

DOUTORAMENTO

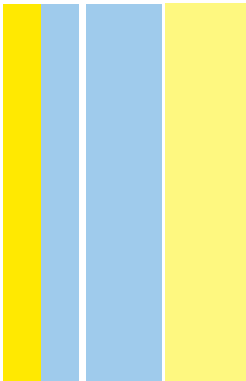
BIOLOGIA BÁSICA E APLICADA

A Metabolic Arms Race Between
Host and Parasite: Amino Acid
Sensing and Response in
Plasmodium

Maria Inês Marreiros

D

2022



Maria Inês Marreiros. A Metabolic Arms Race Between
Host and Parasite: Amino Acid Sensing and Response in
Plasmodium

A Metabolic Arms Race Between Host and Parasite:
Amino Acid Sensing and Response in *Plasmodium*

MARIA INÊS MOREIRA OLIVEIRA LEITE MARREIROS



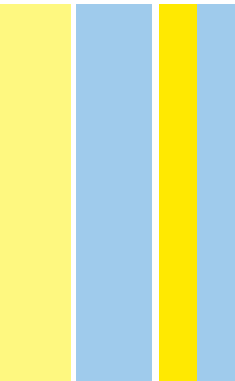
D.ICBAS 2023

SEDE ADMILSTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE CIÊNCIAS

FACULDADE DE MEDICINA



A Metabolic Arms Race Between Host and Parasite: Amino Acid Sensing and Response in *Plasmodium*

Supervisor: Dr. Vanessa Zuzarte-Luís

Co- Supervisors: Prof. Maria Mota
Dr. Valdemar Máximo

MARIA INÊS MOREIRA OLIVEIRA LEITE MARREIROS

Tese de Candidatura ao grau de Doutor em Biologia Básica e Aplicada
submetida ao Instituto de Ciências Biomédicas
Abel Salazar da Universidade do Porto.

“Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference.”

“The Road not Taken”

Robert Frost

This work was supported by a FCT (Fundação para a Ciência e Tecnologia) Ph.D. fellowship PD/BD/135454/2017 awarded to Maria Inês Moreira Oliveira Leite Marreiros through the Graduate Program in Areas of Basic and Applied Biology (GABBA), Universidade do Porto, Portugal. The research conducted at IMM was also supported by the “La caixa” Banking Foundation (HR17-00264-PoEMM). Work at the Weill Cornell Medicine and at the Loyola University Chicago was funded by NIH.



ACKNOWLEDGMENTS

“It always seems impossible until it’s done”. This work would not have been possible without the love and support of many people to whom I am forever grateful. I would like to express my sincere gratitude and deep admiration to everyone who has helped and encouraged me on this arduous journey. I was given so much during my PhD journey that I hope I have been able to give it in return.

I am truly thankful to Maria Mota, who believed in this project and gave me the opportunity to explore the scientific questions I am more passionate about and to be part of this amazing story. Thank you for trusting and supporting my decisions and for giving me critical advice when I needed the most. I was very fortunate to learn from you and to be part of such a bright team.

To my mentor Vanessa Zuzarte-Luís, with whom I worked side by side since the beginning of my scientific path and who taught me everything I know about science. For all your patience and kindness and for inspiring me to be better. For pushing me to achieve great goals. For teaching me the importance of being independent. Thank you for trusting me (even when I doubted) and for cheering up every time I felt discouraged. The scientific and personal growth I have experienced under your guidance is beyond what I imagined for myself.

I would like to extend my appreciation to my co-supervisor, Valdemar Máximo, for his support and guidance throughout this journey. I am grateful for the insights and perspectives he shared with me.

The daily obstacles could not have been overcome without the help of all my colleagues and friends from the Mota lab. I would like to thank each member (former and current) for creating such a vibrant, inspiring, and enjoyable environment, especially to Ana P., Inês B., Ângelo, Lénia, Sofia, Soraia, Yash, Yvonne, Margarida Vigário, Debanjan, João, Viriato, Sónia, Sara and Nuno. To Ana Parreira in particular, thank you for your kind heart and unwavering support. Thank you for all the great moments we shared and for the everlasting friendship; To Ângelo, for keeping me on track, for the countless conversations (and cigarettes), laughs and constant support. To Inês B., for being always so kind and willing to help and for the immense guidance. To Lénia, for the friendship, for being an inspiration and for showing me that life doesn’t get better by chance, but by change. To my “petit estudante” Sofia, the first student I mentored, who ended up teaching me as much (or even more) than I taught her.

A special thank you to the Parasitology core of IMM, everyone in the Prudêncio and Figueiredo labs, for all the help, troubleshooting and advice - but especially to Denise, who taught me everything about *P. falciparum in vitro* culturing, always with great kindness and patience.

To the GABBA scientific commission, Professor Maria de Sousa, Manuel Sobrinho Simões, António Amorim, Fátima Carneiro, João Relvas, Cláudio Sunkel, Alexandre do Carmo and Graça Porto, I am truly thankful for the opportunity and trust when accepting me as a GABBA student and as part of this incredible family. To my GABBA fellow colleagues, especially to those from the GABBA 21st (the last, but certainly not the least edition): To Silvia, Nélinho, Martini, Joana, Filipa, Anaísa, Ana Mary and Catarina, for the friendship and support, for the great moments we shared and for always being there to help, even when apart.

I wouldn't be near the person I am today without the support of my friends, namely Mariana Teixeira, my closest and oldest friend. Thank you for standing by my side when things got hard, for making me laugh when I didn't even want to smile and for celebrating every victory (even the small ones). To Miguel Dinis, my friend forever, because a friend who is like a brother is one worth holding on to. Thank you for all the support, for the great moments, for inspiring me to be better and for always being there. To Marta Machado, for the contagious energy, for all the great moments we shared and for being my partner in most scientific meetings. It is impossible not be happy when any of you is around.

À minha família. O meu maior pilar e suporte. À minha querida avó Lúcia, pelo carinho e por ser um exemplo de força e superação. Por ser tarde de domingo em família. Cheirinho a bolos e scones quentes. Por ser porta aberta e mão estendida, que toca piano e enche o coração. Ao meu avô José, por ser sabedoria e determinação. À minha tia-avó Graça, por todas as 'saías da Carolina' cantadas na cozinha da Chamuscada, por todos os cachecóis em "ponto de liga" e todas as tardes entre agulhas de crochet. E em especial à minha tia (avó) Anicas. Por ser exemplo de dedicação, determinação e disciplina. Sinónimo de lições de vida e generosidade. Por viveres pelos outros e para os outros. Por seres sorriso que aquece o coração. Obrigada pelo carinho, pelas viagens ao Algarve e por reunires toda a família. De quem herdei o gosto pela ciência e que é para mim uma verdadeira inspiração.

À minha irmã, Mariana, por todo o apoio e amor incondicional. Por seres exemplo de bondade e sinceridade. Por seres silêncio que conforta e coração que ouve. Por saberes o que queres, no universo do que não tens. Por sonhares alto e ires atrás, sem medos. Sem saberes, deste-me também essa coragem. Por estares a meu lado desde sempre. Não há um dia que não sinta a tua falta.

Aos meus pais, Alexandra e João, por me fazerem sempre sentir amada e apoiada. Mãe, és força que faz mover o mundo. És mão estendida, palavra que conforta, sorriso que abraça. És inspiração, luz e alegria. Um coração cheio de amor onde há sempre mais espaço para amar. Pai, és gargalhada de encher o coração. Lareira numa noite fria de inverno. Mesa em família e os melhores cozinhados. És muro que protege, tijolo que constrói, ponte que une. Obrigada por me guiarem sempre que me perdi. Devo-vos tudo o que sou e tudo aquilo que consegui alcançar. Tenho saudades vossas todos os dias.

Ao meu querido Di. Por seres porto seguro. Alma calma no meio da minha tempestade. Companheiro das maiores aventuras. Caminho certo. É sobre fazer o mundo parecer pequeno e o tempo curto para o tanto que sonhamos. Sobre ser genuíno e bondoso; braços sempre abertos capazes de mudar o mundo. Mas sobretudo, sobre amor, amor na mais pura essência. À Maggie, meu furacão de 4 patas, de energia inesgotável. Conquistas o mundo com esses olhos meigos – e que conseguem sempre o que querem! Trapalhona como ninguém, viraste o nosso mundo do avesso. És sobre lições diárias de paciência. Mas sobretudo de lealdade e amor.

