Immune responses to malaria - Across early life, chronic exposure, and acute malaria

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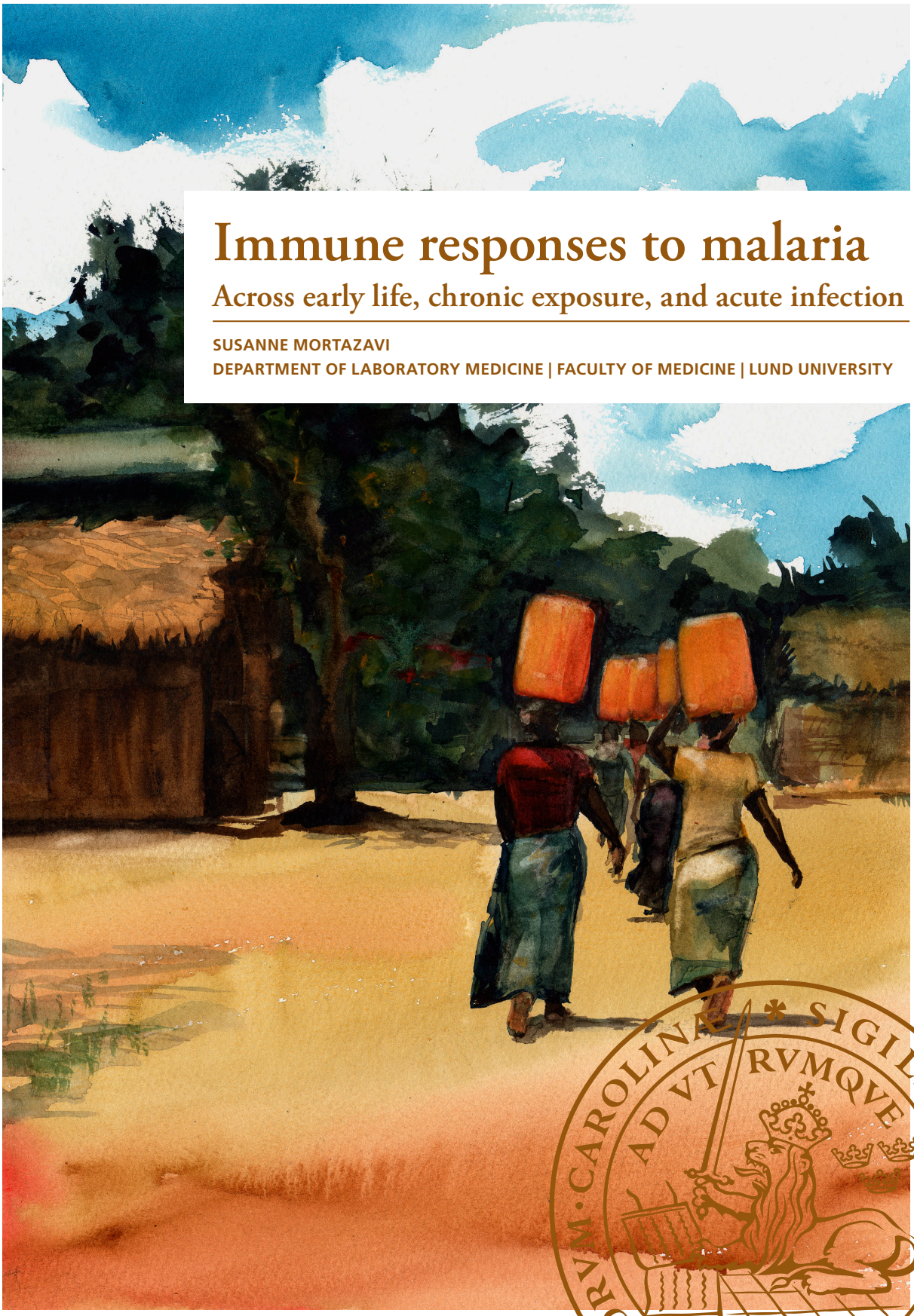

Immune responses to malaria

Across early life, chronic exposure, and acute infection

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DEPARTMENT OF LABORATORY MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY

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About the author

Susanne Mortazavi is a specialist in infectious diseases at Skåne University Hospital in Lund, Sweden, with a particular interest in tropical and mosquito-borne diseases such as malaria. She obtained her medical degree from Karolinska Institute in Stockholm and has since worked clinically in Sweden as well as internationally, including in the Democratic Republic of Congo. She completed her specialist training in Infectious Diseases in Lund. Her doctoral research focuses on immunity to malaria, with particular emphasis on B-cell responses, antibody function, and the role of inflammatory mediators in shaping immune regulation during infection.

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It's not the numbers that are interesting.
It's what they tell us about the lives behind the numbers.

Hans Rosling

Immune responses to malaria

Across early life, chronic exposure, and acute infection

Susanne Mortazavi

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 22 of May at 09.00 in Belfragesalen, BMC, Sölvegatan 17

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Abstract: Malaria caused by *Plasmodium falciparum (P. falciparum)* remains a major cause of morbidity and mortality, particularly in sub-Saharan Africa. Immunity develops gradually after repeated exposure and is largely mediated by antibodies, yet the mechanisms regulating the development and function of humoral immunity, including the roles of B-cell subsets and inflammatory mediators, remain incompletely understood. This thesis therefore investigates humoral immune responses to *P. falciparum*, focusing on antibody function, B-cell differentiation, and immune regulatory pathways across different exposure contexts.

In a longitudinal Ugandan mother-infant cohort (Paper I), C1q-fixing antibodies against *P. falciparum* merozoites were high in infants at birth and comparable to maternal levels, consistent with effective placental transfer. Levels declined during early infancy and increased as endogenous responses developed. C1q-fixing antibodies correlated with schizont-specific IgG and atypical memory B cells, linking functional antibodies to parasite exposure. In the same cohort (Paper II), the immunomodulatory protein osteopontin (OPN) was highest in early infancy and exceeded maternal levels. OPN correlated positively with naïve B cells and B-cell activating factor, and negatively with atypical and IgG memory B cells. No direct effect on parasite growth was observed *in vitro*. These results suggest a role for OPN in immune regulation during early B-cell development rather than direct antiparasitic activity. During acute malaria (Paper III), OPN levels were elevated in children in Uganda and in adults with imported malaria, and correlated with parasitemia. In contrast, adults living in endemic settings showed lower OPN responses. Elevated levels were also observed in non-malarial febrile illness, indicating that OPN reflects general inflammatory activation rather than malaria-specific responses. Finally, PD-1 expression across B-cell subsets (Paper IV) was influenced by both exposure history and infection status. Chronic malaria exposure was associated with expansion of atypical and activated memory B cells. Increased PD-1 expression was observed specifically in individuals with acute malaria and prior exposure, with broad upregulation across B-cell subsets. PD-1 expression was also enriched among *P. falciparum*-binding B cells. Together, these findings suggest a role for PD-1 in longer-term immune regulation.

This thesis provides insight into how antibodies, B cells, and immunomodulatory signals develop and are regulated across different stages of life and infection. Future studies should determine how these immune features influence parasite-specific B-cell responses and functional antibody activity, which are important for understanding the development of naturally acquired immunity to malaria and may inform the design of more effective and durable malaria vaccines.

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MADE IN SWEDEN

Till Mommo

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Preface

From a young age, I was fascinated by parasites and other small organisms, while also aspiring to become a doctor and to work in different parts of the world. These interests followed me into medical school and eventually led me to work as a doctor with Médecins Sans Frontières. During my time in the Democratic Republic of the Congo, I worked at a small hospital that received a constant flow of children suffering from malaria. Many arrived too late, often after walking for several days to reach medical care, and mortality rates were high. Despite the availability of effective medicines, we were often unable to help. This experience gave me a broader perspective on malaria, as I saw how closely the disease was linked to poverty, limited infrastructure, and fragile health systems. It also highlighted the importance of prevention through functioning health systems, early diagnosis, and effective control measures in reducing the burden of malaria.

When I returned to Sweden, I knew that I wanted to continue working with malaria. By chance, I learned about Kristina, and I am grateful that she welcomed me into her research group despite my background as a clinician and my limited laboratory experience at the time. Through this work, I was introduced to the immunology of malaria and to questions I had encountered in the clinic but had not been able to explore scientifically. Studying immune responses became a way for me to approach the same disease from a different perspective.

This thesis begins with an overview of the history and epidemiology of malaria, followed by disease mechanisms and recent developments in diagnostic methods and vaccination. It then focuses on the immunology of malaria, particularly humoral immune responses involved in naturally acquired immunity. The thesis concludes with a summary of methods, major findings, and a general discussion with future perspectives.

Populärvetenskaplig sammanfattning

Malaria är en infektionssjukdom som orsakas av parasiter av släktet *Plasmodium* och sprids till människor genom bett från myggor av släktet *Anopheles*. Sjukdomen orsakar varje år över 200 miljoner sjukdomsfall och nästan 600 000 dödsfall. Den drabbar främst små barn och gravida kvinnor i Afrika söder om Sahara, där den mest sjukdomsframkallande arten, *Plasmodium falciparum*, är vanlig.

När en infekterad mygga biter en människa förs parasiten in i blodet och transporteras till levern, där den förökar sig innan den återvänder till blodet och infekterar röda blodkroppar. Det är den sistnämnda delen av parasitens livscykel som orsakar sjukdomssymptomen, som ofta innefattar feber, frossa, huvudvärk och kraftig trötthet. I svåra fall kan malaria leda till blodbrist, organsvikt, koma och död.

Under flera decennier har globala insatser gjorts för att minska spridningen av malaria, bland annat genom impregnerade myggnät, förbättrad diagnostik och effektivare läkemedelsbehandling. Dessa insatser har räddat många liv, men under de senaste åren har utvecklingen stannat av. Samtidigt uppstår nya utmaningar, såsom resistens mot både insektsmedel och läkemedel samt mutationer hos parasiten som kan göra den svårare att upptäcka med vanliga diagnostiska tester. Därför behövs nya strategier för att minska sjukdomsbördan, och utvecklingen av effektiva vacciner mot malaria har blivit en viktig forskningsprioritet.

För att kunna utveckla ett vaccin behövs en bättre förståelse för hur immunitet mot malaria uppstår. Studier sedan 1960-talet har visat att antikroppar spelar en viktig roll i skyddet mot malaria, men det är fortfarande oklart vilka antikroppssvar som är mest skyddande. Immuniteten utvecklas långsamt och kräver upprepad exponering för parasiten under flera år. Den skyddar framför allt mot sjukdom, men inte nödvändigtvis mot infektion, vilket innebär att personer i malariaområden kan bära på låga nivåer av parasiter i blodet utan tydliga symtom.

Trots omfattande forskning finns det fortfarande stora kunskapsluckor kring hur immunförsvaret reagerar på malaria och vilka mekanismer som bidrar till skydd mot sjukdomen. Särskilt viktigt är att förstå hur antikroppar och B-celler, immunceller som producerar antikroppar, påverkas av infektion och tidigare malariaexponering. Denna avhandling omfattar fyra delstudier som undersöker hur immunförsvaret utvecklas tidigt i livet i malariaendemiska områden, hur det reagerar vid akut och upprepad infektion och hur olika immunologiska signalproteiner är kopplade till dessa processer.

Det första arbetet undersökte utvecklingen av antikroppar som kan aktivera komplementsystemet, en del av immunförsvaret som hjälper till att bekämpa infektioner genom att binda till malariaparasiten och bidra till att den förstörs. I studien följdes mödrar och barn i Uganda från födseln och under de första levnadsåren. Antikropparna överfördes effektivt från mamma till barn under graviditeten och fanns i höga nivåer vid födseln. Nivåerna minskade dock snabbt under de första månaderna efter födseln innan barnets eget immunförsvar gradvis började producera nya antikroppar i takt med att barnet exponerades för malariaparasiten. Detta tyder på att funktionella antikroppssvar mot malaria byggs upp gradvis under barndomen i takt med upprepad exponering för parasiten.

Det andra arbetet fokuserade på ett protein som heter osteopontin (OPN), en signalmolekyl i immunförsvaret som kan påverka hur olika immunceller utvecklas och aktiveras. I studien mättes OPN-nivåer hos mödrar och spädbarn i Uganda för att undersöka hur nivåerna förändras under barnets första levnadsmånader och hur de hänger samman med olika typer av B-celler. Spädbarn hade betydligt högre nivåer av OPN än sina mödrar, särskilt under de första månaderna i livet. Höga nivåer sågs främst när immunförsvaret till stor del bestod av omogna B-celler, vilket kan tyda på att OPN kan påverka hur B-celler och antikroppssvar utvecklas tidigt i livet.

Det tredje arbetet undersökte hur nivåerna av OPN förändras vid akut malaria. Blodprover analyserades från personer med malaria i Uganda och Sverige, vilket gjorde det möjligt att jämföra immunförsvaret hos personer med olika grader av malariaexponering. OPN ökade tydligt vid akut malaria hos barn i Uganda och hos resenärer med malaria i Sverige. Däremot sågs inte samma ökning hos vuxna som levde i malariaområden och hade tidigare exponering för parasiten. Detta tyder på att OPN främst speglar inflammatorisk aktivering av immunförsvaret vid infektion och att denna respons kan vara mer uttalad hos personer som ännu inte utvecklat immunitet mot malaria.

Det fjärde och sista arbetet undersökte uttrycket av det immunreglerande proteinet Programmed Cell Death-1 (PD-1) på olika B-cellsgrupper. PD-1 fungerar som en broms i immunförsvaret och kan påverka hur immunceller aktiveras vid infektion. B-celler analyserades från personer med olika grader av malariaexponering, både vid akut infektion och hos friska individer. PD-1 uttrycktes i högre grad vid en akut infektion hos personer med tidigare malariaexponering. Uttrycket var också högre på B-celler som kunde känna igen malariaparasiten. Detta tyder på att PD-1 kan ha en roll i hur antikroppssvaret formas vid upprepad exponering för malaria.

Sammantaget belyser denna avhandling att utvecklingen av immunitet mot malaria är en komplex process som påverkas både av exponering för malariaparasiten såväl som av immunologiska och reglerande mekanismer. En bättre förståelse för dessa processer kan bidra till kunskap om hur immunitet uppstår och hur mer effektiva vacciner kan utvecklas.

List of Papers

This thesis is based on the following papers, which will be referred in text by their roman numerals (I – IV).

Paper I

Mortazavi SE, Lugaajju A, Nylander M, Danielsson L, Tijani MK, Beeson JG, Persson KEM. Acquisition of complement fixing antibodies targeting *Plasmodium falciparum* merozoites in infants and their mothers in Uganda. *Front Immunol*. 2023 Nov 28;14:1295543.

Paper II

Mortazavi SE, Lugaajju A, Kaddumukasa M, Tijani MK, Kironde F, Persson KEM. Osteopontin and malaria: no direct effect on parasite growth, but correlation with *P. falciparum*-specific B cells and BAFF in a malaria endemic area. *BMC Microbiol*. 2021 Nov 6;21(1):307.

Paper III

Mortazavi SE, Lugaajju A, Danielsson L, Wu B, Norrgren H, Persson KEM. Dynamics of osteopontin levels and correlation with parasitemia in acute malaria in Uganda and Sweden. *BMC Infect Dis*. 2024 Oct 15;24(1):1164.

Paper IV

Mortazavi SE, Lugaajju A, Tijani MK, Wu B, Danielsson L, Norrgren H, Persson KEM. Malaria exposure history shapes PD-1 expression across human B-cell subsets during acute *Plasmodium falciparum* infection. *Submitted.*

Papers not included in this thesis

Lugaajju A, Idro R, Kiwuwa S, Beeson JG, Drew DR, **Mortazavi SE**, Linse S, Persson KEM. Development of high affinity antibodies to *Plasmodium falciparum* merozoite and sporozoite antigens during infancy and adulthood. *Front Immunol*. 2025 Jul 2;16:1562671.

Mortazavi SE, Lugaajju A, Ivarsson A-C, Karlsson Söbirk S, Norrgren H and Persson KEM. (2025). High rate of false positive malaria rapid diagnostic tests in a district hospital in Uganda. *Front Malar*. 3:1545825.

Mortazavi SE, Inghammar M, Christiansen C, Pesola AK, Stenkilsson M, Paulsson M. A retrospective cohort study of the effect of SARS-CoV-2 point of care rapid RT-PCR at the Emergency Department on targeted admission. *BMC Infect Dis*. 2022 Jun 13;22(1):536.

Abbreviations

P. falciparum	*Plasmodium falciparum*
An.	*Anopheles*
WHO	World Health Organization
MDGs	Millenium Development Goals
ITN	Insecticide-treated bed net
RDT	Rapid diagnostic test
ACT	Artemisinin-based combination therapies
ITPp	Intermittent preventive treatment in pregnancy
VFR	Visiting friends and families
CSP	Circumsporozoite protein
PCR	Polymerase chain reaction
iRBC	Infected red blood cells
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
EPCR	Endothelial C receptor
GPI	Glycosylphosphatidylinositol
TNF	Tumour necrosis factor
IFN-γ	Interferon-γ
HRP2	Histidine-rich protein 2
pLDH	*Plasmodium* lactate dehydrogenase
LAMP	Loop-mediated isothermal amplification
ACT	Artemisinin-based combination therapies
AMA1	Apical membrane antigen 1
EBA-175	Erythrocyte-binding antigen-175
PfRH5	*P. falciparum* reticulocyte-binding protein homolog 5

CHMI	Controlled human malaria infection
LLPCs	Long-lived plasma cells
MBCs	Memory B cells
BAFF	B-cell activating factor
atMBCs	Atypical memory B cells
C1q	Complement component 1q
OPN	Osteopontin
SPP1	Secreted phosphoprotein-1
ETA-1	Early T-lymphocyte activation-1
PD-1	Programmed cell death protein-1
NK cells	Natural killer cells
PBMC	Peripheral blood mononuclear cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate-buffered saline
GiRBCs	Ghost-infected red blood cells
Qdots	Quantum dots

Thesis at a glance

Paper	Aim	Method	Key results	Conclusion
I	To describe the development of *P. falciparum* merozoite-specific C1q-fixing antibodies from birth through childhood and their associations with malaria-specific responses.	Prospective longitudinal Ugandan mother-infant cohort; ELISA for merozoite-specific C1q-fixing antibodies.	High levels at birth with rapid decline in infancy and gradual increase with age; associated with malaria-specific IgG and atypical MBCs.	C1q-fixing antibodies are maternally transferred, wane in infancy, and are reacquired with malaria exposure.
II	To examine plasma OPN levels in early life and their associations with immune parameters and parasite growth.	Prospective longitudinal Ugandan mother-infant cohort with adult controls; plasma OPN measured by ELISA; *in vitro* growth inhibition assays.	OPN levels were high in infancy, declined with age, and correlated with B-cell subsets and BAFF but not parasite growth.	OPN levels reflect age-dependent immune development during infancy in a malaria-endemic setting and do not show direct anti-parasitic activity.
III	To examine plasma OPN levels during acute malaria in relation to exposure, parasite burden, inflammation, and convalescence.	Cross-sectional study with follow-up; OPN and IFN-γ measured by ELISA in cohorts from endemic and non-endemic settings.	OPN was elevated in children and imported malaria cases, correlated with parasitemia, and declined during follow-up in children.	OPN is associated with inflammatory responses during acute malaria in individuals with limited immunity.
IV	To characterize PD-1 expression on B-cell subsets in relation to malaria exposure and acute infection, and to evaluate whether PD-1 expression is more frequently expressed in *P. falciparum*-binding B cells than in non-binding B cells.	Cross-sectional study; flow cytometry analysis of B-cell subsets and PD-1 expression.	PD-1 expression differed by exposure and infection, with broad induction during acute malaria in exposed individuals and higher levels on *P. falciparum*-binding B cells.	PD-1 expression on B cells is shaped by prior malaria exposure during acute infection.

