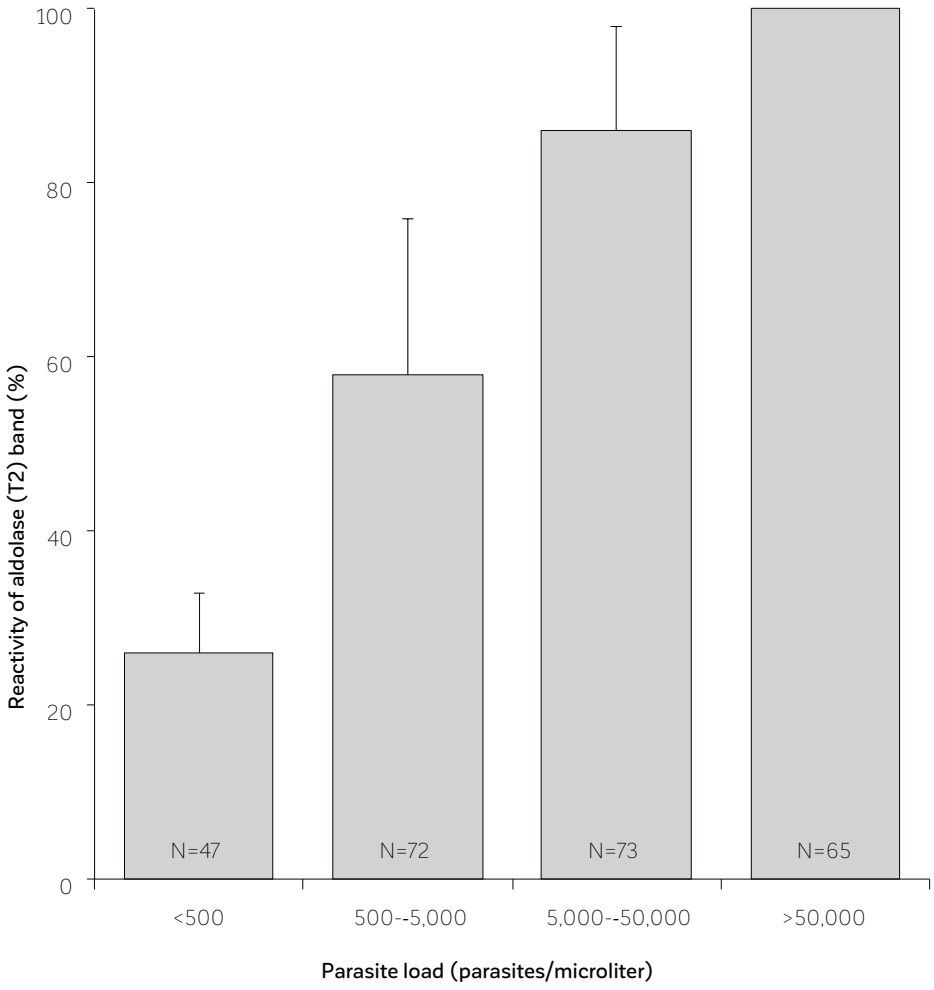


Figure 2. Co-reactivity of aldolase and HRP-2 bands in Binax NOW® Malaria rapid diagnostic test in relation to *P. falciparum* parasitaemia.

Aldolase co-reactivity was consistently present at a parasitaemia above 50,000 asexual parasites/µl, but variably present at lower parasitaemia indicating that absence of aldolase co-reactivity always was associated with a parasitaemia ≤ asexual 50,000 parasites/µl. The RDT findings (absence or presence of aldolase co-reactivity) of the participating three centres are shown in relation to category of parasitaemia and expressed as mean ± SD. The number of patients per category of parasitaemia is given within each respective bar.

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CHAPTER 7

The prognostic value of schizontaemia in imported *Plasmodium falciparum* malaria

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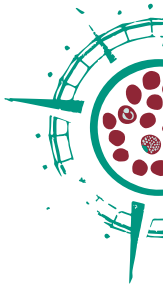
Malar J. 2012;11:301



ABSTRACT

Background: In *Plasmodium falciparum* infection, peripheral parasite counts do not always correlate well with the sequestered parasite burden. As erythrocytes parasitized with mature trophozoites and schizonts have a high tendency to adhere to the microvascular endothelium, they are often absent in peripheral blood samples. The appearance of schizonts in peripheral blood smears is thought to be a marker of high sequestered parasite burden and severe disease. In the present study, the value of schizontaemia as an early marker for severe disease in non-immune individuals with imported malaria was evaluated.

Methods: All patients in the Rotterdam Malaria Cohort diagnosed with *P. falciparum* malaria between 1 January 1999 and 1 January 2012 were included. Thick and thin blood films were examined for the presence of schizontaemia. The occurrence of WHO defined severe malaria was the primary endpoint. The diagnostic performance of schizontaemia was compared with previously evaluated biomarkers C-reactive protein and lactate.

Results: Schizonts were present on admission in 49 of 401 (12.2%) patients. Patients with schizontaemia were more likely to present with severe malaria, a more complicated course and had longer duration of admission in hospital. Schizontaemia had a specificity of 0.95, a sensitivity of 0.53, a negative predictive value of 0.92 and a positive predictive value of 0.67 for severe malaria. The presence of schizonts was an independent predictor for severe malaria.

Conclusion: Absence of schizonts was found to be a specific marker for exclusion of severe malaria. Presence of schizonts on admission was associated with a high positive predictive value for severe malaria. This may be of help to identify patients who are at risk of a more severe course than would be expected when considering peripheral parasitaemia alone.

BACKGROUND

Sequestration of infected erythrocytes in the microvasculature is one of the main pathological features of severe *Plasmodium falciparum* infection (1-4). Particularly, erythrocytes parasitized with mature trophozoites and schizonts have a high tendency to adhere to the microvascular endothelium and are therefore often absent in collected peripheral blood samples. However, the appearance of schizonts in peripheral blood smears is thought to reflect a high sequestered parasite burden and was recognized as a sign of severe disease already in early field studies on malaria (5). The predominance of mature parasites on peripheral smears was found to reflect more severe disease by Silamut and White (6), but only a few other studies on the performance of schizontaemia as a predictor for severe disease have been done (7-9). In addition, data is only available from studies in areas with malaria endemicity where many inhabitants are believed to have some degree of immunity toward malaria (so-called semi-immunity). Data in non-immune individuals are lacking.

Although it is stated in the current WHO malaria guidelines that the presence of schizontaemia may be associated with a poor outcome in *P. falciparum* malaria, presence of schizonts is currently not a well-established WHO criterion for severe disease (9, 10). To further explore the value of schizontaemia as an early marker for severe disease in non-immune individuals with imported malaria, the prognostic value of schizontaemia was assessed in a large cohort of travellers with *P. falciparum* malaria.

METHODS

Patients

The Harbour Hospital is a 161-bed general hospital located in Rotterdam, The Netherlands. It comprises the Institute of Tropical Diseases, which serves as a national referral centre. All patients in the Rotterdam Malaria Cohort diagnosed with *P. falciparum* malaria between 1 January 1999 and 1 January 2012 were included in this observational study. Of all patients identified, demographic, clinical and laboratory data were collected using a standardized form and stored in an electronic database. Patients with a mixed infection of *P. falciparum* with another *Plasmodium* species were included in this study, but pure non-*falciparum* *Plasmodium* infections were excluded. Multiple malaria episodes in a single patient

were only regarded as separate cases if caused by true re-infection; recrudescent infections of *P. falciparum* were excluded.

Laboratory investigations

Available laboratory examinations included red and white blood cell counts, haematocrit, platelet counts, C-reactive protein, serum electrolytes, total bilirubin, serum creatinine and urea, sodium, potassium, liver enzymes, blood glucose and venous plasma lactate.

Detection of *Plasmodium* parasites

The standard procedure to diagnose malaria comprised a quantitative buffy coat (QBC) analysis, a rapid diagnostic test (RDT) for malaria antigens Binax NOW Malaria Test (Binax, Inc, Maine, USA), and thick and thin blood smears using freshly collected blood specimens from finger pricks. The malaria rapid test and the QBC analysis were performed according to the manufacturer's instructions. QBC capillaries were examined independently by two technicians by microscopic analysis of two complete rows of the region between the bottom of the capillary and the polynuclear leukocyte layer using an Olympus BX-60 fluorescence microscope equipped UV- filter and 50x objective and 12.5x oculars (total magnification 625x). These two lanes represented about 100 microscopic fields (at 625x magnification) and took an average examination time of 5 min. Subsequently, the polynuclear and mononuclear cell layer was screened for schizonts, gametocytes, malaria pigment and elderly tro- phozoites of *Plasmodium vivax* and *Plasmodium ovale*.

Thick blood smears were stained with Field's stain (Brunschwig Chemie, Amsterdam, The Netherlands) and thin smears with Diff Quick stain (Medion Diagnostics, Düdingen, Switzerland). Both staining procedures had been optimized for optimal staining of *Plasmodium* parasites as well as Schüffner's dots and Maurer's clefts in infected erythrocytes. Thick and thin smears were examined with regular light microscopes at a total mag- nification of 1,250x. The *Plasmodium* species was identified on morphological characteristics in the thin and thick blood smears. Since a real-time quantitative PCR became available several years ago, a substantial proportion of the samples were additionally confirmed by PCR (without discrepant findings compared to classic light microscopy).

Parasite counting and identification of *Plasmodium falciparum* schizonts

Parasitaemia was determined by parasite counting as described before (11). *Plasmodium falciparum* schizonts were defined as clustered malaria pigment adjacent to two or more nuclei of *P. falciparum* in thick blood smears or QBC capillaries. Compared to thin and thick blood smears QBC analysis is the most sensitive test to detect *Plasmodium* parasites. *Plasmodium falciparum* schizonts can be identified in the erythrocyte layer in the QBC capillary, but identification within the leukocyte layer is difficult due to the intense fluorescent signal of the leukocyte nuclei. For this reason, this layer is also screened for quenched fluorescence by malaria pigment. If malaria pigment was detected in the leukocyte layer of the QBC capillary, the thick and thin blood smears were thoroughly examined for the presence of schizonts by analysis of at least 200 microscopic fields at 625x magnification.

Definitions

Severe malaria

Patients were classified as having severe malaria if they met one or more of the WHO criteria for severe malaria, as published [9], either on presentation or later during hospital admission.

Schizontaemia

Schizontaemia was defined as presence of one or more *P. falciparum* schizonts identified in QBC and/or blood smear examinations from a blood sample taken on admission.

Immunity to malaria.

The degree of immunity to malaria was estimated as previously described (12). In brief, adult immigrants from a malaria-endemic country living in The Netherlands were considered partially immune. Immigrant patients who had been living in a malaria-endemic area for at least two years at the time of diagnosis were presumed semi-immune. Tourists from non-endemic countries who travelled to malaria-endemic regions were considered non-immune.

Statistical analysis.

All data were not normally distributed (Kolmogorov-Smirnov test) and are therefore presented as medians and range. Univariate comparisons were performed using the Mann-Whitney U test and the Fisher's Exact test. Trends between

categorical variables were done with the Chi square test for Trend. Odds ratios were calculated using the approximation of Woolf. For clinical reference, the diagnostic performance of schizontaemia for severe disease was compared to that of the classic biomarkers serum C-reactive protein and plasma lactate (13-15). Binary logistic regression analysis was used to analyse if schizontaemia was independently associated with malaria severity. Malaria severity was entered as the dependent variable. To test its independence from parasite load and the previously evaluated markers lactate and C-reactive protein, these were entered as co-variants. As parasite load was a highly skewed continuous variable with a logarithmic distribution, the data were transformed to log-parasitaemia to make them suitable for logistic regression analysis.

RESULTS

Patient characteristics

Between 1 January 1999 and 1 January 2012, a total of 562 cases of imported malaria were seen. Of these cases, 161 cases were caused by non-*falciparum Plasmodium* species; 401 infections were caused *P. falciparum* infections including three patients who had a mixed infection (two with *P. falciparum* and *P. ovale*, one with *P. falciparum* and *P. vivax*, respectively). No patient with a mixed *P. falciparum* infection presented with schizontaemia on admission. These 401 patients with either a pure *P. falciparum* infection or a mixed *Plasmodium* infection with *P. falciparum* were included in this study.

Schizontaemia

Schizonts were present on admission in 49 of 401 (12.2%) patients. The general characteristics of the patients with presence and absence of schizonts on admission are shown in Table 1. Males were less likely to present with schizontaemia than female patients (Odds ratio 0.37 (95% confidence interval 0.20-0.67), p = 0.0018). Although the number of patients who acquired *P. falciparum* malaria outside of Africa was relatively low (n = 26), these patients tended to present with schizontaemia more frequently than patients who contracted malaria in Africa (Odds ratio 0.26 (95% CI 0.086-0.79), p = 0.0259). There were no differences between patients presenting with and without schizontaemia with regard to their immune status, ethnic background and use of malaria chemoprophylaxis, nor did they differ in duration of symptomatology. As detailed in Table 1, patients with schizontaemia presented more ill on admission: they had higher pulse rates,

were more likely to have impaired consciousness and laboratory parameters were compatible with more severe degree of liver and kidney injury as well as more compromised tissue perfusion given the higher plasma lactate levels. Other laboratory parameters revealed that patients with schizontaemia also presented with significantly higher parasite loads, higher leucocyte counts, higher C-reactive protein levels and significantly lower platelet counts. When the WHO severity criteria for malaria (9) were assessed in both patient groups, patients with schizontaemia were more likely to present with or develop severe and complicated disease and they had to stay significantly longer in hospital than patients without demonstrable schizontaemia on admission (Table 2). Death occurred in two of 401 malaria patients, one with and one without schizontaemia on ad- mission. Presence of schizontaemia on admission significantly increased with increasing parasite loads on admission (Chi square test for Trend, $p < 0.0001$), as shown in Figure 1.

The diagnostic accuracy of schizontaemia as a predictor for severe malaria was characterized by a high specificity, negative predictive value and an acceptable positive predictive value. When compared to the performance of plasma lactate, schizontaemia had a lower sensitivity but a higher specificity and positive predictive value for severe disease. When compared to the performance of C-reactive protein, schizontaemia had a higher specificity and positive predictive value for severe disease (Table 3). Using binary logistic regression analysis, schizontaemia was found to be an independent predictor for severe malaria with odds ratios substantially exceeding that found for C-reactive protein and lactate but not that found for parasite load (Table 4).

DISCUSSION

Sequestration of parasitized erythrocytes in the microvasculature of vital organs is a cardinal feature of *P. falciparum* malaria and is a cause of major pathology in severe disease (1-4). Maturing *P. falciparum* parasites disappear from the peripheral blood after 24-26 hours to complete the asexual life cycle in the microvasculature, as the tendency to adhere to the endothelial wall increases during the maturation process (6). This process of sequestration accounts for the fact that peripheral parasite counts do not always correlate well with the sequestered parasite burden. This discrepancy was noted soon after the use of the peripheral parasitaemia to predict the outcome of *P. falciparum* infection became common

practice in the 1930s (5).
In the microvasculature, rupturing schizonts release up to 32 merozoites (7, 16), causing an exponential rise in parasitaemia. A high schizont count is therefore likely to precede a rise in parasitaemia and might be an early marker for severe disease . As compared to quinine, artemisinin derivatives like artesunate have an anti-malarial efficacy to the broadest spectrum of parasite stages, including the generally less drug-sensitive schizonts (1, 7). This may provide another argument to treat patients with schizontaemia with artemisinin derivatives given the convincing proof of past studies in regions of malaria endemicity showing that artesunate was superior over quinine for treatment of severe malaria in both children and adult patients (17, 18), not only in terms of reduction of mortality and parasite clearance but also in ease of use. Recent studies in non-endemic industrialized regions confirmed that in non-immune travellers with imported malaria, treatment with artesunate results in a comparable rapid parasite clearance to that observed in semi-immune individuals (19, 20).

In the present study schizontaemia was found to be a useful marker for severe malaria. Although schizontaemia was more frequently seen in patients with high-grade parasitaemia, the predictive power appeared to be independent of parasitaemia. In the study by Silamut and White (6) in adult Thai patients and children from The Gambia, it was demonstrated that a predominance of mature parasite forms in the peripheral blood reflect a greater sequestered parasite mass and the presence of $>10^4$ mature trophozoites and schizonts per µL of peripheral blood was found to have a high sensitivity (90%) and specificity (70%) for fatal outcome. The data of the present study confirm the prognostic value of schizontaemia for severe disease in predominantly non-immune travellers, comparable to that observed in semi-immune patients in region of malaria endemicity. However, it should be noted that besides differences in presumed immunity towards malaria, the current study also differed in its design with regard to outcome. In many studies in malaria-endemic regions, the primary outcome measure is usually survival or death and studies are restricted to these severe cases using the WHO severity criteria as an entry criterion. Since case fatalities are less common in the western non-endemic, resource-rich setting (12) and malaria is only seen as a sporadic imported disease, the present study in patients with imported malaria focused on severe disease as the primary outcome, using the WHO criteria to define disease severity.

Unfortunately, for the present study quantitative counts of the different parasite stages were not available. Although quantification would likely have resulted in a

more precise estimation of its prognostic value, the specificity of schizontaemia was found to be a useful tool for exclusion of severe disease. The negative predictive value of schizontaemia is high, and comparable to that of CRP and lactate. Moreover, unlike C-reactive protein and lactate (13-15) schizontaemia on admission was also associated with a high positive predictive value. This may provide the clinician with a helpful tool for decision making in the acute care setting by allowing an early identification of those malaria patients who are at risk of a more severe and complicated course than would normally be expected when considering peripheral parasitaemia alone.

CONCLUSION

Absence of schizonts on admission in travellers with imported *P. falciparum* malaria was found to be a specific marker for exclusion of severe disease; in contrast, presence of schizonts had a high positive predictive value for presence of severe disease and can thus help to identify patients that may be candidates in need of intensive monitoring and timely administration of preferably (parenteral) artemisinin derivatives.

Table 1. General characteristics of patients with imported *Plasmodium falciparum* malaria grouped by presence or absence of schizontaemia on admission

	All patients n=401	Schizontaemia n=49	No schizontaemia n=352	P-value
Demographics				
Age, years	39 (4-78)	41 (4-70)	39 (5-78)	NS
Male, female, n (%)	292 (72.8), 109 (27.2)	26 (53.1), 23 (46.9)	266 (75.6), 86 (24.4)	0.0018
Continent of acquisition				
Africa, n (%)	360 (89.8)	38 (77.6)	322 (91.5)	0.0196
Asia, n (%)	16 (4.0)	5 (10.2)	11 (3.1)	
South and Central America, n (%)	10 (2.5)	3 (6.1)	7 (2.0)	
Unknown, n (%)	15 (3.7)	3 (6.1)	12 (3.4)	
Duration of signs/symptoms				
<8 days, n (%)	259 (64.6)	30 (61.2)	229 (65.1)	NS
8-14 days, n (%)	74 (18.5)	15 (30.6)	59 (16.8)	
15-28 days, n (%)	27 (6.7)	0 (0)	27 (7.7)	
>28 days, n (%)	8 (2.0)	0 (0)	8 (2.3)	
Unknown, n (%)	33 (8.2)	4 (8.2)	29 (8.2)	
Use of malaria chemoprophylaxis				
No chemoprophylaxis, n (%)	272 (67.8)	35 (71.4)	237 (67.3)	NS
Inadequate use, n (%)	72 (18.0)	6 (12.2)	66 (18.8)	
Adequate use, n (%)	39 (9.7)	6 (12.2)	33 (9.4)	
Unknown, n (%)	18 (4.5)	2 (4.1)	16 (4.5)	

Table 1. Continued

	All patients n=401	Schizontaemia n=49	No schizontaemia n=352	P-value
Vital signs on admission				
Body temperature, °C	38.5 (35.5-41.2)	38.7 (35.7-41.2)	38.5 (35.5-41.0)	NS
Pulse rate, beats per minute	95 (45-150)	105 (68-150)	92 (45-150)	0.0002
Systolic blood pressure, mm Hg	120 (64-190)	118 (64-160)	120 (73-190)	NS
Impaired consciousness (GCS<15), n (%)	9 (2.2)	5 (10.2)	4 (1.1)	0.0019
Cerebral malaria (GCS≤11), n (%)	5 (1.2)	2 (4.1)	3 (0.9)	NS
Laboratory data on admission				
Haemoglobin, mmol/L	8.3 (2.5-11.1)	7.8 (3.8-10.9)	8.4 (2.5-11.1)	NS
Leucocyte count, x 109/L	5.1 (1.3-18.5)	6.1 (2.5-18.5)	5.0 (1.3-13.4)	0.011
Thrombocytes, x 109/L	86 (2-385)	38 (10-164)	94 (2-385)	<0.0001
C-reactive protein, mg/L	98 (5-476)	166 (22-476)	87 (5-373)	<0.0001
Serum creatinine, µmol/L	95 (39-1,081)	110 (39-1,081)	95 (47-871)	0.0019
Serum sodium, mmol/L	135 (115-146)	132 (115-142)	135 (119-146)	<0.0001
Lactate dehydrogenase, U/L	275 (118-2,297)	435 (135-2,038)	262 (118-2,297)	<0.0001
Total bilirubin, µmol/L	24 (4-416)	44 (9-269)	23 (4-416)	<0.0001
Plasma lactate, mmol/L	1.6 (0.5-6.2)	2.7 (0.6-6.2)	1.5 (0.5-4.7)	<0.0001
Parasite count				
P. falciparum load (asexual parasites/µL)	8,400 (2-1,380,600)	162,000 (144-1,380,600)	5,430 (2-860,000)	<0.0001
Gametocytes, presence, absence, n (%)	64 (16.0), 337 (84.0)	11 (22.4), 38 (77.6)	53 (15.1), 299 (84.9)	NS

Table 2. Outcome measures of patients with imported *Plasmodium falciparum* malaria, grouped by presence or absence of schizontaemia on admission

	All patients (n=401)	Schizontaemia (n=49)	No schizotaemia (n=352)	P-value	Odds ratio (95% CI)
Case-fatalities, n (%)	2 (0.5)	1 (2.0)	1 (0.3)	NS	
Severe malaria, n (%)	62 (15.5)	33 (67.3)	29 (8.2)	<0.0001	23.0 (11.3-46.6)
ICU admission, n (%)	92 (22.9)	38 (77.6)	54 (15.3)	<0.0001	19.1 (9.2-39.6)
Exchange transfusion, n (%)	40 (10.0)	26 (53.1)	14 (4.0)	<0.0001	27.3 (12.6-59.2)
Renal replacement therapy, n (%)	7 (1.7)	4 (8.2)	3 (0.9)	0.0053	10.3 (2.2-47.7)
Mechanical ventilation, n (%)	4 (1.0)	3 (6.5)	1 (0.3)	0.0063	22.9 (2.3-224.8)
Time in hospital, days (range)	5 (0-56)	7 (0-56)	5 (0-19)	<0.0001	

Table 3. Descriptive statistics of schizontaemia, lactate and C-reactive protein as predictors for severe malaria

	Schizontaemia (presence or absence)	Plasma lactate (mmol/L)	C-reactive protein (mg/L)
Number	401	240	381
Median (range)	N/A	1.6 (0.5-6.2)	98 (5-476)
Mean	N/A	1.9	111
Standard deviation	N/A	1.05	78
Optimal cut-off	N/A	≥1.7	≥142
Sensitivity	0.53 (0.40-0.66)	0.80 (0.67-0.89)	0.76 (0.63-0.86)
Specificity	0.95 (0.92-0.97)	0.63 (0.55-0.70)	0.75 (0.70-0.80)
Youden's index	0.48	0.43	0.51
PPV	0.67 (0.52-0.80)	0.39 (0.30-0.49)	0.35 (0.27-0.45)
NPV	0.92 (0.88-0.94)	0.91 (0.85-0.95)	0.95 (0.91-0.97)

Table 4. Results of binary logistic regression analysis of explanatory variables for severe malaria

	β	S.E. β	Wald's χ	df	P-value	Odds ratio (95% CI)
C-reactive protein	0.01	0.04	8.326	1	0.004	1.01 (1.00-1.02)
Log parasitaemia*	2.65	0.56	22.325	1	<0.001	14.13 (4.71-42.38)
Plasma lactate	0.42	0.27	3.564	1	NS	1.52 (0.89-2.57)
Schizontaemia	1.87	0.62	9.024	1	0.003	6.52 (1.92-22.13)
Constant	-16.63	2.93	32.133	1	<0.001	N/A

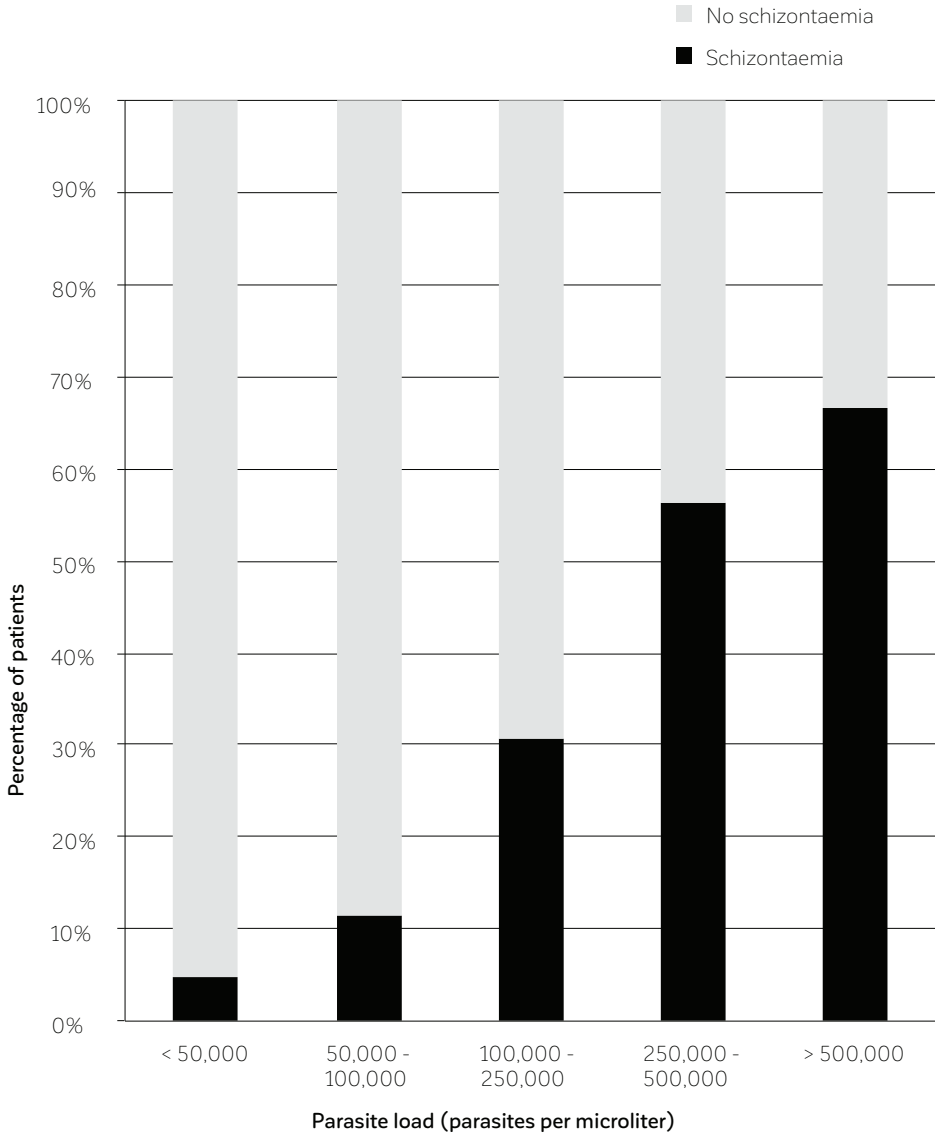


Figure 1. The percentage of patients with schizontaemia on admission, stratified by peripheral parasite count.