Ao Cajú, o melhor (4 patas) que a vida meu deu. Companheiro de todas as horas: de metas alcançadas, de tentativas falhadas. Sinónimo de lealdade, meiguice e bondade na sua mais pura essência. Lufada de ar fresco, raio de sol em dias escuros, sorriso em dias tristes, aconchego em noites frias. É sobre ser abraço que conforta, lambidela que cura, alegria que contagia, silêncio que escuta. É sobre fazer ver o lado bom da vida, ensinar a desfrutar do mais simples e a viver o momento. É sobre estar sempre. É sobre um amor que não se mede nem se explica. Ainda hoje me pergunto se fui eu que te salvei, ou se foste tu que me salvaste a mim 🐾

You all definitely have a special place in my heart. Thank you for the untiring support. This project is as much mine as it is yours!

*To my **parents**, who have always supported me unconditionally and whose good values and examples have taught me to be kind and work hard for the things that I aspire to achieve.*

To my best companion, Cajú, who taught me about unconditional love and how to enjoy the little things of life.

(_____) 6

(_____) 6

// \ \ // \ \

|||| ||||

TABLE OF CONTENTS

ABSTRACT / RESUMO	1
ABSTRACT	3
RESUMO	5
CHAPTER I	9
INTRODUCTION	11
I. Malaria: A Modern Arms Race Against an Old Foe	13
Malaria Through History	13
The Discovery of Malaria Parasites	14
Malaria Paroxysms & Severe Disease	18
The Road to Resistance: Antimalarials with History	19
Rising to the Challenge of Malaria Eradication	26
The Complex Life Cycle of <i>Plasmodium</i> Parasites	33
<i>Plasmodium</i> Schizogony: An Unusual Mode of Replication	37
II. Fork In the Road: Host-Parasite Competition for Resources	39
A Co-Evolutionary Signature: The Phenomenon of Genome Reduction	39
<i>Plasmodium</i> Nutrition: A Minimalist Lifestyle	40
The Building Blocks of Life: Amino Acid Metabolism	46
III. Food For Thought: Nutrient Sensing & Metabolic Decisions	49
Sensing Changes in Host Nutritional Levels	50
Mechanisms of Growth Control and Stress Response	54
THESIS AIMS	61
Thesis Aims & Scope	62
CHAPTER II	65
A non-canonical sensing pathway mediates <i>Plasmodium</i> adaptation to AA deficiency	67
Abstract	71
Introduction	72
Results	74
AA depletion impacts <i>Plasmodium</i> development and replication	74
Polyamines regulate <i>Plasmodium</i> response to MetD	78
Nek4, eIK1 and eIK2 are key in the response to AA limitation	84
Parasite response to low AA implies eIF2 α phosphorylation at different stages of the cell cycle	88
Discussion	91
Materials & Methods	94
Supplementary Information	101
Acknowledgments	113

CHAPTER III	115
<i>Plasmodium</i> parasites exploit liver-specific methionine metabolism to drive high replication rates	117
Abstract	121
Introduction	122
Results	124
SAM is the critical methionine metabolite for parasite replication	124
<i>Plasmodium</i> MAT enzyme (SAMS) is dispensable for parasite replication inside hepatocytes	127
<i>Plasmodium</i> 's high replication capacity in hepatocytes depends on the liver-specific MAT1A enzyme	133
Discussion	134
Materials & Methods	138
Supplementary Information	144
Acknowledgments	169
 CHAPTER IV	 149
Sexual differentiation in human malaria parasites is regulated by competition between phospholipid metabolism and histone methylation	151
Abstract	155
Introduction	156
Results	158
P-cho precursors alter SAM, SAH and sexual commitment	158
Intracellular SAM levels regulate sexual commitment	161
SAM regulates sexual commitment in rodent parasites	162
Heterochromatin is responsive to changes in SAM and SAH	163
Discussion	168
Materials & Methods	170
Supplementary Information	176
Acknowledgments	185
 CHAPTER V	 187
GENERAL DISCUSSION	189
 CHAPTER VI	 207
REFERENCES	211

FIGURE AND TABLE INDEX

CHAPTER I

Figure 1.1- Malaria parasites drawn by Alphonse Laveran in 1880.	15
Figure 1.2 - Original drawing by Ronald Ross of a mosquito salivary gland.	17
Figure 1.3 - Overview of the effect of antimalarials on <i>Plasmodium</i> blood stages.	20
Figure 1.4 - Proportion of patients and distribution of <i>Plasmodium</i> clearance time with ART treatment in Southeast Asia.	22
Figure 1.5 - Map of Southeast Asia and an insert of Africa showing the location of the Tracking Resistance to Artemisinin Collaboration (TRAC) II study.	23
Figure 1.6 - Number of estimated additional deaths per million due to malaria, in different regions of sub-Saharan Africa, due to COVID-19 mitigation strategies.	25
Figure 1.7 - Malaria-free countries since 2000.	26
Figure 1.8 - Overview of the Malaria Drug Accelerator (MalDA)	31
Figure 1.9 - The complex two-host life cycle of <i>Plasmodium</i> parasites.	33
Figure 1.10 - The pre-erythrocytic stage of <i>Plasmodium</i> parasites.	35
Figure 1.11 - <i>Plasmodium falciparum</i> intra-erythrocytic development cycle (IDC).	37
Figure 1.12 - Glucose uptake by <i>Plasmodium falciparum</i> intra-erythrocytic stages.	41
Figure 1.13 - Polyamine metabolism and transporters of trypanosomatid and apicomplexan parasites in comparison to their human host.	45
Figure 1.14 - Schematic representation of the transport of methionine and isoleucine into <i>Plasmodium</i> -infected erythrocytes.	47
Figure 1.15 - Diagram depicting the main players of directed transcriptional responses in <i>Plasmodium</i> spp.	54
Figure 1.16 - <i>P. falciparum</i> response to Ile starvation.	60

CHAPTER II

Figure 2.1 - AA availability impacts parasite intra-erythrocytic development and replication.	74
Figure 2.2 - Overview of the phenotypes induced upon AA depletion in <i>P. berghei</i> ANKA parasites.	76
Figure 2.3 - AA depletion impacts parasite progression through the cell cycle during intra-erythrocytic development <i>ex vivo</i> .	77
Figure 2.4 - AA depletion impacts parasite intra-erythrocytic replication rates.	78
Figure 2.5 - Schematic representation of methionine cycle, the aminopropylation and the transsulfuration pathways in malaria parasites.	80
Figure 2.6 - Polyamines regulate parasite response to methionine depletion.	81
Figure 2.7 - Polyamine biosynthesis is downregulated in <i>P. falciparum</i> upon methionine withdrawal.	84
Figure 2.8 - Polyamines have no impact in IleD-induced phenotypes, as it regards to development and replication.	83

Figure 2.9 - A signaling pathway comprising 3 distinct parasite kinases regulates the stress response to AA depletion.	84
Figure 2.10 - Adaptation to isoleucine depletion also relies on eIK1, eIK2 and nek4.	85
Figure 2.11 - Intra-erythrocytic development of <i>P. falciparum</i> eIK1, eIK2 and nek4 kinases knockout mutants upon AA withdrawal.	86
Figure 2.12 - Replication rates of <i>P. falciparum</i> eIK1, eIK2 and nek4 kinases knockout mutants upon AA withdrawal.	87
Figure 2.13 - Parasite response to AA depletion results in enhanced phosphorylation of the <i>Plasmodium falciparum</i> eIF2 α orthologue.	89
Figure 2.14 - Schematic model for parasite's adaptive response to AA depletion during intra-erythrocytic development.	90
Supplementary Figure 2.1 - AA depletion impacts parasite intra-erythrocytic development but not viability.	101
Supplementary Figure 2.2 - Impact of AA depletion throughout <i>P. falciparum</i> intra-erythrocytic development.	103
Supplementary Figure 2.3 - Impact of AA depletion in <i>P. berghei</i> intra-erythrocytic development using the <i>ex vivo</i> maturation assay.	103
Supplementary Figure 2.4 - Effect of AA depletion <i>in vitro</i> , <i>ex vivo</i> and <i>in vivo</i> .	104
Supplementary Figure 2.5 - Effect of Met-downstream metabolites in <i>Plasmodium</i> development and replication.	111
Supplementary Figure 2.6 - Spermine but not putrescine rescues methionine depletion-induced growth impairment.	108
Supplementary Figure 2.7 - Phenotypic screen of <i>P. berghei</i> kinase knockout mutant parasites cultured <i>ex vivo</i> in AA deficient media.	109
Supplementary Figure 2.8 - Parasite stage progression in wt and kinase mutant knockout lines culture in MetD media, using the <i>ex vivo</i> maturation assay.	110
Supplementary Figure 2.9 - eIK1 and nek4, but not eIK2, regulate parasite development under AA deficiency.	111
Supplementary Figure 2.10 - Impact of AA depletion in <i>P. falciparum</i> kinase knockout mutant parasites.	112