Introduction

Malaria presents a paradox of immunity: despite repeated infection, protective immune responses develop slowly and rarely prevent reinfection. Although extensively studied, the mechanisms underlying protective immunity are still not fully understood. Infection with *Plasmodium falciparum* (*P. falciparum*) induces immune responses that contribute both to parasite control and to disease, and individuals living in endemic areas gradually acquire protection against clinical malaria only after repeated exposure. This protection is incomplete and varies between individuals, indicating that naturally acquired immunity reflects complex and regulated host responses.

Antibodies are widely recognized as important mediators of protection, but their role extends beyond antibody levels alone. Functional properties of antibodies, together with B-cell differentiation and the inflammatory environment, all appear to influence the development and effectiveness of immune responses. Repeated malaria exposure is also associated with alterations in immune-cell populations, suggesting that immune activation and immune regulation develop in parallel.

In this context, the work presented in this thesis examines how humoral immune responses, inflammatory mediators, and regulatory pathways are shaped across early life, cumulative exposure, and acute malaria infection in individuals with different levels of prior immunity.

Malaria

History

Malaria is one of the oldest infectious diseases affecting humans, with both ancient DNA evidence and descriptions of intermittent febrile illnesses found in medical texts from early civilizations, including ancient Egypt (around 4000 BC), China (around 2000 BC), and Greece (around 400 BC) (1-5). Hippocrates described tertian and quartan fevers in his *Books of Epidemics*, noting their periodic nature and association with environmental conditions (6). Malaria has played a major role in shaping human settlement, migration, and mortality, and it is estimated that malaria caused between 150 and 300 million deaths during the 20th century, accounting for approximately 2–5% of global mortality over that period (4). For much of this time, however, the cause of malaria remained unknown and the disease was attributed to environmental factors.

Genomic and phylogenetic studies indicate that the major human malaria parasites originated in Africa through zoonotic transmission from great apes. However, the timing and routes of their early spread remain incompletely resolved (7). Following their emergence in Africa, malaria parasites spread to Asia and Europe through early human migration and trade and were later introduced into the Americas during European colonization and the trans-Atlantic slave trade (7).

For much of history, malaria was explained by environmental theories rather than biological causes. The Italian term *mal-aria* ("bad-air") came into scientific use in the early 19th century, reflecting the prevailing miasma theory that linked intermittent fevers to marshy environments (8). It was not until the late 19th century that the biological cause and mode of transmission of malaria was finally elucidated.

Figure 1. Le fantôme du marais ("The Ghost of the Swamp"), an allegorical depiction of malaria by Maurice Dudevant (Maurice Sand, 1823–1889).
Painted in the late 19th century, the work reflects the contemporary belief that fevers and deaths associated with marshlands were caused by harmful emanations from swamps, before the discovery of *Plasmodium* parasites and mosquito transmission. Source: "An allegory of malaria. Reproduction of an engraving after M Wellcome" is licensed under CC BY 4.0. This file comes from the Wellcome Collection, a website operated by Wellcome Trust, a global charitable foundation based in the United Kingdom.

A major turning point occurred in 1880, when Nobel Prize-winner Alphonse Laveran identified malaria parasites in the blood of febrile patients, providing the first evidence that malaria was caused by a protozoan organism (9). In 1897, Ronald Ross demonstrated that malaria parasites are transmitted by mosquitoes (8), and shortly thereafter Giovanni Grassi showed that human malaria is transmitted specifically by mosquitoes of the genus *Anopheles* (*An.*) (10). Subsequent decades brought many advances in malaria biology, including clarification of the intra-erythrocytic stages, the identification of multiple human-infecting *Plasmodium* species, and the later discovery of the parasite's liver stage (11). Together, these discoveries clarified the malaria transmission cycle and provided the basis for current research in malaria biology, immunology and control.

The recognition of mosquitoes as vectors rapidly transformed malaria control in the early 20th century. In Europe and North America, changes in agricultural practices, drainage of marshlands and improved housing led to major reductions in transmission even before modern insecticides became available (12). The introduction of quinine, and later chloroquine, contributed further to the decline of

malaria in many regions. A new global effort began in 1955 with the launch of the World Health Organization's (WHO) Global Malaria Eradication Programme, which aimed to interrupt transmission through widespread indoor residual spraying with DDT and mass administration of chloroquine (12). While the programme achieved substantial success in many regions outside of Africa, its progress was hindered by insecticide and drug resistance, logistical and financial challenges, and limited effectiveness in the high-transmission settings, ultimately leading to its abandonment in 1969 (4).

In the following decades, malaria mortality and morbidity rose again in many parts of the world, particularly in sub-Saharan Africa, driven largely by the spread of chloroquine-resistant *P. falciparum* (12). A renewed global effort began in 1998 with the launch of the Roll Back Malaria Partnership, initiated by WHO, UNICEF, the World Bank and the United Nations to strengthen coordination and support malaria-endemic countries (13). The Millenium Development Goals (MDGs) set ambitious targets to reduce malaria morbidity and mortality, and expanded investment enabled the wide distribution of insecticide-treated bed nets (ITNs), indoor residual spraying, rapid diagnostic tests (RDTs) and artemisinin-based combination therapies (ACTs). These interventions contributed to substantial reductions in malaria cases and deaths in many regions, although several high-burden countries in sub-Saharan Africa still did not meet the MDG targets (12).

The adoption of the Sustainable Development Goals in 2015, alongside WHO's Global Technical Strategy for Malaria 2016–2030, renewed global efforts and set targets to reduce malaria incidence and mortality by 90% by 2030 (14). These developments bring malaria control efforts up to the present day.

Epidemiology

Global burden of disease

Malaria remains a major global public health problem and a substantial burden on national health systems and socioeconomic development, particularly in endemic regions. The disease disproportionately affects young children and pregnant women, who experience the highest rates of severe disease and death.

According to WHO estimates from 2024, malaria remains endemic in 80 countries, with nearly half of the global population at risk of transmission (Figure 2) (15). In 2024, there were an estimated 282 million malaria cases globally, representing an increase of 9 million compared with 2023. The burden is heavily concentrated in the WHO Africa Region, which accounted for 94% of all cases. Five countries -

Nigeria, DR Congo, Uganda, Ethiopia, and Mozambique - together represented 49.5% of the global burden.

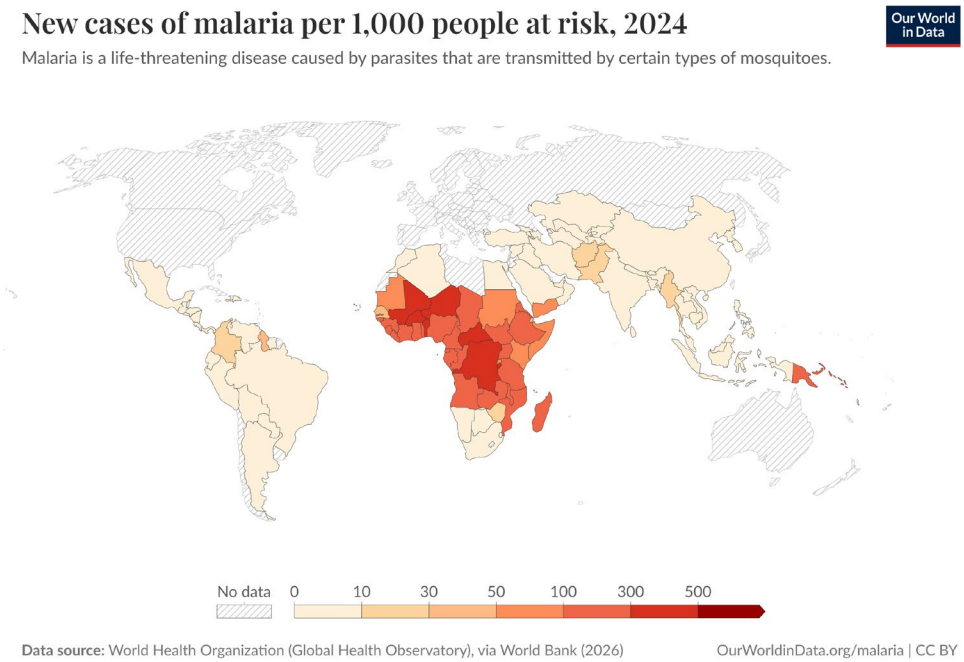

Figure 2. Global malaria incidence (2024).
Map showing the estimated number of new malaria cases per 1,000 population at risk. The burden of malaria is concentrated in sub-Saharan Africa. Source: World Health Organization (Global Health Observatory), via World Bank (2026) - processed by Our World in Data. Licenced by CC BY 4.0.

Mortality followed a similar distribution: 95% of the estimated 610,000 malaria deaths in 2024 occurred in Africa. The global mortality rate was 14.9 deaths per 100,000 individuals, more than three times higher than the level required to meet the Global Technical Strategy for Malaria 2016–2030 target of a 75% reduction in mortality by 2025. Four countries accounted for more than half of all global malaria deaths, with Nigeria alone accounting for 38.6% of all malaria deaths in children under five years of age (15).

In addition to its direct health impact, malaria has major socioeconomic consequences at both national and household levels. Countries with high malaria prevalence have experienced approximately 1.3% lower annual GDP growth per person (16), largely due to reduced labour productivity and increased healthcare costs (17). Malaria control and treatment impose a substantial burden on national health budgets. In some settings, they account for up to 39% of total public health expenditure, thereby limiting investment in other sectors (18, 19).

At the household level, families often shoulder a significant proportion of malaria-related costs, covering up to 70% of total malaria-related expenditure, including costs for treatment, transport, diagnostics, and preventive measures such as bed nets and mosquito control products (18, 20, 21). Lost income due to illness or caregiving further strains resources; for example, malaria accounted for nearly one quarter of all sickness-related absenteeism among horticultural workers in Southeast Ethiopia (21, 22). Beyond these immediate effects, recurrent childhood malaria can hinder cognitive and educational development and contribute to poorer health and reduced work capacity later in life, thereby undermining long-term human capital (23, 24).

At the same time, important progress has been made in global malaria control. Since 2015, ten countries have been certified malaria-free, and approximately 36.5% of malaria-endemic countries reported sustained declines in case incidence in 2024 (15). Compared with 2000, the global mortality rate has been reduced by half, with an estimated 2.3 billion cases and 14 million deaths averted (Figure 3).

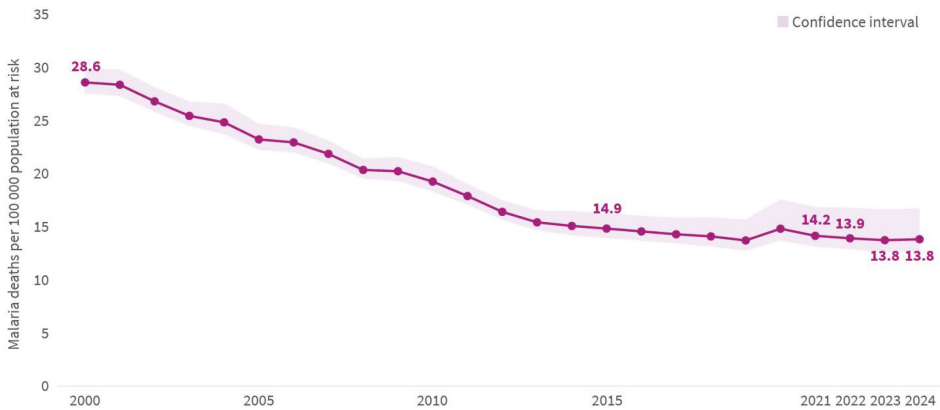

Figure 3. Global trends in malaria mortality, 2000–2024.
Graph showing the global malaria mortality rate from 2000 to 2024, expressed as deaths per 100,000 population at risk. Source: World Malaria Report 2025. Geneva: World Health Organization; 2026. Licence: CC BY-NC-SA 3.0 IGO.

Expanded access to ITNs has played a central role in these gains, with household ownership of at least one ITN in sub-Saharan Africa increasing from 5% in 2000 to 68% in 2024 (15). Additional intervention strategies, including seasonal malaria chemoprevention, intermittent preventive treatment in pregnancy (IPTp), and the introduction of rapid diagnostic tests (RDTs) have further strengthened malaria control. Together, these advancements demonstrate that significant reductions in malaria burden are possible with sustained political commitment, effective surveillance, and broad vector control and treatment strategies.

Despite this progress, global malaria control has stalled since 2015. By 2024, malaria case incidence had increased by 8.5% (15). Insufficient funding for malaria

control remains a major barrier in many African countries: WHO estimates that USD 9.3 billion per year will be required by 2025 to meet GTS targets, yet global malaria funding reached only USD 3.9 billion in 2024, less than half of what is needed. This funding gap has steadily widened over the past five years (15).

In addition to underfunding, several biological and environmental factors further challenge malaria control efforts. Insecticide resistance in *Anopheles* mosquitoes and emerging artemisinin partial resistance in *P. falciparum* reduce the effectiveness of core interventions. The increasing prevalence of *pfhrp2/pfhrp3* gene deletions in *P. falciparum* undermines HRP2-based diagnostic tests (15). Climate variability, including flooding, droughts, and rising temperatures, expands suitable habitats for mosquitoes and alters seasonal transmission patterns (25). Recent modelling studies further suggest that climate change may substantially increase malaria burden in Africa, primarily through the effects of extreme weather events that disrupt control efforts and intensify transmission in already endemic areas (26). Population displacement due to conflict, political instability, or extreme weather disrupts access to prevention and treatment (15, 25). Furthermore, changes in mosquito behaviour and the spread of *Anopheles stephensi* into densely populated urban areas reduce the impact of IRS and ITNs (15). These ongoing challenges highlight the need for additional interventions and control strategies, including the development and implementation of effective malaria vaccines and continued innovation in vector control and diagnostics.

Malaria in Uganda

Uganda carries one of the highest malaria burdens globally, with more than 95% of its population of over 50 million people at risk of *P. falciparum* infection. This is largely due to the country's ecological and climatic conditions, which are highly favourable for malaria transmission. Most regions experience two rainy seasons, from March to May and from September to December, while northern Uganda has a single rainy season. These rainfall patterns drive seasonal increases in malaria transmission (27). Within this environment, malaria transmission is sustained primarily by *An. gambiae* and *An. funestus*, which are widely distributed across the country. Four human malaria parasite species circulate in Uganda, with *P. falciparum* accounting for approximately 97% of infections, while *P. malariae*, *P. vivax* and *P. ovale* occur at much lower frequencies (28).

Within Uganda, transmission intensity varies markedly across regions, with higher malaria transmission in northern and eastern parts of the country (29). To guide the deployment of malaria interventions, the country has been stratified based on parasite prevalence, malaria incidence, and all-cause mortality (30).

As a result, malaria remains the most frequently reported illness in both public and private health facilities. The burden on the healthcare system is substantial: the

disease accounts for 30–50% of outpatient visits, 15–20% of hospital admissions, and up to 20% of inpatient deaths (27). In 2024, Uganda reported an estimated 13.2 million malaria cases and more than 16,000 deaths, placing the country among those with the highest malaria burdens globally (15). Although substantial progress was achieved between 2009 and 2018, when parasite prevalence among children under five declined from 42% to 9.1%, progress has slowed in recent years (15, 28). Since 2015, reductions in malaria incidence have been modest. Case incidence declined from 288.7 per 1,000 population in 2015 to 264.7 per 1,000 in 2024 (15). Beyond its health impact, malaria also imposes a substantial economic cost in Uganda. Annual treatment-related expenses are estimated to exceed USD 500 million (approximately 1.4% GDP), with households bearing more than 70% of both outpatient and inpatient costs (31).

Population-based survey data show a similar pattern. In the 2018-2019 Malaria Indicator Survey, 9% of children under five years of age were parasitemic by microscopy (28). Parasite prevalence increased with age, rising from 3% among infants younger than 6 months to 12% among children aged 4 years. Marked differences were also observed by place of residence, with substantially higher prevalence in rural areas compared with urban settings. In addition, parasite prevalence showed a clear socioeconomic pattern, declining from 17% in the lowest wealth quintile to 1% in the highest, and reaching 13% among children living in refugee settlements (28).

In terms of prevention, coverage of malaria control measures is relatively high, with 83% of households owning at least one insecticide treated net. However, utilization remains suboptimal, as only around 60% of children under five years of age and pregnant women reported sleeping under a net, and only 41% of pregnant women received IPTp during pregnancy (28). Consequently, malaria exposure remains common across much of the country.

Malaria in Sweden

Although malaria is no longer endemic in Europe, it was a major public health problem in the Nordic countries, including Sweden, until the early 20th century (32). Recurrent outbreaks were documented particularly in southern Sweden and the Stockholm region, primarily caused by a former Scandinavian strain of *P. vivax,* with transmission persisting into the early 1900s. Historical sources further suggest that a *Plasmodium* species resembling *P. malariae* may also have circulated during this period (32).