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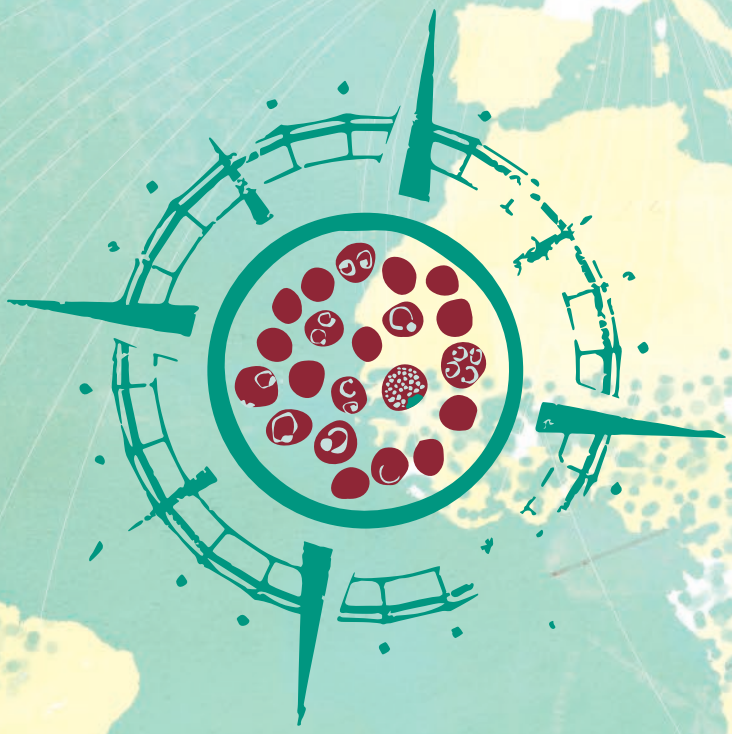
PART II

Acute kidney injury and hyponatraemia in imported malaria



CHAPTER 8

Acute kidney injury in imported *Plasmodium falciparum* malaria

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Malar J. 2015;14:523.

ABSTRACT

Background: Acute kidney injury (AKI) is a known complication of malaria, and is reported to occur in up to 40% of adult patients with a severe *Plasmodium falciparum* infection in endemic regions. To gain insight in the incidence and risk factors of AKI in imported *P. falciparum* malaria, a retrospective analysis was performed on a large cohort of mostly non-immune patients with imported *P. falciparum* malaria. Aiming to include not only severe but also milder forms of renal failure, the KDIGO criteria were used to define AKI.

Methods: Clinical and laboratory data from 485 consecutive cases of imported *P. falciparum* malaria were extracted from the Rotterdam Malaria Cohort database. Acute kidney injury (AKI) was defined using the KDIGO criteria. Univariate and multivariate logistic regression analyses were used to identify risk factors for AKI..

Results: AKI was seen in 39 (8%) of all patients and in 23 (38%) of the 61 patients with severe malaria. Eight patients eventually needed renal replacement therapy (RRT); seven of them already had AKI at presentation. Higher age, higher leucocyte count and thrombocytopenia were independently associated with AKI but their positive predictive values were relatively poor.

Conclusion: AKI was found to be a common complication in adults with imported *P. falciparum* necessitating RRT in only a small minority of patients. The use of the KDIGO staging allows early recognition of a decline in renal function.

BACKGROUND

Acute kidney injury (AKI) is associated with a risk of chronic kidney disease and high mortality (1-3). The clinical course of *Plasmodium falciparum* malaria may be complicated by AKI, contributing to the high mortality rate of severe malaria (4). The pathogenesis of AKI in malaria is still not clearly understood. Blockage of renal microcirculation due to sequestration of infected erythrocytes, immune-mediated glomerular injury and volume depletion are some of the proposed hypotheses (4, 5). The main histopathological finding in malaria-associated AKI is acute tubular necrosis (ATN) with reports of interstitial nephritis and glomerulonephritis (4-7). In malaria-endemic regions, AKI can occur in up to 40% of adult patients with severe *P. falciparum* malaria, and it is associated with a mortality as high as 75% when renal replacement therapy (RRT) is not started in time (8-11). In non-immune travellers with severe *P. falciparum* infection, AKI is reported to occur in 34 to 52% of cases (12-15). In these studies, AKI is usually defined according to the WHO criteria for severe malaria, in which the creatinine threshold is preset at 265 µmol/L (13-15). Using this relatively high threshold, less severe forms of renal failure are not taken into account. The present study, a retrospective analysis on a large cohort of patients with both severe and non-severe imported *P. falciparum* malaria, was designed to include these milder forms of renal failure in the analysis. To detect these, the criteria set by the Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group, in which the dynamics of the GFR changes are taken into account, were used to define renal failure (16). The study further aimed to determine which parameters are associated with the development of AKI.

METHODS

Patients

The Harbour Hospital is a 161-bed general hospital, located in Rotterdam, The Netherlands. It also harbours the Institute for Tropical Diseases, which serves as a national referral centre. The Rotterdam Malaria Cohort consists of all patients diagnosed with malaria at the Institute for Tropical Diseases since 1998. Of all patients, anonymized demographic, clinical and laboratory data are routinely collected and stored in an electronic database. For the present study, data from patients with imported *P. falciparum* malaria who entered the Rotterdam Malaria Cohort before January 1st, 2015 were analysed retrospectively. At that moment, the Rotterdam Malaria Cohort comprised 690 cases of imported malaria, of

which 491 were caused by *P. falciparum*. Six cases were excluded because of missing creatinine values at initial presentation, leaving 485 patients for the analysis. The six excluded cases had a favourable outcome and did not require RRT. As this is a retrospective study of patient files, ethical approval was not necessary, as stated in the Medical Research involving Human Subjects Act (WMO).

Laboratory investigations

All laboratory data were measured on admission with routine procedures, as described before (17). The standard procedure to diagnose malaria comprised a Quantitative Buffy Coat (QBC) analysis, a rapid diagnostic test (RDT) for malaria antigens (Binax NOW® Malaria Test Binax, Inc. Maine, USA), and thick and thin blood smears using freshly collected blood specimens from finger pricks. The RDT and the QBC analysis were performed according to the manufacturer's instructions. QBC capillaries were examined independently by two technicians by microscopic analysis of two complete rows of the region between the bottom of the capillary and the polynuclear leukocyte layer using an Olympus BX-60 fluorescence microscope equipped with UV-filter, 50x objective and 12.5x oculars (total magnification 625x).

Definitions

Acute Kidney Injury

Acute kidney injury (AKI) was defined using the KDIGO criteria, which include three stages of progressive renal dysfunction (*see* Supplementary Table 1) (16).

Since patients may present with renal dysfunction without a known baseline serum creatinine, a classification based on a change of creatinine or estimated glomerular filtration rate (eGFR) from baseline poses a significant limitation. To circumvent this problem, the following method, which was previously described as a suitable method by the ADQI Group (18), was used: in patients without previously measured creatinine levels, a normal premorbid renal function was assumed. A baseline creatinine level was then calculated, using the four-variable modification of diet in renal disease (MDRD) formula with an assumed normal eGFR. These calculated baseline values were used to estimate the change of renal function during infection (*see* Supplementary Table 2).

Severe malaria

Patients were classified as having severe *P. falciparum* malaria if they met the recently updated World Health Organization (WHO) criteria for severe malaria on admission or during hospitalization (*see* Supplementary Table 3) (19).

Immunity to malaria

Adult immigrants from a malaria-endemic country living in The Netherlands were considered partially immune, as they had likely been exposed to *P. falciparum* during childhood. Patients who had been born and raised and were still living in a malaria-endemic area at the time of diagnosis were presumed semi-immune. However, given the low number of semi-immune persons in the Rotterdam Malaria Cohort, they were grouped with partially immune individuals for the statistical analysis. All other patients were considered non-immune.

Statistical analysis

All data were extracted from the original database. For statistical analysis IBM Statistical Package for the Social Sciences (SPSS) version 22 (IBM Inc, Chicago, IL, USA) was used. Differences between the groups "AKI" and "no AKI" were analysed using Fisher's exact test or Chi square test for nominal variables. For continuous variables the unpaired two sample t-test or the Mann-Whitney U test was used. Normality of distribution of variables was tested with the Kolmogorov-Smirnov test. Univariate and multivariate logistic regression analyses were used to identify risk factors for AKI in malaria at initial presentation. Based on the univariate analysis, variables with a *P*-value <0.10 were included in the multivariate logistic regression. The details of the univariate and multivariate regression analysis are available as supplementary files (*see* Supplementary Table 4 and 5). For the variables that were significant in the multivariate analysis, the diagnostic performance for detecting KDIGO-defined AKI was evaluated, using the odds ratio, sensitivity, specificity, negative and positive predictive value at the optimal cut off point (determined using the Youden's index) and the area under the receiver operating characteristics curve (AUROC). All reported *P*-values are two-tailed, and *P*-values less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics and incidence of AKI

The general characteristics on admission of the 485 included patients are shown in Table 1. Fifty-nine patients were treated as outpatient or finished their treatment at home, all other patients were treated as inpatients. None of the patients had serum creatinine levels measured in our hospital prior to admission; the premorbid creatinine levels were therefore calculated according to the described method to subsequently apply the KDIGO classification. AKI, defined as a KDIGO

stage 1, 2 or 3, was seen in 39 (8%) of all 485 patients with imported *falciparum* malaria and in 23 (38%) of the 61 patients with severe malaria. Immunity differed significantly between the groups with and without AKI, with non-immunes being over-represented in the AKI group. The use of potentially nephrotoxic co-medication (non-steroidal anti-inflammatory drugs (NSAIDs), ACE-inhibitors, diuretics or lithium) did not differ significantly between groups. Pulse rate was significantly higher in the AKI group and the systolic blood pressure was significantly lower in comparison to patients without AKI. Apart from creatinine and urea, also leukocytes, C-reactive protein (CRP), creatinine, total bilirubin, lactate, lactate dehydrogenase (LDH), alanine transaminase (ALAT), aspartate transaminase (ASAT) and parasite load levels were all significantly higher in the AKI group, while thrombocytes and serum sodium levels were significantly lower. There was no significant difference in the number of patients with circulatory shock between the two groups (three in the AKI group versus 13 in the no-AKI group. Severe malaria was significantly more common in the AKI group, as might be anticipated since AKI is one of the defining criteria for severe malaria (*see* Supplementary Table 3). There were two deaths, both in the AKI group.

On admission, a concomitant infection was suspected in 64 patients and proven in 31 patients. Fourteen of these concerned a bacterial infection, of which 5 were in the AKI group (two with a pneumonia and three with an urinary tract infection). One of these had a positive blood culture with an *Escherichia coli.* All other blood cultures remained negative.

Deterioration of kidney function during admission

In 27 (69%) of the 39 patients with AKI, kidney injury was already present at presentation; in the other twelve the initial KDIGO stage was 0. A deterioration of kidney function, defined as a progression of KDIGO stage during admission, was seen in 17 (44%) of the 39 AKI patients. Eight patients eventually needed RRT; one of these patients had a normal renal function at initial presentation (Table 2). The maximal creatinine level was reached after a median of
0.3 days (range 0 – 3.4 days) in non-RRT patients. In the 8 RRT patients, creatinine levels rose maximally with 414 µmol (median 209 µmol) after a median of 1.3 days (range 0 – 3.4 days) before they were referred for dialysis.

KDIGO stage versus WHO defined malarial AKI

The 2014 WHO definition for renal failure, as a criteria for severe malaria, uses the serum creatinine level at a cut-off of 265 µmol/L. At initial presentation, this

criterium was met by 19 of the 485 patients (4%) or 31% of patients with WHO defined severe malaria (19/61). Among these 19 patients are 5 of the 8 patients (63%) that eventually needed RRT. Using the KDIGO classification, 7 of these 8 patients (89%) would have been identified; two classified as stage 2 and five as stage 3 at presentation.

Predictors for the outcome AKI

Multivariate logistic regression analysis revealed that age, leucocytes and thrombocytes were independently associated with AKI. However, they all had relatively poor positive predictive values for AKI despite acceptable negative predictive values (Table 3). Creatinine and urea were not entered in the multivariate analysis, as they belong to the defining criteria for AKI.

Characteristics and outcome patients needing RRT

Eight patients were referred for RRT. Their median age was 52.5 (range 36 - 62) and 3 (38%) were female. Half of the patients were non-immune and half partially immune. All were admitted in the ICU and received intravenous treatment. Indications for dialysis were acidosis (4/8 patients), uremia (2/8 patients) and fluid overload (2/8 patients). None of the patients was hyperkalemic.

All patients met more than one of the WHO severity criteria during admission; two met two criteria, one three, two four, one five and two met nine severity criteria.

In five of the eight RRT patients (63%) renal function recovered fully during admission, but two patients progressed to end-stage renal disease (ESRD). In one of them the renal biopsy showed severe ischemic changes and a tubulo-interstitial nephritis with advanced scarring. She switched from hemodialysis to peritoneal dialysis and eventually received a renal transplantation. In the other patient with ESRD the renal biopsy remarkably showed a collapsing glomerulopahy, for which other causes were excluded. She is currently still on hemodialysis and is being prepared for a transplantation. Her case was described in detail previously (20). One patient died of a suspected central pulmonary embolism two weeks after admission, while still on intermittent hemodialysis for the malaria-induced AKI.

DISCUSSION

AKI is a frequently observed complication of malaria in adults, both in malaria-endemic countries and in non-endemic regions (5-15). The overall prevalence of AKI, defined according to the KDIGO classification, in the Rotterdam Malaria Cohort was 8% in all patients with *P. falciparum* infection and 38% in patients with severe disease. Age, leucocytes and thrombocytes were found to be independently associated with AKI.

Some authors report AKI incidence rates as high as 52.5% in patients with imported malaria (15) whereas others found incidences as low as 1% (21-23). There are several explanations for this large variation. Most importantly, different definitions of AKI have been used. Many studies on malaria in endemic regions use the WHO criteria for severe malaria, where the serum creatinine threshold for AKI is preset at 265 µmol/L. Other studies have used the RIFLE (acronym for risk, injury, failure, loss of renal function and end stage renal disease) staging (23, 24). In the current study the KDIGO criteria were used. The optimal serum creatinine threshold for the detection of AKI, defined as a KDIGO stage 1 or higher, was found to be 120 µmol/L, which is much lower than the threshold used in the WHO criteria. The current consensus is to define AKI according to the KDIGO criteria.

Variation in incidence rates can also be explained by differences in the patient populations that are analysed. Many studies have reported the incidence of AKI in cohorts of patients with *P. falciparum* only (10-14, 21, 24), while some used cohorts with malaria caused by any *Plasmodium* species (22, 23, 25, 26), and several studies report on specific subsets of patients like patients with WHO defined severe *P. falciparum* infection (10, 12-14, 24). The immune status of the patient may also influence the risk of AKI. In previous studies it was shown that non-immune individuals with imported *P. falciparum* malaria have a higher risk of severe malaria and its complications including AKI, than partially- or semi-immune subjects (5, 7, 11, 27). In the present study, non-immune patients were overrepresented in the AKI group.

It was previously shown that in AKI in severe malaria a return to baseline creatinine occurs after 17 ± 6 days (10). Most patients with AKI do not require RRT and a return to baseline kidney function is achieved in the vast majority of patients in response to antimalarial therapy and fluid resuscitation alone (5, 10, 28). In line with these observations, only 8 patients of the Rotterdam Malaria Cohort needed

RRT, and 5 of these 8 patients had a full recovery of their renal function during admission. However, the long-term prognosis of recovered AKI patients has been a matter of debate. A meta-analysis of Coca *et al* showed that patients with AKI are more likely to develop chronic kidney disease later on (CKD) (1). As 3 of the 8 RRT patients in our cohort were not residents of the Netherlands and returned to their home countries not long after being discharged, we are not informed about whether or not their recovered renal functions remained stable in the long term.

The most important limitation of this study is that, in the absence of data on premorbid creatinine levels, assumptions had to be made about the baseline kidney function of the included patients. Although these assumptions might be wrong in individual cases, there is no obvious reason to assume that the baseline kidney functions of the patients in this cohort, which consists mostly of international travellers with little comorbidity, are in general any different from the reference values. The method has been proposed as a suitable option in case of missing baseline renal function by the Acute Dialysis Quality Initiative (ADQI) Group and has been used before in similar studies (18, 29). Still, the possibility remains that AKI was overdiagnosed, as some patients may have had some form of chronic kidney disease before admission with malaria. Also, we did not have information about comorbidity available for all patients in the database. The presence of comorbid risk factors might have contributed to the development of AKI in some patients. Another limitation is that follow-up creatinine levels were not available in the majority of patients who had a normal admission creatinine. We cannot exclude that some of these patients developed AKI later on, but, as this concerns patients with mild disease who were discharged after a short admission, we do not expect AKI to be a common complication in this group.

If AKI is detected early, measures can be taken to prevent further deterioration of kidney function and the development of associated complications. These measures include timely institution of proper parenteral antimalarial treatment, fluid replacement, avoidance of nephrotoxic drugs and, if indicated, RRT (5, 9, 10, 28). The use of the KDIGO classification allows for an early detection of changes in GFR, but nevertheless 12 of 39 AKI (31%) patients in the Rotterdam Malaria Cohort had no evidence of kidney injury at first presentation. This observation emphasises that the use of serum creatinine-based prediction models for AKI has limitations. In several animal model studies it has been shown that functional injury of the kidney, reflected in serum creatinine values, is preceded by structural injury (30). Prediction of AKI in malaria could theoretically become more accurate with the

use of markers of structural kidney injury, such as the novel biomarkers Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Kidney Injury Molecule-1 (KIM-1). Recent studies, performed in a broad range of experimental and clinical settings including cardiac surgery, kidney transplantation, contrast-induced AKI and critically ill patients, show that the use of these markers may improve risk assessment (30-35). A study in adult malaria patients in Bangladesh showed that NGAL was not superior to creatinine to predict the requirement of RRT, but patients in this study generally presented severely ill and more than half of them already had a decreased eGFR at admission (36). One could speculate that findings in imported malaria, where patients usually tend to present much earlier in the course of the infection, could be different.

CONCLUSION

AKI is a common complication in adults with imported *P. falciparum* infection, occurring in 8% of cases and in 38% of the patients with severe malaria. RRT was necessary in 8 of the 39 patients with AKI. The use of the KDIGO staging allows an earlier recognition of a decline in renal function in imported malaria as compared to the WHO criteria, but more research is needed to determine whether novel biomarkers can help to detect AKI in an earlier stage.

Table 1. General characteristics on admission

	AKI n=39	No AKI n=446	P-value
Demographic findings			
Age, years	47 (26-70)	39 (4-78)	<0.001
Male gender	27 (69)	325 (73)	0.708
Ethnicity			**0.078**
Caucasian	24 (62)	189 (42)	0.028
African	11 (28)	218 (49)	0.018
Asian	2 (5)	14 (3)	0.374
Other	2 (5)	22 (5)	1.000
Adequate prophylaxis use	0 (0)	42 (9)	0.052
Symptoms ≥ 8 days	16 (41)	111 (25)	0.055
Immunity			**0.038**
Non-immune	28 (74)	218 (53)	0.007
Partially immune	10 (26)	188 (45)	0.060
Semi-immune	0 (0)	9 (2)	1.000
Nephrotoxic co-medication*	5 (20)	24 (5)	0.073
Clinical findings			
Temperature °C	38.5 (1.3)	38,4 (1.3)	0.852^
Systolic blood pressure, mmHg	114 (21.3)	123 (18.3)	0.017^
Pulse rate beats/minute	104 (20.1)	94(17.3)	0.003^
Glasgow Coma Scale	15 (5-15)	15 (9-15)	<0.001
Laboratory findings			
Hemoglobin, mmol/L	8.1 (1.8)	8.3 (1.3)	0.929^
Leucocytes, x 10^9/L	7.5 (3.2)	5.2 (2.0)	<0.001^
Thrombocytes, x 10^9/L	29 (2-188)	91(3-385)	<0.001
CRP, mg/L	185 (16-476)	91 (1-363)	<0.001
Creatinine, µmol/L	166 (77-1,081)	93 (39-175)	<0.001

Table 1. Continued

	AKI n=39	No AKI n=446	P-value
Urea, mmol/L	13.8 (4.1-55.8)	5.0 (1.5 -33.6)	<0.001
Sodium, mmol/L	131 (6.2)	135 (4.0)	<0.001^
Potassium, mmol/L	3.7 (0.6)	3.8 (0.4)	0.422^
Bilirubin total, µmol/L	52 (10-416)	22 (3-304)	<0.001
ASAT, U/L	96 (9-394)	32 (9-326)	<0.001
ALAT, U/L	68 (12-655)	36 (3-265)	<0.001
LDH, U/L	488 (127-2,297)	268 (103-1,833)	<0.001
Lactate, mmol/L	3.0 (1.0-6.2)	1.4 (0-5.5)	<0.001
Parasitemia , parasites/µL	128,000 (240-1,380,600)	8,400 (2-784,000)	<0.001
Concomitant bacterial infection	5 (13)	9 (2)	0.003
Treatment			**<0.001**
quinine iv	19 (49)	54 (12)	<0.001
artesunate iv	13 (33)	27 (6)	<0.001
atovaquone / proguanil	4 (10)	279 (63)	<0.001
halofantrine	1 (3)	54 (12)	0.108
artemeter / lumefantrine	0 (0)	4 (1)	1.000
unknown	2 (5)	28 (6)	1.000
Outcome			
Severe malaria (WHO 2014)	29 (74)	32 (11)	<0.001
Renal replacement therapy	8 (21)	0 (0)	<0.001
Death	2 (5)	0 (0)	0.006

*^ Normally distributed continuous variables, given as mean (standard deviation) with P-values derived from T-tests. All other continuous variables are not Normally distributed and given as median (range) with P-values derived from Mann Whitney U tests. Nominal variables are given as number (percentage). P-values <0.05 are considered significant. * Self-reported use of non-steroidal anti-inflammatory drugs (NSAIDs), ACE-inhibitors, diuretics or lithium*

Table 2. KDIGO score at initial presentation in relation to worst score during admission

	Worst KDIGO stage				
	0	**1**	**2**	**3**	**Total**
Initial KDIGO stage 0	446	8	2	2	458
1		9	3	0	12
2			6	2	8
3				7	7
Total	446	17	11	11	485

Table 3. Diagnostic performance of variables that are independently associated with AKI

Variable	Age	Thrombocytes	Leukocytes	Creatinine
P-value	0.002	0.005	0.005	reference
Cut-off	>43 years	≤32 x 10^9/L	>6.1 x 10^9/L	120 µmol/L
Sensitivity	70 (54-83)	62 (45- 77)	62 (45-77)	85 (70 - 94)
Specificity	63 (59-68)	93 (90- 95)	74 (70-78)	92 (89 - 94)
PPV	15 (10-20)	43 (30- 57)	17 (11-24)	48 (36- 60)
NPV	96 (93-98)	97(94- 98)	96 (93-98)	99 (97-100)
AUROC	0.69 (0.65-0.73)	0.80 (0.77-0.84)	0.73 (0.69-0.77)	0.90 (0.88-0.93)

Supplementary Table 1.