Table 2. 1 - List of primer sequences used.	95
--	----

CHAPTER III

Figure 3.1 - The impact of methionine depletion in <i>Plasmodium</i> liver-stage development is strongly linked to decreased SAM levels..	125
Figure 3.2 - Met/SAM are only required during the replicative phase of <i>Plasmodium</i> development.	127
Figure 3.3 - <i>Plasmodium</i> SAMS enzyme is essential for parasite development inside erythrocytes.	129

Figure 3.4 - <i>Plasmodium</i> SAMS enzyme is dispensable for parasite replication inside hepatocytes.	130
Figure 3.5 - Liver-specific MAT1A sustains parasite high multiplication rates.	135
Supplementary Figure 3.1 - Methionine depletion impacts <i>Plasmodium</i> liver-stage development.	144
Supplementary Figure 3.2 - SAMS is expressed throughout all stages of <i>P. berghei</i> life cycle.	145
Supplementary Figure 3.3 - Host MAT1 expression levels modulate <i>Plasmodium</i> liver-stage development.	146

CHAPTER IV

Figure 4.1 - Phosphocholine precursor availability alters parasite SAM and SAH levels.	159
Figure 4.2 – <i>De novo</i> synthesis of P-cho by PMT is a major sink of SAM and source of SAH.	160
Figure 4.3 - Intracellular SAM abundance regulates the frequency of sexual differentiation in human and rodent malaria parasites.	161
Figure 4.4 - <i>P. berghei</i> SAMS cKD parasites exhibit increased rate of sexual commitment.	163
Figure 4.5 – Reducing intracellular SAM levels impairs heterochromatin maintenance and increases both AP2-G expression and sexual commitment.	164
Figure 4.6 - Increasing intracellular SAH impairs heterochromatin maintenance and increases both AP2-G expression and sexual commitment.	166
Supplementary Figure 4.1 - Dose-dependent metabolic response to LysoPC.	176
Supplementary Figure 4.2 - Schizont <i>ap2-g</i> transcript abundances closely track sexual commitment under various nutrient conditions.	177
Supplementary Figure 4.3 - Changes in SAM/SAH metabolism are specific to parasite metabolism.	178
Supplementary Figure 4.4 - Validation of PMT- <i>glmS</i> knockdown parasite line.	179
Supplementary Figure 4.5 - Methionine depletion does not alter parasite growth.	179
Supplementary Figure 4.6 - Validation of <i>pfsams-glmS</i> knockdown parasite line.	180
Supplementary Figure 4.7 - Validation of <i>pbsams-DD</i> knockdown parasite line.	181
Supplementary Figure 4.8 - Coverage comparisons of H3K4me3 and H3K9me3 at two representative chromosome 6 loci under Low vs. High SAM conditions.	182
Supplementary Figure 4.9 - Dose-response of parasite sexual commitment and growth to 3-DZA.	183
Supplementary Figure 4.10 - Coverage comparisons of H3K4me3 and H3K9me3 at two representative chromosome 6 loci under High vs. Low SAH conditions.	183
Supplementary Table 4.1 – Compositions of the cell culture media used.	184
Supplementary Table 4.2 – Primers used in this study.	184

LIST OF ABBREVIATIONS

AA	<u>A</u> mino <u>A</u> cid
ACT	<u>A</u> rtemisinin-based <u>C</u> ombination <u>T</u> herapies
AMPK	<u>A</u> denosine <u>M</u> onophosphate-activated <u>P</u> rotein <u>K</u> inase
ATP	<u>A</u> denosine <u>T</u> riphosphate
AQ	<u>A</u> modiaquine
ART	<u>A</u> rtemisinin
BSA	<u>B</u> ovine <u>S</u> erum <u>A</u> lbumin
Cho	<u>C</u> holine
CDP-Cho	<u>C</u> ytidine <u>D</u> iphosphate <u>C</u> holine
CQ	<u>C</u> hloroquine
CR	<u>C</u> aloric <u>R</u> estriction
CSP	<u>C</u> ircumsporozoite <u>P</u> rotein
DDT	<u>D</u> ichlorodiphenyltrichloroethane
DFMO	α -difluoromethylornithine
DHA	<u>D</u> ihydro <u>a</u> rtemisinin
DNA	<u>D</u> eoxyribonucleic <u>A</u> cid
eIF2α	<u>E</u> karyotic <u>I</u> nitiation <u>E</u> actor <u>2</u> α
eIK1	<u>e</u> IF2 α <u>K</u> inase <u>1</u>
eIK2	<u>e</u> IF2 α <u>K</u> inase <u>2</u>
ER	<u>E</u> ndoplasmic <u>R</u> eticulum
FACS	<u>F</u> luorescence-activated <u>C</u> ell <u>S</u> orting
FBS	<u>F</u> etal <u>B</u> ovine <u>S</u> erum
GCN2	<u>G</u> eneral <u>C</u> ontrol <u>N</u> onderepressible <u>2</u>
GFP	<u>G</u> reen <u>F</u> luorescent <u>P</u> rotein
GMEP	<u>G</u> lobal <u>M</u> alaria <u>E</u> radication <u>P</u> rogramme
GMP	<u>G</u> lobal <u>M</u> alaria <u>P</u> rogramme
GMS	<u>G</u> lobal <u>M</u> ekong <u>S</u> ubregion
GlcN	<u>G</u> lucosamine
GlcN-NAC	<u>N</u> -acetylglucosamine
GLUT1	<u>G</u> lucose <u>T</u> ransporter <u>1</u>
GSH	Glutathione
G6PD	<u>G</u> lucose- <u>6</u> - <u>P</u> hosphate <u>D</u> ehydrogenase
HA	<u>H</u> emagglutinin

Hb	<u>H</u> emoglobin
hENT1	<u>h</u> uman <u>E</u> quilibrative <u>N</u> ucleobase <u>T</u> ransporter <u>1</u>
hfNT1	<u>h</u> uman <u>F</u> acilitative <u>N</u> ucleobase <u>T</u> ransporter <u>1</u>
Hprt	<u>H</u> ypoxanthine-guanine <u>p</u> hosphor <u>i</u> bosyl <u>t</u> ransferase
hpi	<u>h</u> ours <u>p</u> ost <u>i</u> nfection
HRI	<u>H</u> eme- <u>r</u> egulated <u>I</u> nhibitor Kinase
Hsp70	<u>H</u> eat <u>s</u> hock <u>p</u> rotein <u>70</u>
IDC	<u>I</u> nter-erythrocytic <u>D</u> evelopmental <u>C</u> ycle
Ile	<u>I</u> sole <u>e</u> ucine
IleS	<u>I</u> sole <u>e</u> ucine <u>S</u> ufficient
IleD	<u>I</u> sole <u>e</u> ucine <u>D</u> eficient
i.p.	<u>i</u> nterperitoneal
IPTi	<u>I</u> ntermittent <u>P</u> reventive <u>T</u> reatment for <u>i</u> nfants
IPTp	<u>I</u> ntermittent <u>P</u> reventive <u>T</u> reatment in <u>p</u> regnancy
iRBCs	<u>i</u> nfecte <u>d</u> <u>R</u> ed <u>B</u> lood <u>C</u> ells
IRS	<u>I</u> ndoor <u>R</u> esidual <u>S</u> praying
ITNs	<u>I</u> nsecticide- <u>t</u> reated <u>N</u> ets
i.v.	<u>i</u> nteravenous
KO	<u>K</u> nock <u>o</u> ut
LAMP	<u>L</u> oop-mediated Isothermal <u>A</u> mplification
LLNs	<u>L</u> ong- <u>l</u> asting <u>N</u> ets
LysoPC	<u>L</u> ysophosphatidyl <u>l</u> choline
MaIDA	<u>M</u> alaria <u>D</u> rug <u>A</u> ccelerator
Met	<u>M</u> ethionine
MetS	<u>M</u> ethionine <u>S</u> ufficient
MetD	<u>M</u> ethionine <u>D</u> eficient
MQ	<u>M</u> efloquine
mRNA	<u>m</u> essenger <u>R</u> ibonucleic <u>A</u> cid
mSP1	<u>m</u> erozoite <u>s</u> urface <u>p</u> rotein <u>1</u>
ODC	<u>O</u> rnithine <u>D</u> ecarboxylase
P.	<u>P</u> lasmodium
<i>P. berghei</i>	<u>P</u> lasmodium <u>b</u> erg <u>h</u> ei
PBO	<u>P</u> iperonyl <u>B</u> utoxide
PBS	<u>P</u> hosphate- <u>b</u> uffered <u>S</u> aline
PCR	<u>P</u> olymerase <u>C</u> hain <u>R</u> eaction