The significance of malaria in Sweden is reflected in early scientific reports. In 1735, the Swedish botanist and physician Carolus Linnaeus devoted his doctoral thesis, *Hypothesis nova de febrium intermittentium causa*, to intermittent fevers in Sweden (33). Based on travel observations and regional comparisons, he described

malaria epidemiology and noted the absence of transmission in northern parts of the country. Although Linnaeus incorrectly attributed the disease to environmental factors such as waterborne clay particles, his work illustrates both the widespread impact of malaria in Sweden and the early attempts to understand its causes. He also described the therapeutic use of cinchona bark, indicating that quinine-based treatments were already established at the time (33).

In Sweden today, malaria remains an important public health and clinical concern due to imported cases from malaria-endemic regions. In recent years, approximately 150–200 malaria cases have been reported annually, with some year-to-year variation (34). A notable increase occurred in 2014 and 2015, when 354 and 249 cases were reported, respectively, largely associated with newly arrived refugees from Eritrea and a predominance of *P. vivax* infections (35). During the same period, several other European countries reported increases in *P. vivax* malaria, although Sweden had one of the highest notification rates in Europe in 2014 (36, 37).

Despite substantial global reductions in malaria transmission over recent decades, the number of imported malaria cases in Europe has not declined to the same extent. In 2022, more than 6,000 confirmed malaria cases were reported across Europe, almost all among travellers and migrants arriving from endemic regions (38). Although rare cases of autochthonous transmission have been reported, malaria in Europe remains primarily an imported disease (38).

In Sweden, imported malaria is seen predominantly among two groups: travellers returning from endemic regions and recently arrived migrants. A large proportion of cases occur among individuals born in endemic countries who travel to visit friends and families (VFR) (39). This group is at increased risk of infection due to limited use of chemoprophylaxis and lower uptake of pre-travel advice (40, 41).

Throughout this thesis, the term imported malaria refers to malaria infections acquired in malaria-endemic regions and subsequently diagnosed and managed in Sweden.

The parasite

Plasmodium species and transmission

Malaria in humans is caused by protozoan parasites of the genus *Plasmodium*, which are transmitted through the bite of infected *Anopheles* mosquitoes. Several mosquito species can transmit the parasite, most notably *An. gambiae.* In recent years, *An. stephensi*, historically confined to Asia, has spread to several parts of Africa and is

now recognized by the WHO as a potential threat due to its ability to thrive in both urban and rural environments (15).

Five *Plasmodium* species are traditionally recognized as infecting humans. Among these, *P. falciparum* is responsible for the majority of malaria-related deaths and predominates in sub-Saharan Africa. In contrast, *P. vivax* is the dominant species across Asia and the Americas and is notable for its ability to cause relapsing infections (15). *P. malariae* and the two subspecies *P. ovale curtisi* and *wallikeri* occur at lower prevalence worldwide. Beyond these classical human malaria parasites, zoonotic species also contribute to the malaria burden. *P. knowlesi*, a parasite of macaque monkeys, is now recognized as an important cause of human malaria in parts of Southeast Asia (15, 42). More recently, *P. cynomolgi*, another simian *Plasmodium* species, has also been reported to cause human illness (43). With the increasing use of sensitive molecular diagnostics, including PCR-based methods, infections with non-human *Plasmodium* species are increasingly being detected in both symptomatic and asymptomatic individuals (44).

This thesis focuses on *P. falciparum*, the species accounting for the most severe forms of malaria. The parasite has a complex genome encoding more than 5,000 proteins, many of which are highly polymorphic and variably expressed during infection (45). This antigenic diversity contributes to immune evasion and represents a major challenge for the development of durable immunity and effective vaccines.

Life cycle

Malaria transmission begins when an infected female *Anopheles* mosquito injects sporozoites into the human host during a blood meal (Figure 4). Within minutes to a few hours, the sporozoites migrate to the liver, where they invade hepatocytes and form a parasitophorous vacuole surrounding the parasite. This process is mediated in part by the circumsporozoite protein (CSP) (46) and is followed by a phase of asexual replication. After 5–8 days, this clinically silent liver stage culminates in the release of thousands of merozoites into the bloodstream. In *P. vivax* and P. *ovale*, a subset of parasites can persist in the liver as dormant hypnozoites, giving rise to relapsing infections weeks to months after the initial exposure (47).

Once released into the bloodstream, merozoites rapidly invade red blood cells (RBCs) and initiate the intraerythrocytic developmental cycle. RBC invasion is a rapid, multistep process involving initial attachment, apical reorientation of the merozoite, and formation of a tight junction, resulting in internalisation of the parasite within a parasitophorous vacuole (48). Within infected RBCs, parasites progress through ring, trophozoite, and schizont stages, culminating in rupture of the host cell and the release of new merozoites that infect additional erythrocytes.

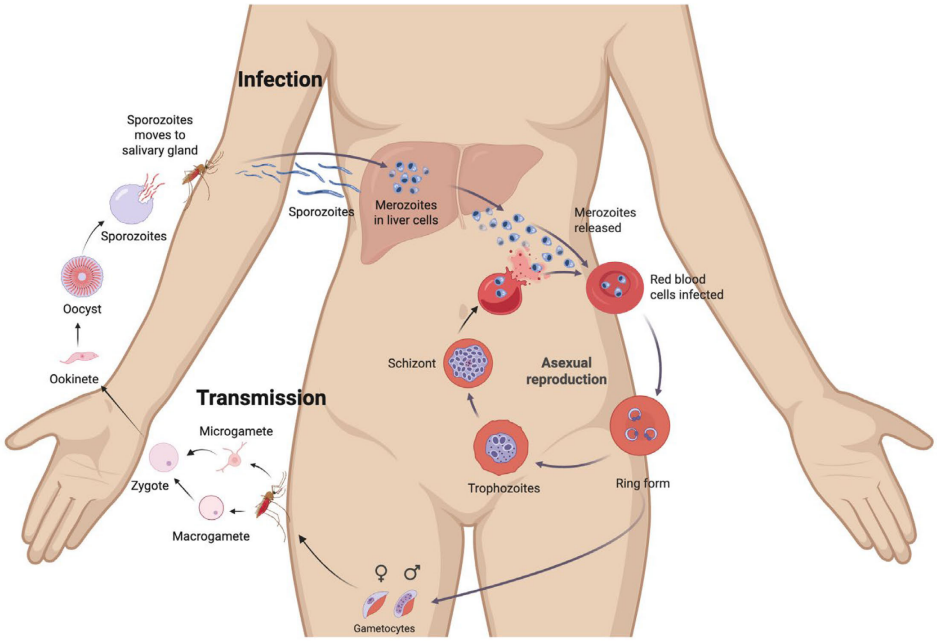

Figure 4. Life cycle of *P. falciparum.*
Schematic overview of the *P. falciparum* life cycle alternating between the human host and the *Anopheles* mosquito vector. Sporozoites are transmitted to humans during a mosquito blood meal and undergo a liver stage before initiating asexual replication in red blood cells. A subset of blood-stage parasites differentiates into gametocytes, which are taken up by mosquitoes and develop into sporozoites that enable onward transmission. Created with Bio-Render by Mortazavi, S (2026).

The duration of the intraerythrocytic cycle varies between *Plasmodium* species and underlies the periodicity of malaria-associated fever (49). In *P. falciparum*, the cycle typically lasts approximately 48 hours, whereas *P. knowlesi*, for example, has a shorter cycle of around 24 hours (50). This cyclical blood-stage replication is responsible for the clinical manifestations of malaria and represents the stage most exposed to the host immune system.

In addition to asexual replication, a small proportion of blood-stage parasites differentiate into sexual forms known as gametocytes. These are taken up by another mosquito during a subsequent blood meal. Within the mosquito midgut, gametocytes mature and undergo sexual reproduction, ultimately giving rise to sporozoites that migrate to the salivary glands, completing the life cycle and enabling onward transmission.

Clinical presentation

Severe and uncomplicated malaria

The clinical spectrum of malaria is broad, ranging from asymptomatic carriage of parasites to clinical disease with varying severity. Uncomplicated malaria is typically characterised by non-specific symptoms such as fever, chills, headache, malaise and myalgia, reflecting the systemic inflammatory response associated with blood-stage parasite replication. While these symptoms can be debilitating, uncomplicated malaria is generally self-limiting with appropriate treatment (51).

Severe malaria, in contrast, is associated with life-threatening complications, including severe anaemia, cerebral malaria, respiratory distress and multi-organ dysfunction (51). Severe disease occurs most frequently in young children and pregnant women in endemic areas and in individuals with little or no prior malaria exposure, such as travellers from non-endemic regions (15). The development of severe malaria is not solely determined by parasite burden but is strongly influenced by host factors, including immune status and the magnitude of the inflammatory response (52-55).

Asymptomatic malaria

In malaria-endemic areas, infection with *P. falciparum* does not always lead to clinical illness, and many individuals carry parasites in their blood without experiencing symptoms. These individuals are therefore classified as having asymptomatic or chronic malaria infection. This state reflects the development of partial or anti-disease immunity that will be discussed later in this thesis (56). Asymptomatic infection is most commonly observed in older children and adults living in endemic regions, particularly in settings with sustained malaria transmission.

These infections are typically characterised by low-density parasitemia that may fluctuate over time and persist for months or even years (57, 58). Parasite levels are often below the detection threshold of routine diagnostic methods such as microscopy and rapid diagnostic tests (RDTs), and may only be detectable using more sensitive techniques such as polymerase chain reaction (PCR) (57). This may partly relate to parasite sequestration in deep tissues, including the spleen (59).

Despite the absence of clinical symptoms, asymptomatic infections are both clinically and epidemiologically relevant because individuals with low-density parasitemia can serve as a reservoir for ongoing transmission. In addition, asymptomatic infections may be associated with chronic low-grade haemolysis,

anaemia, increased risk of invasive bacterial infections, and hyperreactive malarial splenomegaly (60-64).

Pathogenesis

Infection with *P. falciparum* induces innate and inflammatory immune responses that are essential for limiting parasite replication but can also contribute to host tissue damage. Disease severity is influenced by parasite burden, sequestration of infected erythrocytes and host inflammatory responses.

A key feature of *P. falciparum* pathogenesis is the ability of infected RBCs (iRBCs) to adhere to the vascular endothelium. During intraerythrocytic development, the surface of iRBCs is modified through the formation of knob-like structures that express parasite-derived surface ligands mediating adhesion to endothelial cells. Through this process, infected erythrocytes sequester in the microvasculature and thereby avoid splenic clearance (65). Sequestration is strongly associated with severe disease, and its extent is closely linked with parasite biomass and tissue pathology (55, 66). The principal mediator of cytoadhesion is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a highly polymorphic protein encoded by the *var* gene family that binds several endothelial receptors, including ICAM-1, CD36, and endothelial protein C receptor (EPCR) (67). Expression of specific PfEMP1 variants has been associated with severe disease and may influence organ-specific pathology by directing sequestration to distinct vascular beds (55). For instance, variants that bind EPCR have been linked to endothelial activation and severe malaria (68).

In addition to cytoadhesion, several *Plasmodium* species can promote rosetting, whereby iRBCs bind uninfected erythrocytes to form aggregates. In *P. falciparum*, rosetting contributes to microvascular obstruction and impaired blood flow, while also reducing splenic clearance and phagocytosis. Rosetting has additionally been proposed to limit exposure of late-stage parasites to antimalarial drugs (67, 69).

Together, these processes contribute to both mechanical and inflammatory injury. Obstruction of the microcirculation impairs tissue perfusion and creates focal sites where parasite-derived products and host inflammatory mediators accumulate. Parasite components released from iRBCs, including hemozoin and glycosylphosphatidylinositol (GPI) anchors, can trigger innate immune responses and cytokine production (55, 70). Host pro-inflammatory mediators, including tumour necrosis factor (TNF) and interferon-γ (IFN-γ), promote endothelial activation and upregulation of adhesion molecules such as ICAM-1, further reinforcing sequestration. In turn, endothelial activation leads to vascular permeability, microvascular thrombosis, and impaired tissue perfusion. These mechanisms lead to tissue hypoxia, metabolic acidosis, inflammation, and organ

dysfunction that characterize severe malaria (71, 72). Cytokine responses therefore play a dual role in malaria pathogenesis, as TNF and IFN-γ are critical for parasite control but, when excessive or prolonged, contribute to immunopathology and severe disease (55, 71).

Another major complication of malaria infection is severe malarial anaemia. This condition results from both parasite-driven and host-mediated processes. Destruction of iRBCs contributes directly to anaemia, while splenic clearance also removes uninfected erythrocytes. Inflammatory responses lead to suppressed erythropoiesis and reduce RBC survival, further contributing to reduced haemoglobin levels (55).

Diagnostics and treatment

Current WHO guidelines recommend that all suspected cases of malaria be confirmed by parasitological testing using RDTs, light microscopy or molecular diagnostic methods (73). Accurate diagnosis and species identification are essential for appropriate case management, as treatment regimens differ between *Plasmodium* species.

In many endemic settings, RDTs are the most widely used diagnostic tool. These tests detect parasite antigens in peripheral blood, most commonly *P. falciparum* specific histidine-rich protein 2 (HRP2) or pan-*Plasmodium* markers such as aldolase and *Plasmodium* lactate dehydrogenase (pLDH). Due to their ease of use and low cost, RDTs have been widely implemented since the early 2000s and have substantially improved access to malaria diagnosis in endemic settings. Their introduction has contributed to a reduction in presumptive antimalarial treatment of non-malarial febrile illnesses (74). However, RDTs generally have a detection limit of approximately 100 parasites/μL. Reduced sensitivity at low parasite densities, together with false-negative results associated with *pfhrp2/pfhrp3* deletions, remain important limitations of current RDTs (15, 75). False-positive RDT results may also occur, contributing to unnecessary antimalarial treatment and increased drug pressure (76).

Light microscopy of Giemsa-stained blood smears remains an important diagnostic tool, as it allows parasite detection, quantification, and species identification. The typical detection limit is approximately 50–100 parasites/μL, but the method requires trained personnel and laboratory infrastructure (75). Molecular diagnostic methods, including PCR and loop-mediated isothermal amplification (LAMP), offer substantially higher sensitivity and can detect parasitemia as low as 1–2 parasites/μL (75, 77). Because of their technical and logistical requirements, these methods are mainly used in reference laboratories and in non-endemic countries such as Sweden. Even with improved diagnostic capacity, low-density and asymptomatic infections

remain difficult to detect in routine clinical practice, particularly in high-transmission settings.

Treatment of malaria is guided by diagnostic findings, including species identification and disease severity. Uncomplicated *P. falciparum* malaria is treated with artemisinin-based combination therapies (ACTs), while severe malaria requires parenteral artesunate followed by a full course of oral ACT. Treatment of *P. vivax* and *P. ovale* malaria additionally requires clearance of dormant liver-stage hypnozoites using primaquine or tafenoquine to prevent relapse. Although effective therapies are available, treatment does not prevent reinfection in endemic areas. In addition, the emergence of partial artemisinin resistance, first identified in Southeast Asia and now reported in parts of Africa, poses a growing challenge to sustained malaria control (15).

Malaria Vaccines

As outlined earlier, global progress in reducing malaria cases has stalled in recent years. Several factors contribute to continued transmission, including the spread of insecticide and drug resistance, *pfhrp2/pfhrp3* deletions affecting diagnostic performance, the expansion of *An. stephensi* into urban settings, and the persistence of asymptomatic infections. These challenges have renewed interest in additional preventive tools, including effective vaccines. In 2006, the WHO and its partners formalized this goal through the Malaria Vaccine Technology Roadmap (78).

Developing vaccines against malaria has historically been regarded as exceptionally difficult. The complex life cycle of *P. falciparum*, extensive antigenic diversity, and multiple immune evasion strategies all complicate vaccine design. In addition, reliable immune correlates of protection are lacking, and naturally acquired sterile immunity is rare (79). Despite these obstacles, the first vaccines targeting *P. falciparum* have now been introduced. RTS,S/AS01 was implemented in WHO pilot programmes in 2021, followed by R21/Matrix-M in 2023, marking an important step forward in malaria control.

Both vaccines target the pre-erythrocytic stage by inducing antibodies against the circumsporozoite protein, a major surface antigen on sporozoites that is essential for hepatocyte invasion. Anti-CSP antibodies are thought to protect primarily by neutralising sporozoites before liver infection is established, thereby reducing subsequent blood-stage infection, although Fc-mediated effector mechanisms may also contribute (80). However, vaccine efficacy has been moderate. In phase III trials conducted in African settings, RTS,S demonstrated 55% (95% CI 51–59) efficacy against clinical malaria over one year in children aged 5–17 months, with protection declining to 39% (34–43) with longer follow-up despite booster vaccination (81, 82). In comparable paediatric age groups, R21/Matrix-M showed

efficacy estimates of 78% (73–82) over one year (83). However, longer-term durability data and results from perennial high-transmission settings remain limited, particularly for R21. Notably, RTS,S showed only 30% efficacy in infants aged 6–12 weeks (84), and both vaccines are therefore currently recommended only for children aged 5–17 months, leaving younger infants without vaccine protection.

In parallel with these pre-erythrocytic approaches, vaccine development has also focused on blood-stage antigens with the aim of reducing parasite replication and disease severity rather than preventing infection. Numerous candidate antigens have been evaluated, including merozoite surface proteins and invasion ligands such as apical membrane antigen 1 (AMA1) (85), erythrocyte-binding antigen-175 (EBA-175) (86), and *P. falciparum* reticulocyte-binding protein homolog 5 (PfRH5) (87). Among these, PfRH5 has emerged as a promising target. Phase 2b studies of the RH5.1/Matrix-M vaccine have demonstrated an acceptable safety profile and an estimated efficacy of approximately 55% against clinical malaria in young children, with ongoing follow-up to assess durability (88).

Another strategy involves transmission-blocking vaccines, which aim to interrupt parasite development within the mosquito. Candidate antigens such as Pfs230 have shown encouraging results in early-phase clinical studies (89). Because these vaccines do not directly protect vaccinated individuals from clinical disease, they are primarily considered as tools to reduce transmission at the population level (90).

More recently, the modest and time-limited protection achieved by current vaccines has prompted interest in multistage and combination vaccine strategies targeting different phases of the parasite life cycle (91). While the introduction of malaria vaccines represents an important advance, the difficulty in inducing long-lasting protection emphasizes the need for a deeper understanding of the immune mechanisms underlying durable immunity in malaria.