KDIGO criteria

Stage	Serum creatinine	Urine output
1	1.5–1.9 times baseline OR ≥0.3 mg/dl (≥26.5 mmol/l) increase	< 0.5 ml/kg/h for 6–12 hours
2	2.0–2.9 times baseline	< 0.5 ml/kg/h for ≥12 hours
3	3.0 times baseline OR Increase in serum creatinine to ≥4.0 mg/dl (≥353.6 mmol/l) OR Initiation of renal replacement therapy OR, In patients <18 years, decrease in eGFR to <35 ml/min per 1.73 m2	<0.3 ml/kg/h for ≥24 hours OR Anuria for ≥12 hours

Table adapted from [16]

Supplementary Table 2.

Estimated baseline serum creatinine levels in mg/dL (and in μmol/L within brackets)

Age (years)	Black males	Other males	Black females	Other females
20–24	1.5 (133)	1.3 (115)	1.2 (106)	1.0 (88)
25–29	1.5 (133)	1.2 (106)	1.1 (97)	1.0 (88)
30–39	1.4 (124)	1.2 (106)	1.1 (97)	0.9 (80)
40–54	1.3 (115)	1.1 (97)	1.0 (88)	0.9 (80)
55–65	1.3 (115)	1.1 (97)	1.0 (88)	0.8 (71)
>65	1.2 (106)	1.0 (88)	0.9 (80)	0.8 (71)

Estimated glomerular filtration rate = 75 (mL/min per 1.73 m2) = 186 × (serum creatinine [sCr]) - 1.154 × (age) - 0.203 × (0.742 if female) × (1.210 if black) = exp(5.228 - 1.154 × In [sCr]) - 0.203 × In(age) - (0.299 if female) + (0.192 if black). Adapted from Bellemo et al. [18]

Supplementary Table 3.
WHO criteria for severe malaria (2014)

2014 WHO malaria severity criteria	
For epidemiological and research purposes, severe malaria is defined as one or more of the following, occurring in the absence of an identified alternative cause, and in the presence of P. falciparum asexual parasitemia:	
Impaired consciousness	A Glasgow Coma Score <11 in adults or a Blantyre coma score <3 in children
Acidosis	A base deficit of >8 meq/L or, if unavailable, a plasma bicarbonate of <15 mmol/L or venous plasma lactate >5 mmol/L. Severe acidosis manifests clinically as respiratory distress – rapid, deep and laboured breathing
Hypoglycemia	Blood or plasma glucose <2.2 mmol/L (<40 mg/dL)
Severe malarial anemia	A hemoglobin concentration <5 g/dL or a hematocrit of <15% in children <12 years of age (<7 g/dL and <20%, respectively, in adults) together with a parasite count >10,000/μL
Renal impairment (acute kidney injury)	Plasma or serum creatinine >265 μmol/L (3 g/dL) or blood urea >20 mmol/L
Jaundice	Plasma or serum bilirubin >50 μmol/L (3 mg/dL) together with a parasite count >100,000/μL
Pulmonary oedema	Radiologically confirmed, or oxygen saturation <92% on room air with a respiratory rate >30/min, often with chest indrawing and crepitations on auscultation
Significant bleeding	Including recurrent or prolonged bleeding from nose gums or venepuncture sites; hematemesis or melena
Shock	Compensated shock is defined as capillary refill ≥3 sec or temperature gradient on leg (mid to proximal limb), but no hypotension. Decompensated shock is defined as systolic blood pressure <70 mm Hg in children or <80 mm Hg in adults with evidence of impaired perfusion (cool peripheries or prolonged capillary refill)
Hyperparasitemia	*P. falciparum* parasitemia >10%. A 4% parasitemia in non-immune children or adults should be considered an indicator of high risk requiring supervised management but not by itself a criterion of severe malaria

Table adapted from WHO 2014 criteria for severe malaria [19]

Supplementary Table 4.
Descriptive statistics of diagnostic accuracy of various parameters at initial presentation for acute kidney injury (AKI)

Parameter	Cut-off value	Sensitivity	Specificity	PPV	NPV	AUROC	Youden	P-value*
Creatinine	>120	85 (70 - 94)	92 (89 - 94)	48 (36- 60)	99 (97-100)	0.90 (0.88-0.93)	0.765	reference
Age	>43	70 (54-83)	63 (59-68)	15 (10-20)	96 (93-98)	0.69 (0.65-0.73)	0.332	<0.0001
Platelets	≤32	62 (45- 77)	93 (90- 95)	43 (30- 57)	97(94-98)	0.80 (0.77-0.84)	0.543	0.0996
Leucocytes	>6.1	62 (45-77)	74 (70-78)	17 (11-24)	96 (93-98)	0.73 (0.69-0.77)	0.354	0.0004
CRP	>141	78 (62 - 90)	74 (69-78)	20 (14-28)	98 (95- 99)	0.78 (0.74-0.82)	0.519	0.0347
Sodium	≤130	56 (40-72)	88 (85-91)	30 (20- 42)	96 (93- 98)	0.74 (0.70-0.78)	0.450	0.0125
Bilirubin	>35	76 (59 - 88)	79 (75 - 83)	24 (17 - 33)	97 (95 - 99)	0.81 (0.78-0.85)	0.550	0.4778
Urea	>6.8	90 (76 - 97)	83 (79 - 87)	32 (24 - 42)	99 (97-100)	0.91 (0.88-0.93)	0.729	0.8990
Lactate	>1.6	94 (79 - 99)	21 (14 - 28)	21 (14 - 28)	99 (96-100)	0.85 (0.81-0.89)	0.561	0.4778
LDH	>338	78 (61 - 90)	74 (70 - 78)	20 (14 - 27)	98 (95 - 99)	0.81 (0.78-0.85)	0.518	0.0437
ALAT	>49	76 (60-89)	69 (64-73)	17 (12- 24)	97 (95- 99)	0.74 (0.70-0.78)	0.449	0.0110
ASAT	>58	76 (59- 88)	79 (75- 83)	23 (16- 32)	98 (9 - 99)	0.81 (0.77-0.84)	0.548	0.1228
Parasitemia	>101,400	59 (42-74)	84 (80-87)	25 (16-34)	96 (93-98)	0.74 (0.70-0.78)	0.429	0.0101

*Data are given as mean (95% confidence interval). PPV = positive predictive value, NPV = negative predictive value, AUROC = Area Under the ROC curve. Youden = Youden's index. *P-values of pair-wise comparison of Area Under ROC curves are given (with creatinine ROC curve as comparator).*

Supplementary Table 5.
Multivariate logistic regression analysis of predictors for outcome Acute Kidney Injury (AKI) in imported *P. falciparum* malaria

Variable		P-value	Odds ratio (CI 95%)
Age	years	0.002	1.076 (1.028-1.127)
Immunity	Non- or partial	0.357	0.837 (0.573-1.223)
Systolic blood pressure	mm Hg	0.336	0.987 (0.961-1.014)
Pulse rate	beats/min	0.182	1.022 (0.990-1.056)
Glasgow coma score	EMV score	0.091	0.576 (0.304-1.093)
Thrombocytes	x 10^9/L	0.005	0.976 (0.959-0.993)
Leucocytes	x 10^9/L	0.005	1.396 (1.106-1.762)
CRP	mg/L	0.876	1.001 (0.993-1.008)
Sodium	mmol/L	0.406	0.954 (0.852-1.067)
Bilirubin total	µmol/L	0.275	1.007 (0.995-1.018)
LDH	U/L	0.441	0.999 (0.996-1.002)
ALAT	U/L	0.706	0.998 (0.987-1.009)
ASAT	U/L	0.137	1.009 (0.997-1.020)
LogParasitemia	parasites/uL	0.496	0.819 (0.461-1.456)

95% CI = 95% confidence interval (CI). Significant outcomes are given in bold.

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CHAPTER 9

Neutrophil Gelatinase-Associated Lipocalin (NGAL) predicts the occurrence of malaria-induced acute kidney injury

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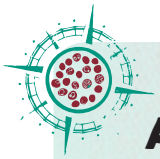


ABSTRACT

Background: Acute kidney injury (AKI) is a frequently encountered complication of imported *Plasmodium falciparum* infection. Markers of structural kidney damage have been found to detect AKI earlier than serum creatinine-based prediction models but have not yet been evaluated in imported malaria. This pilot study aimed to explore the predictive performance of Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Kidney Injury Molecule-1 (KIM-1) for AKI in travellers with imported P *falciparum* infection.

Methods: Thirty-nine patients with imported *P. falciparum* malaria from the Rotterdam Malaria Cohort with available serum and urine samples at presentation were included. Ten of these patients met the criteria for severe malaria. The predictive performance of NGAL and KIM-1 as markers for AKI was compared with that of serum creatinine.

Results: Six of the 39 patients (15%) developed AKI. Serum and urine NGAL and urine KIM-1 were all found to have large area's under the receiver operating characteristic curves (AUROC's) for predicting AKI. Urine NGAL was found to have an excellent performance with a positive predictive value (PPV) of 1.00 (95% CI 0.54-1.00), a negative predictive value (NPV) of 1.00 (95% CI 0.89-1.00) and an AUROC of 1.00 (95% CI 1.00-1.00).

Conclusion: A good diagnostic performance of NGAL and KIM-1 for AKI was found. Particularly urine NGAL was found to have an excellent predictive performance. Larger studies are needed to demonstrate whether these biomarkers are superior to serum creatinine as predictors for AKI in *P. falciparum* malaria.

BACKGROUND

Malaria-induced acute kidney injury (AKI) is an important contributing factor to the high mortality of severe malaria (1). In endemic regions, AKI occurs in up to 40% of adult patients with severe *Plasmodium falciparum* infection and it is associated with high mortality (2-5). In Western countries, AKI is also a frequently encountered complication of imported *P. falciparum* infection. In a recent study in travellers with imported *P. falciparum* infection, AKI, defined according to the criteria set by the Kidney Disease: Improving Global Outcomes (KDIGO) AKI Work Group (6), was seen in 39 (8%) of all 485 patients and in 23 (38%) of the 61 patients with severe malaria (7). Although the KDIGO classification allows for an early detection of changes in glomerular filtration rate (GFR), the use of serum creatinine-based prediction models for AKI has limitations. Substantial rises in serum creatinine are only observed 48 to 72 hours after the initial injury to the kidney, and because of factors such as enhanced tubular creatinine secretion, significant renal injury can occur without an important rise of serum creatinine (8). This is illustrated by a recent study in which 12 (31%) of 39 patients with malaria-induced AKI had a normal serum creatinine at presentation (7). Because structural kidney injury precedes loss of renal function, a marker of structural kidney damage could theoretically detect AKI much earlier, in a way that has been compared to the role of troponin in the detection of myocardial injury (9).

A promising marker of structural kidney damage in AKI is Neutrophil Gelatinase-Associated Lipocalin (NGAL), also known as lipocalin 2. This protein was initially discovered in activated neutrophils and was later found to be produced by many tissues, including renal tubular epithelial cells. Under physiologic conditions, NGAL is present at very low concentrations in the urine and plasma (10, 11, 12). Studies in animals showed NGAL to be one of the most upregulated genes in the kidney very early after AKI. As a consequence of the induction of NGAL-expression in the distal nephron and reduced proximal tubular re-uptake due to tubular damage, both urine and plasma NGAL concentrations rise quickly in the event of AKI (10, 11). The value of NGAL as a predictor of AKI has been established in a large number of studies, predominantly performed in critically ill patients, after cardiac surgery and after kidney transplantation (11, 13, 14). In studies in endemic regions, urine NGAL has been shown to rise with increasing severity of AKI in severe malaria (2) but was not superior to creatinine in predicting in hospital requirement for renal replacement therapy (RRT) (15). However, NGAL has not yet been evaluated as a marker for AKI in patients with imported malaria.

Another frequently used biomarker for kidney injury is Kidney Injury Molecule-1 (KIM-1). KIM-1 is a transmembrane protein in tubular kidney cells that is undetectable in plasma of patients with undamaged kidneys. In damaged tubular epithelial cells undergoing dedifferentiation and proliferation, KIM-1 is induced and can become detectable in plasma and urine (16). The exact function of KIM-1 is unknown and reports have suggested its involvement in epithelial damage as well as in protection and restoration of the tubular epithelium (16). Urinary KIM-1 concentrations were found to increase much earlier than blood urea nitrogen and plasma creatinine in studies in which proximal tubular injury was induced by cadmium or ischemia (8, 17, 18). Its performance as an early marker for AKI has been evaluated in several clinical settings, including contrast-induced nephropathy and sepsis (19-21) but not during malaria.

The aim of this pilot study was to explore the predictive performance of NGAL and KIM-1 in AKI in travellers with imported *P. falciparum* infection.

METHODS

Patients

The Harbour Hospital is a 161-bed general hospital, located in Rotterdam, The Netherlands. It also harbours the Institute for Tropical Diseases, which serves as a national referral centre. The Rotterdam Malaria Cohort consists of all patients diagnosed with malaria at the Institute for Tropical Diseases since 1998. Of all these patients, anonymized demographic, clinical and laboratory data are routinely collected and stored in an electronic database. On January 1st, 2015, the Rotterdam Malaria Cohort comprised 690 cases of imported malaria, of which 491 were caused by *P. falciparum*.

The present pilot study focussed on the diagnostic accuracy of serum NGAL (sNGAL), urine NGAL (uNGAL), and urine KIM-1 (uKIM-1) concentrations as predictive biomarkers for AKI. Patients with imported *P. falciparum* malaria that were included in the Rotterdam Malaria Cohort between February 2010 and February 2012 with available serum and urine samples at their initial presentation were included. The findings were compared with the performance of serum creatinine as a marker for AKI in *P. falciparum* malaria.

Laboratory investigations

All laboratory parameters were measured on admission with routine procedures, as described before (22). The standard procedure to diagnose malaria comprised a Quantitative Buffy Coat (QBC) analysis, a rapid diagnostic test (RDT) for malaria antigens (Binax NOW® Malaria Test Binax, Inc. Maine, USA), and thick and thin blood smears using freshly collected blood specimens from finger pricks. The RDT and the QBC analyses were performed according to the manufacturer's instructions (23, 24). QBC capillaries were examined independently by two technicians by microscopic analysis of two complete rows of the region between the bottom of the capillary and the polynuclear leukocyte layer using an Olympus BX-60 fluorescence microscope equipped with UV-filter, 50× objective and 12.5× oculars (total magnification 625×).

Measurements of sNGAL, uNGAL and uKIM-1 were done using residual patient material in November 2012. Samples of serum and urine had been collected at initial presentation and stored at -80 °C until analysis with a maximum storage time of two years and nine months, during which stability of NGAL and KIM-1 levels can be assumed (25). sNGAL, uNGAL and uKIM-1 were quantitatively determined with commercially available sandwich ELISA kits (NGAL Quantikine and KIM-1 Quantikine kits from R&D Systems, Inc., Minneapolis, USA).

Definitions

Acute Kidney Injury

AKI was defined using the KDIGO criteria, which include three stages of progressive renal dysfunction (see Supplementary Table 1) (6). Since patients may present with renal dysfunction without a known baseline serum creatinine, a classification based on a change of creatinine or estimated GFR (eGFR) from baseline poses a significant limitation. To circumvent this problem, the following method, which was previously described as a suitable method by the ADQI Group (26), was used: in patients without previously measured creatinine concentrations, a normal pre-morbid renal function was assumed. A baseline, creatinine concentration was then calculated, using the four-variable modification of diet in renal disease (MDRD) formula with an assumed normal eGFR. These calculated baseline values were used to estimate the change of renal function during infection (see Supplementary Table 2).

Severe malaria

Patients were considered as having severe *P. falciparum* malaria if they met the recently updated World Health Organization (WHO) criteria for severe malaria on admission or during hospitalization (see Supplementary Table 3) (27).

Statistical analysis

All data was extracted from the original secured database and reviewed for inconsistencies and missing values. For statistical analysis, IBM Statistical Package for the Social Sciences (SPSS) version 22 (IBM, inc, Chicago, IL, USA) was used. Differences between the groups having AKI or no AKI were analysed using the Fisher's exact test or Chi square test for nominal variables. For continuous variables the Mann-Whitney U test was used. The diagnostic performance of sNGAL, uNGAL, the ratio between uNGAL and urine creatinine (uNGAL/uCreat) and the ratio between uKIM-1 and urine creatinine (uKIM-1/uCreat) was reported as sensitivity, specificity, positive and negative predictive value for AKI with their corresponding 95% confidence intervals (with the use of MedCalc version 15.6). Of each parameter a Receiver Operating Characteristics (ROC) curve was constructed as a summary statistic and the area under the ROC curve (AUROC) and its corresponding 95% confidence intervals were calculated. The AUROC of each of the variables was compared to that of reference comparator variable serum creatinine in a pair-wise comparison using the method of Hanley and McNeil (28). Optimal cut-off points were determined using the Youden index. All reported *P*-values are two-tailed. *P*-values less than 0.05 were considered statistically significant.

Ethics approval and consent to participate

Ethical clearance for the storage and use of residual serum and urine samples for scientific purposes was granted by the Medical Ethics Review Board of the Erasmus MC, University Medical Center Rotterdam, the Netherlands (MEC 2012-047).

RESULTS

Patient characteristics and AKI at presentation and during admission

During the study period, 50 patients with *P. falciparum* malaria were included in the Rotterdam Malaria Cohort. Ten of these patients met the criteria for severe malaria and seven had AKI. Of 39 of these patients, including the ten patients with severe malaria and six of the patients with AKI, residual urine and serum samples

were available for analysis. The general characteristics on admission of these 39 patients are shown in Table 1.

Six patients (15%) developed AKI, defined as KDIGO stage 1, 2 or 3, during the course of their infection. In four of these patients (67%) AKI was already identified on admission; one patient was classified in KDIGO stage 1, two in stage 2 and one in stage 3. Two of these patients later progressed to a more advanced KDIGO stage. Two patients did not meet the KDIGO criteria at presentation but progressed during admission to KDIGO stage 3 (see Table 2).

The non-AKI group contained more men than the AKI group but other demographic findings did not differ significantly. Pulse rate, parasitaemia and serum concentrations of creatinine, urea, lactate, sNGAL, uNGAL and uKIM-1 were significantly higher in the AKI group. Serum sodium and potassium concentrations were significantly lower in the AKI group. The ratios uNGAL/sNGAL and of uNGAL/uCreat were significantly higher in the AKI patients. Intensive care unit admission was significantly more common in the AKI group. Since AKI is one of the defining criteria for severe malaria [23] severe malaria was also more common in the AKI group.

Identification of AKI by NGAL and KIM-1

Five out of six patients with AKI presented with elevated sNGAL. uNGAL was elevated in all six (Table 2). In contrast, only one of the AKI patients showed elevated uKIM-1 at presentation. All patients that were classified as KDIGO stage 3, either at presentation or later during admission, already showed elevated concentrations of both sNGAL and uNGAL at presentation.

In seven patients, high concentrations of sNGAL, uNGAL or uKIM-1 on admission were found despite a seemingly normal kidney function throughout their admission; one had elevated sNGAL, uNGAL and uKIM-1, two patients showed elevated sNGAL and uNGAL with normal uKIM-1, three patients presented with only an elevated uNGAL, and in one patient only uKIM-1 was elevated. These data are available as a supplementary file (Supplementary Table 4).

Diagnostic performance of sNGAL, uNGALand uKIM-1 at presentation for AKI

Analysis of the diagnostic accuracy of sNGAL, uNGAL, uKIM-1 and the ratios of uNGAL and uKIM-1 to urine creatine was performed by pair-wise comparison of

the AUROCs of these parameters to the AUROC of serum creatinine. All parameters showed large AUROCs. However, none of these differences were statistically significant when compared to the AUROC of creatinine, likely due to the small sample size of this study. uNGAL was found to have an excellent performance with PPV of 1.00 (95% CI 0.54-1.00), an NPV of 1.00 (95% CI 0.89-1.00) and an AUROC of 1.00 (95% CI 1.00-1.00; Table 3). The ROC curves of serum creatinine, sNGAL, uNGAL, uKIM-1 are available as a figure (see Supplementary Figure 1).

Diagnostic performance sNGAL, uNGAL and uKIM-1 at presentation for KDIGO stage 3

Analysis of the diagnostic accuracy of creatinine, NGAL and KIM-1 for the most severe form of AKI, KDIGO stage 3, was also performed. uNGAL (0.99, 95% CI 0.88-1.0) and sNGAL (0.97, 95% CI 0.86-1.00) were found to perform excellently. The AUROC of creatinine was found to be 0.75 (95% CI 0.59-0.87; p). Again, differences were not statistically significant (see Supplementary Table 5).

Renal replacement therapy in relation to sNGAL, uNGAL and uKIM-1 at presentation

Three patients developed a need for RRT. One of these patients did not classify as AKI on admission but later progressed to KDIGO stage 3, one was in KDIGO stage 2 and one in stage 3. However, all three presented with elevated sNGAL and uNGAL and one also showed an elevated urine KIM-1 on admission.

Follow-up measurements during admission

For two patients, sNGAL and uNGAL concentrations were available for follow-up during admission. Both patients showed elevated uNGAL at initial presentation, while serum creatinine concentrations were still normal (Supplementary Figure 2). One of the patients (patient 1, Supplementary Figure 2) started RRT on admission day 2, the other patient did not need RRT. No major changes of sNGAL concentrations were seen during admission.

DISCUSSION

In the present pilot study it is demonstrated that sNGAL and uNGAL have an excellent predictive performance for malaria-induced AKI.

Biomarkers for the early detection of AKI are under intense investigation and studies have revealed promising results in a broad variety of settings including

contrast-induced nephropathy, critically ill patients, cardiac surgery, and kidney transplantation (29-35). In several studies, both urine and serum NGAL concentrations were found to be superior to the serum creatinine concentration for the prediction of AKI (33, 36).

Early detection of AKI in malaria could help to prevent further deterioration of kidney function by appropriate fluid management, avoidance of nephrotoxic drugs and close monitoring to assess the need for RRT (3, 4, 37). However, very few studies on biomarkers for AKI have been performed in malaria. The value of uNGAL was previously studied in adult patients with severe *P. falciparum* infection in Bangladesh. uNGAL was shown to increase with increasing severity of AKI (2), but creatinine was found to be superior to uNGAL in predicting the need for RRT (15). However, patients in this study generally presented severely ill and more than half of them already had a decreased eGFR on admission. One could speculate that the benefit of an early marker for AKI can be larger in the very different population of travellers with imported malaria, who tend to present in a much earlier stage of the infection.