P-cho	<u>Phospho-cho</u> line
PERK	pKR-like <u>E</u> ndoplasmic <u>R</u> eticulum <u>K</u> inase
P-etn	<u>Phospho-e</u> thanola <u>m</u> ine
PFA	<u>Para</u> forma <u>l</u> dehyde
<i>P. falciparum</i>	<i>Plasmodium</i> <u>falciparum</u>
PfCRT	<i>P. falciparum</i> <u>C</u> hloroquine <u>R</u> esistance <u>T</u> ransporter
PfHRP-2	<i>P. falciparum</i> <u>H</u> istidine-rich <u>P</u> rotein <u>2</u>
PfHT	<i>P. falciparum</i> <u>H</u> exose <u>T</u> ransporter
PIKK	<u>Phosphatidyl</u> inositol <u>K</u> inase-related <u>K</u> inase
PKR	<u>P</u> rotein <u>K</u> inase <u>R</u> / RNA-activated Protein Kinase
pLDH	<i>Plasmodium</i> <u>L</u> actate <u>D</u> ehydrogenase
PMT	<u>Phospho</u> ethanolamine <u>M</u> ethyl <u>t</u> ransferase
PPM	<u>Parasite</u> <u>P</u> lasma <u>M</u> embrane
PPQ	<u>P</u> ipera <u>q</u> uine
PQ	<u>P</u> rima <u>q</u> uine
Ptd-cho	<u>Phosphatidyl</u> cho <u>l</u> ine
Putr	<u>P</u> utrescine
PV	<u>Parasitophorous</u> <u>V</u> acuole
PVM	<u>Parasitophorous</u> <u>V</u> acuole <u>M</u> embrane
qPCR	quantitative <u>P</u> CR
RBCs	<u>R</u> ed <u>B</u> lood <u>C</u> ells
RDT	<u>R</u> apid <u>D</u> iagnostic <u>T</u> est
RNA	<u>R</u> ibonucleic <u>A</u> cid
SAH	<u>S</u> - <u>a</u> denosyl <u>h</u> omocysteine
SAHH	<u>S</u> - <u>a</u> denosyl <u>h</u> omocysteine <u>H</u> ydrolase
SAM	<u>S</u> - <u>a</u> denosyl <u>m</u> ethionine
SAMS	<u>S</u> - <u>a</u> denosyl <u>m</u> ethionine <u>S</u> ynthetase
SD	<u>S</u> tandard <u>D</u> eviation
SDC	<u>S</u> AM <u>D</u> ecarboxylase
SEM	<u>S</u> tandard <u>E</u> rror of the <u>M</u> ean
Ser	<u>S</u> erine
SMC	<u>S</u> easonal <u>M</u> alaria <u>C</u> hemoprevention
Snf1	<u>S</u> ucrose <u>n</u> on-fermenting <u>1</u>
SP	<u>S</u> ulfadoxine- <u>P</u> rimethamine
SpD	<u>S</u> permi <u>d</u> ine

SpdS	<u>S</u> permidine <u>S</u> ynthase
SpM	<u>S</u> permine
SpmS	<u>S</u> permine <u>S</u> ynthase
TACTs	<u>T</u> riple <u>A</u> rtemisinin-based <u>C</u> ombination <u>T</u> herapies
TCA	<u>T</u> ricarboxylic <u>A</u> cid
TMP	<u>T</u> rimethoprim
TOR	<u>T</u> arget <u>O</u> f <u>R</u> apamycin
uRBCs	<u>u</u> ninfected <u>R</u> ed <u>B</u> lood <u>C</u> ells
WHO	<u>W</u> orld <u>H</u> ealth <u>O</u> rganization
WMR	<u>W</u> orld <u>M</u> alaria <u>R</u> eport
wt	<u>w</u> ild-type
3-DZA	<u>3</u> - <u>D</u> eaza- <u>a</u> denosine

ABSTRACT / RESUMO

ABSTRACT

Malaria, a life-threatening febrile illness, stands as one of the most prevalent infectious diseases worldwide. The disease is caused by protozoan parasites belonging to the *Plasmodium* genus, which are transmitted through the bite of an infected female *Anopheles* mosquito. Malaria parasites have a complex life cycle shared between two hosts, requiring transmission through distinct metabolic environments and establishment in multiple niches. Even when developing within a single host, the environment is greatly dynamic as parasites invade distinct cell types. As an obligate intracellular parasite, *Plasmodium* relies on the host to supply the nutrients required to sustain such a highly proliferative lifestyle. While an in-depth characterization of the mechanisms underlying nutrient exploitation in the liver stage of infection has yet to be established, it is generally accepted that in the symptomatic blood stage of infection, *Plasmodium* takes up most nutrients through digestion of the host's erythrocyte hemoglobin. However, since hemoglobin does not contain all required nutrients, parasites face some challenges by selecting to reside within mature but metabolically inactive erythrocytes. These include some essential amino acids such as isoleucine, the sole amino acid that is absent in erythrocytes, and methionine, the least represented, which must therefore be obtained from the serum.

To elucidate how *Plasmodium* parasites respond to amino acid starvation, we monitored the intra-erythrocytic growth of the parasite under isoleucine- or methionine-limiting conditions. While Babbitt *et al.* first suggested a dormancy-like response of *P. falciparum* to isoleucine deficiency, McLean and Jacobs-Lorena showed that parasites growing under isoleucine-deprived conditions do not cease their cell cycles; rather, they cycle at a slower rate, completing schizogony, and invading new erythrocytes. In this dissertation, evidence is presented that supports a unified paradigm that reconciles the two opposing viewpoints: while most parasites growing under AA-limiting conditions exhibit growth arrest, as reported by Babbitt *et al.*, some parasites successfully progress through the cell cycle and complete schizogony, thereby developing mature schizonts. Under AA-deficient circumstances, however, schizogony results in fewer offspring, with less merozoites being produced per schizont. While the master regulator of growth, the TOR complex, has not yet been identified in *Plasmodium*, the AA-starvation responsive GCN2/eIF2 α signaling cascade is functionally conserved in the malaria parasite. Thus, we next focused on whether this response was an active adaptive mechanism orchestrated by the parasite to survive under amino acid scarcity. As previously observed for isoleucine depletion, we have found that this signaling pair exhibits remarkable

specificity in detecting amino acid deficiency, with eIK1 phosphorylating its downstream effector eIF2 α in response to nutrient starvation during ring-stage development. Additionally, we also revealed a role for eIK2 in AA sensing, albeit only at later stages of parasite development (schizont stage), disclosing a stage-specific and temporally coordinated sensing network in the parasite. Notably, the kinome screening analysis revealed that a third kinase, Nek4, is also involved in such an active response, coordinating both development and replication rates under AA starvation. We further extended our knowledge on the parasite's response to AA withdrawal during liver stage and gametocyte development, with a particular focus on methionine and its downstream metabolite SAM - the chief methylation donor in virtually all living cells and the precursor of polyamines and glutathione. By chemical, genetic and metabolomic approaches we have observed that i) methionine metabolism lies at the heart of such high replication rates achieved inside hepatocytes, with parasites totally depending on the host liver-specific MAT enzyme (which converts methionine into SAM) and also that ii) SAM levels regulate investment into gametocyte production, by shifting the metabolic competition for the SAM-derived methyl groups between distinct methyltransferases, ultimately resulting in profound changes at gene expression level. In summary, this work establishes the role of AA metabolism (particularly methionine and isoleucine) in parasite survival during intra-erythrocytic development and, most importantly, the mechanisms underlying parasite's active adaptation to AA scarcity. This dissertation discloses hitherto that blood stage parasites possess an efficient amino acid sensing pathway that coordinates development and replication, allowing a suitable adaptation and ultimately, survival under nutritional scarcity. Additionally, we have disentangled the relevance of methionine metabolism for *Plasmodium* intra-hepatic development as well as for commitment to sexual development.

RESUMO

A malária é uma doença febril, potencialmente fatal, e uma das doenças infecciosas mais prevalentes no mundo. É causada por parasitas protozoários do gênero *Plasmodium*, os quais são transmitidos aquando da picada de um mosquito *Anopheles* fêmea infectado. Os parasitas da malária têm um ciclo de vida complexo, partilhado entre dois hospedeiros distintos, o que implica a transmissão através de ambientes metabólicos distintos bem como o estabelecimento em diferentes nichos. Contudo, mesmo quando se desenvolve dentro de um único hospedeiro, o ambiente é bastante dinâmico dado que os parasitas invadem diferentes tipos de células. Sendo um parasita intracelular obrigatório, o Plasmodio depende dos nutrientes do seu hospedeiro para sustentar as suas elevadas taxas de multiplicação. Enquanto que durante a fase hepática da infeção os mecanismos subjacentes à obtenção de nutrientes a partir hospedeiro são ainda desconhecidos, durante a fase sanguínea e sintomática da infeção está bem estabelecido que o *Plasmodium* adquire a maioria dos nutrientes através da digestão da hemoglobina presente nos eritrócitos do hospedeiro. No entanto, por residir dentro de eritrócitos maduros, mas metabolicamente inativos, os parasitas enfrentam alguns desafios pois a hemoglobina não contém todos os nutrientes necessários. Entre estes destacam-se alguns aminoácidos essenciais como a isoleucina, que está totalmente ausente, ou a metionina, que está muito pouco representada na hemoglobina, tendo por isso que ser obtidos através do soro.