Immunity to malaria

> "Malaria immunity may be defined as the state of resistance to the infection brought about by all those processes which are involved in destroying the plasmodia or by limiting their multiplication. Natural (innate) immunity to malaria is an inherent property of the host, a refractory state or an immediate inhibitory response to the introduction of the parasite, not dependent on any previous infection with it. Acquired immunity may be either active or passive. Active (acquired) immunity is an enhancement of the defence mechanism of the host as a result of a previous encounter with the pathogen (or parts thereof). Passive (acquired) immunity is conferred by the prenatal or postnatal transfer of protective substances from mother to child or by the injection of such substances."
>
> Bruce-Chwatt, 1980 (92)

The rationale for malaria vaccine development stems from the observation that individuals living in malaria-endemic areas gradually acquire protection against disease following repeated exposure to *Plasmodium* parasites. However, naturally acquired immunity to malaria is rarely sterilizing, except under certain experimental conditions (93). Individuals with prior exposure may still become infected but are less likely to develop severe disease or high parasite densities. This form of protection is therefore often described as partial immunity or semi-immunity, reflecting the ability to tolerate infection and limit disease rather than prevent parasite establishment entirely (94, 95).

Naturally acquired immunity to malaria is commonly divided into two related components: anti-disease immunity and anti-parasite immunity. Anti-disease immunity reduces the pathological consequences of infection and protects against severe disease despite the continued presence of parasites. Anti-parasite immunity, in contrast, limits parasite replication and reduces parasite densities in the blood. As a result, individuals living in endemic areas may harbour parasites without developing fever or other clinical symptoms, and asymptomatic infections are common. This state has sometimes been described as disease tolerance or premunition (94-97).

Evidence that immunity develops through repeated infections comes from both historical and experimental studies. In the 1940s and 1950s, before the introduction of effective antibiotics, malaria infection was induced in tens of thousands of patients with neurosyphilis as a form of fever therapy (98). Observations from these

treatments showed that subsequent infections were associated with reduced fever and lower parasitemia, indicating the gradual acquisition of both anti-disease and anti-parasite immunity. It was also noted that protection was strain-specific and declined relatively quickly in the absence of continued exposure (98).

More recently, controlled human malaria infection (CHMI) studies have provided further insight. In these studies, volunteers are deliberately exposed to *P. falciparum* through infected mosquito bites or by inoculation of infected erythrocytes, allowing immune responses and clinical outcomes to be closely monitored. Repeated exposure in CHMI studies has been shown to reduce clinical symptoms despite similar parasite densities, supporting the concept that anti-disease immunity can develop relatively rapidly (99, 100).

The development of immunity is strongly influenced by host age as well as by the intensity and duration of transmission (94, 101, 102). In highly endemic regions, children are exposed early in life and frequently experience multiple infections during their first years. The risk of severe malaria is highest during the first few infections and is therefore concentrated in young children (101, 102). Protection against severe disease is acquired relatively rapidly, although symptomatic malaria episodes may continue for several years (103). In contrast, broader immunity that limits parasite densities and prevents symptomatic infection develops more slowly and may require many years of repeated exposure (97). The rate at which immunity develops also depends on transmission intensity. In moderate to high-transmission settings, substantial protection from disease may be acquired by late childhood, whereas in low-transmission settings individuals may remain susceptible into adolescence or even adulthood (97, 101, 102).

Naturally acquired immunity to malaria is not static and may decline in the absence of continued exposure. Individuals who move away from endemic areas can become susceptible again, and several studies have reported a rapid decline in malaria-specific antibody levels following parasite clearance or treatment in both children and adults (104, 105). These observations led to the widely held view that malaria antibody responses are relatively short-lived and require repeated parasite exposure to be maintained, a phenomenon often referred to as premunition (97). However, other studies have shown that malaria-specific antibodies can persist for many years in the absence of detectable infection, suggesting that some components of the immune response are long-lived (106-108). Evidence for such durability has been reported in Madagascar, where individuals exposed decades earlier experienced less severe disease compared with younger individuals without prior exposure following re-emergence of malaria transmission (109).

The slow and incomplete acquisition of malaria immunity remains incompletely understood. One explanation relates to the extensive antigenic diversity of *P. falciparum*. The parasite expresses highly variable surface antigens, such as PfEMP1, and can switch antigen expression during infection, allowing parasites to

evade immune recognition (110). As a result, the immune system must be exposed to a broad range of parasite variants before effective protection can develop. In addition, it has long been speculated that malaria infection itself may influence host immune responses, thereby altering the development or maintenance of protective immunity toward dysfunctional B-cell memory responses (97, 111, 112). Host genetic and immunological differences also appear to influence susceptibility (97, 113). For example, certain ethnic groups such as the Fulani in West Africa have been reported to carry lower parasite densities and experience reduced malaria risk compared with neighboring populations living under similar environmental conditions (113).

Pregnancy represents a unique situation, as women with prior immunity to malaria become susceptible again during pregnancy (114). This induced susceptibility is caused by the ability of *P. falciparum*-infected erythrocytes to adhere to chondroitin sulfate A in the placenta through the expression of a specific PfEMP1 variant known as VAR2CSA (115, 116). Immunity to pregnancy-associated malaria develops over successive pregnancies as women acquire antibodies that block this interaction, illustrating how immunity to malaria can be directed against specific parasite variants (117).

The complexity of malaria immunity is further illustrated by the difficulty of developing vaccines that achieve similar efficacy in malaria-exposed populations as in malaria-naïve individuals in CHMI trials (118-122). Similar variation in vaccine responses has also been observed for vaccines against other infectious diseases, particularly between low- and high-income settings, suggesting that environmental and host factors can shape vaccine-induced immunity (123). These findings raise important questions about whether continuous exposure to parasites alters immune responses in ways that affect the ability to respond to vaccination. Several explanations have been proposed. One possibility is that the CSP variant used in the RTS,S vaccine does not fully represent parasite strains circulating in Africa (124). Another is that vaccination schedules may be suboptimal in endemic settings (82). It has also been suggested that pre-existing antibodies may interfere with vaccine-induced immune responses (125, 126). Consistent with this, RTS,S shows lower efficacy in younger infants than in older infants (81, 84), who have lower levels of maternally transferred antimalarial antibodies. However, the extent to which maternal antibodies alone account for this difference remains unclear.

Despite decades of research, the mechanisms underlying naturally acquired immunity remain only partly understood. Epidemiological observations clearly demonstrate the development of protection, but the immune mechanisms involved have not been fully defined. In particular, reliable immune correlates of protection remain elusive, and it is still unclear why immunity develops slowly despite repeated infections. These unresolved questions are central to understanding malaria and to improving strategies for prevention and control.

Immunology of malaria

Immunity to *P. falciparum* is acquired gradually, depends on repeated exposure, and does not provide complete protection against infection.

Infection induces strong host immune responses that contribute both to parasite control and to disease pathogenesis. Innate immune activation plays an important role in early control of parasitemia but also drives much of the clinical symptomatology through the production of inflammatory mediators. In parallel, adaptive immune responses contribute to parasite clearance and limitation of parasite replication through antibody-mediated effector mechanisms, while regulatory pathways shape the magnitude and balance of these responses.

This section outlines key immunological mechanisms relevant to malaria, with a particular focus on humoral immunity and immune regulation during blood-stage infection.

Humoral immunity in malaria

B cells in malaria

B cells are central components of adaptive immunity and contribute to host defence primarily through antibody production. Following antigen recognition, they differentiate into short-lived plasmablasts, long-lived plasma cells (LLPCs), or memory B cells (MBCs) (127). LLPCs maintain circulating antibody levels over time, whereas MBCs provide rapid recall responses upon re-exposure. Through affinity maturation and class-switch recombination, B cells generate antibodies with increased specificity and functional capacity. In addition to antibody production, B cells also act as antigen-presenting cells, secrete regulatory cytokines, and respond to soluble mediators that regulate their survival and differentiation, including B-cell activating factor (BAFF), thereby shaping their magnitude and quality of immune responses (127).

During blood-stage *P. falciparum* infection, B cells contribute to host defence through the generation of parasite-specific antibodies and maintenance of immunological memory. However, unlike many viral and bacterial infections,

durable protective antibody responses to malaria develop slowly and generally require repeated exposure (128). This has raised questions about how effectively LLPCs and MBCs are generated and maintained following infection.

Despite the gradual acquisition of clinical immunity, several studies show that *Plasmodium*-specific MBCs are generated after infection at levels comparable to those induced by conventional vaccines (129). In mouse models, a single infection can induce antigen-specific MBCs that persist long after parasite clearance (130, 131). In humans, parasite-specific MBCs have been detected in previously exposed individuals, including travellers and migrants, for years after infection and sometimes in the absence of detectable circulating antibodies (107, 108, 132). Importantly, these long-lived memory B cells include specificities capable of inhibiting the parasite, indicating preserved functional potential (133).

However, the relationship between MBCs, circulating antibodies, and protection from reinfection is not straightforward. Whereas MBC populations can remain relatively stable, circulating antibody levels often decline more rapidly, suggesting that LLPC maintenance may be suboptimal following malaria infection (95, 128). At the same time, rapid boosting of antibody titres after re-exposure documented in travellers and in areas of seasonal transmission supports the presence of functional memory responses (106, 108).

Malaria infection is also associated with pronounced perturbations of the B-cell compartment. Acute infection often induces strong polyclonal activation, transient plasmablast expansion and increased antibody secretion (134). As parasite burden decreases, this activation phase contracts, with reduced frequencies of activated B cells (128, 134).

A characteristic feature of repeated malaria exposure is the expansion of atypical memory B cells (atMBCs). Initially described in HIV infection as “exhausted” memory B cells, this population is now recognized in malaria, other chronic infections, and auto-immune diseases such as SLE (104, 135-139). Atypical MBCs are commonly defined by low or absent expression of CD21 and CD27 and often express markers such as CD11c, T-bet, FCRL5 and inhibitory markers including PD-1 (134, 140-143). Their frequency increases with age and cumulative *P. falciparum* exposure and is elevated in both adults and children living in endemic areas, including during asymptomatic infection (104, 129, 136, 142, 144-146). Atypical MBCs constitute a substantial proportion of *P. falciparum*-specific B cells in exposed individuals. Early studies suggested that these cells represent a dysfunctional population, based on reduced proliferative capacity, impaired B-cell receptor signalling, and diminished differentiation into antibody-secreting cells (135, 140). However, more recent data indicate that atypical MBCs may instead represent an exposure-adapted phenotype. Under certain conditions, they can contribute to antibody production and possess distinct functional properties rather than reflecting simple immune exhaustion (128, 144). Thus, although their

expansion is consistently associated with malaria exposure, their precise role in protective immunity and long-term B-cell memory remains unresolved.

Antibody responses and effector functions

Early passive-transfer studies demonstrated that immunoglobulin from malaria-immune adults reduced parasitemia and disease severity in infected individuals (147, 148), providing direct evidence that antibodies contribute to protection against malaria. Subsequent epidemiological studies further showed that higher levels of antibodies targeting blood-stage parasite antigens are associated with reduced risk of clinical disease (149-151). Together, these observations established antibodies as key mediators of naturally acquired immunity to *P. falciparum*.

Antibodies act against malaria parasites through both direct and indirect mechanisms. Direct effects include inhibition of sporozoite invasion of hepatocytes and merozoite invasion of erythrocytes (152). In addition, Fc-dependent mechanisms recruit host immune components, promoting opsonization, phagocytosis, antibody-dependent cellular cytotoxicity, and activation of the complement cascade, including C1q-fixation (45, 153-156). The effectiveness of these responses depends not only on antibody levels but also on qualitative features such as isotype, subclass distribution, antigenic breadth, affinity, and Fc glycosylation.

Cytophilic IgG subclasses, particularly IgG1 and IgG3, bind Fcγ receptors and complement components efficiently and have consistently been associated with reduced parasitemia and lower risk of clinical disease (153, 156-158). In contrast, non-cytophilic subclasses such as IgG2 and IgG4 engage these pathways less effectively and show weaker or less consistent associations with protection (157, 158). IgM responses to blood-stage antigens have also been linked to reduced risk of clinical malaria (159, 160), supporting a functional role beyond early primary responses. Consistent with this, functional assays measuring opsonic phagocytosis and complement fixation often correlate more strongly with protection than antibody concentration alone (156, 161, 162). However, which functional features that best predict protection is unclear and may vary with antigen specificity, transmission intensity, and age.

Antibody breadth increases with age and cumulative exposure, and has been linked to protection, reflecting the need to recognize a wide array of parasite antigens (163, 164). However, increased exposure does not uniformly improve antibody quality, as broader responses may be accompanied by lower avidity (146). At the same time, malaria antibody responses are often relatively short-lived following infection, particularly in the absence of re-exposure. In endemic settings, parasite-specific antibody levels can decline rapidly after infection or reduced exposure, sometimes becoming undetectable within months (105, 165, 166). Estimated half-lives of

certain merozoite-specific IgG responses are shorter than those observed for antibodies against many viral or bacterial antigens, which may persist for decades after a single exposure (105, 166, 167). However, considerable inter-individual variation exists, and some previously exposed individuals retain detectable parasite-specific antibodies for years (106, 107, 168).

The age dependence of malaria antibody immunity is particularly evident early in life. Infants are born with maternally transferred IgG that provides transient protection during the first months of life, together with factors such as foetal haemoglobin and lower exposure (169). Nevertheless, infection can still lead to severe disease (170, 171). Around six months of age, maternally derived antibody levels decline while endogenous humoral responses are still maturing, creating a period of increased susceptibility (171). Studies examining maternal antibodies and infant malaria have yielded conflicting results, with detectable antibodies sometimes reflecting exposure rather than protection (171, 172). In young children, parasite-specific antibody responses are typically lower in magnitude and narrower in breadth than in adults, although their functional properties are not fully defined (163, 172, 173). Differences in early-life immunity are also suggested by RTS,S vaccine studies, in which efficacy is low in young infants (84), possibly reflecting immunological immaturity and/or interference from maternal antibodies (174).

Protective antibody-mediated immunity to malaria therefore reflects a combination of specificity, breadth, functional activity, and persistence rather than any single antibody measurement. Despite strong evidence that antibodies contribute to protection, no universally accepted antibody correlate of protection has yet been identified. Understanding which antibody features best predict protection remains an important step toward defining immune correlates and improving malaria vaccine development.

Complement activation and malaria

The complement system is a key component of both innate and adaptive immunity and represents an effector pathway through which antibodies can enhance parasite control. Complement activation occurs via the classical, lectin, and alternative pathways, of which the classical pathway is triggered by antigen-antibody complexes (175). Binding of complement component C1q to immune complexes, referred to as C1q fixation, initiates a proteolytic cascade that promotes opsonization, inflammation, and, in some settings, formation of the membrane attack complex, which inserts into target cell membranes and induces lysis (176).

In malaria, complement-fixing antibodies have emerged as a functional feature associated with protective humoral immunity. During blood-stage infection, antibodies targeting merozoite surface antigens can recruit C1q and activate the classical pathway, which has been shown to enhance inhibition of erythrocyte

invasion and, in some cases, contribute to parasite lysis *in vitro* (154). Complement activity is thought to operate primarily during the brief extracellular phase when merozoites transition between red blood cells (154). Experimental *in vitro* studies show that IgG from malaria-exposed individuals can inhibit invasion more effectively in the presence of active complement, and in some cases inhibitory activity is only detectable when complement is present (154). Complement-fixing activity, particularly C1q fixation, has been shown to correlate with deposition of downstream complement components such as C3 and the terminal complement complex, supporting its use as a functional readout of complement activation (162). The predominant subclasses of naturally acquired anti-merozoite antibodies, IgG1 and IgG3, are efficient complement activators and have been shown to correlate strongly with complement fixation against both merozoites and sporozoites (154, 162, 177).

Complement-dependent activity is also observed during pre-erythrocytic infection. Antibodies can promote deposition of C1q and C3 on sporozoites, which has been associated with reduced motility, impaired hepatocyte invasion, and parasite lysis (162, 178). Sporozoites appear relatively resistant to complement in the absence of antibody, suggesting that efficient targeting requires activation of the classical pathway (162, 178). In addition to downstream complement activation, C1q binding itself has been suggested to contribute to inhibition, potentially through steric interference with invasion processes or by facilitating interactions with complement receptor-expressing phagocytes (154, 156, 179).

Multiple *P. falciparum* antigens have been identified as targets of complement-fixing antibodies, including merozoite surface proteins (MSP1, MSP2, MSP6) and apical organelle proteins such as PfRH5, AMA1, and EBA family ligands (154, 177). Antibodies directed against different antigens vary in their capacity to recruit complement, likely reflecting differences in epitope specificity, subclass distribution, and antibody functional properties that influence C1q engagement (156, 162).

Epidemiological and functional studies frequently report associations between complement-fixing activity and protection from clinical malaria. High levels of C1q-fixing antibodies have been linked to reduced parasite density and lower risk of symptomatic disease in endemic populations (154, 162, 180), as well as protection against placental malaria (181). CHMI studies further support this relationship, as individuals who develop strong complement-fixing responses following RTS,S vaccination have been shown to be less likely to develop parasitemia (161). In several settings, complement-fixing activity shows stronger associations with protection than growth inhibition measured by standard *in vitro* assays, suggesting that complement recruitment may reflect a functionally relevant aspect of antibody quality, highlighting that antibody function, rather than antibody quantity alone, may be a more relevant determinant of protective immunity (177, 182). However, complement activation does not necessarily translate into effective parasite clearance, as *P. falciparum* can evade complement-mediated killing. Recent work suggests that while antibodies from both children and adults can fix C1q to merozoites, differences in downstream

complement activity may reflect variation in antibody-mediated interference with parasite evasion mechanisms (183).

Despite accumulating evidence for its protective role, most data derive from older children or adults with established immunity. Far less is known about how complement-fixing antibody responses develop during infancy, when susceptibility to severe disease is highest and humoral immunity is still maturing. Understanding how these functional responses arise during early immune development is therefore important for improving our understanding of how malaria immunity is acquired.