In the present pilot study on biomarkers of AKI in imported malaria, highly promising results were observed for uNGAL, which was able to identify all 6 AKI patients at initial presentation. Follow-up measurements in two AKI patients revealed that uNGAL increased before serum creatinine started to rise (Supplementary Figure 2). The AUROCs of sNGAL, uNGAL and uKIM-1 were large but, due to the very small sample size, the study was underpowered to demonstrate statistically significant differences. This is a major limitation of the present study and larger studies should be performed to demonstrate whether these biomarkers truly outperform serum creatinine.

The suggestion that uNGAL performs better than sNGAL, is an important discussion in literature. A meta-analysis conducted by Haase *et al.* showed that both serum and urine NGAL concentrations appear to perform similarly well and provide a relevant advantage as compared with serum creatinine (13). It deserves further investigation whether this conclusion also applies to the performance of serum and urine NGAL in imported *P. falciparum* malaria. These results are important since oliguria is a common event in malaria-induced AKI (37) and the use of urine NGAL for diagnosis of malaria-induced AKI therefore has its limitations. Another ongoing debate is whether urine biomarker concentrations are better reported as absolute values or as a ratio to creatinine concentration (38). In the present study, the absolute values seemed to perform somewhat better than the ratio.

Although uKIM-1 performed well when the cut-off level was set at 1.83 ng/ml, only one of the six patients with AKI had a uKIM-1 level above the upper limit of normal, which is 5.33 ng/ml, while all six patients had markedly raised uNGAL levels. The patient who did have an elevated uKIM-1 was already in KDIGO stage 3 at presentation. These findings raise the question whether the rise of KIM-1 levels occurs later in the course of early AKI than NGAL. A study in children undergoing cardiac surgery suggests that this might indeed be the case (39). However, this study was performed in a study population which was much different from the present, and more research is needed to confirm this finding in other clinical settings.

Several patients were found to have elevated sNGAL, uNGAL or uKIM-1 but did not progress to a KDIGO stage 1 or higher. The clinical significance of increased NGAL concentrations without detectable changes in creatinine, previously interpreted as a shortcoming of the biomarker, has been found to indicate subclinical AKI and to be of prognostic importance in critically ill adult patients (40). NGAL can be influenced by extra-renal production by neutrophils caused by factors like concomitant systemic inflammation or malignancy (41). However, it is unlikely that such bias influenced the findings in the present study to a large degree as there was no evidence of co-infection or malignancy in any of the patients. Follow-up of patients with elevated NGAL concentrations deserves further investigation, as this would increase insight into the correlation of NGAL and the resolution of AKI or the development of chronic kidney disease.

An important limitation of biomarkers like NGAL and KIM-1 is that their availability is usually limited to resource-rich settings where malaria is only seen as an imported disease; in the regions that carry the largest burden of malaria, laboratory diagnostics are often limited to basic measurements. Even in resource-rich settings, studies on the cost-effectiveness of these biomarkers are still to be done (42).

CONCLUSION

This pilot study reveals good diagnostic value of sNGAL, uNGAL and uKIM-1. Especially uNGAL was found to have an excellent predictive performance for AKI. Larger studies are needed to demonstrate whether these biomarkers are superior to serum creatinine as predictors for AKI in *P. falciparum* malaria.

Table 1. General characteristics

	AKI n=6	No AKI n=33	P-value
Demographic findings			
Age, years	43 (29-62)	46 (28-69)	0.89
Male gender	3 (50%)	30 (91%)	<0.05
Ethnicity			1.00
Caucasian	2 (33%)	9 (27%)	1.00
African	4 (67%)	22 (67%)	1.00
Asian	0 (0%)	2 (6%)	1.00
Adequate prophylaxis use	0 (0%)	0 (0%)	1.00
Symptoms ≥ 8 days	2 (33%)	6 (19%)	0.58
Risk factors for chronic kidney disease*	1 (17%)	4 (12%)	1.00
Nephrotoxic co-medication**	1 (17%)	4 (12%)	1.00
Clinical findings			
Temperature °C	39.5 (38.3-40.1)	38.4 (35.5-40.7)	0.11
Systolic blood pressure, mmHg	100 (73-140)	125 (95-170)	0.08
Pulse rate beats/minute	111 (100-140)	95 (56-129)	<0.05
Glasgow Coma Scale	15 (15-15)	15 (11-15)	0.54
Laboratory findings			
Creatinine, µmol/L	196 (77-1081)	96 (39-133)	<0.01
Urea, mmol/L	14.8 (9.1-55.8)	5.6 (2.7-33.6)	<0.001
Sodium, mmol/L	129 (120-136)	137 (127-141)	<0.01
Potassium, mmol/L	3.2 (2.9-3.9)	3.7 (3.3-4.5)	<0.05
Lactate, mmol/L	4.5 (1.9-5.8)	1.5 (0.6-4.6)	<0.001
Parasitemia, parasites/µL	386600 (45900-1380600)	39200 (64-678400)	<0.05
sNGAL, ng/ml	700 (174-2287)	91 (19-204)	<0.001

Table 1. Continued

	AKI n=6	No AKI n=33	P-value
uNGAL, ng/ml	5792 (376-22028)	9 (1-240)	<0.001
uKIM-1, ng/ml	3.9 (1.8-7.8)	1.2 (0.1-8.7)	<0.01
uNGAL/ sNGAL	10.1 (0.4-25.1)	0.1 (0.0-2.6)	<0.001
uNGAL/uCreatinine	815.0 (5.4-2172.7)	0.8 (0.1-42.5)	<0.001
uKIM-1/uCreatinine	0.04 (0.02-0.08)	0.1 (0.0-2.9)	0.04
Concomitant bacterial infection	1 (17%)	0 (0%)	0.15
Outcome			
Severe malaria (WHO 2014)	5 (83%)	5 (15%)	<0.01
Renal replacement therapy	3 (50%)	0 (0%)	<0.01
ICU admission	4 (67%)	4 (12%)	<0.05
Death	1 (17%)	0 (0%)	0.15

Continuous variables are given as median (range). Nominal variables are given as number (percentage). P-values <0.05 are considered significant.

** Hypertension (n=4), type 2 diabetes mellitus (n=1)*

*** Self-reported use of non-steroidal anti-inflammatory drugs (NSAIDs), ACE-inhibitors, diuretics or lithium*

Table 2. KDIGO stage at initial presentation and during admission in relation to serum and urine NGAL and urine KIM-1 for all six patients with AKI

KDIGO stage at presentation	Highest KDIGO stage during admission	RRT*	Serum NGAL (≤177 ng/ml)	Urine NGAL (≤72 ng/ml)	Urine NGAL/ Urine creat ratio	Urine KIM-1 (≤5.33 ng/ml)	Urine KIM-1/ Urine creat ratio
0	3	Yes	**899**	**22028**	1368.2	1.84	0.11
0	3	No	**501**	**8691**	2172.7	3.88	0.97
1	2	No	174	**376**	5.4	4.40	0.06
2	2	No	**2287**	**6452**	1955.1	3.92	1.19
2	3	Yes	**205**	**5132**	261.8	3.87	0.20
3	3	Yes	**2014**	**838**	72.1	**7.75**	0.67

*Bold: Elevated laboratory measurements. Reference ranges in brackets. *renal replacement therapy.*

Table 3. Descriptive statistics of diagnostic accuracy of various biomarker measurements at presentation for the presence or subsequent development of acute kidney injury (AKI)

Parameter and cut-off value^	Sensitivity	Specificity	PPV	NPV	Youden index	AUROC	P-value*
sCreatinine ≥ 128 µmol/L	0.83 (0.36-1.00)	0.97 (0.84-1.00)	0.83 (0.36-0.99)	0.97 (0.84-1.00)	0.80	0.85 (0.60-1.00)	
sNGAL ≥ 168 ng/ml	1.00 (0.54-1.00)	0.88 (0.72-0.97)	0.60 (0.26-0.88)	1.00 (0.88-1.00)	0.88	0.98 (0.93-1.0)	0.389
uNGAL ≥ 308 ng/ml	1.00 (0.54-1.00)	1.00 (0.89-1.00)	1.00 (0.54-1.00)	1.00 (0.89-1.00)	1.00	1.00 (1.00-1.00)	0.299
uNGAL/sNGAL ≥ 1.9	0.83 (0.36-1.00)	0.97 (0.84-1.00)	0.83 (0.36-1.00)	0.96 (0.84-1.00)	0.80	0.95 (0.85-1.00)	0.544
uNGAL/uCreat ≥ 5.1 ng/mmol	1.00 (0.54-1.00)	0.88 (0.72-0.97)	0.60 (0.26-0.88)	1.00 (0.88-1.00)	0.88	0.98 (0.93-1.00)	0.390
uKIM-1 ≥ 1.83 ng/ml	1.00 (0.54-1.00)	0.72 (0.54-0.87)	0.40 (0.16-0.68)	1.00 (0.86-1.00)	0.72	0.87 (0.75-0.99)	0.919
uKIM-1 /uCreat ≤ 0.085 ng/ mmol	1.00 (0.54-1.00)	0.58 (0.39-0.74)	0.30 (0.12-0.54)	1.00 (0.82-1.00)	0.58	0.76 (0.61-0.92)	0.810

*Data are given as mean (95% confidence interval). Optimal cut-off values were determined using the Youden index. PPV = positive predictive value, NPV = negative predictive value, AUROC = Area Under the ROC curve. *P-values of pair-wise comparison of Area Under ROC curves are given (with creatinine ROC curve as comparator).*

Supplementary Table 1.
KDIGO criteria

Stage	Serum creatinine	Urine output
1	1.5–1.9 times baseline OR ≥0.3 mg/dl (≥26.5 mmol/l) increase	< 0.5 ml/kg/h for 6–12 hours
2	2.0–2.9 times baseline	< 0.5 ml/kg/h for ≥12 hours
3	3.0 times baseline OR Increase in serum creatinine to ≥4.0 mg/dl (≥353.6 mmol/l) OR Initiation of renal replacement therapy OR, In patients <18 years, decrease in eGFR to <35 ml/min per 1.73 m2	<0.3 ml/kg/h for ≥24 hours OR Anuria for ≥12 hours

Table adapted from Khwaja et al (6). eGFR estimated glomerular filtration rate

Supplementary Table 2.

Estimated baseline serum creatinine concentration

Age (years)	African ethnicity, males µmol/L	Other males µmol/L	African ethnicity, females µmol/L	Other females µmol/L
20–24	133	115	106	88
25–29	133	106	97	88
30–39	124	106	97	80
40–54	115	97	88	80
55–65	115	97	88	71
>65	106	88	80	71

Estimated glomerular filtration rate = 75 (mL/min per 1.73 m2) = 186 × (serum creatinine [sCr]) - 1.154 × (age) - 0.203 × (0.742 if female) × (1.210 if black) = exp(5.228 - 1.154 × In [sCr]) - 0.203 × In(age) - (0.299 if female) + (0.192 if black). Adapted from Bellemo et al. (26)

Supplementary Table 3.
WHO criteria for severe malaria (2014)

2014 WHO malaria severity criteria	
For epidemiological and research purposes, severe malaria is defined as one or more of the following, occurring in the absence of an identified alternative cause, and in the presence of P. falciparum asexual parasitemia:	
Acidosis	A base deficit of >8 meq/L or, if unavailable, a plasma bicarbonate of <15 mmol/L or venous plasma lactate >5 mmol/L. Severe acidosis manifests clinically as respiratory distress – rapid, deep and laboured breathing
Hypoglycemia	Blood or plasma glucose <2.2 mmol/L (<40 mg/dL)
Severe malarial anemia	A hemoglobin concentration <5 g/dL or a hematocrit of <15% in children <12 years of age (<7 g/dL and <20%, respectively, in adults) together with a parasite count >10,000/µL
Renal impairment (acute kidney injury)	Plasma or serum creatinine >265 µmol/L (3 g/dL) or blood urea >20 mmol/L
Jaundice	Plasma or serum bilirubin >50 µmol/L (3 mg/dL) together with a parasite count >100,000/µL
Pulmonary oedema	Radiologically confirmed, or oxygen saturation <92% on room air with a respiratory rate >30/min, often with chest indrawing and crepitations on auscultation
Significant bleeding	Including recurrent or prolonged bleeding from nose gums or venepuncture sites; hematemesis or melena
Shock	Compensated shock is defined as capillary refill ≥3 sec or temperature gradient on leg (mid to proximal limb), but no hypotension. Decompensated shock is defined as systolic blood pressure <70 mm Hg in children or <80 mm Hg in adults with evidence of impaired perfusion (cool peripheries or prolonged capillary refill)
Hyperparasitemia	*P. falciparum* parasitemia >10%. A 4% parasitemia in non-immune children or adults should be considered an indicator of high risk requiring supervised management but not by itself a criterion of severe malaria

Table adapted from WHO 2014 criteria for severe malaria (27)

Supplementary Table 4.
Patients without AKI but with abnormal NGAL or KIM-1 levels

Patient	Serum NGAL (≤177 ng/ml)	Urine NGAL (≤72 ng/ml)	Urine NGAL/ Urine creat ratio	Urine KIM-1 (≤5.33 ng/ml)	Urine KIM-1/ Urine creat ratio
1	204	128	42.5	8.72	2.91
2	184	99	3.4	3.26	0.11
3	184	129	4.8	1.86	0.07
4	138	39	1.7	5.43	0.23
5	175	240	13.4	1.64	0.09
6	41	105	3.6	1.82	0.06
7	136	98	16.6	0.56	0.09

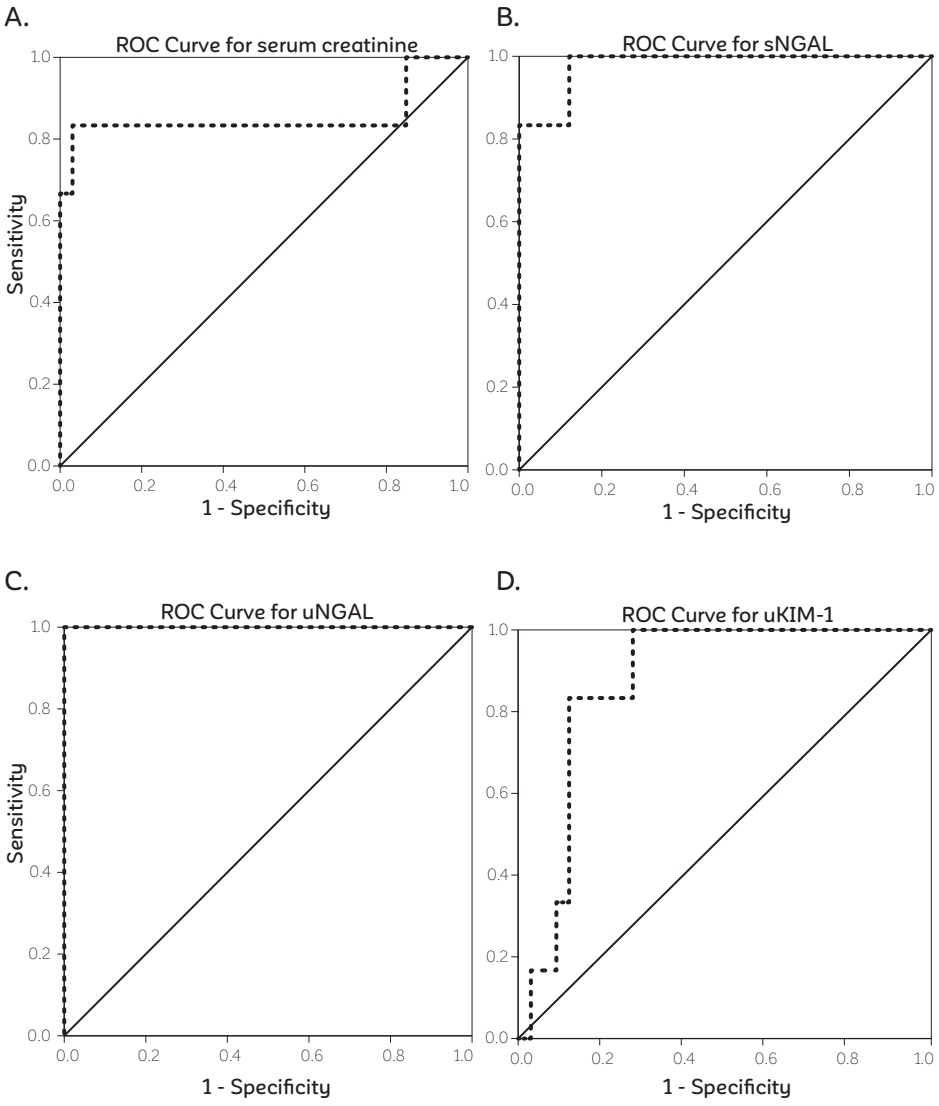
***Bold**: Elevated laboratory measurements. Reference ranges in brackets*

Supplementary Table 5.

Descriptive statistics of diagnostic accuracy of various laboratory measurements at presentation for the presence or development of AKI - KDIGO stage 3

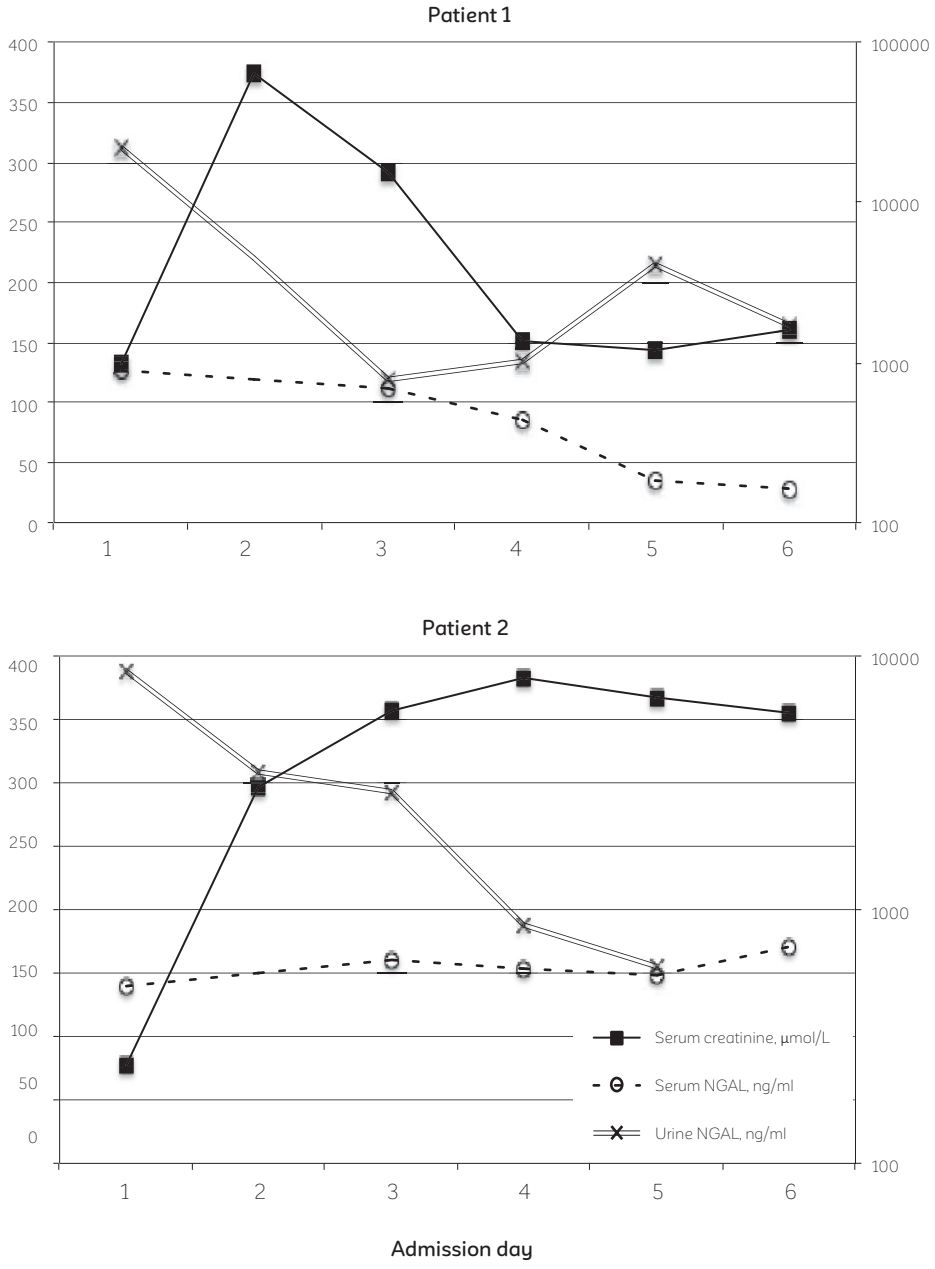
Parameter and cut-off value^	Sensitivity	Specificity	PPV	NPV	Youden index	AUROC	P-value*
sCreatinine ≥ 124 µmol/L	0.75 (0.19-0.99)	0.91 (0.77- 0.98)	0.50 (0.12-0.88)	0.97 (0.84-1.00)	0.66	0.75 (0.59-0.87)	
sNGAL ≥ 204 ng/ml	1.00 (0.40-1.00)	0.97 (0.85-1.00)	0.80 (0.28-1.00)	1.00 (0.90-1.00)	0.97	0.97 (0.86-1.00)	0.199
uNGAL ≥ 376 ng/ml	1.00 (0.40-1.00)	0.97 (0.85-1.00)	0.80 (0.28-1.00)	1.00 (0.90-1.00)	0.97	0.99 (0.88-1.0)	0.170
uNGAL/sNGAL ≥ 2.8	0.75 (0.19-0.99)	1.00 (0.90-1.00)	1.00 (0.29-1.00)	0.97 (0.86-1.00)	0.75	0.92 (0.78-0.98)	0.359
uNGAL/uCreat ≥ 42.5 ng/mmol	1.00 (0.40-1.00)	0.97 (0.85-1.00)	0.80 (0.28-1.00)	1.00 (0.90-1.00)	0.97	0.98 (0.87-1.0)	0.173
uKIM-1 ≥ 1.82 ng/ml	1.00 (0.40-1.00)	0.68 (0.50-0.83)	0.27 (0.08-0.55)	1.00 (0.85-1.00)	0.74	0.82 (0.67-0.93)	0.679
uKIM-1 / uCreat ≤ 0.54 ng/mmol	1.00 (0.40-1.00)	0.66 (48-81)	0.25 (0.07-0.52)	1.00 (0.85-1.00)	0.66	0.85 (0.70-0.94)	0.603

*Data are given as mean (95% confidence interval). Optimal cut-off values were determined using the Youden index. PPV = positive predictive value, NPV = negative predictive value, AUROC = Area Under the ROC curve. *P-values of pair-wise comparison of Area Under ROC curves are given (with creatinine ROC curve as comparator).*



Supplementary Figure 1.

ROC- curves for serum creatinine, sNGAL, uNGAL and uKIM-1 for the prediction of AKI.



Supplementary Figure 2.
The course of the serum creatinine, serum NGAL and urine NGAL concentrations for two patients with AKI during admission.

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CHAPTER 10

Hyponatraemia in imported malaria is common and associated with disease severity

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ABSTRACT

Background: (serum sodium <135 mmol/L) has long been recognized as a complication of malaria. However, few studies have been done in non-immune adult populations. It has not been investigated previously how hyponatraemia is distributed among the various *Plasmodium* species, and its association with malaria severity is unknown. The aim of this retrospective cohort study was to determine the prevalence of hyponatraemia and its association with malaria severity in a large cohort of patients with imported malaria caused by various *Plasmodium* species.

Methods: All patients that were diagnosed with malaria in the Harbour Hospital and Institute for Tropical Diseases in Rotterdam in the period 1999-2009 and who had available serum sodium on admission were included. Severe malaria was defined according to the modified WHO criteria. Prevalence of hyponatraemia and its association with malaria severity were investigated by univariate comparison, ROC analysis and multivariate logistic regression analysis.

Results: A total of 446 patients with malaria (severe *falciparum* malaria n=35, non-severe *falciparum* malaria n=280, non-*falciparum* malaria n=131) was included. Hyponatraemia was present in 207 patients (46%). Prevalence and severity of hyponatraemia were greatest in severe *falciparum* malaria (77%, median serum sodium 129 mmol/L), followed by non-severe *falciparum* malaria (48%, median serum sodium 131 mmol/L), and non-*falciparum* malaria (34%, median serum sodium 132 mmol/L). Admission serum sodium <133 mmol/L had a sensitivity of 0.69 and a specificity of 0.76 for predicting severe malaria. Multivariate logistic regression showed that serum sodium <131 mmol/L was independently associated with severe *falciparum* malaria (odds ratio 10.4, 95% confidence interval 3.1-34.9). In patients with hyponatraemia, hypovolaemia did not appear to play a significant role in the development of hyponatraemia when prerenal azotaemia and haematocrit were considered as surrogate markers for hypovolaemia.