De modo a compreender como o *Plasmodium* responde à escassez de aminoácidos, começámos por estudar o crescimento do parasita nos eritrócitos em condições limitantes de isoleucina ou metionina. Dados previamente obtidos por Babbitt *et al.*, suportam um modelo de dormência em *P. falciparum* em resposta à ausência de isoleucina. No entanto, McLean e Jacobs-Lorena revelaram que na ausência de isoleucina os parasitas não cessam o ciclo celular, progredindo a um ritmo mais lento. Não obstante, os parasitas conseguem completar com sucesso o processo de esquizogonia, invadindo novos eritrócitos. Os resultados apresentados na presente dissertação propõem um modelo que une estes dois pontos de vista divergentes: enquanto a maioria dos parasitas cessa a sua progressão pelo ciclo celular, alguns conseguem completar o mesmo com sucesso, desenvolvendo esquizontes maduros que contêm merozoitos individualizados. No entanto, em condições limitantes de aminoácidos, o processo de esquizogonia gera menos descendência, com um menor número de merozoitos a ser produzido por esquizonte. Embora o regulador central do

crescimento celular - a proteína alvo da Rapamicina (mTOR, *mechanistic target of rapamycin em inglês*) - não tenha sido ainda identificado em *Plasmodium*, o par de sinalização GCN2/eIF2 α , o qual é ativado durante a deficiência de aminoácidos, está funcionalmente conservado no parasita. Assim, começámos por testar se a resposta do parasita à escassez de aminoácidos constituía um mecanismo adaptativo ativo para sobreviver em condições limitantes de recursos. Em concordância com dados previamente obtidos para a privação de isoleucina, verificámos que durante o estágio de anel, a via de sinalização GCN2/eIF2 α exibe uma especificidade significativa para a deteção dos níveis de aminoácidos, com a quinase eIK1 a fosforilar eIF2 α em resposta à carência de aminoácidos. Adicionalmente, observámos que uma segunda quinase, eIK2, tem também um papel crucial na deteção da escassez de aminoácidos, atuando especificamente em estágios de desenvolvimento mais tardios (esquizonte). Estes dados revelam, portanto, que no parasita da malária, o mecanismo de deteção e resposta à limitação de aminoácidos é temporalmente regulado, sendo específico de cada estadio de desenvolvimento. Notavelmente, a análise do quinoma do parasita demonstrou que esta resposta ativa é ainda coordenada por uma terceira quinase, Nek4, a qual demonstramos pela primeira vez, ser um regulador central do crescimento do parasita em condições de privação de aminoácidos – tanto a nível do desenvolvimento como da replicação.

Em paralelo, estudámos a resposta do parasita à escassez de aminoácidos durante a fase hepática da infeção bem como durante o desenvolvimento de gametócitos, as formas sexuais do parasita, com particular foco na metionina e na S-adenosilmetionina (SAM) – o principal dador de grupos metilo em todas as células e também o precursor de poliaminas e glutatono. Através de métodos químicos e genéticos e de análise metabolómica revelamos que i) o metabolismo da metionina é a chave para as elevadas taxas de replicação alcançadas dentro dos hepatócitos – sendo o desenvolvimento do parasita dependente da enzima hepática do hospedeiro MAT (que converte metionina em SAM); e ainda que ii) os níveis de SAM regulam a taxa de conversão do parasita em formas sexuais e transmissíveis, ao modularem a competição metabólica entre diferentes metiltransferases pelos grupos metilos derivados da SAM, o que resulta em alterações profundas ao nível da expressão génica.

Em suma, os nossos resultados demonstram a relevância do metabolismo dos aminoácidos (em particular a isoleucina e metionina) para a sobrevivência do parasita durante a fase hepática e sanguínea da infeção, bem como para o desenvolvimento de

gametócitos. Os dados obtidos nesta dissertação permitem estabelecer a existência de uma via de sinalização não canônica no parasita da malária, a qual permite detetar de modo eficiente os níveis de aminoácidos. Tal é essencial para que o parasita regule as suas taxas de replicação bem como o seu desenvolvimento (ciclo celular), assegurando assim uma adaptação adequada e a sobrevivência em ambientes nutricionalmente desfavoráveis.

CHAPTER I

CHAPTER I

INTRODUCTION

I. Malaria: A Modern Arms Race Against an Old Foe

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium* and transmitted through the bite of an infected *Anopheles* mosquito. Despite more than a century since Laveran first identified *Plasmodium* spp. as the causative agent of malaria [1] and Ross's discovery of the female *Anopheles* mosquito as its vector [2,3], malaria still remains a leading cause of morbidity and mortality worldwide [4]. In humans, natural infections can be caused by six *Plasmodium* species: *P. falciparum*, *P. knowlesi*, *P. malariae*, *P. vivax*, *P. ovale curtisi* and *P. ovale wallikeri* - the latter two being closely related but genetically distinct [5]. Among these, *P. falciparum* and *P. vivax* account for 95% of all malaria infections and pose the greatest public health challenge [6]. While *P. falciparum* is the most pathogenic spp., causing severe pathology and therefore the highest mortality rates [7]; *P. vivax* presents a wider geographical distribution as it can develop in *Anopheles* mosquitoes that inhabit more temperate regions and higher altitudes [8–10]. Interestingly, *P. vivax* parasites can develop into hepatic cryptic forms known as hypnozoites which lie dormant inside liver cells for extended periods of time. These parasitic forms are potential reservoirs of infection and lead to disease recurrence even after treatment of the initial symptomatic infection [8,11–13].

Malaria is an acute febrile illness whose symptoms emerge 7 to 15 days after the infective mosquito bite in nonimmune individuals. The first symptoms to appear are fever, headache, chills and vomiting, which are similar to other viral and bacterial infections [14]. If not treated promptly, *P. falciparum* malaria can quickly progress to severe pathology, often culminating in coma and death. Severe malaria is mainly observed in children in endemic areas causing symptoms such as severe anemia, dyspnea, metabolic acidosis and eventually cerebral malaria [15,16]. In endemic regions, partial immunity develops over time, reducing the risk of severe disease, yet sterile immunity is never achieved [16].

Malaria Through History

The earliest evidence of malaria can be traced back to ancient times based on its clinical features such as recurrent periodic fever and splenomegaly [17,18]. The earliest records were found in a Chinese document from 2700 BC, clay tablets in Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC and also in Hindu texts as far back as the 6th century BC [17,19–21]. Such historical records, however, require careful consideration, but as we step into later centuries we begin to tread on firmer ground. Numerous other ancient Chinese texts provided more convincing evidence, documenting not only fever-

induced spleen enlargement, but also the use of the antimalarial drug Qinghaosu for malaria treatment [19]. Two other documents dating from 476 to 221 BC described how mosquito bites caused malaria infection, which occurred particularly in late summer and early autumn [22]. The early Greeks, including Homer around 850 BC, Empedocles of Agrigentum in 550 BC, and Hippocrates later in 400 BC, were aware of the marked poor health, fever, and enlarged spleens observed in individuals living near marshy places [20,23]. The first detailed clinical description of malaria fell to the physician Hippocrates, who described the malarial paroxysm. Paroxysms appear as the disease progresses and consist of three distinct, but sequential stages characterized by chills, fever, and sweating (when febrile episodes cease) [17,23]. However, the origin of the disease remained elusive for centuries. The idea that malarial fever was caused by miasmas rising from swamps persisted for over 2,500 years, a correlation recognized by the Romans. The term *malaria*, as used today, derives from the Italian *mal'aria*, meaning bad, evil or spoiled air [17,22]. Numerous synonyms have been used throughout history to describe malaria including: mal'aria, ague, paludism, febris ardens, paludal fever, marsh fever, seasonal fevers, fever of summer/fall (estivo-autumnal fever), autumnal fever, intermittent fever, periodical fever, the quakes, the Bailiff of the Marshes, Lord John's fever and Old Johnny Axey [17,23]. "Malaria", the finally settled word for the disease, was stated for the first time by Francesco Puccinotti in his book "*Storia delle febbri intermittenti di Roma*" in the first half of the 19th century [17].

The Discovery of Malaria Parasites

The discovery of bacteria in 1676 by Antoni van Leeuwenhoek and the development of the germ theory of infection by Louis Pasteur and Robert Koch in 1878-1879, which recognized microorganisms as the causative agents of infectious diseases, had intensified the search for a bacterial cause of many diseases, including malaria [20].