Inflammation and immune regulation in malaria

Inflammatory mediators shaping immune responses

Inflammation is a central feature of *P. falciparum* infection and plays a dual role in both parasite control and disease pathogenesis (184). Recognition of blood-stage antigens induces innate immune activation, leading to the production of pro-inflammatory cytokines such as IL-1β, IL-6, TNF, and IFN-γ (185). These mediators promote parasite control by activating phagocytic cells and enhancing antigen presentation, but they also contribute to many of the clinical manifestations of malaria, including fever, endothelial activation, and tissue inflammation (184, 186). Dysregulated systemic cytokine responses have been associated with severe manifestations such as cerebral malaria and severe anaemia (186, 187). Effective control of infection therefore requires not only inflammatory activation but also appropriate regulation.

Inflammatory responses vary across age groups and exposure histories. Malaria-naïve individuals typically mount strong systemic cytokine responses during primary infection, whereas individuals living in highly endemic settings often show more attenuated inflammatory responses during acute disease (95, 96, 188). In these populations, older children tend to have lower circulating levels of pro-inflammatory cytokines during acute infection compared with younger children at a similar stage of infection, indicating that repeated exposure modifies the inflammatory environment (189). Consistent with this, asymptomatic parasitemia in endemic populations is frequently associated with relatively muted systemic inflammatory responses despite ongoing infection (95). These observations support the development of anti-disease immunity, in which inflammatory responses become more tightly regulated despite continued parasite exposure. Several molecular pathways likely contribute to this regulation, including cytokine-mediated signaling and immune checkpoint pathways.

Osteopontin as an immunomodulatory mediator

One mediator implicated in the regulation of inflammatory responses is osteopontin (OPN). OPN, also known as secreted phosphoprotein-1 (SPP1) or early T-lymphocyte activation-1 (ETA-1), is a multifunctional phosphoglycoprotein originally identified in bone matrix but now recognized as a regulator of immune activation and inflammatory signaling (190-193). It is produced by a wide range of immune and non-immune cells, including macrophages, dendritic cells, T cells, and B cells. OPN exists in both secreted (sOPN) and intracellular (iOPN) forms, with distinct immunological functions (194, 195). Secreted OPN interacts with several receptors, particularly integrins and CD44, whereas intracellular OPN can function as a signaling adaptor within pattern-recognition and interferon signaling pathways (195-199). Post-translational modification and proteolytic cleavage further generate fragments with different receptor affinities and biological activities (200), contributing to the range of immune processes associated with OPN.

Several studies describe both pro-inflammatory and regulatory effects of OPN, suggesting that its function is highly dependent on cellular context and receptor expression rather than reflecting a single immunological role (200-203). Within innate immunity, OPN enhances myeloid activation by promoting macrophage migration, phagocytosis, cytokine production, and differentiation from monocytes (204-206). It also increases dendritic-cell IL-12 production, thereby strengthening type-1 T-cell polarization (207, 208), and stimulates neutrophil migration (209). Through these effects, OPN can amplify early inflammatory responses and enhance pathogen clearance, although excessive activation may contribute to tissue damage.

In adaptive immunity, OPN shapes both T- and B-cell responses. It has been reported to promote type-1 responses through IL-12 and IFN-γ-related pathways while suppressing anti-inflammatory cytokines such as IL-10 (210, 211). In B cells, OPN has been associated with both activating and regulatory features. It can enhance immunoglobulin production and polyclonal activation (212), and promote B-cell aggregation within inflamed tissues (201), features typically linked to ongoing immune activation. Conversely, OPN has also been shown to reduce expression of co-stimulatory molecules such as CD80 and CD86 and to shift cytokine production toward lower IL-6 and higher IL-10 levels (201), findings more consistent with regulatory or dampening functions.

Evidence also indicates that OPN participates in immune development. Circulating OPN concentrations are particularly high during early life and are abundant in breast milk, cord blood, and infant plasma, where higher levels have been associated with immune maturation and reduced susceptibility to infection (213-215). Experimental studies further suggest that intracellular OPN can influence hematopoietic differentiation in the bone marrow, promoting shifts toward lymphoid rather than myeloid lineages under inflammatory conditions (198).

Consistent with its immunoregulatory properties, OPN contributes to host defence in several infection models. OPN-deficient mice show impaired immune responses to intracellular pathogens, including *Listeria monocytogenes*, *Mycobacterium bovis*, herpes simplex virus-1, and *Plasmodium chabaudi* (210, 216, 217). At the same time, excessive or sustained OPN expression has been linked to immune-mediated pathology. Elevated OPN levels are associated with autoimmune diseases such as SLE (218), Crohn's disease (219) and multiple sclerosis (220), as well as with tumour progression in several cancers (221). These findings suggest that OPN is required for appropriate immune activation, but that its dysregulation may contribute to pathology. Tight control of OPN expression therefore appears critical for maintaining balanced immune responses.

Because OPN levels change during inflammation, circulating concentrations have been explored as biomarkers in several infectious diseases, including tuberculosis, HIV, viral hepatitis, dengue and COVID-19, where elevated levels frequently correlate with disease severity or progression (200, 222). However, interpretation of circulating OPN is complex. Its biological activity depends not only on total concentration but also on isoform composition, post-translational modification, and tissue-specific context. Circulating OPN levels may therefore reflect overall inflammatory activity but do not fully capture its functional effects.

Despite growing evidence in other infectious and inflammatory conditions, the role of OPN in malaria remains incompletely defined, particularly in human infection. Available data nevertheless suggest functional relevance. OPN expression has been associated with enhanced type-1 cytokine responses and reduced parasite densities in malaria patients (223), and experimental models indicate impaired parasite control in its absence (217). At the same time, elevated OPN concentrations have been detected in cerebrospinal fluid of children with cerebral malaria compared with neurologically symptomatic but malaria-negative controls, supporting an association with neuroinflammation and malaria (224).

These observations suggest that OPN may be linked to both protective immune activation and inflammatory pathology. This distinction is particularly relevant in malaria, where protective immunity requires a balance between parasite-clearing inflammation and mechanisms that limit tissue damage. Several known properties of OPN indicate that it could influence this balance, including its effects on type-1 immunity, B-cell activation, hematopoietic regulation, and inflammatory signalling. However, direct evidence from human studies is still limited, particularly across individuals with different levels of prior exposure or at different stages of immune development.

Immune checkpoint regulation and PD-1

Immune checkpoint pathways are central regulators of immune activation and homeostasis during infection. Programmed cell death protein-1 (PD-1, CD279) was first described in the 1990s as a molecule upregulated in apoptotic T cells but is now recognized as a key inhibitory co-receptor regulating immune activation (225). Structurally, PD-1 is a type I transmembrane protein belonging to the CD28/CTLA-4 family and contains intracellular inhibitory motifs that mediate downstream signalling (226). It is expressed at low levels on resting immune cells and is rapidly induced following activation across multiple immune cell types, including T cells, B cells, natural killer (NK) cells and some myeloid populations (225).

PD-1 interacts with two ligands, PD-L1 (CD274) and PD-L2 (CD273), which differ in distribution and regulation (227, 228). PD-L1 is broadly expressed on both hematopoietic and non-hematopoietic cells, whereas PD-L2 is more restricted and mainly found on antigen-presenting cells and activated lymphocytes (225, 229, 230). Both ligands are induced by inflammatory signals, particularly interferons. As a result, immune activation itself promotes PD-1 engagement, which in turn dampens further immune responses (231).

The PD-1 pathway has been most extensively studied in T cells. Persistent antigen exposure, such as during chronic viral infection or cancer, drives sustained PD-1 expression and is associated with T-cell exhaustion, characterized by reduced proliferation and impaired effector function (232). However, PD-1 expression is not synonymous with exhaustion. It is also transiently induced during acute activation and is present on subsets of memory and regulatory T cells (225, 233). PD-1 expression may therefore reflect activation or regulation rather than dysfunction per se. Experimental blockade of PD-1 can enhance antimicrobial responses, but may simultaneously increase immune-mediated pathology (225, 234). This balance is illustrated by the development of autoimmunity in PD-1-deficient mice (235), and by the therapeutic use of PD-1 blockade to enhance anti-tumour immunity in cancer (236). These observations indicate that PD-1 signaling contributes to balancing pathogen control with protection of host tissues.

In contrast to T cells, the role of PD-1 in B-cell biology is less well defined. *In vitro* studies show that PD-1 signaling inhibits B-cell receptor-mediated activation, proliferation, and cytokine production, and that PD-1 can cluster with the B-cell receptor, supporting a direct co-signalling function (237). Blocking PD-1 engagement enhances B-cell proliferation and antibody production *in vitro* (237). However, findings from *in vivo* systems indicate a more complex role. Although PD-1 deficiency can enhance certain aspects of B-cell activation (238), both humans and mice lacking PD-1 or its ligands exhibit reduced numbers of long-lived plasma cells, impaired B-cell memory and altered antibody affinity (239, 240). These data indicate that PD-1 may regulate not only the magnitude but also the quality and durability of humoral responses.

Evidence from infectious disease research further highlights the relevance of PD-1 signaling in host defence. In chronic viral infection such as hepatitis B, PD-1 blockade enhances virus-specific T-cell proliferation *ex vivo* (241) and reduces viremia (242), whereas PD-1-deficiency may increase mortality to *Mycobacterium tuberculosis* in mice (243, 244). In malaria, checkpoint pathways appear to modulate antiparasitic immunity across both adaptive and innate compartments. Increased PD-1 expression has been observed on $CD4^+$ T cells during acute *Plasmodium* infection in humans, and elevated expression of PD-1 and other inhibitory receptors has also been reported on T cells and atypical memory B cells in chronically exposed individuals (136, 141, 245, 246). Longitudinal studies in semi-immune individuals undergoing controlled malaria infection further show expansion of PD-1^+ B-cell populations following exposure (247). PD-1 expression is also transiently induced on NK cells during acute *P. falciparum* infection, where it has been associated with reduced natural cytotoxicity but enhanced antibody-dependent cellular cytotoxicity, showing that PD-1 modulates NK cells function, rather than simply suppressing it (248).

Experimental studies provide additional insight into how PD-1 signaling can influence malaria outcomes in different directions. In some models, PD-1 deficiency or PD-L1 blockade enhances parasite clearance and improves survival (245, 249, 250). In vaccination settings, PD-1 deficiency promotes germinal-center B-cell and T follicular helper responses, leading to increased parasite-specific antibody production (251). However, inhibition of PD-1 signaling does not consistently improve outcomes. PD-L1 and PD-L2 appear to exert distinct and potentially opposing effects: PD-L1 expressed on dendritic cells may suppress immune responses, whereas PD-L2 has been associated with enhanced T-cell activation and lower parasitemia (252). This suggests that the outcome of PD-1 signaling depends on the balance between its ligands (252). Moreover, in some experimental malaria systems, inhibition of PD-1 signaling aggravates disease through excessive Th1 activation and interferon-γ–mediated suppression of humoral immunity (253, 254). Together, these findings indicate that PD-1 signaling calibrates immune responses rather than acting solely as a brake on immunity. As a result, there is growing interest in whether therapeutic modulation of checkpoint pathways could improve antiparasitic immunity or vaccine responses, although such strategies remain largely unexplored in human malaria (243).

Despite increasing recognition of PD-1 pathway involvement in malaria, important questions remain. The relative contributions of PD-1 signaling across immune cell types are still not well defined, and much of the current understanding comes from animal models or *in vitro* systems. It remains unclear how PD-1 expression during acute malaria is shaped by prior exposure, how it relates to protection or pathology, and whether it primarily reflects activation, regulatory adaptation or dysfunction. Clarifying the role of PD-1 is therefore important both for interpreting immune responses in malaria and for evaluating whether checkpoint modulation could enhance protection without increasing pathology.

Aims and Rationale

The overall aim of this thesis was to improve our understanding of how humoral immune responses to *Plasmodium falciparum* are shaped across early life, malaria exposure, and acute infection, with a focus on antibody function, B-cell phenotypes, and immune regulation.

The specific aims of this thesis were:

I. To describe the development of *P. falciparum* merozoite-specific C1q-fixing antibodies from birth through early childhood and to examine their associations with maternal antibodies, malaria-specific responses, and B-cell subsets.

II. To examine plasma OPN levels in infants and mothers in a malaria-endemic region, their associations with malaria-specific antibody responses, B-cell subsets, and BAFF, and to assess whether OPN directly affects *P. falciparum* growth *in vitro*.

III. To examine plasma OPN levels during acute malaria in children and adults with differing malaria exposure, changes following convalescence, and their associations with parasite burden and IFN-γ.

IV. To characterize PD-1 expression across circulating B-cell subsets in relation to malaria exposure and acute infection, and to evaluate whether PD-1 expression is more frequently expressed in *P. falciparum*-binding B cells than in non-binding B cells.

Rationale

Paper I

Infants represent a unique immunological period in malaria-endemic settings. During early life, circulating antimalarial antibodies are largely maternally derived and decline over the first months of life, while the infant's own immune responses gradually develop with to exposure. Although the decay of maternal IgG and the acquisition of parasite-specific antibodies have been described, much less is known about how functional complement-fixing antimalarial antibodies develop during infancy, whether they are transferred from mother to child, and how they relate to B-cell populations and markers of malaria exposure.

Paper II

During infancy, the immune system undergoes rapid maturation as maternal immune factors decline, and the child's own immune responses begin to develop. In malaria-endemic settings, this process occurs alongside repeated exposure to *Plasmodium falciparum*, yet most studies of malaria immunity in early life have focused on antibodies and cellular responses, while much less is known about how soluble immune mediators behave during this period. OPN has been implicated in immune activation and regulation in several infectious and inflammatory conditions, but data on circulating OPN levels during infancy, particularly in malaria-endemic populations, remain limited. It therefore remains unclear whether OPN reflects age-related immune development, malaria exposure, or has direct effects on the parasite itself.

Paper III

Although OPN has been implicated in immune regulation and has been associated with protection in experimental malaria models, its role during acute malaria in humans remains poorly defined. Findings from Paper II showed that plasma OPN levels vary with age and associate with some malaria-specific immune parameters, raising questions about how OPN behaves during active infection and whether responses vary between individuals with different levels of naturally acquired immunity. In addition, the relationship between circulating OPN, parasite burden, and inflammatory activation during acute malaria is not well characterized.

Paper IV

Individuals living in malaria-endemic regions gradually acquire tolerance to infection, characterized by reduced clinical symptoms despite persistent parasitemia, suggesting a role for immune regulatory mechanisms in anti-disease immunity. While PD-1 is a key inhibitory receptor known to regulate immune activation during both acute and chronic infections, studies in malaria have focused mainly on T cells, and how PD-1 expression is distributed across B-cell subsets in relation to malaria exposure and infection remains incompletely understood.

General Methodology

Study design

	I	II	III	IV
Study type	Prospective longitudinal study of mother-infant pairs in a malaria-endemic setting.	Prospective longitudinal study of mother-infant pairs in a malaria-endemic setting.	Cross-sectional study with follow-up.	Cross-sectional study with follow-up in a subset.
Study population	Ugandan mother-infant pairs: mothers at delivery (n = 131) and 9 months (n = 129); infants at birth (n = 129), 2.5 (n = 95), 6 (n = 104), and 9 months (n = 112); subset at 5–6 years (n = 39).	Ugandan mother-infant pairs (n = 80), sampled longitudinally from birth to 9 months (mothers at delivery and 9 months) plus adult controls: Ugandan malaria-exposed males (n = 35) and Swedish malaria-naïve males (n = 20)	Uganda: individuals with acute malaria recruited at Iganga General Hospital (n = 81; n = 42 follow-up) and febrile non-malaria (n = 46) plus healthy controls (children n = 24; adults n = 81); Sweden: adults with imported malaria (n = 14) and malaria-naïve controls (n = 13).	Uganda: malaria-exposed adults (healthy donors n = 23; acute malaria n = 15, with n = 9 paired convalescent samples); Sweden: adults with imported malaria (n = 13) and malaria-naïve controls (n = 13).
Data	Plasma ELISA for *P. falciparum* merozoite-specific C1q-fixing antibodies	Plasma OPN concentrations measured by ELISA; in vitro parasite growth inhibition assay	Plasma OPN and IFN-γ concentrations measured by ELISA	Flow cytometry of circulating B-cell subsets, PD-1 expression, and *P. falciparum*-binding B cells.
Outcomes	Longitudinal changes in *P. falciparum*-specific C1q-fixing antibody levels and correlations with schizont-specific IgG/IgM, B-cell subsets, BAFF, and OPN.	Longitudinal patterns of OPN in infancy and correlations with schizont-specific IgG/IgM, B-cell subsets, BAFF, parasitemia, and parasite growth	Differences in plasma OPN levels by age, infection status, and immunity background during acute malaria, and associations with parasitemia and IFN-γ.	PD-1 expression across circulating B-cell subsets in relation to malaria exposure history, antigen specificity, and inflammatory markers.

Study populations

In this thesis, we examined immune responses in populations with different degrees of malaria exposure, allowing comparisons between malaria-naïve individuals, malaria-exposed controls, and participants with acute malaria.

Mother-infant cohort (Paper I and II)

Papers I and II were based on blood samples collected from mothers and their newborn infants who were prospectively enrolled during the third trimester at the Kasangati Health Centre antenatal clinic in Uganda between March 2012 and July 2013. Kasangati is a peri-urban town located approximately 20 km northeast of Kampala and is characterized by mesoendemic malaria transmission with two annual rainy seasons.

Blood samples from mother-infant pairs were collected at birth, at three consecutive follow-up visits during infancy (at 2.5, 6 and 9 months), and at a follow-up visit when the children were five to six years of age. Maternal blood samples were obtained at delivery and again nine months postpartum. Inclusion criteria were normal deliveries and healthy newborns, and study participants were consecutively enrolled. Clinical examinations were performed at each visit, and relevant data were recorded using a standardized study questionnaire.

Control cohorts (Uganda and Sweden; Papers II, III and IV)

Healthy malaria-exposed control samples were obtained from adult male blood donors residing in Kampala and Wakiso District, Uganda. These individuals were anonymous, reported good health at the time of donation, and were assumed to have prior exposure to malaria and some degree of naturally acquired immunity based on residence in malaria-endemic areas. Blood samples from this cohort were used as healthy malaria-exposed controls in Papers II and IV.

In Paper III, an additional geographically matched Ugandan control group was included, consisting of healthy adults accompanying children to the hospital who reported no current symptoms.

Healthy malaria-naïve control samples were obtained from adult volunteers residing in Sweden with no prior travel to tropical or malaria-endemic regions. These individuals were sampled once and reported no history of malaria. This cohort served as malaria-naïve controls in Papers II, III and IV.