Conclusion: Hyponatraemia is common in imported malaria and is associated with severe *falciparum* malaria. From a clinical point of view, the predictive power of hyponatraemia for severe malaria is limited. The precise pathophysiological mechanisms of hyponatraemia in malaria require further study

BACKGROUND

Hyponatraemia has long been recognized as a complication of malaria (1). The incidence of hyponatraemia in malaria has mostly been studied in endemic areas focusing on children with severe *Plasmodium falciparum* malaria and was approximately 55% (1-3). Two studies in adults in Bangladesh and Thailand found incidences of 57% and 37%, respectively (4, 5). However, few studies have been performed in non-immune populations or in patients infected with other *Plasmodium* species.

The pathophysiology of hyponatraemia in malaria remains unclear but several studies have suggested that increased secretion of vasopressin, either appropriately or inappropriately, plays an important role (4, 6-10). Although a recent study suggested that the outcome of patients with malaria and hyponatraemia is good (4), cerebral oedema may still occur in rare cases (11).

The aim of this retrospective cohort study was to investigate the prevalence and severity of hyponatraemia in a large cohort of predominantly adult non-immune travellers with imported malaria caused by various *Plasmodium* species and its relationship with malaria severity.

METHODS

Patients

The Harbour Hospital is a 161-bed general hospital located in Rotterdam, The Netherlands. It also harbours the Institute of Tropical Diseases, which serves as a national referral centre. All patients diagnosed with malaria in our hospital in the 10-year-period between January 1st 1999 and January 1st 2009 were included. Patients were identified by screening the malaria database of the department of Parasitology. Of all patients thus identified, demographic, clinical and laboratory data were collected using a standardized form, and subsequently stored in an electronic database.

Laboratory investigations

Available laboratory examinations included red and white blood cells, haematocrit, platelets, serum electrolytes, C-reactive protein (CRP), total bilirubin, serum creatinine and urea, liver enzymes, lactate dehydrogenase (LDH), blood glucose

and plasma lactate. Serum sodium concentration was measured using indirect potentiometry (Beckman Synchron UniCel DxC 600 analyser). Blood smears (thin and thick films) were obtained from finger pricks and stained with Giemsa for parasite counts. Malaria was diagnosed by Quantitative Buffy Coat analysis, *P. falciparum* Histidine-Rich-Protein 2 screening (now ICT Malaria,Binax) and conventional microscopy with subsequent specification of the *Plasmodium* species. Multiple malaria episodes in a single patient were only regarded as separate cases if caused by true re-infection; recrudescent infections of *P. falciparum* and *Plasmodium malariae* and relapses of *Plasmodium vivax* and *Plasmodium. ovale* were excluded. Patients with a mixed infection of *P. falciparum* with another *Plasmodium* species were considered as having *P. falciparum* malaria.

Definitions

Severe malaria

Patients were considered as having severe *P. falciparum* malaria if they met predefined modified World Health Organization (WHO) criteria for severe malaria on admission or during hospitalization (12) ("severity criteria"):

- A Glasgow Coma Scale (GCS) score < 11 (indicating cerebral malaria) *or*
- Anaemia (haematocrit < 0.20 L/L with parasite count > 100.000/µL) *or*
- Jaundice (serum bilirubin > 50 µmol/L with parasite count > 100.000/µL) *or*
- Renal impairment (urine output < 400 mL/24h and serum creatinine > 250 µmol/L) *or*
- Hypoglycaemia (blood glucose < 2.2 mmol/L) *or*
- Hyperparasitaemia (> 10% parasitaemia) *or*
- Shock (systolic blood pressure < 80 mm Hg with cold extremities)

Hyponatraemia.

Hyponatraemia was defined as a serum sodium concentration of less than 135 mmol/L.

Statistical analysis

All data were not Normally distributed (Kolmogorov-Smirnov test) and are, therefore, presented as medians and range. Univariate comparisons were performed using the Kruskall-Wallis test (three groups) with Dunn's post-hoc tests, or the Mann-Whitney test (two groups). Correlations were analyzed using Spearman rho (r_s) and Wilcoxon signed rank test. The prognostic value of serum sodium for malaria severity was determined by a receiver operating characteristic (ROC) analysis. To analyse if hyponatraemia was also independently associated with

malaria severity, a logistic regression analysis was performed using a backward stepwise conditional approach. In the latter analysis only patients with *falciparum* malaria were included (because only the *falciparum* species can cause severe malaria) and the presence of severe malaria was defined as the outcome.

RESULTS

Patient characteristics

Serum sodium concentration on admission was available for 446 of the 477 malaria cases (93.5%), and they comprised the study population. Infection was most commonly acquired in Africa (75%) and Asia (14%). Infections with *P. falciparum* accounted for the majority of cases (n = 315, 70.6%). *P. falciparum* infection was classified as severe (n=35) or non-severe (n=280). One hundred and thirty-one patients had non-*falciparum* malaria, which consisted of *P. vivax* (n=92, 70%,), *P. ovale* (n=33, 25%) and *P. malariae* (n=6, 5%). True re-infection occurred in 13 patients, and four patients had a mixed infection of *P. falciparum* and another *Plasmodium* species. Table 1 shows the comparison of the demographic characteristics, vital signs, and laboratory data on admission in the groups with severe and non-severe *falciparum* malaria, and non-*falciparum* malaria.

Characteristics of patients with severe malaria

Thirty-two patients fulfilled one or more of the severity criteria at initial presentation (GCS<11 n=3; anaemia with a parasite count exceeding 100,000 trophozoites per µL n=3; icterus with a parasite count exceeding 100,000 trophozoites per µL n=17; acute oliguric renal insufficiency n=4; hypoglycaemia n=0; hyperparasitaemia n=12 and shock n=2, respectively). Three patients did not fulfil the criteria for severe disease on admission but their clinical course deteriorated shortly hereafter. Admission plasma lactate levels were significantly increased in patients with severe malaria as compared with patients with uncomplicated *falciparum* malaria and patients with non-*falciparum* malaria (Table 1). Eventually, at admission to the intensive care unit (ICU), all 35 patients fulfilled one or more of the severity criteria (cerebral malaria or impaired conscious level n=9; anaemia with a parasite count exceeding 100,000 trophozoites per µL n=3; icterus with a parasite count exceeding 100,000 trophozoites per µL n=19; acute oliguric renal insufficiency n=5; shock n=2, respectively). The first arterial blood gas analysis on ICU showed a median bicarbonate level of 21 mmol/L (range 17 to 26 mmol/L) and a median base deficit of 2 (range -3 to 9). Two patients needed mechanical

ventilation, whereas three patients needed haemodialysis. Twenty-four patients received exchange transfusion. Details of this standardized adjunct treatment have been published elsewhere (13). One patient with severe *falciparum* malaria died of cerebral malaria.

Hyponatraemia in imported malaria

Hyponatraemia was present in 207 patients (46%). The distribution of serum sodium in the whole population seemed to be shifted to lower values (Figure 1). Figure 2 shows the distribution of serum sodium in patients with severe *P. falciparum*, non-severe *P. falciparum*, and non-*falciparum* malaria. Only two patients (0.4%) had a serum sodium concentration exceeding 145 mmol/L. When only patients with hyponatraemia were considered, hyponatraemia was more severe in severe *falciparum* malaria (median serum sodium 129 mmol/L) compared with both non-severe *falciparum* and non-*falciparum* malaria (median serum sodium 131 and 132 mmol/L, respectively, $p < 0.05$). In the whole population, prerenal azotaemia (defined as a serum urea : creatinine ratio > 1:10) was not more common (11/207 or 5.3% vs. 7/239 or 2.9%, $p = 0.2$) and haematocrit values (median 0.39 vs. 0.39, $p = 0.6$) were not higher in patients with hyponatraemia and malaria. Other causes of hyponatraemia appeared rare. Only three patients with malaria and hyponatraemia also used drugs associated with hyponatraemia (1 thiazide diuretic, 1 several diuretics, 1 risperidone) (14). None of the patients had severe heart or liver failure, and none of the patients had known pre-existing hyponatraemia. A significant correlation was demonstrated between sodium levels and CRP levels on admission for all patients with imported malaria ($n=428$; $r_s=-0.36$; $p<0.0001$) but also for patients with infections solely caused by *Plasmodium falciparum* ($n=298$; $r_s= -0.41$; $p<0.0001$).

Hyponatraemia and malaria severity

The area-under the ROC-curve for the predictive value of serum sodium concentration for malaria severity was 0.72 for *P. falciparum* infections and 0.74 for all patients with malaria (Figure 3). A cut-off of 133 mmol/L was identified as having the best discriminatory performance (sensitivity 0.69, specificity 0.76, positive predictive value 0.20, negative predictive value 0.97). Variables significant on univariate analysis that were not severity criteria (Table 1) were entered as dichotomous variables in the multivariate model using different cut-off points. The following parameters were independently associated with severe *falciparum* malaria: serum sodium < 131 mmol/L, CRP > 175 mg/L, LDH > 750 U/L, thrombocytes < 20×10^9/L and leukocytes > 6.5×10^9/L (Table 2). When the severity criteria were also entered in the

model, only serum sodium < 131 mmol/L and CRP > 175 mg/L remained independent predictors of severe *falciparum* malaria (both p < 0.05).

Clinical course and outcome

The median duration of hospital stay is shown in Table 1 Three of the 35 patients with severe *falciparum* malaria met the criteria for severe malaria only hours after admission when their parasite counts increased to above cut-off levels or their clinical course deteriorated. Interestingly, two of them were hyponatraemic on admission (serum sodium 130 and 132 mmol/L). Follow-up serum sodium concentrations were available for 24 of 27 patients with severe *falciparum* infection and hyponatraemia. Serum sodium normalized within 24 hours in seven patients. It took between 24 and 96 hours in seven patients, and more than 96 hours in eight patients. In three patients the time in which serum sodium normalized was unknown. Patients with more severe hyponatraemia on admission remained hyponatraemic for a longer period of time (p < 0.01). There was no significant correlation between sodium levels and GCS in patients with severe malaria (n=35; r_s= -0.036; p=0.84).

DISCUSSION

The present study shows that hyponatraemia is common in patients with imported malaria, and is associated with severe disease in *P. falciparum* malaria. The incidence of hyponatraemia in this study (77% severe *P. falciparum*, 48% non-severe *P. falciparum*, and 34% non-*falciparum*) is comparable to that found in other studies. Holst *et al* (7) found hyponatraemia in 13 of 17 (76%) non-immune travellers with severe *P. falciparum* infection whereas Kockaerts *et al* (15) reported an incidence of 53% in a cohort of 101 patients with imported malaria, although no differentiation according to *Plasmodium* species or severity of malaria was made. In children with imported malaria, lower incidences were found (5/20 or 25% by Viani *et al* (16), 16/192 or 8% by Ladhani *et al* (2)).

Interestingly, in the present study, a serum sodium < 131 mmol/L, CRP > 175 mg/L, LDH > 750 IU/L, thrombocyte count < 20 x 10^9/L, and leukocyte count > 6.5 x 10^9/L were all independently associated with severe *falciparum* malaria (as defined by the modified WHO severity criteria (12)). Some of these parameters like thrombocytopaenia (17) and leukocytosis (18) have been reported as risk factors for severe malaria or a fatal outcome but this has not been a universal finding (18, 19).

When the severity criteria were also entered in the regression model, serum sodium < 131 mmol/L and CRP > 175 mg/L were the only remaining independent variables with Odds ratio's of 10.4 (95% Confidence Interval 3.1 – 34.9) and 4.8 (95% CI 1.6 – 15.0) for severe malaria, respectively, suggesting that determination of these parameters might contribute to an early recognition of patients with severe malaria on admission.

However, there are some important drawbacks, which may limit its usefulness in clinical practice. First, sodium had a poor positive predictive value (0.20) for severe malaria. The observations that hyponatraemia may be seen in imported malaria caused by any *Plasmodium* species as well as in other infectious diseases suggest that hyponatraemia *per se* is unlikely to represent an exclusive feature of *falciparum* malaria but merely reflects the effects of severity of any disease rather than malaria itself. Second, hyponatraemia is a well recognized indicator of disease severity and predictor of mortality regardless of its cause, as has been observed in several studies with hospitalized adult patients (20, 21). As such, the recognition that lower sodium occurs more frequently in severe malaria will probably not significantly change the monitoring and management of the patient since most physicians would already have a higher index of suspicion for a complicated course. Third, the finding that sodium is an independent risk factor for severe malaria contrasts with the findings of two recent studies. In the first study (22), performed in several malaria-endemic countries, involving more than 1000 adults individuals with severe malaria (defined by the same modified WHO criteria) only base deficit and cerebral malaria (measured with GCS) but not sodium were found to be main independent predictors of outcome. Moreover, in the other study (23) involving 482 individuals with imported *falciparum* malaria, sodium was not identified as an independent risk factor for severe *falciparum* malaria. The reason for this discrepancy with the present findings is not immediately apparent but might relate to differences in the number of elderly patients (24) and differences in ethnicity (23). For example, of the 482 individuals with imported malaria (23), 68% of the patients were of black ethnicity, which was associated with a reduced risk of developing WHO-defined severe *falciparum* malaria, whereas white patients had odds that were 8-fold higher. In the present study patients of black ethnicity constituted only a minority of the patients. Fourth and probably most important from a clinical point of view, other parameters like coma and parameters of impaired tissue perfusion (such as acidosis (25), elevated lactate level (25, 26), or base deficit (22)) have been reported as more powerful risk factors for severe malaria or a fatal outcome than sodium.

Even though the predictive power of hyponatraemia for severe malaria may be limited in clinical practice, its pathophysiology is certainly puzzling. Suggested mechanisms are absolute sodium deficit due to cerebral salt wasting, excessive sweating or loss in the gastrointestinal or urinary tract (3), and the secretion of vasopressin which can be either appropriate, in case of volume depletion (4), or inappropriate as in the syndrome of inappropriate antidiuretic hormone secretion (10) or reset osmostat (8). There is, however, no consensus as to their relative contributions Although it has been suggested that hyponatraemia is associated with worse outcome and should be corrected (27), a recent study found that hyponatraemia is associated with preserved consciousness and even a decreased mortality in severe *P. falciparum* malaria (4). In the setting of hypovolaemia, the hyponatraemia was likely related to a continued oral hypotonic fluid intake in those patients who had preserved consciousness and the hyponatraemia required no therapy beyond rehydration given the observation that plasma sodium normalized with crystalloid rehydration within 24 hours after admission (4).

Although the present study was not designed to investigate the pathophysiology of hyponatraemia in malaria, a number of observations might argue against a role for hypovolaemia in the pathophysiology of hyponatraemia in the present study. When prerenal azotaemia and haematocrit were considered as surrogate markers for hypovolaemia, prerenal azotaemia was not more common and haematocrit was not higher in hyponatraemic patients as compared with normonatraemic individuals. In addition, hyponatraemia persisted for days in one third of the patients despite rapid volume resuscitation with normal saline. An explanation for this difference could be that the patients described by Hanson *et al* (4) presented late to hospital and were more ill on admission as evidenced by the high number of comatose patients and the higher mortality rate. The inverse relationship between plasma sodium and GCS could not be reproduced in the present study but might be underpowered by the relatively low number of comatose patients. Although speculative, an other mechanism may have contributed to hyponatraemia in the present study. It is certainly interesting to note that the pro-inflammatory cytokine interleukin-6 is elevated in malaria and also implicated in the non-osmotic release of vasopressin (28, 29). The delayed normalization of serum sodium concentration as was observed in the present study, might reflect the persistent elevation of inflammatory cytokines, which are known to remain increased for several weeks in some patients with severe malaria (30). Previously, a relationship between the development of in-hospital hyponatraemia and a rise in CRP was demonstrated, which is not only another illustration of this mechanism (20) but also in line with

the observed correlation between sodium levels and CRP levels in patients with imported malaria in the present study. Additional studies are needed to further unravel the intriguing pathophysiology of hyponatraemia in malaria.

CONCLUSION

Hyponatraemia is common in imported malaria and associated with severe *falciparum* malaria. From a clinical point of view, however, the predictive power of hyponatraemia for severe malaria is limited. The precise pathophysiological mechanisms of hyponatraemia in malaria require further study.

Table 1. Patient characteristics at initial presentation

	Severe *P. falciparum* (n = 35)	Non-severe *P. falciparum* (n = 280)	Non-*falciparum* (n = 131)	P-value*
Demographics				
Age, years	44 (4 – 70)	39 (4 – 78)	35.5 (8 – 77)	0.007[B]
Male, female, n (%)	21 (58), 14 (42)	202 (72), 78 (28)	87 (66), 44 (34)	N.S.
Vital signs on admisson				
Body temperature, °C	38.2 (36.2 – 41.2)	38.6 (35.7 – 41.0)	38.6 (35.0 – 41.5)	N.S.
Pulse rate, beats per minute	101 (50 – 150)	92 (45 – 150)	89 (58 – 138)	0.02[A]
Systolic blood pressure, mm Hg†	115 (80 – 160)	120 (80 – 90)	120 (70 – 196)	N.T.
GCS < 11, n (%)†	3 (9)	0 (0)	0 (0)	N.T.
Laboratory data on admission				
C-reactive protein, mg/L	184 (91 – 265)	77 (5 – 320)	71 (8 – 213)	< 0.0001[A]
Haematocrit, L/L†	0.35 (0.17 – 0.51)	0.40 (0.19 – 0.54)	0.38 (0.22 – 0.53)	N.T.
Leucocyte count, x 109/L	7.1 (3.2 – 18.5)	4.9 (1.3 – 13.2)	5.3 (1.9 – 15.3)	< 0.0001[A]
Thrombocytes, x 109/L	36 (3 – 188)	98 (11 – 433)	94 (10 – 292)	< 0.0001[A]
Serum sodium, mmol/L	130 (115 – 146)	135 (119 – 145)	136 (124 – 148)	< 0.0001[A]
Serum potassium, mmol/L	3.7 (2.7 – 4.7)	3.8 (2.5 – 5.1)	3.8 (2.7 – 5.4)	N.S.
Serum creatinine, µmol/L†	114 (48 – 871)	94 (41 – 228)	87 (46 – 477)	N.T.
Serum glucose, mmol/L	6.3 (4.1 – 12.5)	6.6 (4.4 – 33.8)	6.4 (4.7 – 22.1)	N.S.
Total bilirubin, µmol/L†	53 (15 – 416)	21 (4 – 262)	21.5 (3 – 91)	N.T.
Alanine-aminotransferase, U/L	58 (11 – 655)	35 (3 – 265)	31 (6 – 198)	0.001[A]
Lactate dehydrogenase, U/L	890 (308 – 4113)	455 (224 – 2724)	428 (185 – 1395)	< 0.0001[A]
Plasma lactate (mmol /L)	2,3 (1,2 – 5,8)	1,4 (0,5 – 4,4)	1,3 (0,7 – 3,0)	<0.001[B]
Parasite load (trophozoites / µL) †	239,200 (520 – 1,110000)	5300 (2 – 385,000)	N/A	N.T.
Duration hospitalisation, days	8 (3 – 19)	5 (1 – 16)	2 (1 – 11)	<0.0001[A+C]

** Using Kruskall-Wallis and Dunn's post-hoc tests. Parameters that are included in the modified WHO severity criteria for severe falciparum malaria (indicated by † (12)) were not tested (N.T.). N.S. denotes 'not significant'.* A group 1 vs. group 3; B group 1 vs. groups 2 and 3; C group 2 vs. group 3

Table 2. Logistic regression analysis showing independent predictors of severe *falciparum* malaria

Variable	β	SE β	Wald's χ	df	P-value	Odds ratio (95% CI)
Constant	-6.1	1.32	21,400	1	< 0.001	N/A
Serum sodium < 131 mmol/L	2.3	0.62	14,286	1	< 0.001	10.4 (3.1 – 34.9)
C-reactive protein > 175 mg/L	1.6	0.58	7,472	1	0.006	4.8 (1.6 – 15.0)
Lactate dehydrogenase > 750 U/L	1.8	0.58	9,288	1	0.002	5.9 (1.9 – 18.7)
Thrombocytes < 20 x 109/L	3.7	1.03	12,761	1	< 0.001	40.0 (5.3 (302.8)
Leukocytes > 6.5 x 109/L	2.3	0.60	14,384	1	< 0.001	9.7 (3.0 – 31.5)

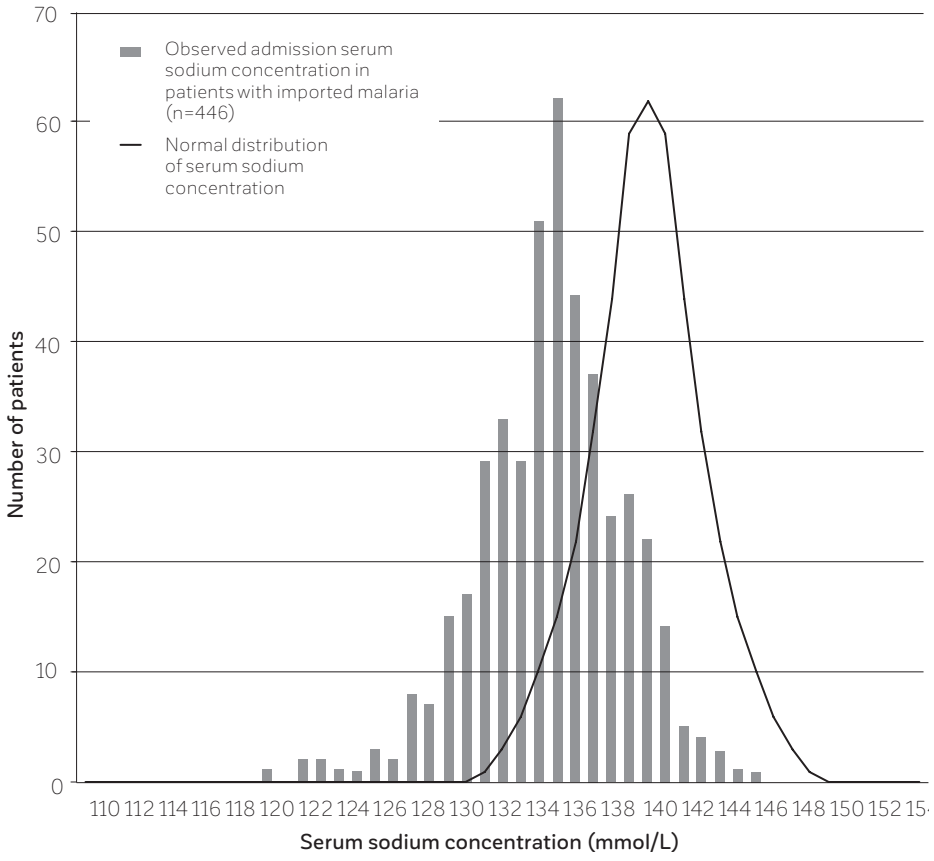

Figure 1. Distribution of serum sodium in patients with imported malaria

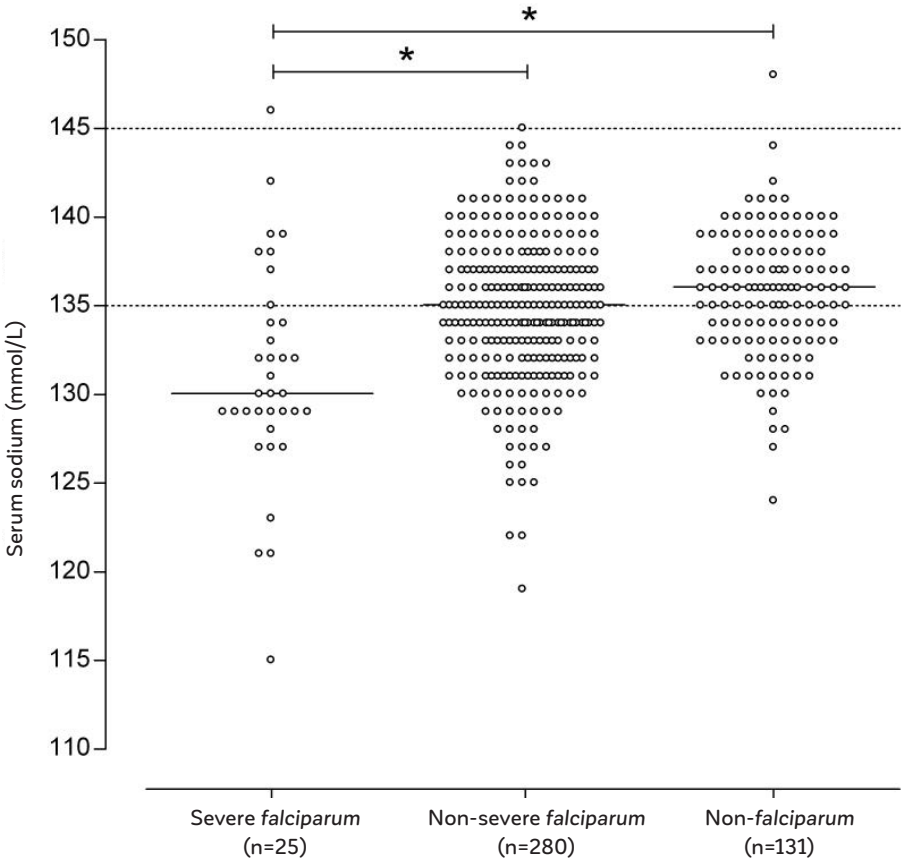

Figure 2. Distribution of serum sodium in malaria patients grouped by causative *Plasmodium* species

Hyponatraemia was present in 27/35 (77%) of patients with severe P. falciparum infection, in 135/280 (48%) of patients with non-severe P. falciparum infection, and in 45/131 (34%) of patients with non-falciparum infection. Dashed lines indicate normal reference range. Bars indicate the median.