The erythrocytic stage

By 1879 the miasma theory had been abandoned and two new theories of malaria etiology had emerged, one advocating airborne transmission and inhalation [24] and the other supporting waterborne transmission or ingestion [25]. The prevailing theory was advanced by Tommasi-Crudeli and Klebs, who claimed to have identified the bacterium *Bacillus malariae* in swampy waters, which caused febrile illness and enlarged spleens in rabbits, mimicking a malaria infection [26]. This theory was refuted in 1880 by

Alphonse Laveran, a French military doctor working in Algeria [1]. Aware of the presence of a dark pigment in the spleens of malaria patients, Laveran searched for such a pigment in fresh, unstained blood of patients during episodes of malaria fever, having observed it first in leucocytes and later in erythrocytes. Deeper investigation allowed Laveran to identify distinct forms of erythrocytic organisms, including: curved-shaped bodies resembling a crescent, spherical motionless or moving pigment-containing bodies and also pigmented bodies exuding flagella-like structures - all of which were proposed to reside outside erythrocytes (Figure 1.1) [27–29].

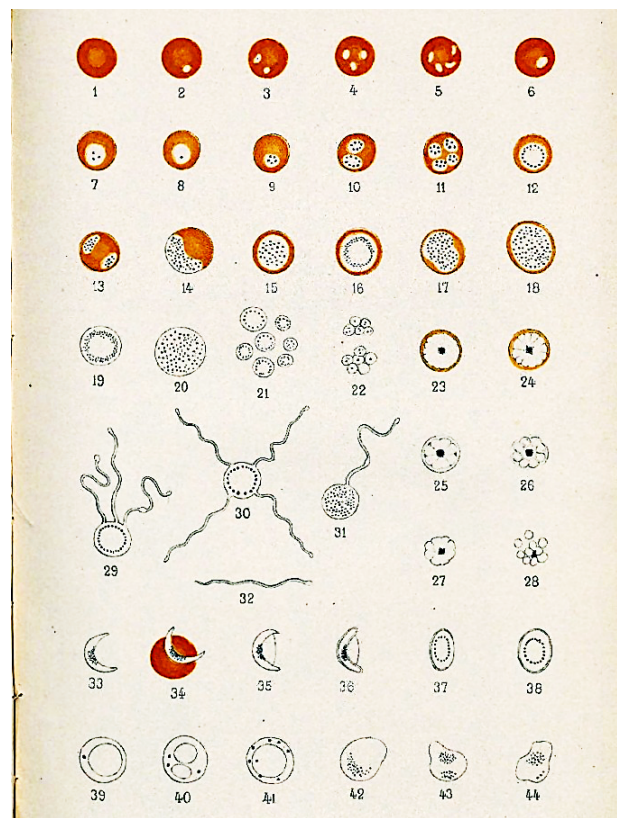


Figure 1.1- Malaria parasites drawn by Alphonse Laveran in 1880. Laveran's illustration of the distinct stages of malaria parasites as seen in fresh blood smears. Dark pigment is observed and represented in most stages. The first four rows show the asexual blood stages. Exflagellating male gametocytes, producing filamentous elements are shown in the fifth row. The lower sections represent the sexual blood stages of parasite development. From [29].

Laveran proposed that these events would follow a chronological order, starting with non-pigmented structures that grew and acquired pigment filling the corpuscle, which then burst along with malaria-related fevers. Laveran quickly recognized that he had found a parasitic protozoa which he named *Oscillaria malariae* [27]. However, when this discovery was presented to the French Academy of Medical Sciences in December 1880, it was initially met with skepticism [20,30]. Only by 1884, Laveran was able to convince the leading Italian malariologists including Bignami, Golgi and Marchiafava that

malaria was caused by a protozoan and not a bacterium [20,27]. The miasma theory was abandoned, paving the way for contemporary germ theory. Laveran is regarded as the father of protistological parasitology and was awarded the Nobel Prize in Medicine in 1907 [27,30]. Unbeknownst to Laveran, the Russian physiologist Vassily Danilewsky had studied the blood of birds and reptiles and in 1885 discovered the three most common types of intraerythrocytic parasites: *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* [20]. Thereafter, the quest for other malaria parasites in mammals, birds, and reptiles had become more extensive. Dmitri Romanowsky's accidental discovery of the methylene blue-eosin stain in 1891 contributed significantly to this endeavor [20,31]. This dye, which still serves as the basis for contemporary blood stains including Leishman, Giemsa, and Wright stains, gained prominence in the early 20th century [20]. In 1890, owing to the work of Marchiafava, Bigmani and Golgi, malaria was discovered to be spread by a unicellular parasite that invaded erythrocytes, grew in size, and gave rise to daughter parasites that reinvaded erythrocytes [32]. Comprehensive monitoring of the parasite's erythrocytic stages allowed for the distinction between tertian malaria, which has a 48-hour periodicity, and quaternary malaria, which has a 72-hour periodicity [20,29,32]. *P. vivax* and *P. falciparum* cause benign and malignant tertian malaria, respectively, whereas *P. malariae* causes benign quartan malaria [33]. While doing research in West Africa in 1920, John Stephens identified a fourth species, which he named *P. ovale*, that resembled *P. vivax* and produced benign tertiary malaria [20].

The mosquito stage: unveiling parasite transmission

While Laveran's seminal work uncovered the causative agent of malaria, no malariologist could explain how the parasite spread from one human to another. Evidence linking mosquitoes to malaria had surfaced over the centuries, but it was not until 1883 that an American physician, Albert King, had assembled the bulk of the evidence supporting the mosquito-malaria doctrine [34]. The definitive proof that mosquitoes transmit malaria parasites was provided by Ronald Ross who discovered that *Culicine* mosquitoes transmit the avian malaria parasite *Plasmodium relictum* while working in India in 1897 [3,17,20,29]. By dissecting mosquitoes that had fed on malaria-infected birds, Ross discovered that after ingestion of male and female gametocytes, parasites fertilize in the gut and mature into spores, today referred to as oocysts. Oocysts grow in size and develop rod-shaped structures that migrate to the mosquito's salivary glands (sporozoites), where they reside until subsequent injection into a new host (Figure 1.2) [3,35]. Aside from elucidating the whole life cycle of *Plasmodium relictum*, Ross

suggested that mosquitoes are also likely to transmit human malaria parasites. It was only later in 1899, while working in Sierra Leone, that Ross was able to confirm his theory by observing that *Anopheles* mosquitoes that fed on malaria patients also contained parasites in their guts. The pioneering work of Ross, which earned him the 1902 Nobel Prize in Physiology or Medicine, was instrumental in establishing mosquito-borne malaria transmission [3,17]. Bignami and Grassi also observed that mosquitoes transmit malaria while working near Rome and Sicily, where malaria was widespread. By feeding local *Anopheles claviger* mosquitoes to *P. vivax*-infected patients, they could subsequently transmit the infection to uninfected individuals via the bite of the same mosquitoes [17,18,20,30]. Over the next two years, Bignami, Grassi, and Bastianelli collected evidence that only female *Anopheles* mosquitoes could transmit the disease and successfully characterized the full life cycle of three *Plasmodium* species: *P. vivax*, *P. falciparum*, and *P. malariae* [17,18,20,36]. Such insights into malaria transmission were key to controlling the disease by reducing exposure to infected mosquitoes, a malaria prevention strategy still employed nowadays [18,20].

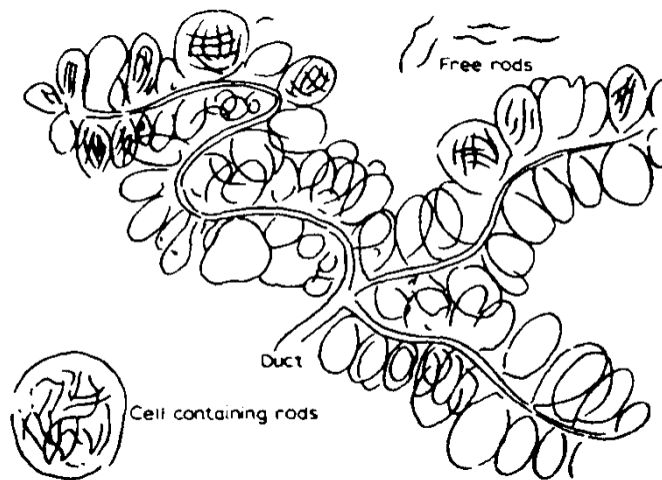


Figure 1.2 - Original drawing by Ronald Ross of a mosquito salivary gland. Ross pioneered the discovery of *Plasmodium* sporozoites in the salivary glands of *Anopheles* mosquitoes in 1897. From [35].