Acute malaria and febrile illness cohorts in Uganda (Papers III and IV)

For Papers III and IV, individuals with acute malaria were prospectively enrolled at the outpatient department of Iganga General Hospital, located approximately 120 km east of Kampala, Uganda, between March and December 2022, covering two malaria transmission seasons. At enrolment, malaria diagnosis was based on clinical

presentation together with a positive malaria RDT. Inclusion criteria were a history of fever within the previous 24 hours and/or an axillary temperature ≥ 37.5 °C. Exclusion criteria included age below six months, severe anaemia (haemoglobin < 5 g/dl), blood transfusion within the preceding three months and known chronic conditions such as sickle cell disease, severe malnutrition, cancer, or HIV infection. Both adults and children were followed up 42 days after enrolment, at which time repeat blood sampling was performed.

In addition, for Paper III, adults and children presenting with febrile non-malarial illness were recruited at the same site.

Imported malaria cohort in Sweden (Papers III and IV)

Adults diagnosed with acute malaria following travel to malaria-endemic regions were prospectively enrolled at the Department of Infectious Diseases, Skåne University Hospital, Lund and Malmö, Sweden, between 2018 and 2022. Blood samples were collected during the acute phase of infection. Information on previous malaria exposure, country of origin, and duration of residence in Sweden was obtained, and clinical data were extracted from medical records.

Laboratory methods

Malaria diagnostics

For Papers I and II, malaria parasitemia in blood samples collected from mother-infant pairs was initially assessed using a malaria RDT (pLDH/HRP2 Combo, Premier Medical Corporation Limited, India), with positive samples subsequently confirmed by light microscopy. Thick and thin blood smears were examined by experienced laboratory technicians at the Makerere University Biomedical Cross-cutting Laboratory in Uganda.

For Papers III and IV, participants in Uganda were enrolled based on clinical presentation in combination with a positive malaria RDT (RightSign Malaria Ag HRPII/Pan *Plasmodium* Aldolase, Hangzhou Biotest Biotech Co., Ltd., China). All blood samples were subsequently assessed for parasitemia by either light microscopy at the Makerere University Biomedical Cross-cutting Laboratory in Uganda or by loop-mediated isothermal amplification (LAMP) using the HumaTurb C + A Loopamp™ Malaria Pan Detection Kit (Human Diagnostics Worldwide, Wiesbaden, Germany), according to the manufacturer's instructions and as previously described (255). LAMP analyses were performed at the Clinical Microbiology Laboratory of Region Skåne in Sweden.

In Sweden, blood samples from participants with imported malaria were assessed at the time of diagnosis using either malaria RDT (Carestart Malaria HRP2/pLDH Pf/Pan Combo test) or LAMP, with subsequent species determination and parasitemia confirmed by light microscopy performed by a doctor or an experienced laboratory technician at Skåne University Hospital in Lund and Malmö.

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood for downstream analyses. PBMCs were isolated by density-based separation of cellular blood components, allowing enrichment of mononuclear cells while removing erythrocytes and granulocytes. A schematic of PBMC isolation by density gradient centrifugation is shown in Figure 5.

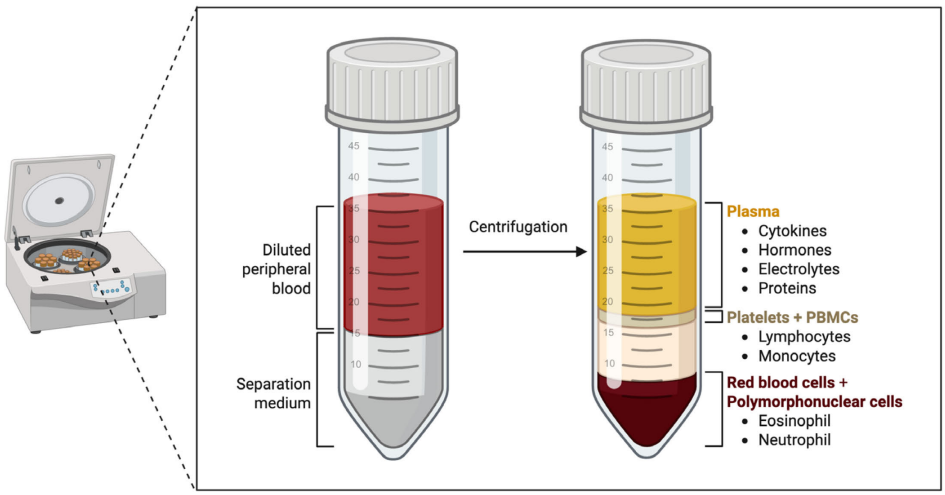

Figure 5. Schematic of density gradient centrifugation illustrating PBMC isolation and cellular layers.
Following centrifugation, PBMCs form a distinct layer at the plasma-separation medium interface, enabling separation from granulocytes and erythrocytes. Created in BioRender. Mortazavi, S (2026).

In Uganda, venous blood samples were collected in EDTA-coated tubes (5 mL in adults and 2 mL in children) at enrolment and day 42 post-infection. Samples were transported to the Makerere University Biomedical Cross-cutting Laboratory for processing, where PBMCs and plasma were isolated by density gradient centrifugation using Ficoll-Hypaque and cryopreserved according to established protocols.

In Sweden, venous blood samples were collected in EDTA and serum tubes and transported to the laboratory at Skåne University Hospital in Lund for processing. PBMCs and plasma were isolated using LeukoSep (Greiner Bio-One, Kremsmünster, Austria) and PluriMate II (pluriSelect Life Science, Leipzig,

Germany) tubes following the manufacturers' instructions and cryopreserved in liquid nitrogen.

Enzyme-linked immunosorbent assay (ELISA)

In this thesis, ELISA was one of the principal methods employed to assess antibody responses to *P. falciparum* merozoites and to quantify circulating inflammatory mediators. The specific ELISA formats applied are described in the following sections.

ELISAs are plate-based immunoassays used to detect and quantify antibodies, antigens, or soluble proteins in biological samples. Since their introduction in the 1970s, ELISAs have become a routinely used method in both research and clinical diagnostic laboratories (256, 257). The method is based on the immobilization of an antigen or capture antibody onto a solid surface, typically a 96-well plate. Binding of the target molecule is detected using an enzyme-conjugated antibody, which catalyses a reaction with a suitable substrate to generate a measurable optical or luminescent signal. Depending on the assay design, this signal can be quantified using a microplate reader, fluorometer or luminometer. Several ELISA formats have been developed to address different analytical questions (258). In indirect ELISAs, antigens are immobilized on the assay plate, allowing antigen-specific antibodies present in the sample to bind and subsequently be detected using enzyme-conjugated secondary antibodies. This format is commonly used to assess antibody responses against defined antigens. In sandwich ELISAs, the analyte is captured between two antibodies, providing high specificity and sensitivity for quantification of soluble proteins such as cytokines. While multiplex platforms, including proteomic approaches, are increasingly used for simultaneous measurement of soluble proteins, ELISA remains well suited for quantitative analysis of individual analytes across large sample sets and allows standardized and reproducible comparison between study groups (258). For antigen-specific antibody responses and functional assays such as C1q fixation, ELISA-based approaches remain essential, as proteomic methods do not directly measure antigen-specific or functional binding.

Complement-fixing ELISA for anti-merozoite antibodies (Paper I)

In Paper I, a complement-fixing ELISA was used to assess the capacity of plasma antibodies to bind the complement component C1q when bound to *P. falciparum* merozoite antigens (Figure 6). Crude *P. falciparum* merozoite extract, generated from parasites cultured *in vitro* in our laboratory according to established protocols, was used as antigen (259, 260). ELISA plates were coated with merozoite extract, blocked to reduce non-specific binding, and incubated with plasma samples diluted 1:50. Purified human C1q was subsequently added, and bound C1q was detected using anti-C1q antibodies followed by an enzyme-conjugated secondary antibody. The signal was developed using TMB substrate and measured as absorbance at 450

nm using a microplate reader. A positive reference sample and negative controls (malaria-naïve plasma and buffer) were included on each plate to monitor assay performance and background signal. All samples were analysed in duplicate, and samples with high variability were re-assessed. Longitudinal samples from the same individuals were analysed on the same ELISA plate where feasible to reduce inter-assay variation. Follow-up samples at 5 years were analysed on separate plates, and potential inter-assay variability cannot be excluded. Results are presented as optical density values reflecting the relative C1q-fixing activity of anti-merozoite antibodies.

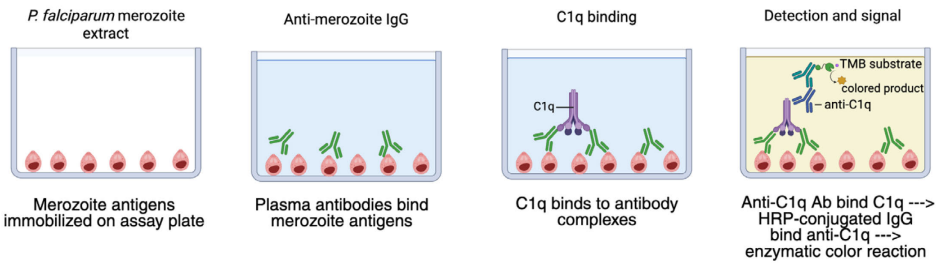

Figure 6. Schematic overview of the C1q-fixing ELISA used to assess anti-merozoite antibody responses.
Plasmodium falciparum merozoite antigens are immobilized on an ELISA plate and incubated with plasma samples, allowing antigen-specific antibodies to bind. The complement component C1q binds to Fc regions of antigen-bound antibodies and is detected using anti-C1q antibodies coupled to an enzyme-conjugated secondary antibody. Created in BioRender. Mortazavi, S (2026).

OPN ELISA (Paper I–IV)

Plasma concentrations of OPN were measured using a commercial sandwich ELISA (Quantikine Human Osteopontin Immunoassay; R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Plasma samples were analysed in duplicate and diluted 1:25, or 1:100 when sample volume were limited. OPN concentrations were quantified using the standard curve provided in the kit ranging from 0.313 to 20 ng/mL. To minimize inter-assay variation, longitudinal samples from the same individuals were analysed on the same ELISA plate where feasible. Optical density was measured at 450 nm using a SpectraMax 340PC384 microplate reader, and OPN concentrations were calculated using SoftMax Pro.

INF-γ ELISA (Paper II –IV)

Plasma concentrations of IFN-γ were measured using a commercial sandwich ELISA kit (BMS228; Invitrogen, USA) according to the manufacturer's instructions. Plasma samples were diluted 1:2 and analysed in duplicate. IFN-γ concentrations were quantified using an in-kit standard curve ranging from 1.6 to 100 pg/mL. To minimize inter-assay variation, baseline and follow-up samples from the same individuals were analysed on the same ELISA plates where feasible.

Optical density was measured at 450 nm using a Multiscan Sky microplate reader, and IFN-γ concentrations were calculated using Microsoft Excel and GraphPad Prism.

P. falciparum culture and growth inhibition assay with OPN (Paper II)

The continuous *in vitro* culture of *P. falciparum* asexual blood stages was first established in the 1970s and has since become a standard method for studying parasite growth and erythrocyte invasion under controlled conditions (261). In this study, a growth inhibition assay was used to evaluate whether OPN influences *P. falciparum* growth *in vitro*. *P. falciparum* FCR3S1.2 parasites were maintained in continuous culture using human O^+ erythrocytes according to established protocols (260, 262), and synchronized prior to experimental use. Recombinant human OPN (298 aa; Sigma-Aldrich) was added to late trophozoite-stage parasite cultures at a starting parasitemia of 1% and a haematocrit of 1%. OPN was tested at concentrations ranging from 0.2 to 12.8 µg/mL in duplicate wells in 96-well plates. Heparin (100 µg/mL) and phosphate-buffered saline (PBS) were included as inhibitory and growth controls, respectively. Parasitemia was determined at approximately 90 hours by acridine orange staining, and the stained cells were analyzed by flow cytometry. The assay was performed in independent experiments, and mean parasitemia from four replicate wells was used for the final analysis at each OPN concentration.

Flow cytometry

In this thesis, multiparametric flow cytometry was one of the main methods used to study immune cell populations in peripheral blood. Flow cytometry is a widely applied technique for the analysis of cells in suspension and enables simultaneous measurement of multiple cellular markers. The method is particularly well suited for immunological studies, as it allows detailed characterization of heterogeneous cell populations based on defined combinations of surface and intracellular markers.

The principle of flow cytometry is based on the passage of individual cells in a fluid stream through a laser. As cells pass the laser, light scatter signals are generated that provide information on cell size and internal complexity (granularity). In addition, fluorochrome-conjugated antibodies bound to specific surface or intracellular antigens emit fluorescence at defined wavelengths. These signals are detected and quantified, allowing identification and characterization of cell populations based on marker expression. By combining several fluorochromes within a single staining panel, multiple parameters can be analysed simultaneously for each cell. A schematic overview of the flow cytometry workflow used in this thesis, from cryopreserved cells to data analysis, is shown in Figure 7.

Flow cytometry data are analysed using a gating strategy, in which cell populations are defined stepwise based on scatter characteristics and marker expression. This approach allows exclusion of debris, dead cells, and cell doublets before focused

analysis of defined immune cell populations. Compensation is applied to correct for spectral overlap between fluorochromes, to ensure accurate interpretation of multicolour data. In addition, fluorescence minus one (FMO) controls are used to define gating boundaries by accounting for background fluorescence, particularly for markers with low or continuous expression.

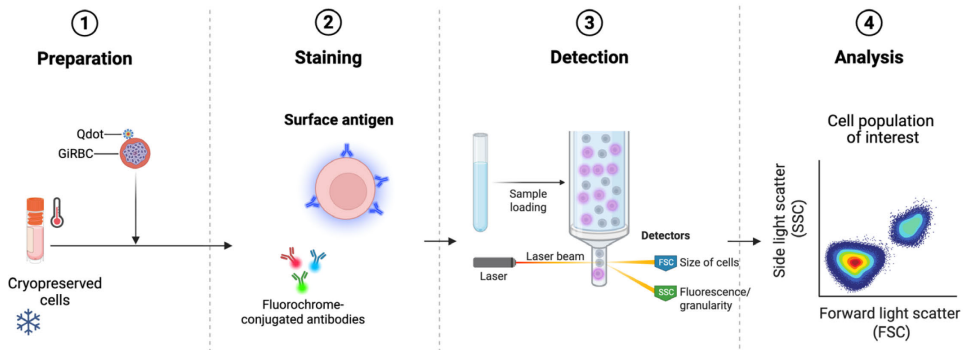

Figure 7. Schematic overview of the flow cytometry workflow in this thesis.
Cryopreserved PBMCs are thawed and stained with fluorochrome-conjugated antibodies to define B-cell subsets and assess expression of regulatory markers. Cells are subsequently analyzed by flow cytometry, where individual cells pass through a laser beam and light scatter and fluorescence signals are detected. Where applicable, *P. falciparum*-specific B cells are identified using fluorescent parasite-derived probes generated from ghost-infected red blood cells conjugated to carboxyl quantum dots (Qdot-GiRBCs). Flow cytometry data are analyzed using a stepwise gating strategy to define immune cell populations. The figure illustrates the experimental workflow and principles rather than quantitative results. Created in Biorender. Mortazavi, S (2026).

Flow cytometry also has some limitations. The method relies on predefined marker panels and therefore requires prior knowledge of which markers to include, which means that only markers included in the panel can be analysed. In addition, spectral overlap and increasing panel complexity can complicate data interpretation and require careful panel design. Another challenge is that gating strategies are not always standardized, which can complicate comparisons between studies. Alternative methods such as mass cytometry and single-cell RNA sequencing allow analysis of a larger number of parameters and provide detailed characterization of cells, but are more resource-intensive and less suited for routine use due to higher cost and more complex workflows.

Immunophenotyping of B-cell subsets, PD-1 expression, and P. falciparum-specific B cells

For Paper IV, multiparametric flow cytometry was used to characterize peripheral blood B-cell subsets, including *P. falciparum*-specific B cells, and assess expression of the inhibitory receptor PD-1 across different B-cell populations. Cryopreserved PBMCs were thawed and processed for flow cytometric analysis according to

standard protocols. Cells were stained with fluorochrome-conjugated monoclonal antibodies. The staining panel included CD19-BUV, IgD-V500, CD27-PE-CF594, CD10-PE, CD21-APC, CD38-APC-H7, IgM-FITC, IgG-BUV496, and PD-1-PE-Cy7. A viability dye (7-AAD) was included to exclude dead cells from analysis. To identify *P. falciparum*-specific B cells, *P. falciparum* parasites were cultured *in vitro* and enriched schizont-stage parasites were used to generate ghost-infected red blood cells (GiRBCs), which retain parasite-derived membrane components. GiRBCs were conjugated to carboxyl quantum dots (Qdots) and incubated with PBMCs prior to antibody staining, as previously described (260, 263). These were used as fluorescent probes to detect *P. falciparum*-specific B cells by flow cytometry. Following staining, cells were acquired on a flow cytometer and data were analysed using FlowJo v10.10.0 (BD). Compensation and fluorescence-minus-one (FMO) controls were applied to ensure accurate gating.

Statistical analysis

Statistical analyses across all studies were primarily based on non-parametric methods, reflecting the distributional characteristics of the data and the relatively small sample sizes in several cohorts. Continuous variables were summarized as means with standard deviations or as medians with interquartile ranges, while categorical variables were expressed as counts and percentages. All statistical tests were two-sided, and a p-value <0.05 was considered statistically significant. Comparisons between two independent groups were performed using the Mann-Whitney U test, while paired comparisons within individuals were assessed using the Wilcoxon matched-pairs signed-rank test. For more than two groups, the Kruskal-Wallis test with Dunn's post hoc correction was applied. For repeated measures within individuals, the Friedman test with post hoc correction was used. For analyses involving repeated measurements over time, longitudinal data were analysed using linear mixed-effects models to account for within-individual correlations. These models were used to assess changes over time, and where relevant, adjust for baseline values. Associations between continuous variables, including antibody levels, PD-1 expression, OPN, IFN-γ, and parasitemia, were evaluated using Spearman's and Pearson's correlation coefficients. Adjustment for multiple testing was performed using false discovery rate-based methods or Dunn's post hoc correction, as appropriate. For the growth inhibition assay in Paper II, differences between experimental conditions were assessed using analysis of variance (ANOVA). All statistical analyses were performed using R and GraphPad Prism.