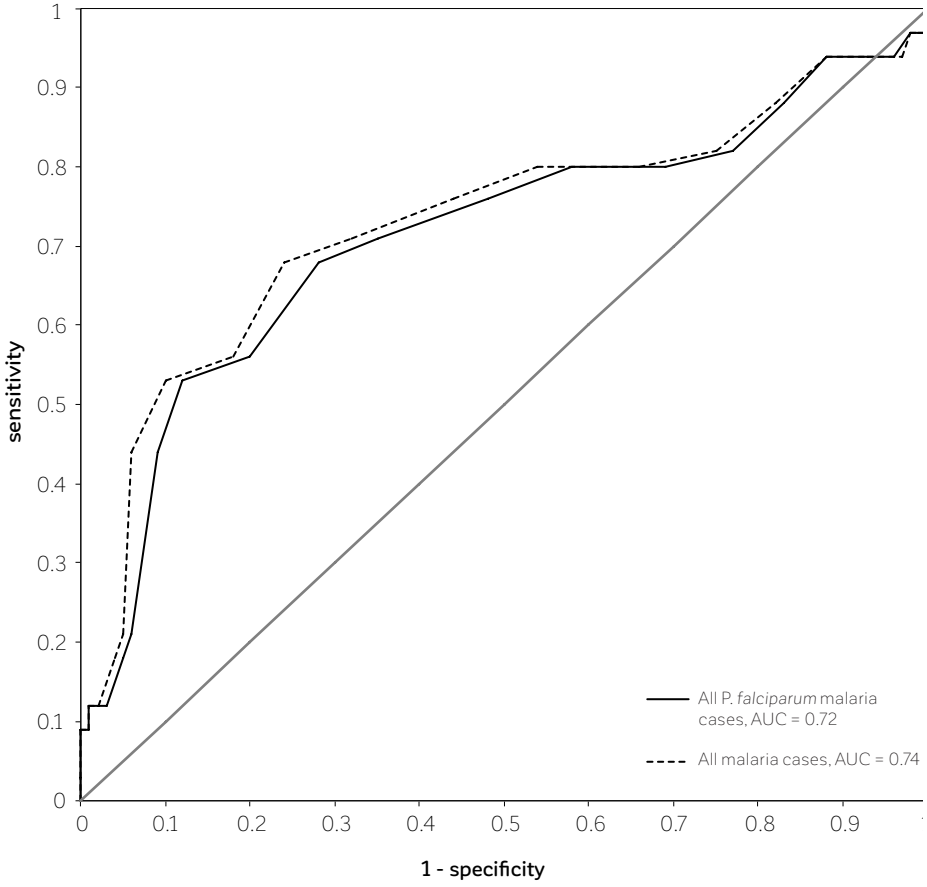

Figure 3. Receiver operating characteristics curve of serum sodium concentration for malaria severity

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CHAPTER 11

Hyponatraemia in imported malaria: The pathophysiological role of vasopressin

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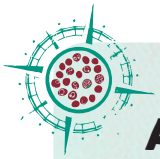


ABSTRACT

Background: In the pathophysiology of hyponatraemia in malaria, the relative contribution of appropriate and inappropriate arginine vasopressin (AVP) release is unknown; the trigger for inappropriate AVP release is also unknown.

Methods: Serum copeptin, a stable and sensitive marker for AVP release, was analysed in a large cohort of patients with imported malaria (204 patients) and in a small prospective substudy (23 patients) in which urine sodium and osmolality were also available. Hyponatraemia was classified as mild (serum sodium 131-134 mmol/l) and moderate-to-severe (< 131 mmol/l).

Results: Serum copeptin on admission was higher in patients with moderate-to-severe hyponatraemia (median 18.5 pmol/L) compared with normonatraemic patients (12.7 pmol/L, $p < 0.05$). Despite prompt fluid resuscitation, the time to normalization of serum sodium was longer in patients with moderate-to-severe hyponatraemia (median 2.9 days) than in patients with mild hyponatraemia (median 1.7 days, $p < 0.001$). A poor correlation was found between serum sodium and copeptin levels on admission ($rs = -0.17$, $p = 0.017$). Stronger correlations were identified between serum C-reactive protein and copeptin ($rs = -0.36$, $p < 0.0001$) and between serum C-reactive protein and sodium ($rs = 0.33$, $p < 0.0001$). Data from the sub-study suggested inappropriate AVP release in seven of 13 hyponatraemic malaria patients; these patients had significantly higher body temperatures on admission.

Conclusion: In hyponatraemic patients with imported malaria, AVP release was uniformly increased and was either appropriate or inappropriate. Although the exact trigger for inappropriate AVP release remains unknown, the higher body temperatures, correlations with C-reactive protein and long normalization times of serum sodium, suggest an important role of the host inflammatory response to the invading malaria parasite.

BACKGROUND

Hyponatraemia is a common finding in imported malaria and associated with severe *Plasmodium falciparum* malaria (1). Nevertheless, its pathophysiology remains incompletely understood. Hyponatraemia is primarily a water balance disorder and usually caused by increased secretion of arginine vasopressin (AVP). With regard to hyponatraemia in malaria, some studies found evidence for "appropriate" vasopressin release due to hypovolaemia (2) whereas other studies found evidence for "inappropriate" vasopressin release (3, 4). There is, however, no consensus regarding the relative contributions of these mechanisms in the pathophysiology of hyponatraemia in malaria.

AVP is a key hormone in maintaining fluid balance and vascular tone (5). Despite these important physiological functions, mature AVP is rarely measured clinically, because the assay is difficult and subject to preanalytical and analytical errors (6). Recently, copeptin, a 39-amino acid glycopeptide that comprises the C-terminal moiety of the AVP precursor (CT-proAVP) was demonstrated to be a stable and sensitive marker for AVP release (6-9). Furthermore, a number of studies have now shown that measurement of serum copeptin or calculation of the serum copeptin to urine sodium ratio is useful in the differential diagnosis of fluid and electrolyte disorders (10, 11). In the present study, serum copeptin levels were evaluated in a large cohort of patients with imported malaria to further explore the role of AVP in the pathophysiology of hyponatraemia in malaria.

METHODS

Patients

The Harbour Hospital is a 161-bed general hospital located in Rotterdam, The Netherlands. It also harbours the Institute for Tropical Diseases, which serves as a national referral centre. The Rotterdam Malaria Cohort consists of all patients diagnosed with malaria at the Institute for Tropical Diseases in Rotterdam. In the period 1999-2010 the Rotterdam Malaria Cohort comprised 519 cases of imported malaria. Of all patients, anonymized demographic, clinical and laboratory data are routinely collected and stored in an electronic database. Moreover, in a large number of patients serum samples taken on admission were stored. For the present study, anonymized data from patients who entered the Rotterdam Malaria Cohort between January 1999 and December 2010 were used to estimate

the prevalence of hyponatraemia in imported malaria at time of first presentation as well as for the follow-up of sodium levels after treatment during admission. For those patients with stored serum samples, copeptin levels were measured retrospectively. In a small sub-study serum copeptin levels were measured prospectively in addition to urinary sodium and osmolality.

Laboratory investigations

All available laboratory data were measured on admission with the use of routine procedures. In contrast, copeptin levels were retrospectively measured in stored serum samples with a commercial sandwich immunoluminometric assay (Brahms Copeptin, Thermo Fisher Scientific, Hennigsdorf/Berlin, Germany) as described (9). Normal values for serum copeptin in healthy volunteers range between 1.70 and 11.25 pmol/L (9). Blood smears (thin and thick films) were obtained from finger pricks and stained with Giemsa for parasite counts. Malaria was diagnosed by Quantitative Buffy Coat analysis, *P. falciparum* Histidine-Rich-Protein 2 screening (now ICT Malaria, Binax) and conventional microscopy with subsequent specification of the *Plasmodium* species.

Definitions

Severe malaria

Patients were considered as having severe *P. falciparum* malaria if they met the recently updated World Health Organization (WHO) criteria for severe malaria on admission or during hospitalization (12).These criteria differ from the preset criteria (13) that were used to define severe malaria in previous studies (1).

Coma Acidosis Malaria (CAM) score

Of each patient with severe disease an admission CAM score, a 5-point (0-4 points) score calculated as the sum of the base deficit score (0-2 points) and Glasgow Coma score (0-2 points), was given (14).

Hyponatraemia

Hyponatraemia was defined as a serum sodium concentration of less than 135 mmol/L. Mild hyponatraemia was defined as a serum sodium concentration 131-134 mmol/L, whereas moderate hyponatraemia was defined as a serum sodium concentration 125-131 mmol/L. The threshold of 131 mmol/L was chosen because a previous study found that a sodium level below 131 mmol/L was an independent predictor for severe disease in imported malaria (1). Severe hyponatraemia was defined as a serum sodium level below 125 mmol/L. Given the low

number of samples of patients with severe hyponatraemia in the copeptin study, these patients were grouped with the patients with moderate hyponatraemia to form the moderate-to-severe hyponatraemia group.

Inappropriate vs appropriate AVP secretion

A recent study found that the serum copeptin to urine sodium ratio may be used to differentiate normovolaemic hyponatraemia (ratio ≤ 30 pmol/mmol) from hypovolaemic hyponatraemia (ratio > 30 pmol/mmol) (11). The most common example of normovolaemic hyponatraemia is the syndrome of inappropriate antidiuresis (15) and, therefore, a ratio ≤ 30 pmol/mmol was used to define inappropriate AVP release. Conversely, AVP release during hypovolaemic hyponatraemia is considered "appropriate" and, therefore, appropriate AVP release was defined as a ratio > 30 pmol/mmol.

Statistical analysis

All data are reported as medians (range). Univariate comparisons were performed using the Kruskall-Wallis test (three groups) with Dunn's post-hoc tests, or the Mann-Whitney test (two groups) for not normally distributed data. Normally distributed data were compared with unpaired t-tests or unpaired t-tests with Welch correction, as appropriate. Correlations were analysed using Spearman rho (r_s) and Wilcoxon's signed rank test. Kaplan-Meier survival curves for resolution of hyponatraemia after treatment were analysed with the Mantel-Cox log-rank test.

RESULTS

Prevalence of hyponatraemia in imported malaria and its distribution among the various Plasmodium species

Of the 519 cases in the Rotterdam Malaria Cohort 1999-2010, 10 (1.9%) patients had severe hyponatraemia on admission, 60 (11.6%) patients had moderate hyponatraemia, whereas 166 (32.0%) malaria patients had mild hyponatraemia on admission, respectively. In the remaining 283 (54.5%) patients the sodium level on admission was normal. Of the 54 *P. falciparum* malaria patients fulfilling the criteria for severe disease, 5 (9.3%) patients had severe hyponatraemia on admission, 20 (37.0%) patients had moderate hyponatraemia, whereas hyponatraemia was mild in 18 (33.3%) patients with severe malaria. Eleven (20.4%) patients with severe malaria had a normal sodium on admission, including the two patients who died. Of the 312 patients with uncomplicated *P. falciparum* malaria,

severe hyponatraemia was present on admission in 4 (1.3%) patients, moderate hyponatraemia in 33 (10.6%) patients and mild hyponatraemia was found in 105 (33.7%) patients on admission, respectively. Serum sodium concentrations were normal in the remaining 170 (54.5%) patients with uncomplicated *P. falciparum* malaria. Of the 153 patients with non-*P. falciparum* malaria, severe hyponatraemia was present in 1 (0.7%) patient, moderate hyponatraemia in 7 (4.6%) patients, mild hyponatraemia in 43 (28.1%) patients, and 102 (66.7%) patients had normal serum sodium concentrations on admission, respectively.

Characteristics of the patients with severe malaria

When focusing on the 54 patients with severe malaria, these patients presented with the following severity criteria: hypotension (n=1); impaired consciousness (n=8) or unrousable coma characterized by a GCS ≤9 (n=3); severe anaemia characterized by a haemoglobin level ≤3.0 mmol/L (n=2) or a packed cell volume <0.20 (n=6); blackwater fever (n=1); renal impairment characterized by a creatinine level ≥265 μmol/L (n=6); liver impairment characterized by a total bilirubin level ≥50 mmol/L (n=29); hyperlactataemia characterized by a lactate ≥5 mmol/L (n=6); hyperparasitaemia characterized by a parasite load ≥5% (n=34; on admission to the intensive care unit n=40) and schizontaemia (n=27), respectively.
Of 30 patients with severe malaria a CAM score could be calculated on admission. The median CAM score was 1, and the scores ranged from 0 to 3. Nine patients had a CAM score of 0, 16 patients a CAM score of 1, four patients had a CAM score of 2, whereas 1 patient had a CAM score of 3.

Thirty-six patients received intravenous treatment with quinine, 11 patients were treated with intravenous artesunate. Four patients were solely treated with oral anti-malarials and the treatment mode was unknown in three patients. Thirty-two patients received exchange transfusion. Details of this adjunct therapy for severe malaria are published elsewhere (16). Sixteen of 54 patients were referred from surrounding hospitals. There were no statistically significant differences between blood glucose levels (glucose 6.3 ± 1.7 mmol/L vs 7.8 ± 5.0 mmol/L, p = 0.1099) and serum sodium levels on admission (sodium 133 ± 7 mmol/L vs 130 ± 4 mmol/L, p = 02491) between patients referred from other hospital (n = 16) and those patients directly referred to the Institute for Tropical Diseases (n = 38), making a significant contribution of dextrose or glucose infusion on serum sodium levels on admission unlikely in the referred patients.

Follow-up of hyponatraemia during hospitalization

In a subset of 151 malaria patients with hyponatraemia from the Rotterdam Malaria Cohort, serum sodium levels were measured consecutively during hospitalization. Fifty-eight patients had moderate-to-severe hyponatraemia (16 with severe *P. falciparum* malaria; 38 patients with uncomplicated *P. falciparum* malaria; 4 non-*falciparum* malaria) and hyponatraemia was mild in 93 patients (11 patients with severe *P. falciparum* malaria; 76 with uncomplicated *P. falciparum* infection and 6 non-*falciparum* infections). As shown in Figure 1, time to normalization of serum sodium was significantly longer in patients with moderate-to-severe hyponatraemia (median time to normalization of sodium 2.9 days) than in patients with mild hyponatraemia on admission (median time to normalization of sodium 1.7 days, $p<0.001$). In approximately 20% of the malaria patients with moderate-to-severe hyponatraemia, serum sodium levels did not normalize after 1 week of antimalarial treatment and infusion of isotonic saline.

Evaluation of the role of copeptin in the pathophysiology of hyponatraemia in imported malaria

In 204 malaria patients from the Rotterdam Malaria Cohort copeptin was measured in stored serum samples and related to previously established laboratory parameters on admission. The general characteristics of these patients are shown in Table 1. In general, patients with moderate-to-severe hyponatraemia presented more ill, as illustrated by their higher body temperatures, pulse rates, CRP and creatinine levels. Elevated creatinine levels were frequently observed: in 12 of 31 (38.7%) patients with moderate-to-severe hyponatraemia, in 10 of 68 (14.7%) patients with mild hyponatraemia and in 20 of 105 (19.0%) of normonatraemic patients, respectively. Only two patients with severe *P. falciparum* malaria had a creatinine level exceeding the WHO threshold of 265 μmol/L for renal impairment (12). These patients both presented with moderate-to-severe hyponatraemia on admission. Serum copeptin levels on admission were significantly higher in patients with moderate-to-severe hyponatraemia (median 18.5 pmol/L) as compared with normonatraemic patients (12.7 pmol/L) but not with malaria patients who presented with mild hyponatraemia (13.2 pmol/L, Figure 2). Copeptin levels exceeding the 97.5 percentile of normal healthy individuals (corresponding to a copeptin level of 11.25 pmol/L) were significantly ($p=0.0099$, Chi square test for trend) more often observed in malaria patients presenting with moderate-to-severe hyponatraemia (25 of 31 [81%] patients) than in patients with patients with mild hyponatraemia (47 of 68 [69%] patients) and normonatraemia (60 of 105 [57%] patients). A poor correlation was found between serum sodium and copeptin levels on admission

(Figure 3, r_s=-0.17, p=0.017). In contrast, stronger correlations were demonstrated between CRP and copeptin (Supplementary Figure 1, r_s=-0.36, p<0.0001) on the one hand and between CRP and serum sodium (Supplementary Figure 2, r_s=0.33, p<0.0001) on the other hand.

Urine biochemistry

In a subset of 23 malaria patients urine sodium and urine osmolality were measured in parallel with measurement of serum copeptin. The main outcome measures are shown in Table 2. In none of the 13 hyponatraemic patients, serum glucose exceeded 10 mmol/L, excluding hyperglycaemia as a significant cause of hyponatraemia. When the 13 hyponatraemic malaria patients were categorized according to the serum copeptin to urine sodium ratio, 7 hyponatraemic patients fulfilled the criteria for inappropriate AVP secretion. There were no significant differences in creatinine, haematocrit or urea to creatinine ratio between hyponatraemic malaria patients with inappropriate or appropriate AVP secretion. Urine osmolality values were significantly higher in patients with inappropriate AVP release (median 780 mOsm/kg) than in patients with appropriate AVP release (480 mOsm/kg) or normonatraemic patients (484 mOsm/kg). Patients with inappropriate AVP release also had significantly higher body temperatures on admission than hyponatraemic patients with appropriate AVP release.

DISCUSSION

Copeptin, the C-terminal glycopeptide domain of pro-vasopressin, is co-secreted with AVP from the posterior pituitary in hyperosmolar states and upon multiple non-osmotic stimuli, such as hypotension, pain, and other non-specific causes of stress (6, 8). Circulating copeptin levels are therefore thought to reflect the activity of the neuroendocrine stress axis. To gain more insight in the pathophysiology of hyponatraemia in malaria and in particular the role of AVP, serum copeptin was measured in a large cohort of 204 patients with imported malaria. In malaria patients the median serum copeptin levels were three to five-fold higher than the median level of 4.2 pmol/L observed in 359 healthy volunteers (8). In fact, the proportion of malaria patients with copeptin levels above the 97.5th percentile of normal significantly increased with decreasing sodium levels (Figure 3). Moreover, in absolute terms, patients with moderate-to-severe hyponatraemia also had significantly higher copeptin levels than normonatraemic malaria patients on admission (Figure 2). Because the physiological stimulus

for AVP release is hypertonicity, elevated AVP or copeptin levels in the context of hyponatraemia indicates a pathological setting. That is, during normal physiology, the development of hyponatraemia ought to suppress AVP release and to result in a maximally dilute urine with a low urine osmolality (5, 17).

A recent study found that the ratio of serum copeptin to urine sodium may be used to differentiate inappropriate from appropriate AVP secretion (11). To further investigate the antidiuretic effect of AVP at the level of the target organ, urine sodium and osmolality were prospectively studied in parallel with measurements of serum copeptin levels in a subset of 13 hyponatraemic and 10 normonatraemic malaria patients on admission. Based on the serum copeptin to urine sodium ratio, six patients had appropriate AVP release, while seven patients had inappropriate AVP release. Hyponatraemic patients with inappropriate AVP release had significantly higher urine osmolality values than observed in patients with an appropriate AVP response or in normonatraemic patients. This suggests active water reabsorption by the kidneys in malaria patients with inappropriate AVP release. Why inappropriate AVP release results in a higher urine osmolality than appropriate AVP release is unclear. One could speculate that in the group with appropriate AVP release, the renin angiotensin system was likely also activated, leading to increased renal sodium reabsorption. Because urine sodium is a major determinant of urine osmolality, a lower urine sodium would therefore result in a lower urine osmolality in malaria patients with appropriate AVP release.

From a pathophysiological point of view there may be two possible explanations for the increased serum copeptin levels despite the presence of hypotonicity. First, volume regulation may have overruled osmoregulation if there was true hypovolaemia (18) or a low effective arterial blood volume (19). This mechanism is mediated via baroreceptors in the vasculature and is often referred to as "appropriate" AVP release. In 6 of 13 hyponatraemic patients with available urine biochemistry data, AVP release was considered appropriate based on the serum copeptin to urine sodium ratio. However, in a substantial number of patients with moderate-to-severe hyponatraemia, the hyponatraemia persisted for more than seven days despite infusion of isotonic saline, rendering persistence of hypovolaemia an unlikely explanation (Figure 1). Hence, other mechanisms must apply in a substantial part of the malaria patients with hyponatraemia.

The second explanation for elevated copeptin levels despite the presence of hypotonicity may involve activation of central osmoreceptors leading to vasopressin

release and subsequent development of hyponatraemia. This alternative mechanism could have been mediated through cytokines (20) and resembles the syndrome of inappropriate antidiuresis, a common cause of hyponatraemia (15). In fact, in 7 of 13 hyponatraemic malaria patients an inappropriate release of AVP appeared to be present. Of potential relevance, in this regard, is the observation that the pro-inflammatory cytokine interleukin-6 is elevated in malaria and is also implicated in the non-osmotic release of AVP (21, 22). The delayed normalization of serum sodium concentration, as was observed in the present study, might be the consequence of the persistent elevation of inflammatory cytokines, as has been shown for patients with severe malaria (23). Previously, a relationship between a rise in CRP and the development of hospital-acquired hyponatraemia was demonstrated (24). This is not only another illustration of a presumed cytokine-driven non-osmotic release of AVP (20) but also in line with the observed inverse relationship between serum sodium and CRP levels on the one hand, and CRP and copeptin levels on the other hand (Supplementary Figures). Although several drugs, such as opiates, non-steroidal anti-inflammatory drugs, and diuretics, can contribute to hyponatraemia, these drugs were rarely used in our cohort, and it is our policy not to administer these drugs to malaria patients because of their potentially adverse effects. Although thyroid and adrenal function were not formally tested, which is recommended before diagnosing inappropriate AVP release, the response of hyponatraemia to malaria treatment was highly suggestive of a causal relationship.

The distinction between appropriate and inappropriate AVP release in hyponatraemic malaria patients may be relevant with regard to selecting the optimal intravenous fluid regimen. Because previous studies did not separate hyponatraemic malaria patients on the basis of appropriate or inappropriate AVP secretion, future studies are necessary to give clinical guidance. In general, however, hypovolaemia causes appropriate AVP release and should therefore be treated with isotonic fluids. A caveat, however, is that serum sodium may rise too rapidly during treatment of hypovolaemic hyponatraemia with isotonic fluids (25). The risk of exceeding recommended correction rates is osmotic demyelination, although few cases in malaria patients have been reported. Conversely, during inappropriate AVP release, the emphasis of therapy should perhaps be more on aggressive anti-malaria treatment, given the association with a stronger cytokine response. In this setting, a restrictive intravenous fluid regimen may prove beneficial, because even isotonic saline can worsen hyponatraemia during the syndrome of inappropriate antidiuresis (26). In this regard, a recent study

advocating restrictive IV-fluid therapy in children with malaria is also of interest, although no serum sodium values were reported (27).