The enigmatic pre-erythrocytic stage

While the erythrocytic stage of parasite development has been widely investigated since its discovery by Laveran, little was known about what happened following sporozoite injection until a detectable blood infection emerged. It was only 50 years later that the full life cycle of the parasite was revealed [37]. In the 1890s, Battista Grassi hypothesized that the life cycle of *Plasmodium* must comprise an additional developmental stage in cells other than erythrocytes, possibly white blood cells. One such hypothesis was based on the morphological differences observed in the nuclei of sporozoites compared to young trophozoites developing in erythrocytes [20]. However, Fritz Schaudinn, an influential German scientist, disregarded Grassi's theory in 1903 after he demonstrated that *P. vivax* sporozoites directly invaded erythrocytes [20,29]. Such a phenomena is now recognized among malariologists as the "Schaudinn's fallacy" due to the inability to corroborate and reproduce these results [20]. By the late 1930s, it was widely recognized that malaria parasites in birds undergo a phase of replication in various nucleated cells before (and after) their appearance in the blood [20,38]. After the end of World War II in 1945, research on malaria had intensified and two years later Henry Shortt and Cyril Garnham showed that the development of *P. cynomolgi* parasites in the blood of infected rhesus monkeys was preceded by a period of division in the liver [39]. Shortly thereafter, Shortt and Garnham observed liver-stage parasite forms (known as exoerythrocytic forms) of *P. vivax* and, later, *P. falciparum* and *P. ovale* in human volunteers [40–42]. It took nearly 70 years to fully elucidate the life cycle of human malaria parasites [20], with the final piece of the puzzle being the discovery of *P. vivax* hypnozoites in the liver by Wojciech Krotoski in 1982. These parasite forms can remain dormant in liver cells for days, months, or even years before resuming schizogony and causing a blood stage infection [43,44].

Malaria Paroxysms & Severe Disease

Hemolysis of infected red blood cells (iRBCs) during *Plasmodium* development is associated with the onset of malaria disease. The first symptom to appear and the hallmark of malaria is fever. The fever begins irregularly but eventually becomes periodic as the parasites synchronize after a few rounds of replication [45]. Depending on the *Plasmodium* spp., fever can occur every 48 hours (*P. falciparum*, *P. ovale* and *P. vivax*) or every 72 hours (*P. malariae*) [46]. The classic malaria paroxysms occur as the disease progresses, with episodes of the disease alternating with asymptomatic periods [14,47,48]. Malaria paroxysms consist of three stages: a cold stage, a heat stage, and a

sweating stage and are often followed by non-specific symptoms such as chills, headache, fatigue, vomiting, diarrhea and impaired consciousness which, if left untreated, can lead to severe pathology [14,23,46–50]. Most cases of malaria are uncomplicated, with less than 1-2% of those infected developing and succumbing to severe disease, resulting in over 400,000 fatalities each year [4]. Severe pathology occurs mostly in non-immune *P. falciparum*-infected patients and children under the age of five, the latter accounting for 60% of cases [16,47,51]. Severe malaria is multi-syndromic, affecting the central nervous system (cerebral malaria), the pulmonary system (shortness of breath) and also the hematopoietic system (severe anemia) [51–54]. Such syndromes can arise singly or in combination and if not treated immediately, multiple organ failure occurs, leading to significantly high mortality rates [55]. In addition to children, pregnant women are also at greater risk of developing severe malaria, with an increased risk of maternal mortality, miscarriage, and low birth weight, as well as associated risks of neonatal death [16]. Severe malaria is particularly common in young children residing in areas of high transmission, such as endemic areas, as adults develop partial immunity over years of repeated exposure to the parasite [16,56,57]. While partial immunity shields against serious disease, it does not protect against chronic, mild, or secondary infections [58,59]. As a result, older children and adults in endemic areas often have asymptomatic parasitemia, or the presence of parasites in the blood without clinical manifestations [56,59,60]. On the other hand, in places with low transmission, immunity to malaria develops slowly, and malaria affects people of all ages [61].

The Road to Resistance: Antimalarials with History

The discovery of quinine was a significant milestone in the history of malaria prevention and treatment. Quinine was first isolated in 1820 by Pelletier and Caventou from the bark of the cinchona tree native to South America [18,62]. Cinchona was first used by Jesuit missionaries in Peru in the 17th century to treat fevers, then imported to Europe and later spread around the globe. For almost 300 years, quinine was the only compound available to treat malaria [18,62,63]. The antimalarial properties of quinine derive from its quinoline moiety, which targets blood stage parasites by blocking the formation of an inert crystal known as the malarial pigment, or hemozoin, in the parasite's digestive vacuole. This is a critical step in the detoxification of free heme produced during the digestion of host hemoglobin [64–66]. However, a thorough understanding of its mode of action is still lacking. Interestingly, the anti-plasmodial quinine moiety is present in a

variety of later synthesized analogues, including chloroquine and primaquine [66–69]. Chloroquine (CQ) is a quinoline analogue that was discovered during World War II as part of a collaborative research program in which thousands of compounds were synthesized and tested for their antimalarial activity [18,62,66]. However, towards the end of the war it turned out that the same compound (then under the name Resochin) was being studied by German researchers in Bayer laboratories, but was rejected due to its toxicity to birds and therefore considered too dangerous for practical use in humans [18,31,70]. The drug was withheld by Bayer for more than a decade, an incident known as the “Resochin error”. Meanwhile, German researcher Johann “Hans” Andersag tweaked the drug to reduce its toxicity. Resochin was rediscovered in the following years and renamed CQ in 1945 by EK Marshall [31]. Despite its enormous importance, the mechanism of action of CQ, as a first-line treatment for malaria, is still a matter of debate. CQ accumulates thousands of times in the digestive vacuole and, like quinine, binds to toxic heme and prevents it from polymerizing into hemozoin (Figure 1.3) [67,71,72].

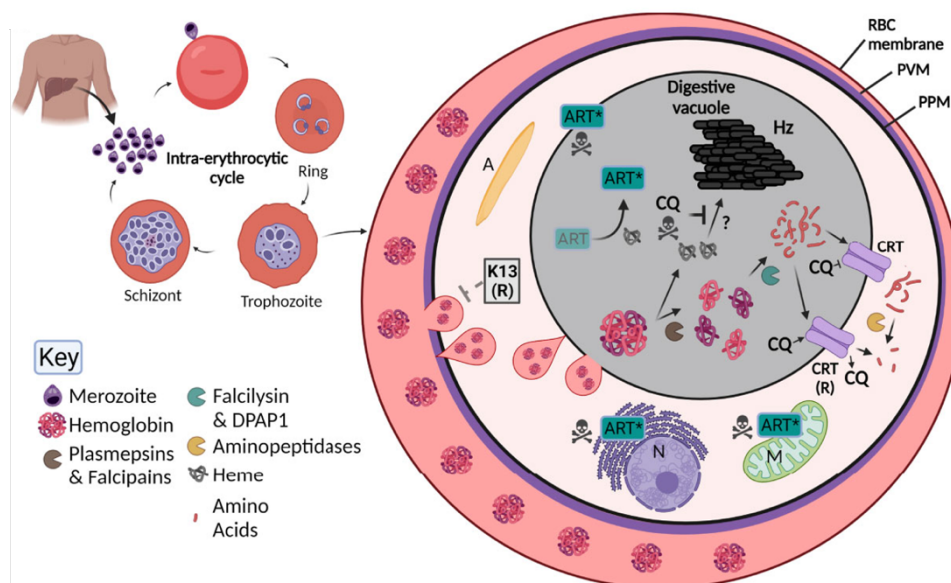


Figure 1.3 - Overview of the effect of antimalarials on *Plasmodium falciparum*. The breakdown of hemoglobin in the digestive vacuole (DV, grey) produces protonated basic amino acids and the toxic ferriprotoporphyrin IX (heme) which is detoxified by polymerization to hemozoin (Hz). Chloroquine (CQ) kills parasites by blocking the formation of Hz. This can be circumvented, nonetheless, by parasites carrying mutant versions of CRT, a transmembrane protein capable of transporting CQ outside of the DV. Artemisinin (Art), on the other hand, is activated (Art*) by heme, causing damage to many organelles including the nucleus and mitochondria. Adapted from [73].

CQ became the cornerstone of the WHO Global Eradication Program for the treatment and prevention of malaria in 1955 [74,75]. However, the massive and indiscriminate use of this drug led to ineffective dosing and the emergence of parasite-resistant strains [68,72,76–78]. Drug resistance was first observed in Southeast Asia and South America in the 1960s, but quickly spread to all malaria-endemic areas [76,78,79]. The critical determinant for CQ resistance was later identified as the *P. falciparum* CQ resistance transporter (*PF*CRT), a transmembrane protein of the digestive vacuole involved in CQ secretion [77,80].