Ethical considerations

All studies included in this thesis were conducted in accordance with the Declaration of Helsinki and relevant national and institutional guidelines. Written informed consent was obtained from all adult participants prior to enrolment. For studies involving children, written informed consent was obtained from parents or legal guardians. The studies conducted in Uganda were approved by ethics committees at Makerere University, including the School of Medicine Research and Ethics Committee and the School of Biomedical Sciences Research and Ethics Committee. National regulatory approval was obtained from the Uganda National Council of Science and Technology. Relevant approval numbers include 2011-114, SBS-REC-765, and HS1267ES. Studies involving participants in Sweden were approved by the Swedish Ethical Review Authority (Stockholm and Lund), with approval numbers 2014/478-32, Dnr 2015/7, 2014/478-32, and 2022-03160-01.

Author contributions

For Papers I and II, I performed the OPN ELISA analyses and conducted the C1q-fixing ELISA for the five-year follow-up samples. I had primary responsibility for statistical analyses and manuscript drafting, except for the linear mixed-effects models, which were developed in collaboration with a statistician. Ethical approval for Papers I and II had been obtained prior to my involvement. For Papers III and IV, I jointly led the ethical application process together with Allan Lugaajju and Kristina Persson and had a leading role in participant recruitment and data collection in Sweden. For Paper III, I had a lead role in data curation, methodology, statistical analysis, and manuscript drafting, reviewing, and editing. For Paper IV, I contributed to data curation, methodology, statistical analysis, and manuscript drafting, reviewing, and editing. I was involved in flow cytometry experiments and protocol development and performed the data analysis, while flow cytometry gating was conducted by other co-authors.

Major findings

Paper I

Participants

Mother-infant pairs were followed longitudinally from birth through infancy. Due to limitations in available plasma volume, the number of samples analysed for *P. falciparum* merozoite-specific C1q-fixing antibodies varied between time points. Samples were collected from infants at birth (n = 129), 2.5 months (n = 95), 6 months (n = 104), and 9 months (n = 112), and from mothers at delivery (n = 131) and 9 months postpartum (n = 114). A subset of children was sampled again at 5–6 years of age (n = 39).

Key findings

We first assessed *P. falciparum* merozoite-specific C1q-fixing antibody levels in infants at birth and compared these with maternal levels at delivery. Antibody levels in infants were high and comparable to those observed in maternal plasma (median 0.36 [IQR 0.26–0.57] vs 0.40 [IQR 0.28–0.61]). We next examined changes in antibody levels during infancy and observed a significant decline during the first months of life, reaching the lowest levels at 6 months of age (Figure 8). This period coincides with the expected waning of maternally derived antibodies and precedes the age at which infants typically begin to experience increased malaria susceptibility. From 6 to 9 months of age, antibody levels increased modestly, although this increase did not reach statistical significance. By 5-6 years of age, antibody levels were comparable to those observed at birth. Statistical adjustment for maternal C1q-fixing antibody concentrations at delivery did not change the observed decline in antibody levels during early infancy and the subsequent increase over time, indicating that maternal antibodies contribute substantially to antibody levels at birth but do not appear to influence the subsequent trajectory during infancy.

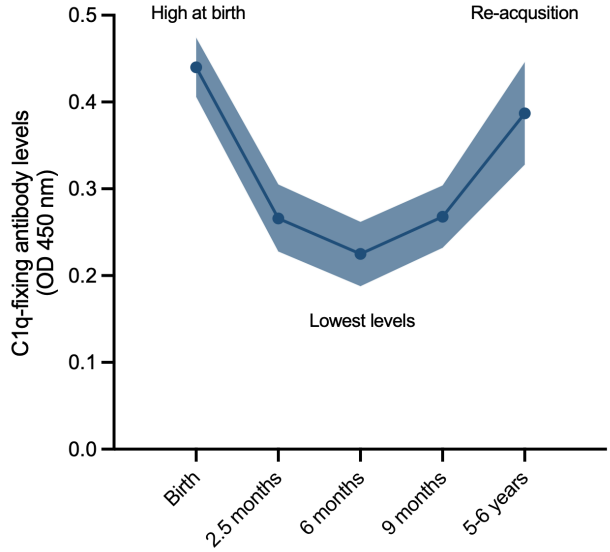

Figure 8. Longitudinal development of C1q-fixing antibody levels during early childhood.
Estimated mean levels of C1q-fixing antibodies in infant plasma are shown from birth to 5–6 years of age, derived from linear mixed models. Antibody levels were highest at birth, declined during early infancy with the lowest levels observed at 6 months of age, and increased thereafter. Shaded areas represent 95% confidence intervals. Time points are equally spaced for visualization purposes.

In contrast to the age-dependent changes observed in infants, C1q-fixing antibody levels in mothers remained stable between delivery and 9 months postpartum (median 0.40 [IQR 0.28-0.61] vs 0.38 [IQR 0.29-0.54]).

When infants were further stratified according to C1q-fixing antibody levels at birth, those in the highest quartile exhibited a more rapid decline during the first months of life compared with those with lower initial levels. By the time infants reached 6 months of age, antibody levels were similar across groups.

To explore how *P. falciparum* merozoite-specific C1q-fixing antibody levels related to malaria exposure markers, B-cell subsets, and inflammatory mediators, correlation analyses were performed. In infants, C1q-fixing antibody levels correlated with schizont-specific IgG levels at birth and at 9 months of age. In mothers at delivery, C1q-fixing antibody levels correlated with schizont-specific IgG and IgM. At 9 months of age, infant C1q-fixing antibody levels showed several associations with immune cell subsets. Specifically, antibody levels were negatively correlated with total B cell frequencies and positively correlated with atypical MBCs and *P. falciparum*-specific atypical MBCs. These associations were not observed at earlier time points. In addition, a negative correlation between C1q-fixing antibody levels and plasma OPN concentrations was observed at 9 months of age.

Overall, *P. falciparum* merozoite-specific C1q-fixing antibody levels in early life were high at birth, declined during infancy, and increased again during childhood, with age-specific associations with markers of malaria exposure and immune maturation.

Paper II

Participants

This study was conducted in the same longitudinal Ugandan mother-infant cohort described in Paper I. Plasma OPN concentrations were analysed in 80 mother-infant pairs followed from birth to 9 months of age. Due to sample availability, not all participants were analysed at each time point. In addition, plasma samples from healthy adult malaria-exposed males in Uganda (n = 35) and malaria-naïve males in Sweden (n = 20) were analysed for comparison.

Key findings

We first examined OPN concentrations across infancy and compared these levels with maternal levels. Plasma OPN concentrations were markedly higher in infants than in mothers throughout the study period. At birth, infants had an estimated mean OPN concentration of 332 (95 % CI 292–373) ng/mL, compared with 73 (95 % CI 56–91) ng/mL in maternal plasma at delivery. Infant OPN concentrations increased during early infancy, reaching a peak at 2.5 months (estimated mean 433 (95 % CI 388–478) ng/mL), and subsequently declined towards 9 months of age (Figure 9A). In contrast, maternal OPN concentrations remained low and stable between delivery and 9 months postpartum (Figure 9B).

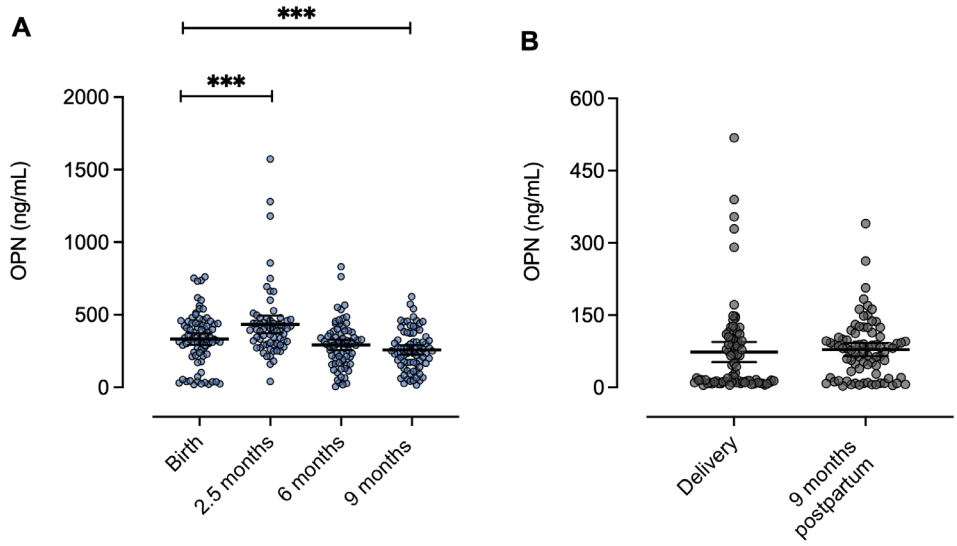

Figure 9. Longitudinal plasma OPN concentrations in infants and mothers.
Plasma OPN concentrations are shown for (A) infants and (B) their mothers at each time point in the longitudinal Ugandan mother-infant cohort. Each dot represents an individual plasma OPN concentration. Horizontal lines indicate estimated mean concentrations derived from linear mixed models, with whiskers representing 95% confidence intervals. OPN concentrations were analysed at birth, 2.5 months, 6 months, and 9 months in infants, and at delivery and 9 months postpartum in mothers. *** $P < 0.001$.

We next used linear mixed models to account for repeated measurements within individuals and to assess whether maternal circulating OPN levels influenced infant OPN concentrations over time. Adjustment for maternal OPN concentration at delivery did not change the age-related pattern observed in infants. In line with this, further analyses showed that OPN concentrations were significantly higher in infants than in mothers both at birth and at 9 months postpartum.

To place these findings in a broader context, we compared OPN concentrations in the study cohort with those measured in healthy adult controls. OPN concentrations in adults were similar between Ugandan and Swedish individuals, with median concentrations of 72 ng/mL in both groups. OPN concentrations did not differ between women and men, as adult OPN levels in both control groups in Uganda and Sweden were similar to those observed in mothers from the study cohort.

We next examined whether OPN concentrations were associated with malaria infection or parasite burden. No association was observed between OPN concentrations and *P. falciparum* parasitemia in this cohort. However, parasitemia was detected in only a small number of individuals. To further assess whether OPN could directly affect parasite growth, we performed growth inhibition assays in which recombinant OPN was added to *P. falciparum* cultures at concentrations

ranging from 200 to 12 800 ng/mL. Under these conditions, OPN did not affect parasite growth or invasion over two life cycles.

We further explored associations between OPN concentrations and immune parameters. OPN concentrations in infants were inversely associated with the frequency of atypical and IgG^+ MBCs and positively associated with naïve B cells, with the strongest associations at 9 months of age. At this time point, these associations were also evident for *P. falciparum*–specific (Pf^+) B-cell subsets, including Pf^+ atypical and Pf^+ naïve B cells. In mothers, inverse correlations between OPN concentrations and atypical MBCs were observed at delivery but not at 9 months postpartum. OPN concentrations were positively correlated with BAFF concentrations in both infants and mothers at multiple time points. Associations between OPN and schizont-specific antibody levels were limited; in infants, no consistent correlations were observed, whereas in mothers OPN concentrations correlated with schizont-specific IgG at delivery and at 9 months.

Overall, OPN levels are high during early infancy, vary with age, and are independent of maternal circulating OPN levels. OPN was not associated with parasitemia and did not affect parasite growth *in vitro* but was associated with the distribution of B-cell subsets, including *P. falciparum*-specific B cells and with BAFF. These associations were most evident during later infancy.

Paper III

Participants

This study included individuals with acute malaria, febrile non-malarial illness, and healthy controls from Uganda and Sweden. The Ugandan malaria cohort comprised 81 individuals with confirmed malaria, including 41 children (<15 years) and 40 adults (≥15 years), of whom a subset (n = 42; 24 children and 18 adults) was sampled again 42 days after treatment. As site-matched comparators, 46 individuals (23 children and 23 adults) presenting with febrile non-malarial illness were included. In addition, healthy Ugandan adults (n = 81) from the same study site and healthy Ugandan children (n = 24) from the follow-up visit were included as control samples. The Swedish cohort comprised 14 adults with imported malaria together with malaria-naïve healthy controls (n = 13).

Major findings

We first examined plasma OPN levels during acute malaria in children and adults in Uganda and compared these with healthy controls (Figure 10). In the Ugandan cohort, children with acute malaria had significantly higher plasma OPN levels than healthy children (median 159 ng/mL [IQR 66–304] vs 65 ng/mL [IQR 45–126], $P < 0.01$), whereas no difference was observed between adults with and without

malaria (Figure 10A). In addition, OPN levels did not differ between younger (≤5 years) and older children.

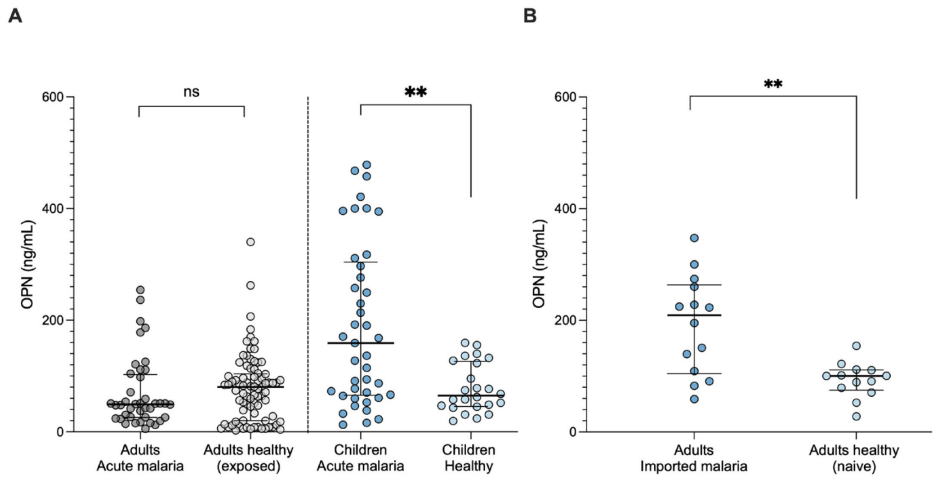

Figure 10. Plasma OPN levels during acute malaria in relation to age, infection status and immunity background.

(A) Plasma OPN levels in Ugandan children and adults with acute malaria and in corresponding healthy control groups. (B) Plasma OPN levels in adults with imported malaria in Sweden and in healthy malaria-naïve adult controls. Each dot represents one individual. Horizontal lines indicate medians with interquartile ranges. Plasma OPN concentrations are shown in ng/mL. ** P < 0.01.

To further examine the influence of immune status, we assessed OPN levels in adults with imported malaria (Figure 10B). Plasma OPN levels were significantly elevated in adults with imported malaria compared with healthy Swedish controls (median 209 ng/mL [IQR 105–264] vs 100 ng/mL [IQR 75–111], P = 0.0047). OPN levels in imported malaria cases were higher than those observed in Ugandan adults but did not differ from levels observed in Ugandan children with acute malaria. These data suggest that elevated OPN levels during acute malaria are associated with limited or absent malaria immunity rather than age alone.

We next explored whether OPN levels reflected parasite burden and inflammatory activation. Across all malaria cases, plasma OPN levels correlated positively with parasite density (Spearman's rho = 0.41, P <0.0001). Plasma IFN-γ levels also correlated with parasitemia, with the strongest association observed in Ugandan children (rho = 0.33, P = 0.036). In addition, OPN and IFN-γ levels were positively correlated in the overall study population, with the strongest correlation observed in Ugandan adults (rho = 0.59, P <0.0001). During convalescence, paired analyses showed that plasma OPN levels declined significantly in children at 42 days follow-up (P = 0.0039), while no change was observed in adults.

Finally, we assessed whether elevated OPN levels were specific to malaria by comparing individuals with acute malaria to those with non-malarial febrile illness. Plasma OPN levels did not differ between the two groups. As observed in malaria, children <15 years of age with non-malarial febrile illness consistently exhibited higher OPN levels than adults (median 123 ng/mL [IQR 63–221] vs 30 ng/mL [IQR 17–49], P <0.0001).

Overall, plasma OPN levels were increased during acute malaria in individuals without established malaria immunity, including children and adults with imported malaria, but not in semi-immune adults in endemic areas. OPN levels were associated with parasite burden and IFN-γ but were not specific to malaria infection.

Paper IV

Participants

This study analysed PBMCs from 64 individuals. The analysis included individuals with acute malaria recruited in Uganda (n = 15) and Sweden (n = 13), as well as healthy malaria-exposed adults in Uganda (n = 23) and malaria-naïve adults in Sweden (n = 13). Paired convalescent samples were available from nine Ugandan individuals.

Major findings

Baseline B-cell composition differed between malaria-naïve and malaria-exposed individuals. Malaria-exposed adults had lower frequencies of transitional/mature-naïve B-cells and higher frequencies of memory B-cell subsets, including atypical MBCs. During acute malaria, B-cell responses differed by exposure background, with expansion of activated and atypical MBCs in Ugandan cases and a dominant plasmablast response in Swedish cases.

To examine whether PD-1 expression differed by malaria exposure, the proportion of PD-1^+ cells was assessed across major circulating B-cell subsets. During acute malaria in previously exposed Ugandan individuals, the proportion of PD-1^+ cells was broadly increased across multiple B-cell subsets compared with healthy malaria-exposed controls. In contrast, individuals with imported malaria did not show a similar increase, with proportions remaining comparable to those in malaria-naïve controls. Differences between Ugandan acute malaria cases and control groups were statistically significant across several B-cell subsets.

Additional analyses within the memory B-cell compartment showed that PD-1 expression differed by differentiation state, with higher proportions of PD-1^+ cells observed among non-class-switched (IgD^+/IgM^+) MBCs compared with IgG^+ class-switched MBCs.

To determine whether PD-1 expression differed between antigen-specific and non-specific B cells, we compared *P. falciparum*-binding (Pf^+) and non-binding (Pf^-) B cells within individuals. PD-1 expression was higher on Pf^+ B cells than on Pf^- B cells across participants.

Parasitemia showed modest negative correlations with PD-1 expression on transitional/mature-naïve B cells and activated MBCs ($r = -0.46$, $p = 0.041$ and $r = -0.45$, $p = 0.04$, respectively), while other subsets showed no significant associations. Plasma OPN showed similarly modest subset-specific associations with PD-1 expression, whereas IFN-γ showed no consistent correlations. In paired analyses of Ugandan participants with acute and convalescent samples, neither B-cell subset distribution nor PD-1 expression showed major changes 42 days after treatment.