A potential limitation of our study is that it remains debatable whether urine sodium can be considered a reliable parameter for the establishment of hypovolaemia in malaria, since circulating cytokines have also been implied in causing tubular injury and therefore natriuresis (28). The evidence for the pathogenetic role of AVP in the pathophysiology of hyponatraemia in malaria is, however, substantial. The results of the small urine substudy suggest that appropriate (2, 19) and inappropriate (3, 29) AVP secretion may both occur in the pathophysiology of hyponatraemia in imported malaria. Although speculative, the urine substudy indicated that patients with an inappropriate AVP release had significantly higher body temperatures on admission than patients with appropriate AVP release, which suggests that - at least in part - the extent of the host inflammatory response to the invading malaria parasite may play a pivotal role in the aetiology of cytokine-driven non-osmotic release of AVP.

Table 1. Characteristics of 204 malaria patients in the copeptin study.

	Moderate-to-severe hyponatraemia (n=31)	Mild hyponatraemia (n=68)	Normonatraemia (n=105)	P-value*
Demographics				
Age, years	42 (11-64)	40 (13-69)	39 (8 – 70)	n.s.
Male, female, n (%)	22 (71), 9 (29)	52 (76), 16 (24)	77 (73), 28 (27)	n.s.
Malaria species				
falciparum, non-*falciparum*, n (%)	26 (84), 5 (16)	54 (79), 12 (21)	61 (58), 44 (42)	0.0008
Severe malaria, n (%)	10 (40)	10 (40)	5 (20)	<0.0001
Parasite load#, parasites/µL	85900 (400 – 567000) B<0,001	11032 (2 – 860000)	4600 (30 – 1380600)	0.0013
Vital signs on admission				
Body temperature, °C	39.0 (35.7 – 40.8)	38.9 (35.7 – 41.2)	38.2 (36.0 – 41.2)	0.0315
Pulse rate, beats per minute	100 (58 – 140) B <0,01	95 (64 – 130)	85 (60 - 130)	0.0109
Systolic blood pressure, mm Hg	120 (80 – 147)	120 (88 – 165)	120 (95 – 196)	n.s.

Table 1. Continued

	Moderate-to-severe hyponatraemia (n=31)	Mild hyponatraemia (n=68)	Normonatraemia (n=105)	P-value*
Laboratory data on admission				
C-reactive protein, mg/L	159 (32 - 352) $^{A<0,01; B<0,001}$	101 (7 – 310) $^{C<0,05}$	78 (7 - 407)	<0.0001
Haematocrit, L/L	0.35 (0.15 – 0.50) $^{A<0,01}$	0.41 (0.12 – 0.52)	0.39 (0.26 – 0.53)	0.0006
Serum glucose, mmol/L	6.9 (4.1 – 26.0) $^{B<0,05}$	7.0 (4.2 – 10.3) $^{C<0,001}$	6.3 (4.1 – 14.9)	0.0003
Serum creatinine, µmol/L	111 (70 – 1081) $^{B<0,05}$	97 (55 – 135)	93 (46 – 208)	0.0180
Serum urea, mmol/L	6.4 (3.6 – 55.8) $^{B<0,01}$	5.2 (2.2 – 13.5)	4.9 (2.7 – 21.1)	0.0061
Prerenal azotaemia[&], n (%)	2 (6)	2 (3)	2 (2)	n.s.
Copeptin, pmol/L	18.5 (3.3 – 91.5) $^{B<0,05}$	13.2 (1.6 – 71.2)	12.7 (1.6 – 82.9)	0.0268
Duration hospitalisation, days	6 (1 – 13) $^{B<0,001}$	5 (0 – 11) $^{C<0,001}$	3 (0 – 12)	<0.0001

Parameters at initial presentation are shown in relation to serum sodium level on admission

** Univariate comparison were performed using Kruskall-Wallis (P-values are given in the column) and Dunn's post-hoc tests (P-values in superscript). A: comparison of severe hyponatraemia vs mild hyponatraemia; B: comparison of severe hyponatraemia vs normonatraemia; C: comparison of mild hyponatraemia vs normonatraemia. # P.falciparum parasite load only. & defined as ratio serum urea/serum creatinin > 1:10; n.s. = denotes "not significant difference". Medians with range are shown.*

Table 2. Results of parallel measurements of urine and blood samples from hyponatraemic and normonatraemic malaria patients on admission

Parameter	Hyponatraemic patients (n=13)			Normonatraemic patients (n=10)	
	Inappropriate AVP secretion@ (n=7)	Appropriate AVP secretion (n=6)	P-value#		P-value*
Vital signs on admission					
Body temperature, °C	38.9 (37.6-41.1)	37.4 (35.7-38.6)	P=0.0153	38.3 (36.0-40.1)	n.s.
Pulse rate, beats per minute	96 (72-121)	105 (91-120)	n.s.	93 (72-125)	n.s.
Laboratory data on admission					
C-reactive protein, mg/L	158 (60-176)	236 (71-352)	n.s.	95 (18-407)	n.s.
Haematocrit, L/L	0.39 (0.15-0.44)	0.40 (0.19-0.50)	n.s.	0.46 (0.36-0.51)	n.s.
Serum Urea:creatinine ratio	0.06 (0.04-0.11)	0.07 (0.05-0.14)	n.s.	0.06 (0.03-0.09)	n.s.
Serum copeptin > P97.5, n (%)	4 (57)	5 (83)	n.s.	6 (60)	n.s
Serum sodium, mmol/L	132 (131-134)	128 (124-132)	P=0.012	138 (135-141)	n.a.
Urine osmol, mosmol/kg	780 (540-924)	480 (298-532)	P=0.0022	484 (234-906)	P=0.047
Urine sodium, mmol/L	49 (38-154)	9 (9-47)	n.a.	32 (9-164)	n.a.

*Legend to the table: @ = inappropriate AVP secretion was defined as a serum copeptin to urine sodium ratio of ≤ 30 pmol/mmol. #P-values of comparison of hyponatraemic patients with inappropriate vs appropriate AVP secretion. *P-values of univariate analysis using Kruskall Wallis followed by Dunn's post hoc tests; n.s. = not significant difference; n.a. = not applicable (defining criterion). Medians with range are shown.*

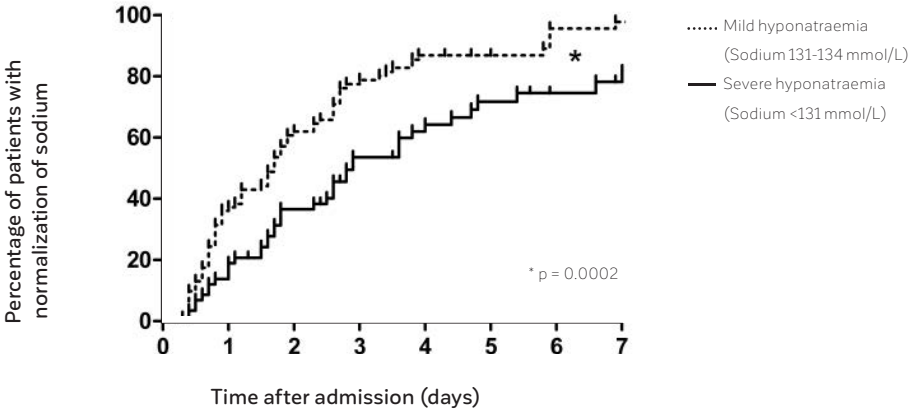


Figure 1. Kaplan-Meier survival curve of restoration of hyponatraemia throughout hospitalization for malaria.

Separate curves for patients with mild and moderate-to-severe (labelled as "severe") hyponatraemia on admission are given. Mantel-Cox log-rank test showed a significant difference between the two curves (p=0.002).

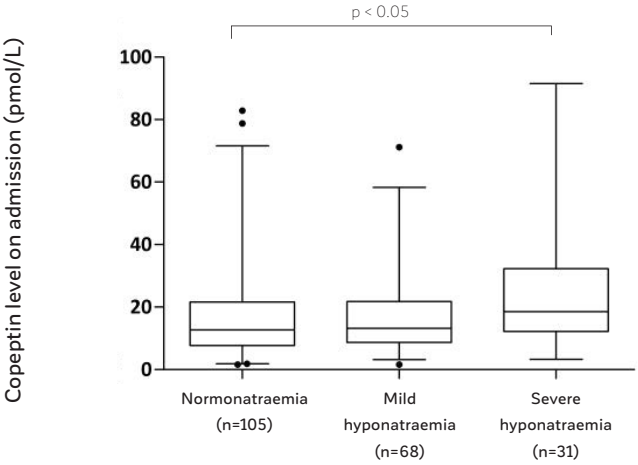


Figure 2 Box- and whiskerplot of copeptin levels in malaria patients with a normal serum sodium concentration on admission (n=105) and in patients with mild hyponatraemia (n=68) and moderate-to-severe (labelled as "severe") hyponatraemia (n=31) on admission.

The box indicates the lower and upper quartiles and the central line represents the median; the end of the whiskers reflect the 2.5th en 97.5th percentile of copeptin in malaria patients.

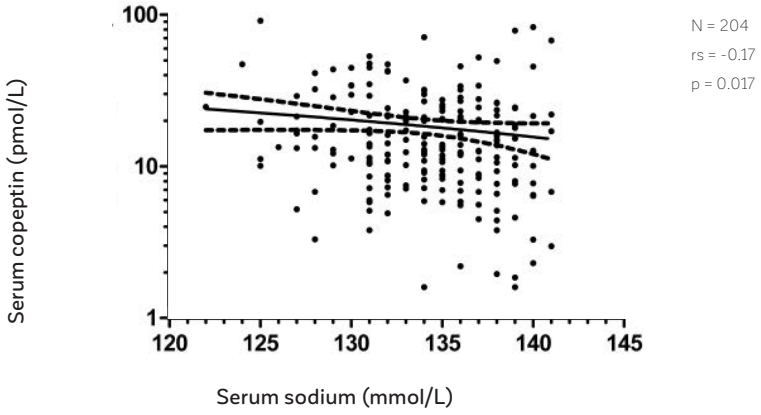
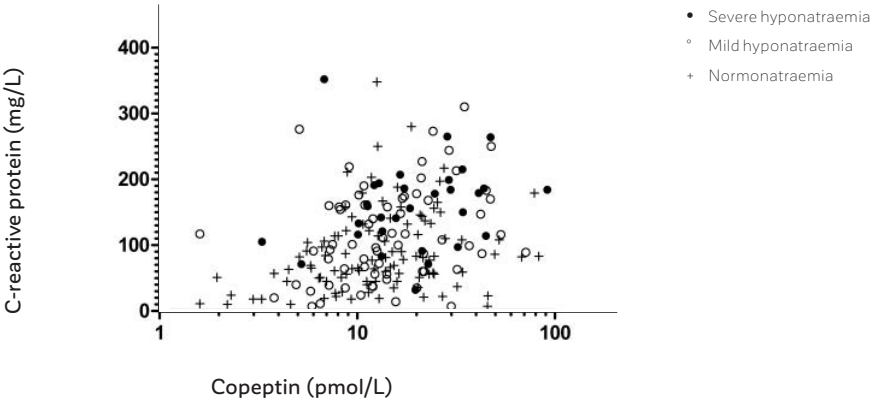


Figure 3. Dot plot of relationship between serum copeptin and serum sodium.

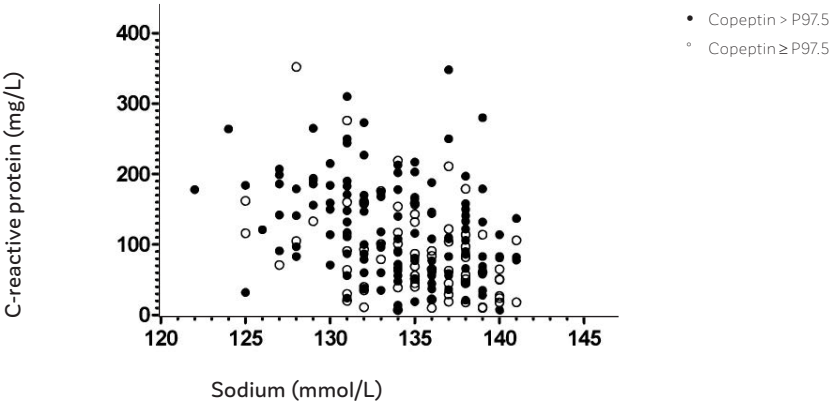
A weak but significant inverse relationship was observed (r_s=-0.17, p=0.017).



Supplementary Figure 1.

Dot plot of relationship between serum C-reactive protein and serum Copeptin on admission as a function of sodium level on admission.

A significant correlation between C-reactive protein and serum Copeptin was present (r_S=0.33, p<0.0001). Patients with moderate or severe hyponatraemia were grouped (labelled as "severe").



Supplementary Figure 2.

Dot plot of relationship between serum C-reactive protein and serum sodium on admission as a function of copeptin on admission.

Copeptin levels above and below the 97.5th percentile of normal are separately given. A significant inverse correlation was present between C-reactive protein and sodium on admission (r_S=-0.36, p<0.0001)

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APPENDIX

CHAPTER 12
Summarizing discussion

Malaria remains one of the most important causes of fever after international travel and must be considered in all patients who fall ill after returning from endemic areas. However, clinicians in non-endemic regions often have little experience with diagnosing and treating this infection and it has been demonstrated that lack of experience with malaria patients increases the case fatality rate (1, 2). Recognizing patients at risk for a severe course of the infection and awareness of the possible complications is essential. In this thesis we aimed to find tools to improve this assessment.

ASSESSING AND PREDICTING THE SEVERITY OF IMPORTED MALARIA

Definition of severe malaria

In the year 1990, the World Health Organisation (WHO) first introduced a set of criteria to define severe *Plasmodium falciparum* infection. The purpose of this case definition was primarily to facilitate clinical and epidemiological studies, but it also aimed to offer guidance in the clinical management of cases. The WHO recommends that patients fulfilling one or more of these criteria should be treated in an intensive care unit (ICU) (3-5). The set of criteria is based on clinical studies and expert opinion (6, 7), and has been modified and updated several times, most recently in 2014 (8). It has indeed been demonstrated that the mortality rate increases when a higher number of severity criteria is met (9). However, the frequency at which the different criteria occur differs largely between patient cohorts in endemic and non-endemic countries and between adults and children (10-12).

Predicting severity in two different worlds of malaria

In resource-limited settings, clinicians often face severe limitations in the capacity of ICU facilities, necessitating strict triage of patients. In this context,

the WHO definition for severe malaria is deemed too broad by some authors (6). As the WHO criteria have been found to have large disparities in their individual predictive value for outcome (6, 9, 13), *Hanson et al.* introduced the 5-point Coma Acidosis Malaria (CAM) score, that uses the two main independent predictors of mortality, acidosis and Glasgow Coma Score, which they derived from the Southeast Asia-located SEAQUAMAT trial (6, 14). The score performed well in predicting mortality and selecting patients with a need for ICU admission. However, in the low CAM-score group, which selects patients who may be safely monitored on a general ward, the mortality was still 4.2%. Moreover, in a London-based study the CAM-score was found to have had limited value in patients with severe imported malaria needing intensive care monitoring (15). These findings illustrate that predictors and scoring systems that are highly useful in endemic settings may not be applicable in non-endemic regions, and vice versa.

In non-endemic countries, an important factor that limits the use of the WHO definition to guide therapeutic decisions is the fact that the definition requires a quantitative *Plasmodium falciparum* load. Facilities for reliable quantification of parasitemia are not available in all hospitals at all hours outside endemic regions. In these situations, rapid diagnostic tests (RDT's) are often used to diagnose malaria. A marker that reliably can identify patients at risk for severe malaria would be of great help in these situations. Therefore, in Part I of this thesis, we aimed to identify parameters that may improve the identification of malaria patients at risk for a severe infection in non-endemic countries.

Host-related biomarkers as predictors for severe imported malaria

In recent years, biomarkers have become increasingly important within the field of infectious diseases and many biomarkers have been evaluated as predictors of outcome in bacterial sepsis. Since similarities in the pathogenesis and clinical presentation of bacterial sepsis and severe malaria exist (16), we evaluated whether biomarkers that have proven successful in sepsis could be equally valuable in the prediction of severity in malaria.

Three of those biomarkers were studied in Chapter 2. Procalcitonin is an extensively evaluated marker for bacterial infections that has been found to reliably predict mortality in patients with sepsis and respiratory infections (17, 18) and it can safely guide therapeutic decisions in septic patients on the ICU (19). Neopterin, a marker of activated cell mediated immunity, is produced by activated monocytes, macrophages, and dendritic cells (20), and was found to be

excreted after exposure to *Plasmodium falciparum* antigens in vitro (21). The triggering receptor expressed on myeloid cells (TREM)-1 is a marker that belongs to the immunoglobulin superfamily and is expressed on the surface of neutrophils, monocytes, and macrophages (22). Soluble TREM-1 yielded promising results in predicting severity of sepsis (23, 24). However, in this thesis we found that TREM-1 levels did not discriminate severe from uncomplicated disease in patients with imported malaria. Travellers with severe *P. falciparum* malaria did have significantly higher levels of neopterin and procalcitonin on admission than travellers with uncomplicated malaria. Both neopterin and procalcitonin had good areas under the receiver operating characteristic curve (AUROC's) of 0.85 and 0.78 respectively (Table 1). Although these biomarkers have a very high negative predictive value (NPV), the positive predictive value (PPV) of both markers was poor (Table 1).

In Chapter 3, we evaluated the glycopeptide copeptin, which forms the C-terminal part of the arginine vasopressin (AVP) precursor and is a stable and sensitive surrogate marker for AVP release (25, 26). In studies in patients with bacterial sepsis and in patients with leptospirosis, it was shown that copeptin levels correlate better with disease severity and outcome than previously evaluated biomarkers like C-reactive protein (CRP) and procalcitonin (27, 28). As one of the key similarities between leptospirosis and severe malaria is the frequent occurrence of hyponatraemia (29), which can be induced by increased AVP secretion (30, 31), we studied the correlation between copeptin levels and the severity and outcome of malaria. In contrast to studies in patients with severe bacterial sepsis and severe leptospirosis however, copeptin was not found to be superior to CRP in imported malaria.

These four biomarkers, procalcitonin, neopterin, TREM-1 and copeptin, all had good negative predictive values, and the first two even outperformed CRP. However, the PPV's were found to be poor (Table 1). In a clinical context this means that particularly procalcitonin and neopterin can be safely used to exclude severe disease and are more accurate than CRP. However, these biomarkers will overestimate disease severity in a large number of patients with malaria. Although one could argue that, in a resource-rich setting, an inclusive ICU-admission policy is defendable, the low PPV limits the usefulness of these biomarkers. An additional drawback is that these parameters are usually not routinely measured and their limited additional value to the routinely used parameter CRP does not warrant the extra cost and effort in clinical practice.

We therefore wondered if we could identify a routinely measured parameter that could help to improve the identification of severely ill patients. Lymphocytopenia has frequently been described in malaria patients, both in endemic and non-endemic areas (32-36), but results of studies on the correlation between lymphocyte count and malaria severity have been conflicting (32, 33, 37, 38). Both lymphocytopenia and the neutrophil-to-lymphocyte count ratio (NLCR) were found to be excellent predictors of bacteraemia and of severity and outcome of community acquired pneumonia (CAP), with higher prognostic accuracy than traditional infection markers (39, 40). Therefore, these parameters are interesting candidate predictors for disease severity in malaria.

As little is known about the dynamics of differential leukocyte count changes and the NLCR in malaria and the correlation between the differential leukocyte counts and *P. falciparum* parasitemia is not clear, we studied this in a proof-of-concept study. In Chapter 4, we investigated the dynamics of leukocyte count changes and the NLCR in a group of malaria-naïve Dutch volunteers, that were exposed to the bites of infected mosquitos in the setting of a controlled human malaria infection (CHMI). During the blood phase a marked decrease in total leukocyte count, lymphocyte count and neutrophil count and a rise of the NLCR were observed. Only the lymphocyte count and the NLCR correlated with parasitemia. As the leukocyte count changes were not observed in the subjects in the non-parasitemic control group, who did receive a full course of atovaquone / proguanil, a treatment-related effect could be excluded. We also found a rise in total leukocyte count and differential lymphocyte and monocyte count during the liver phase; as the liver phase is traditionally regarded as immunologically silent this is a surprising finding that has not been described before. In Chapter 5 we explored the prognostic value of the differential leukocyte count changes and the NLCR in a clinical setting. The NLCR was found to correlate with parasitaemia, but both lymphocytopenia and the NLCR were inferior to CRP as markers of severe disease in patients with imported malaria in direct back-to-back comparisons (Table 1).

The biomarkers discussed so far are all host-related and reflect the non-specific inflammatory response to an invading micro-organism. It is clear form our studies that markers which outperform traditional parameters like CRP in bacterial sepsis do not perform equally well in malaria. This is possibly a consequence of differences in pathogenesis. Although important similarities exist between sepsis and malaria, the latter obviously has its own specific pathophysiological mechanisms, most prominently the extensive microvascular obstruction by sequestered

parasites that may occur in severe malaria. Possibly, parasite-related parameters could be more accurate. We therefore studied two of these parameters: the aldolase co-reactivity of the rapid diagnostic tests and the presence of schizonts in peripheral blood.

Parasite-related biomarkers as predictors for severe imported malaria

Rapid diagnostic tests (RDTs) are highly sensitive tools to diagnose malaria in returning travellers (41), but are not designed to quantify parasitaemia, one of the major determinants of disease severity (42). In Chapter 6, we describe a multicentre operational laboratory study in which we have shown that the widely used Binax NOW® Malaria Test can be used for the semi-quantitative assessment of parasitaemia by differentiating between reactivity of only the *P. falciparum* specific *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP-2) or both the *Pf*HRP-2 and the pan-plasmodial aldolase. Co-reactivity of aldolase and *Pf*HRP-2 was present in all patients with a parasitaemia above 50,000 asexual parasites/µl and also in all patients with severe malaria. While microscopic determination of peripheral blood smears might underestimate the total parasitaemia due to sequestration of parasitized erythrocytes in the microvasculature, the reactivity of *Pf*HRP-2 and aldolase seems to be preserved. Aldolase co-reactivity had a high NPV but a poor PPV for severe malaria, as aldolase co-reactivity was also present in the majority of patients with uncomplicated malaria (Table 1). However, in settings where RDT's are performed in absence of possibilities for conventional quantification of the parasite load, it can be safely used to exclude high parasitaemia or severe malaria.

The second parasite-related biomarker that we evaluated is the presence of schizonts in peritherpal blood. During the erythrocytic cycle of *P. falciparum*, infected erythrocytes tend to adhere to the vascular endothelium and therefore the peripheral parasite count does not always correlate well with the total parasite load (43). The appearance of schizonts in peripheral blood smears is thought to reflect a high volume of sequestered parasites and, as rupturing schizonts release up to 32 merozoites, can be expected to precede a rise in parasitaemia (44-46). In Chapter 7 we found the presence of schizonts in peripheral blood smears to be associated with the need for ICU admission, renal replacement therapy, mechanical ventilation and a longer hospital stay. Schizontaemia has a high NPV for presence of severe disease and, as compared to other parameters, a good PPV. Schizontaemia was shown to be an independent predictor for severe malaria in a binary logistic regression analysis. As artemisinin derivatives like artesunate have

an anti-malarial efficacy to the broadest spectrum of parasite stages, including the generally less drug-sensitive schizonts (44, 47, 48), the presence of schizonts warrants the use of this class of antimalarial drugs.

With the evaluation of these two parasite-related biomarkers we showed that valuable additional information can be obtained from routinely performed diagnostic tests. In settings where quantification of parasitaema cannot be obtained, the aldolase co-reactivity on the widely used Binax NOW® Malaria Test can be used as a surrogate marker of parasitaemia as its absence reliably excludes high parasitaemia or severe disease. If blood slide microscopy can be performed, the presence of schizonts is an independent predictor for severe malaria that should elicit vigilance for complications of the infection, even at low peripheral parasitaemia. Schizontaemia should be used as an additional argument for treatment with intravenous artesunate.

ACUTE KIDNEY INJURY AND HYPONATRAEMIA

In Part II of this thesis, we focused on two frequently observed complications in patients with malaria; acute kidney injury (AKI) and hyponatraemia.

AKI occurs in up to 40% of adult patients with severe *P. falciparum* malaria in endemic areas and is reported in 34 to 52% of non-immune travellers with severe *P. falciparum* infection (12, 15, 49-54). In these studies, AKI is usually defined according to the WHO criteria for severe malaria, with a creatinine threshold of 265 µmol/L. This threshold however, is relatively high and consequently less severe or early forms of renal failure are not taken into account in these studies. In Chapter 8 we analysed a large cohort of 485 patients with an imported *P. falciparum* infection using the Kidney Disease: Improving Global Outcomes (KDIGO) staging, which includes early or less severe forms of AKI. We found KDIGO-defined AKI to occur in 8% of all malaria patients and in 38% of patients with severe malaria. Renal replacement therapy (RRT) was needed in only a small minority of eight patients, and in five of them renal function recovered fully during admission.

In our study, 12 (31%) of 39 patients with malaria-induced AKI had a normal serum creatinine at presentation. This finding illustrates the limitation of serum creatinine-based prediction models; significant renal injury can already have occurred while the serum creatinine level is still normal, as this starts to increase

substantially only 48 to 72 hours after the initial injury to the kidney (49). As structural kidney injury precedes this functional decline, a marker of structural damage could be expected to detect AKI much earlier, and for this reason markers of structural kidney damage have been under intense investigation for the early detection of AKI in recent years (56). In Chapter 9, we describe the predictive value of two markers of structural kidney damage: Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Kidney Injury Molecule-1 (KIM-1). good diagnostic value of serum NGAL, urine NGAL and urine KIM-1 for AKI in patients with imported malaria was demonstrated. Particularly urine NGAL at presentation was found to have an excellent predictive performance for the occurrence of AKI during admission, with a PPV of 1.00 (95% CI 0.54-1.00) and a NPV of 1.00 (95% CI 0.89-1.00). However, as only a small number of patients was evaluaterd, larger studies are needed to confirm our findings and to evaluate whether routine measurement of NGAL should be introduced for patients with malaria.

Although the pathophysiology of AKI is not fully understood, blockage of renal microcirculation by sequestered erythrocytes, immune-mediated glomerular injury and volume depletion are likely to contribute (57, 58). One could expect that a more rapid clearance of parasitaemia will abate the injuring processes and reduce the risk of the development or progression of AKI. Whether the use of artemisinine derivates, which results in faster clearance of parasitaemia than other classes of antimalarial drugs, reduces progression of AKI in patients with an elevated urine NGAL at presentation, would be an interesting question to be answered in future research.

In Chapter 10 we focus on hyponatraemia in malaria. Although hyponatraemia has previously been described as a complication of *P. falciparum* infection (59), the incidence has mostly been studied in endemic areas (59-63) and few studies have been performed in non-immune populations or in patients infected with other *Plasmodium* species. We studied the occurrence of hyponatraemia in patients with imported *P. falciparum* but also in non-*falciparum* infections and a high incidence in all patient groups: 77% in severe *P. falciparum* infection, 48% in non-severe *P. falciparum* infection, and 34% in patients with infections with non-*falciparum* species. Serum sodium <131 mmol/L was independently associated with severe disease. Despite its common occurrence, the pathophysiology of hyponatraemia in malaria remains a matter of debate. Hyponatraemia can be considered to be a water balance disorder, and is usually caused by increased secretion of arginine vasopressin (AVP). AVP is released by the posterior pituitary, most

commonly in response to hyperosmolarity or hypotension, which is regarded as appropriate AVP release. Its secretion can also be stimulated by pain and other non-specific causes of stress, which is seen as inappropriate AVP secretion (30, 31). In hyponatraemic patients with malaria, some studies found evidence for appropriate vasopressin release due to hypovolaemia (62) whereas other studies found evidence for inappropriate vasopressin release (64, 65). In Chapter 11 we evaluate the role of AVP in the pathophysiology of hyponatraemia in a large cohort of 204 patients with imported malaria by measuring copeptin. AVP release was found to be uniformly increased, and in a small prospective substudy, AVP release was found to be appropriate seven of 13 hyponatraemic patients. The inappropriate AVP release in the other hyponatraemic patients is possibly triggered by the host cytokine response to the parasite. The distinction between appropriate and inappropriate AVP release in hyponatraemic malaria patients may be relevant with regard to selecting the optimal intravenous fluid regimen. The subject of fluid resuscitation in malaria is controversial; some studies have advocated aggressive fluid resuscitation in severe malaria as it was hypothesized to play an important role in the development of acidosis (67), but more recent studies found acidosis to correlate more closely with the level of sequestration and demonstrated that liberal fluid resuscitation did not improve acidosis and even leads to an increased risk for acute pulmonary oedema (66, 67). These studies however, did not separate hyponatraemic malaria patients on the basis of appropriate or inappropriate AVP secretion. In general, patients with appropriate AVP release will benefit from treatment with isotonic fluids. One could hypothesize that this could also be the case for malaria patients with appropriate AVP release, while restrictive fluid therapy and more aggressive anti-malaria treatment would be preferable for patients with inappropriate AVP release. Further studies are needed to test this hypothesis.

IMPLICATIONS AND FUTURE PERSPECTIVES

Malaria is a potentially lethal infection and patients clearly benefit from specific medical expertise and the availability of appropriate laboratory facilities and medication. Therefore, the care for patients at risk for a severe infection should be centralised, while patients with a low risk for a severe course of the disease can be managed on the ward of a non-specialised centre, on condition that it's staff feels sufficiently capable to care fore a malaria patient. How do we select these patients at risk? In our studies we have demonstrated that the aldolase co-reactivity on the malaria RDT is a particularly useful tool for this. This parameter has a high

NPV for severe disease, and, as the RDT is routinely performed, does not require specific laboratory skills or extra costs. In settings where procalcitonin is part of the routine laboratory examinations, this can be involved in the clinical decision making as well. If high parasitaemia can be excluded by a negative aldolase co-reactivity on the RDT, and, if available, a procalcitonin < 0.9 ng/ml, patients can be safely treated with oral antimalarials on the ward of a non-specialised centre. However, if at risk for a severe infection, patients should be transferred to a facility with possibilities for reliable species determination, quantification and detection of peripheral schizontaemia, and with intravenous artesunate in stock. There, if after blood slide examination the patient meets the WHO severity criteria or, as this is an independent predictor for severity, schizonts are seen in the peripheral blood slide, the treatment should involve intravenous artesunate.

The search for more accurate parameters to predict malaria severity is ongoing. Possibly, combining existing biomarkers can improve the predictive performance. A promising novel parasite-related parameter is the quantitative load of *Pf*HRP-2, of which the plasma levels were found to correlate better with severity than parasitemia in studies in endemic areas (68-70). A recent study yielded promising results in imported malaria as well (71) and a proof-of-concept study demonstrated diagnostic potential of quantitative *Pf*HRP-2 levels in saliva, which would enable non-invasive measurements, but these findings need confirmation in larger clinical studies. A pitfall for these *Pf*HRP-2-based models however, is that *P. falciparum* parasites that lack the *Pf*HRP-2 protein, which have predominantly been found in South America (72), will not be detected. Molecular detection methods are also increasingly in use. Real-time polymerase chain reaction (PCR) based quantification of parasitaemia has been demonstrated to correlate closely with microscopy-based quantification and multiplex PCR methods can be particularly useful for the identification of mixed plasmodial infections (73) . However, as these methods take at least several hours to perform and are usually not available out of hours, they are currently of limited value in the clinical setting and are still predominantly used in clinical trials (74) and for the confirmation of species identification (75). Further research is needed to show which novel biomarkers or combinations of biomarkers will lead to a more accurate prediction of severity and complications of imported malaria.

Table 1 Summary of the performance of the analyzed parameters.

Parameter	Cut-off value	Sensitivity	Specificity	PPV	NPV	AUROC
CRP	>141 mg/L	0.80 (0.67-0.89)	0.76 (0.71-0.80)	0.33 (0.25-0.42)	0.96 (0.93-0.98)	0.84 (0.79-0.89)
Neopterin	≥ 10 ng/ml	0.93 (0.64-1.00)	0.67 (0.54-0.78)	0.38 (0.23-0.56)	0.98 (0.86-1.00)	0.85 (0.76-0.94)
Procalcitonin	≥ 0.9 ng/ml	1.00 (0.70-1.00)	0.53 (0.40-0.66)	0.30 (0.17-0.47)	1.00 (0.87-1.00)	0.78 (0.66-0.91)
Copeptin	≥ 21 pmol/L	0.60 (0.39-0.78)	0.73 (0.66-0.79)	0.31 (0.25-0.38)	0.93 (0.87-0.96)	0.66 (0.59-0.72)
Total leukocyte count	≥ 6.5 x 109/L	0.59 (0.46-0.71)	0.75 (0.71-0.80)	0.28 (0.21-0.37)	0.92 (0.88-0.95)	0.70 (0.63-0.78)
Neutrophil count	≥ 3.4 x 109/L	0.64 (0.51-0.76)	0.56 (0.51-0.61)	0.19 (0.14-0.25)	0.91 (0.86-0.94)	0.61 (0.53-0.69)
Lymphocyte count	< 0.7 x 109/L	0.33 (0.22-0.46)	0.72 (0.67-0.76)	0.16 (0.10-0.24)	0.87 (0.83-0.90)	0.51 (0.43-0.59)
NLCR	≥ 2.8	0.77 (0.64-0.86)	0.44 (0.39-0.49)	0.18 (0.14-0.24)	0.92 (0.87-0.96)	0.57 (0.50-0.64)
Aldolase coreactivity	N/A	1.00 (0.85-1.00)	0.32 (0.25-0.40)	0.21 (0.11-0.25)	1.00 (0.93-1.00)	N/A
Schizontaemia	N/A	0.53 (0.40-0.66)	0.95 (0.92-0.97)	0.67 (0.52-0.80)	0.92 (0.88-0.94)	N/A

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CHAPTER 13
Nederlandse samenvatting

Malaria is een van de belangrijkste oorzaken van koorts na internationale reizen en moet worden overwogen bij alle patienten die ziek worden na terugkeer uit een malaria-endemisch gebied. Medisch personeel in niet-endemische gebieden hebben echter vaak maar weinig ervaring met het vaststellen en behandelen van malaria, en het is aangetoond dat een gebrek aan ervaring met malaria bij de behandelaars het risico op sterfte bij de patient doet toenemen. Het herkennen van patienten met een verhoogd risico voor een ernstig beloop van de infectie en kennis over de mogelijke complicaties is essentieel. In dit proefschrift hebben we gezocht naar methoden om deze herkenning te verbeteren.

HET INSCHATTEN EN VOORSPELLEN VAN DE ERNST VAN IMPORTMALARIA

In 1990 introduceerde de World Health Organisation (WHO) een lijst met criteria om een ernstige *Plasmodium falciparum* infectie te definiëren. Het doel van deze casusdefinitie was primair het faciliteren van klinische en epidemiologische studies, maar diende ook als leidraad bij de behandeling van patiënten. De WHO adviseert om patiënten die aan een of meer van deze criteria voldoen te behandelen op een intensive care (IC) afdeling. In niet-endemische gebieden wordt het gebruik van de WHO definitie echter beperkt door het feit dat deze een kwantitatieve bepaling van de *P. falciparum* parasitemie vereist. Faciliteiten hiervoor zijn niet in alle ziekenhuizen op elk moment van de dag beschikbaar, en vaak worden er alleen sneltesten gebruikt om malaria vast te stellen. Een parameter die op een betrouwbare manier de patiënten met een verhoogd risico op een ernstig beloop zou kunnen identificeren zou in deze situaties van belangrijke toegevoegde waarde kunnen zijn.

In Deel I van dit proefschrift hebben we gezocht naar dergelijke parameters.

Gastheer-gerelateerde biomarkers

Biomarkers zijn de laatste jaren steeds belangrijker geworden binnen de infectieziekten en reeds veel verschillende biomarkers zijn geëvalueerd als voorspellers van uitkomst in patiënten met bacteriële sepsis. Omdat er belangrijke overeenkomsten bestaan in de pathogenese en het klinisch beeld van bacteriële sepsis en ernstige malaria, hebben we onderzocht of biomarkers die succesvol zijn gebleken bij septische patiënten ook bruikbaar kunnen zijn in de voorspelling van de ernst van malaria. Drie van deze biomarkers werden bestudeerd in Hoofdstuk 2, te weten procalcitonine, neopterine en triggering receptor expressed on myeloid cells (TREM)-1. In onze studie kon met TREM-1 geen onderscheid worden gemaakt tussen ernstige en ongecompliceerde malaria. Wel hadden reizigers met ernstige *P. falciparum* infecties bij opname significant hogere waarden van neopterine en procalcitonine dan reizigers met ongecompliceerde malaria. Zowel neopterine als procalcitonine hadden een zeer goede negatief voorspellende waarde, maar hun positief voorspellende waarde was beperkt.

In hoofdstuk 3 evalueerden we copeptine, een glycopeptide dat deel uitmaakt van precursor van arginine vasopressin (AVP) en een stabiele en sensitieve marker is voor productie van AVP. In studies in patiënten met sepsis en leptospirose correleerde copeptine beter met ernst van de infectie dan C-reactive protein (CRP) en procalcitonine. Een opvallende overeenkomst tussen leptospirose en malaria is het frequente optreden van hyponatriemie, hetgeen meestal het gevolg is van toegenomen AVP productie. In tegenstelling tot de studies in sepsis en leptospirose bleek copeptine in onze studie echter niet superieur aan CRP voor het voorspellen van ernst van importmalaria.

Ondanks de hoge negatief voorspellende waarde van de geëvalueerde biomarkers, waarbij die van procalcitonine en neopterine zelfs hoger was dan die van CRP, wordt hun bruikbaarheid beperkt door het feit dat ze niet routinematig worden gemeten. We zochten daarom verder naar bruikbare markers die wel routinematig worden bepaald.

Lymfocytopenie is frequent beschreven bij malaria, zowel in endemische gebieden als bij importmalaria, maar studies naar de correlatie tussen het lymfocytenaantal en de ernst van malaria hebben tegenstrijdige resultaten laten zien. Zowel lymfocytopenie als de verhouding tussen neutrofielen en lymfocyten, de neutrophil-to-lymphocyte count ratio (NLCR) zijn uitstekende voorspellers van de aanwezigheid van een bacteriëmie en van ernst en uitkomst van bacteriële

luchtweginfecties, met hogere prognostische waarde dan traditionele markers. Omdat er weinig bekend is over de dynamiek van de het totale aantal leukocyten en leukocytensubpopulaties en van de NLCR bij malaria hebben we dit onderzocht in de setting van een gecontroleerde malariainfectie, een controlled human malaria infection (CHMI) in hoofdstuk 4. We keken hierbij naar de veranderingen van de totale en differentiële leukocytenaantallen en hun ratio's in een groep malaria-naïeve Nederlandse vrijwilligers, die werden blootgesteld aan de beten van met malaria geïnfecteerde muggen. Er werd een afname van het totale aantal leukocyten en de lymfocyten en neutrofielen gezien tijdens de bloedfase, met een stijging van de NLCR, en er was een correlatie tussen de parasitemie enerzijds en het lymfocytenaantal en de NLCR anderzijds. Naast deze bevindingen werd er ook een stijging van het aantal leukocyten, monocyten en lymfocyten tijdens de leverfase gevonden. Deze bevindingen zijn niet eerder beschreven. In hoofdstuk 5 hebben we de prognostische waarde van de differentiële leukocytenaantallen en hun ratio's bij patiënten bekeken. De NLCR correleerde ook hier met parasitemie, maar was inferieur aan CRP als marker voor ernstige infectie bij importmalaria.

Parasiet-gerelateerde biomarkers

Sneltesten zijn zeer sensitief voor het vaststellen van malaria bij reizigers, maar zijn niet gemaakt om de parasitemie ook te kwantificeren. Een hoge parasitemie is echter een belangrijke determinant van een ernstige infectie. In hoofdstuk 6, beschrijven we een we een multicenter laboratoriumstudie waarin we hebben aangetoond dat de veel gebruikte Binax NOW® Malaria Test kan worden gebruikt voor een semi-kwantitatieve bepaling van de parasitemie door te differentiëren tussen reactiviteit van alleen het *P. falciparum* specifieke *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP-2) bandje of zowel het *Pf*HRP-2 als het pan-plasmodiale aldolase bandje. Co-reactiviteit van aldolase en *Pf*HRP-2 werd gezien bij alle patiënten met een parasitemie boven de 50,000 aseksuele parasieten/µl en ook bij alle patiënten met ernstige malaria. Aldolase co-reactiviteit had een hoge negatief voorspellende waarde maar een matige positief voorspellende waarde doordat aldolase co-reactiviteit ook aanwezig was bij de meerderheid van de patienten met ongecompliceerde malaria. Echter, in een situatie waar er sneltesten worden gebruikt zonder dat er mogelijkheden zijn voor conventionele kwantificatie van de parasitemie, kan het veilig worden gebruikt om een hoge parasitemie of ernstige malaria uit te sluiten.

De tweede parastiet-gerelateerde biomarker die we onderzochten is de aanwezigheid van schizonten in het perifere bloed. Rode bloedcellen die geparasiteerd

zijn door *P. falciparum* hebben de neiging vast te plakken aan het vasculaire endotheel en daarmee uit het perifere bloed te verdwijnen. Daarom correleert de perifere parasitemie niet altijd goed met de totale parasietenload. Het verschijnen van schizonten in het perifere bloed wordt gezien als een teken van een hoog volume van gesekwestreerde geparasiteerde erythrocyten, en aangezien ruptur erende schizonten tot 32 dochter-merozoïeten kunne vrijlaten, kan het verschijnen van schizonten ook voorafgaan aan een sterke stijging van de parasitemie. In hoofdstuk 7 vonden we dat de aanwezigheid van schizonten geassocieerd was met de noodzaak tot IC opname, nierfunctievervangende therapie, kunstmatige beademing en een langere opnameduur. Schizontemie is een onafhankelijke risicofactor voor ernstige malaria. Omdat artemisininederivaten een goede schizontocide werking hebben gaat de voorkeur bij schizontemie uit naar behandeling met deze klasse middelen.

ACUTE NIERINSUFFICIENTIE EN HYPONATRIËMIE

In het tweede deel van dit proefschrift richten we ons op twee veelvoorkomende complicaties bij importmalaria: acute nierinsufficiëntie en hyponatriëmie.
In hoofdstuk 8 onderzochten we de incidentie van nierinsufficiëntie in een groot cohort patiënten met importmalaria. Anders dan in de meeste eerdere studies gebruikten we hiervoor de Kidney Disease: Improving Global Outcomes (KDIGO) stadiering, waarmee niet alleen ernstige maar ook vroege of minder ernstige nierinsufficiëntie wordt geincludeerd. We vonden dat nierinsufficiëntie optrad bij 8% van alle patiënten met malaria en bij 38% van de patiënten met ernstige malaria. Nierfunctievervangende therapie was in slechts een klein aantal patienten nodig en bij de meerderheid van hen herstelde de nierfunctie nog tijdens opname volledig. Ook in onze studie echter had 31% van de patiënten met een acute nierinsufficiëntie bij opname nog een normale kreatininewaarde. Deze bevinding illustreert de beperking van het gebruik van deze waarde: de kreatinineconcentratie begint pas 48 tot 72 uur na het ontstaan van de structurele nierschade te stijgen. In hoofdstuk 9 onderzoeken we daarom de diagnostische waarde van twee markers van structurele nierschade: Neutrophil Gelatinase-Associated Lipocalin (NGAL) en Kidney Injury Molecule-1 (KIM-1). We vonden een goede diagnostische waarde van serum NGAL, urine NGAL en urine KIM-1. Met name urine NGAL bij presentatie had een uitstekende voorspellende waarde voor het ontstaan van acute nierinsufficiëntie tijdens opname met een positief voorspellende waarde van 1.00 (95% CI 0.54-1.00) en een negatief voorspellende

waarde van 1.00 (95% CI 0.89-1.00). Omdat dit een pilot-studie was met slechts een klein aantal patiënten zijn er grotere studies nodig om deze bevindingen te bevestigen.

In hoofdstuk 10 onderzoeken we hyponatriëmie bij malaria. Hoewel hyponatriëmie eerder is beschreven als complicatie van *P. falciparum* infecties in endemische gebieden is er weinig bekend over de incidentie bij importmalaria of bij patiënten met non-*falciparum* infecties. In onze studie naar het optreden van hyponatriëmie bij importmalaria vonden we een hoge incidentie in alle patientengroepen; 77% bij ernstige *P. falciparum* infecties, 48% bij ongecompliceerde *P. falciparum* infecties en 34% bij non-*falciparum* malaria. Serum natrium <131 mmol/L was een onafhankelijke voorspeller voor ernstige malaria.

In hoofdstuk 11 evalueren we de rol van het antidiuretisch hormoon, ook wel bekend als arginine vasopressine (AVP) in de pathofysiologie van hyponatriemie in malaria. Hyponatriëmie kan worden beschouwd als een verstoring van de waterbalans, en wordt meestal veroorzaakt door waterretentie door een toegenomen secretie van AVP. Deze AVP afgifte kan 'terecht' zijn, zoals bij hyperosmolaliteit of hypotensie, maar kan ook 'onterecht' worden afgegeven als respons op aspecifieke stimuli als stress en pijn. In cohort van 204 patiënten met importmalaria vonden we een uniform verhoogde AVP afgifte. Bij een kleine prospectieve substudie bleek dat er bij ongeveer de helft van de patiënten met hyponatriëmie sprake was van een terechte, en bij de andere helft van een onterechte AVP afgifte. Dit onderscheid zou van belang kunnen zijn in het bepalen van het optimale rehydratiebeleid.

CHAPTER 14
About the Author

CURRICULUM VITAE

Marlies van Wolfswinkel was born in Leiden, the Netherlands, on January 11th, 1983 and was raised in Rijswijk. She finished secondary school at the Gymnasium Haganum in The Hague in 2000 and started studying medicine at the Leiden University in the same year. A clinical rotation in a rural hospital in Mugumu, Tanzania left her with a fascination for infectious diseases and a love for the African continent, where she would return many times for work and travel. After receiving her medical degree she started working as a junior doctor at the internal medicine department of the Harbour Hospital and Institute for Tropical Diseases in Rotterdam, the Netherlands. Before applying for the specialist training in internal medicine, she worked for a year as a registrar at the internal medicine department of the Queen Elizabeth Central Hospital in Blantyre, Malawi. After this year, she returned to the Harbour Hospital in Rotterdam to start her specialist training in 2010 (supervisor: P.J. Wismans). In this period, she also started her research on imported malaria, which eventually led to this thesis. In 2012 she worked for four months at the department of pulmonary medicine and tuberculosis of the Groote Schuur Hospital in Cape Town, South Africa, and continued her training the Maasstad Hospital (supervisor: M.A. van den Dorpel) and the Erasmus Medical Centre (supervisors: J.L.C.M. van Saase and S.C.E. Klein Nagelvoort-Schuit), in Rotterdam, the Netherlands. In 2014 she started her specialisation in infectious diseases at the Erasmus Medical Centre (supervisor: J.L. Nouwen). In 2014, during the Ebola epidemic, she joined Médecins sans Frontières to work in an Ebola Treatment Centre in Sierra Leone for six weeks, and in 2015 she spent a trimester in the Academic Hospital Paramaribo in Suriname. She finished her specialisation in 2016. In January 2017 she moved from Rotterdam to Maastricht, where she currently works as an internist-infectious diseases specialist at the Maastricht University Medical Centre.

PHD PORTFOLIO

Courses and seminars	
Biostatistics for Clincians, Netherlands Institute of Health Sciences, Rotterdam, the Netherlands	2015
Statistics in Medicine, Stanford University online course	2016
Evidence Based Medicine, Erasmus University, Rotterdam, the Netherlands	2014
Teach the Teacher, Erasmus University, Rotterdam, the Netherlands	2016
Medical Ethics, Erasmus University, Rotterdam, the Netherlands	2014
Research seminars, Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam, the Netherlands	2014-2016
Congresses	
Internistendagen, Maastricht, the Netherlands	2010- 2017
Infectious Diseases (ID) week, Philadelphia, United States	2015
Symposium Nederlandse vereniging voor arts-assistenten medische microbiologie (NVAMM), Utrecht, the Netherlands	2015
European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Barcelona, Spain	2015
Internistendagen, Maastricht, the Netherlands	2016
European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, The Netherlands	2016
Teaching and supervision	
Classes in clinical decision making for master-phase students	2014-2016
Clinical supervision of medical students and residents	2010-2017

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CHAPTER 15
Dankwoord

Toen ik als arts-assistent interne geneeskunde in het Havenziekenhuis kwam werken, was promoveren niet direct een doel. Wel waren er boeiende patiënten, interessante ziektebeelden en vragen. Zo begon ik aan mijn eerste artikel over importmalaria. Na dat eerste artikel volgden er meer en zo ontstond dit proefschrift eigenlijk nog voordat ik mij er zelf bewust van was.

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