Overall, PD-1 expression on circulating B cells during malaria varied by malaria exposure history, with increased proportions of $PD\text{-}1^+$ cells during acute malaria in individuals with continuous prior exposure in Uganda.

Discussion

Development of humoral immunity in early life

During early life, protection against malaria is influenced by a combination of maternally derived antibodies, the maturation of the infant immune system, and exposure to the parasite. How these factors interact remains central to understanding how immunity develops and to designing vaccination strategies for this age group. The studies included in this thesis address two aspects of early humoral immunity in a longitudinal cohort of mother-infant pairs living in a malaria-endemic region in Uganda: the development of functional C1q-fixing antibodies against *P. falciparum* and the immunoregulatory mediator OPN in relation to B-cell subsets during early immune development.

A central finding from Paper I was that complement-fixing antibodies were present in cord blood at levels similar to those in the mothers. This most likely reflects maternal transfer, given the limited foetal IgG production at birth (264), and the well-established transplacental transfer of IgG during late pregnancy (265). This indicates that newborns not only receive maternal antibodies, but also antibodies with the capacity to engage complement, an important mechanism of parasite inhibition that has been associated with protection against malaria (154, 162, 177, 178, 181).

However, these responses declined rapidly during the first months of life. This timing coincides with the well-described period during which maternal antibodies wane while the infant immune system is still developing, creating a window of susceptibility to malaria (171, 266-269). In our cohort, C1q-fixing antibody levels increased again alongside schizont-specific IgG, consistent with the gradual acquisition of humoral immunity following parasite exposure. By five years of age, levels approached those observed in adults. Since most previous studies linking complement-fixing antibodies to protection have focused on older children or adults with established immunity, these findings show that such functional antibody responses are already acquired during early life.

Interestingly, infants with higher levels of complement-fixing antibodies at birth showed a faster decline over time. Similar patterns have been reported for maternal antibodies against *P. falciparum* as well as other pathogens (270-272), although contrasting results also exist (273). One explanation relates to IgG homeostasis, where higher antibody concentrations are associated with increased catabolism (271).

Another possibility is that antibody levels at birth reflect differences in maternal exposure. Mothers with high antibody levels are more likely to have experienced frequent infections, and their infants may therefore be exposed to higher transmission intensity. In such settings, antibodies may be consumed more rapidly through binding to parasites during early, often asymptomatic, infections (270). This also reflects a broader challenge in infant-malaria immunology: whether maternally transferred malaria antibodies confer protection or primarily act as markers of maternal exposure (171, 274-276). Our results do not resolve this question directly, as the study was not designed to assess clinical protection.

The acquisition of complement-fixing antibodies occurred alongside changes in the B-cell compartment. As discussed earlier in this thesis, repeated malaria exposure is associated with substantial remodelling of B-cell populations, including expansion of atypical MBCs (104, 136, 142). In this study, complement-fixing activity correlated with both total and *P. falciparum*-specific atypical MBCs. Although atypical MBCs have often been described as dysfunctional or "exhausted," increasing evidence indicates that they may represent an exposure-adapted phenotype capable of participating in parasite-specific immune responses (131, 144). The observed associations are consistent with such a role, but they do not establish causality. It remains unclear whether atypical MBCs directly contribute to complement-fixing antibody production or if they instead reflect cumulative exposure and immune activation.

The complement-fixation assay in this study used whole merozoite extract rather than individual recombinant antigens. This approach captures responses to a broad range of parasite proteins and may better reflect naturally acquired immunity, but it does not allow identification of which antigens contribute most strongly to complement recruitment. IgG subclasses were not measured, which is relevant because subclasses such as IgG1 and IgG3 differ both in complement-fixing capacity and in placental transfer efficiency (162, 265). It is therefore not possible to determine which antibody populations primarily drive the complement-fixing activity observed at birth and during childhood. In addition, the complement-fixation assay measures the capacity to recruit C1q *in vitro* but does not directly demonstrate downstream complement activation or parasite inhibition. The present findings therefore point to functional antibody potential rather than direct evidence of protection.

In Paper II, infants had significantly elevated circulating OPN concentrations compared with their mothers, with levels peaking during the first months of life before declining by nine months. Similar age-dependent patterns have been reported previously, with higher OPN concentrations in infants than in adults (213, 277, 278). In our cohort, infant OPN levels were independent of maternal concentrations, indicating that OPN is produced by the foetus or placenta rather than transferred from the mother, as is the case for IgG. This supports the view that high OPN levels represent a feature of early immune development (213-215). In line with this, higher OPN concentrations were associated with increased frequencies of naïve B cells and

reduced frequencies of atypical and IgG MBCs at selected time points during the study period. This suggests that OPN reflects the developmental state of the immune system during early life, rather than being driven solely by infection. Its decline later in infancy may accompany maturation of the B-cell compartment as naïve cells differentiate into memory and atypical MBCs.

The strong positive correlation between OPN and BAFF further links OPN to pathways regulating B-cell development. BAFF is a key regulator of B-cell survival, maturation, and antibody production, and elevated BAFF levels have been reported during malaria infection as well as in other inflammatory conditions (279-281). Experimental studies also show that BAFF signaling can induce OPN expression in B cells, indicating that these molecules operate within related regulatory networks (282). The consistent association between OPN and BAFF in both mothers and infants supports a role for OPN in shaping the environment in which B-cell differentiation and antibody responses develop. Whether this relationship is specific to malaria-endemic settings or reflects a more general feature of immune regulation remains unclear and warrants further investigation.

Although earlier reports have suggested that OPN might contribute to parasite control in malaria (223), our assays did not demonstrate any direct inhibitory effect of OPN on parasite growth in *vitro*. This was not unexpected, as OPN is more likely to influence host immune responses than parasite replication directly. Any effects of OPN on parasite control are therefore likely to require assay systems that include relevant immune cells rather than parasite cultures alone.

In this study, circulating OPN was measured in EDTA plasma using a commercial ELISA. As OPN exists in multiple modified and cleaved forms with different biological activities, and the assay does not distinguish between intact and cleaved forms, the measurements reflect total detectable OPN rather than isoform-specific activity.

The findings from Papers I and II suggest that early humoral immunity to *P. falciparum* develops gradually, from maternally derived functional antibodies to endogenous responses. This transition occurs within a developing B-cell compartment associated with mediators such as OPN and BAFF, which may influence how humoral immunity is established during early life.

Immune activation and regulation during malaria infection and exposure

While the previous section focused on the development of humoral immunity during early life, immune responses to *P. falciparum* are also shaped by the inflammatory and regulatory environment during infection and repeated parasite exposure. In

endemic settings individuals gradually develop partial immunity, in which parasitemia may persist despite limited symptoms, and immune regulation plays an important role in balancing parasite control and inflammation. Papers III and IV therefore examined immune activation and regulation during malaria infection, focusing on OPN and the immune checkpoint receptor PD-1 expressed on B-cell subsets.

In Paper III, circulating OPN levels were examined during acute malaria in individuals with different levels of malaria exposure. Plasma OPN concentrations were elevated during acute malaria in Ugandan children and in adults with imported malaria in Sweden, in line with observations from other infectious diseases where OPN increases during systemic inflammation (200). In contrast, malaria-exposed Ugandan adults did not show a comparable increase. Differences in inflammatory responses according to exposure history are well described: malaria-naïve individuals typically mount strong systemic cytokine responses during primary infection, whereas individuals in endemic settings develop more regulated responses despite ongoing parasitemia (95, 96, 188). In this context, the higher OPN levels in children and in adults with imported malaria reflect stronger inflammatory activation in individuals who have not yet developed, or who have lost, partial immunity, while the absence of a similar increase in malaria-exposed adults points to more regulated inflammatory responses with repeated parasite exposure. Longitudinal analysis further supports this interpretation. OPN levels declined during convalescence in children but remained relatively stable in exposed adults, indicating that the elevated levels in children were linked to acute infection rather than reflecting baseline age-related differences.

In our cohort, parasitemia correlated with both IFN-γ and OPN levels, in line with previous studies showing that parasite burden is associated with pro-inflammatory cytokine responses, including IFN-γ, TNF and IL-6, which contribute both to parasite control and to malaria pathology (184, 186, 283-285). OPN has been linked to several of these pathways, including the promotion of Th1 responses and IFN-γ production (207, 286). As parasitemia is associated with disease severity, and OPN has been reported to be elevated in cerebral malaria (224), the association between OPN levels and parasite density could also relate to disease severity. However, most individuals in this study had uncomplicated malaria, which limited our ability to assess this directly. In subgroup analyses, the association between parasitemia, IFN-γ and OPN remained evident in children, likely reflecting less developed anti-parasite and anti-disease immunity and the stronger inflammatory responses typically observed at this age. In contrast, the relationship between OPN and IFN-γ differed across exposure groups. A correlation was observed only in malaria-exposed Ugandan adults, whereas it was absent in children and in the imported malaria cohort. These findings suggest that OPN is part of inflammatory responses that become more tightly regulated with repeated malaria exposure. However, these subgroup analyses should be interpreted with caution given the relatively small sample sizes.

Elevated OPN levels were also observed in individuals with non-malarial febrile illness. Together with the absence of a direct inhibitory effect of OPN on parasite growth *in vitro* shown in Paper II, this suggests that circulating OPN primarily reflects systemic inflammatory activation rather than a malaria-specific immune mechanism, arguing against its use as a specific biomarker for acute malaria.

The cohorts in this study differed in age, exposure history, and geographical setting, which complicates direct comparisons. In particular, the imported malaria cohort likely represents a heterogeneous population with varying degrees of prior malaria exposure, and detailed infection histories were not available. Despite this, the elevated OPN levels observed during acute malaria in this group resembled the pattern seen in Ugandan children rather than in malaria-exposed adults, consistent with limited or incomplete immunity.

It is well known that repeated exposure to *P. falciparum* alters both the composition and regulation of the B-cell compartment, including expansion of atypical MBCs and increased expression of inhibitory receptors (134, 140, 141, 143). However, the functional significance of these phenotypes remains debated. In Paper IV, PD-1 expression across circulating B-cell subsets was examined to explore how immune checkpoint regulation relates to B-cell responses during malaria infection. Although PD-1 has been extensively studied in T cells, its role in regulating human B-cell responses in malaria remains less well defined, particularly across different exposure histories.

Differences between exposure groups were evident in B-cell subset composition. Malaria-exposed individuals showed higher frequencies of atypical and activated MBCs compared with malaria-naïve individuals, consistent with previous reports from malaria-endemic regions (104, 129, 136, 142, 144-146). Importantly, acute malaria in malaria-exposed individuals was associated with a broad increase in PD-1 expression across circulating B-cell subsets. In contrast, this increase was not observed in individuals with imported malaria or in healthy exposed and non-exposed individuals. This suggests that PD-1 expression during malaria is influenced by exposure history and may reflect how regulatory pathways are engaged during infection. However, resolving this will require functional characterization of these B-cell populations.

Previous studies similarly report increased PD-1 expression within the B-cell compartment following malaria exposure, particularly among atypical memory B-cell populations (141). A recent CHMI study by Requena et al further show that PD-1 expression varies depending on exposure history and the experimental context (247). Semi-immune individuals displayed higher baseline frequencies of PD-1$^+$ atypical MBCs compared with malaria-naïve participants, while experimental infection induced increases in PD-1 expression on selected B-cell subsets, particularly in naïve or vaccinated individuals (247). This differs from our results, where baseline PD-1 expression did not vary markedly between exposure groups. However, differences in

cohort composition, cumulative exposure, and flow cytometry panels may contribute to variation between studies. Controlled infections also differ from naturally acquired malaria in several respects, including infection dose and early treatment, which may influence the dynamics of immune activation and PD-1 expression.

PD-1 expression also differed across immunoglobulin-defined memory B-cell subsets, with higher frequencies in IgD^+ MBCs compared with class-switched IgG^+ MBCs. As IgD^+ MBCs are less differentiated, this suggests that PD-1 expression is associated with earlier stages of B-cell differentiation, rather than with more differentiated or exhausted B cells, and points toward a role for PD-1 in activation and regulation rather than exhaustion alone. This is consistent with previous studies showing that PD-1 can be transiently induced during activation and contribute to immune regulation (225, 232, 233).

In addition, PD-1 expression was higher on *P. falciparum*-binding B cells compared with non-binding B cells within the same individuals. Although the number of parasite-binding events was limited, this observation suggests that checkpoint signaling is preferentially engaged within antigen-specific B-cell populations. Given that PD-1 signaling dampens B-cell receptor-mediated activation and regulates proliferation and differentiation (237), this could represent a mechanism to control antigen-driven responses. In the context of repeated exposure, such regulation might help limit excessive immune activation while preserving the ability to generate parasite-specific humoral responses.

Associations between parasitemia, OPN, IFN-γ and PD-1 expression were limited and varied across B-cell subsets. This contrasts with findings from CHMI studies, where $PD\text{-}1^+$ B-cell subsets have been associated with inflammatory cytokine responses (247). Nevertheless, PD-1 expression and B-cell subset composition did not differ significantly between acute malaria and one-month convalescence, suggesting that these regulatory features persist beyond parasite clearance and are not primarily driven by acute inflammatory responses.

Together, the findings from Papers III and IV indicate that OPN and PD-1 reflect distinct aspects of the immune response to malaria. OPN is linked to inflammatory activation and is not malaria-specific, whereas PD-1 expression appears less dependent on acute inflammation and varies with malaria exposure, suggesting a role in longer-term immune regulation. Further studies are needed to define the functional role of PD-1-expressing B-cell populations, particularly whether they contribute to regulation of parasite-specific responses or reflect altered B-cell function following repeated exposure.

Conclusions

- Complement-fixing antibodies against *P. falciparum* are efficiently transferred from mother to infant and are gradually re-acquired during childhood with increasing parasite exposure.
- Elevated OPN concentrations characterize early immune development and are associated with B-cell maturation and BAFF-related pathways, without directly influencing parasite growth.
- OPN levels increase during acute malaria as part of systemic inflammatory responses, with responses differing according to prior malaria exposure.
- PD-1 expression on B-cell subsets varies with malaria exposure history, B-cell differentiation state and antigen-specificity, supporting a role for immune checkpoint pathways in regulating humoral responses during repeated malaria infections.

Future Perspectives

The long-term vision of the WHO and the global malaria community is a world free of malaria. Despite the implementation of multiple control measures, malaria continues to impose a substantial burden in many parts of the world. Looking ahead, several emerging challenges may complicate elimination efforts, including the impact of climate change on malaria transmission patterns and geographic distribution (26), as well as the continued emergence of drug and insecticide resistance. At the same time, recent advances, including vaccines and novel preventive and therapeutic strategies such as monoclonal antibodies (287), new antimalarial drugs (288), and improved diagnostic tools (289), provide reasons for cautious optimism. However, sustained progress will depend not only on the development of new interventions, but also on their effective implementation, requiring continued investment in health systems, infrastructure, and access to care in endemic regions.

Within this broader context, a deeper understanding of host immunity remains central to both disease control and the development of effective interventions. Despite decades of research, many aspects of naturally acquired immunity to *P. falciparum* remain unresolved, particularly regarding how protective immune responses are generated, maintained, and regulated over time.

A particularly important area for future research is the development of immunity in early life. Infants represent a group at high risk of severe malaria and are a key target population for vaccination strategies, yet the transition from maternal protection to endogenous immunity remains poorly understood. Future studies should aim to clarify the impact of maternally transferred antibodies on infant immune responses, as well as to identify the factors underlying the reduced vaccine efficacy observed in young infants. Longitudinal studies with detailed immunological profiling during the first years of life will be essential to define how protective immunity emerges and how it can be optimized.

An additional area of increasing interest is the role of functional antibody responses as potential correlates of protection (290). Findings from this thesis, together with previous studies, support the relevance of complement-fixing antibodies, which appear to be acquired early in life through both maternal transfer and endogenous responses. However, it remains unclear to what extent such responses reflect protective immunity rather than cumulative exposure, particularly in infancy.

Addressing this will require longitudinal studies combining functional antibody measurements with detailed clinical follow-up, as well as integration into vaccine trials to determine their relevance as correlates of protection.

Osteopontin (OPN) has immunomodulatory effects, including roles in B-cell activation and antibody responses (201, 212), and may be linked to BAFF signalling (282). In this thesis, OPN was associated with BAFF and naïve B-cell subsets, but it remains unclear whether OPN directly influences B-cell differentiation and malaria-specific immune responses or instead reflects general immune activation. Addressing this will require experimental studies examining its effects on B-cell activation, survival, and differentiation, alone or in combination with BAFF, as well as longitudinal studies to determine how these relationships develop over time and relate to susceptibility to infection. These questions are also relevant for vaccination, where early-life immune regulation may influence the development and durability of B-cell-mediated responses. If OPN contributes to shaping these processes, it could represent an additional factor influencing how effective immune responses are generated and maintained.

Similarly, immune regulatory pathways such as PD-1 are likely to play an important role in shaping B-cell responses during malaria infection. The findings presented in this thesis suggest that PD-1 expression on B cells is influenced by both exposure history and B-cell differentiation state, but its functional consequences remain unclear. Further work is needed to determine how checkpoint signaling affects B-cell activation, differentiation, and antibody production, and whether PD-1 primarily limits protective responses or contributes to their regulation. In a broader perspective, this may also be relevant for vaccination, where both effective activation and appropriate regulation of B-cell responses are required, and where modulation of checkpoint pathways may represent a potential approach to enhance vaccine-induced antibody responses.

Related to this is the still unresolved question of the role of atypical memory B cells in malaria immunity. Although their expansion in malaria-exposed populations has been consistently observed, their contribution to protective immunity is still unclear. Advances in single-cell technologies, antigen-specific B-cell sorting, and functional assays provide new opportunities to address this question and to determine whether these cells represent dysfunctional populations or adaptive responses to chronic antigen exposure.

Overall, a better understanding of how functional antibody responses, B-cell differentiation, and immune regulatory pathways interact during malaria infection may help explain why immunity to malaria develops slowly and remains incomplete despite repeated parasite exposure. Such knowledge may help identify immune mechanisms that are important for protection and could inform the rational design of more effective and durable malaria vaccines.

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