Primaquine (PQ) is an 8-aminoquinoline developed by the US Army in the 1950's and an estimated 200 million people have been administered PQ since then [81–83]. The first mass use of PQ occurred during the Korean War when over 250,000 US soldiers received a 14-day radical regimen to eliminate long-latency *P. vivax* infections [81,84]. Noteworthy, PQ is the only clinically available drug that targets *P. vivax* and *P. ovale* hypnozoites in the liver [83,85–87]. PQ has also shown potent activity against blood- and liver-stage *P. falciparum* parasites [55,66,88,89]. Despite its overall potency, PQ has a short plasma half-life (approximately 6 h) due to oxidative deamination to carboxyprimaquine [66,90]. This results in high blood toxicity and an increased risk of hemolytic anemia, particularly in patients carrying the glucose 6-phosphate dehydrogenase deficiency (G6PD) - an X-linked trait with a prevalence of 5-20% in malaria endemic regions [81,91,92]. Recent evidence has uncovered another drawback of PQ: a mutation in the cytochrome P-450 2D6 enzyme, which seriously impairs drug metabolism and prevents its anti-relapse activity [8,93].

Artemisinin (ART) was discovered in the 1970s during the Chinese “Cultural Revolution” and derives from a plant called *Artemisia annua*, also known as Qinghaosu [18,94]. The discovery of ART is credited to Tu Youyou, who led the research group that successfully extracted and crystallized its main antimalarial component, Qinghaosu (nowadays known as Artemisinin) in 1972 [95]. Such a landmark discovery earned Professor Tu Youyou the 2015 Nobel Prize in Physiology or Medicine [96]. ART is currently one of the most effective antimalarials with a rapid onset of action and no major side effects. It shows potent nanomolar activity against multidrug-resistant strains of *P. falciparum* and also broad stage specificity for different stages of the parasite life cycle, including asexual blood stages [95] but also gametocytes (sexual stages) [97]. However, it has low solubility in water and oil, incomplete absorption and rapid elimination with a half-life of 2 to 5 hours [66,94,97–102]. This prompted the search for the synthesis of derivatives,

including dihydro-artemisinin (DHA), the water-soluble artesunate, and the lipophilic alkyl ether artemether that exhibit enhanced pharmacological properties and improved antimalarial activity [18,66,103]. Nowadays, ART-based combination therapies (ACTs) are the cornerstone of antimalarial treatment in order to overcome the short half-lives of circulating ART and ART derivatives [4,104]. ACTs consist of a potent, short-acting ART derivative co-administered with one or more longer-acting partner drugs with independent modes of action. The effectiveness of ACT is determined by the drug working together with the ART derivative and for artesunate-mefloquine, artemether-lumefantrine and dihydroartemisinin-piperaquine it exceeds 95% [100,103,105–107]. However, ART resistance has been described clinically [108–110] as well as for three of its partner drugs [111–113]. Genome-wide analysis of artemisinin-resistant *P. falciparum* strains revealed that the propeller domain of the *Kelch13* gene is the most promising molecular marker for resistance to ART and ART derivatives [109,114]. Dozens of different *kelch13* point mutations have been reported in *P. falciparum* around the world, with the first ART resistance outbreak identified in western Cambodia [115], which quickly spread to Southeast Asia (Figure 1.4) [116].

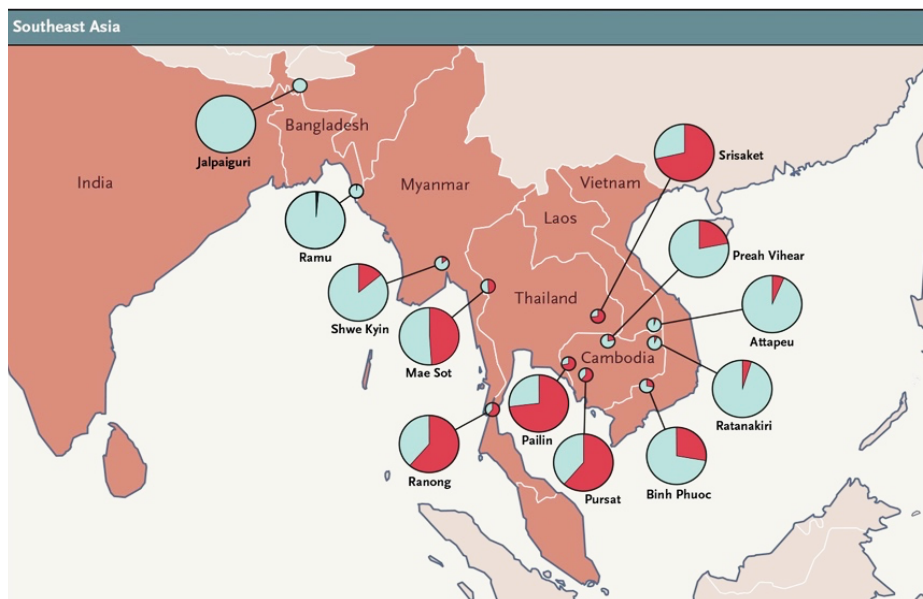


Figure 1.4 - Proportion of patients and distribution of *Plasmodium* clearance time with ART treatment in Southeast Asia. Artemisinin resistance was defined by a parasite clearance half-time greater than 5 hours, with some parasite isolates having *kelch13* polymorphisms. Adapted from [29].

Surprisingly, mutations associated with ART resistance have been rare in Africa, but recent evidence suggests that resistance has emerged in Rwanda, Uganda and also Eritrea [117–119]. Artemisinin-based triple combination therapies (TACTs) have been used to address the recent high failure rates of ACTs and to combat antimalarial drug

resistance, particularly in the Greater Mekong Subregion (GMS) of Southeast Asia, which historically is the origin of antimalarial drug resistance [120]. TACTs combine artemisinin with two currently available partner drugs and offer one of the last remaining safe and effective treatments for *P. falciparum* malaria. Such triple combinations are now standard for the treatment of other infectious diseases such as tuberculosis and HIV infections [121,122]. When combined with a single partner drug, all recommended ACTs have shown reduced efficacy in some GMS countries. Compared to ACTs, triple combinations could maintain the efficacy of partner drugs for longer periods and delay or prevent the emergence of resistance. Two such TACTs were recently evaluated for safety, tolerability, and efficacy in the recent Tracking Resistance to Artemisinin Collaboration II (TRACII) study (Figure 1.5) [120]. TACTs have proven highly effective in some areas of Cambodia, Vietnam, and Thailand – regions of the world where most conventional ACTs are no longer effective. These are therefore an effective option for treating multidrug-resistant malaria or in areas where ART or partner drug resistance has not yet emerged, to delay the emergence and spread of malaria drug resistance [115,120]. Because these therapies use existing, affordable, and already approved antimalarial drugs, they may be made widely available in a near future.

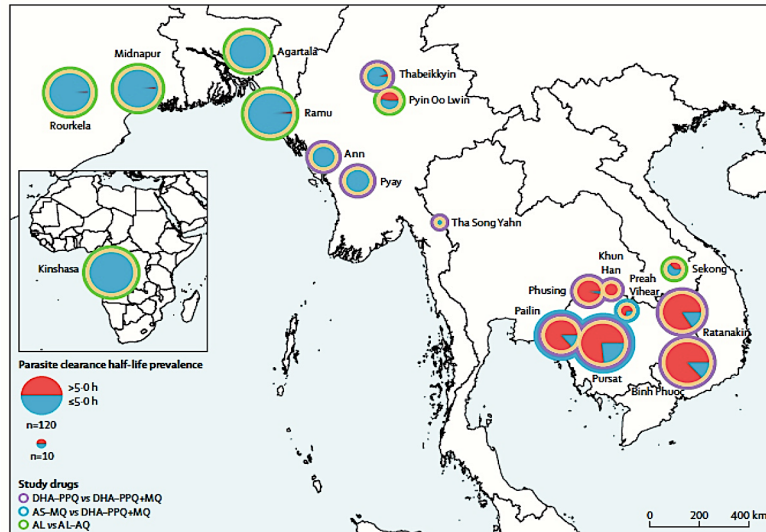


Figure 1.5 – Map of Southeast Asia and an insert of Africa showing the location of the Tracking Resistance to Artemisinin Collaboration (TRAC) II study. This is an open-label, randomized trial in 1100 patients with uncomplicated *falciparum* malaria conducted in 18 sites in 8 countries, namely Cambodia, Thailand, Myanmar, India, Laos, Vietnam, Bangladesh and Democratic Republic of the Congo. From [123].

The Economic, Social and Health Challenge

Malaria is a curable and preventable disease, yet it is still endemic in the tropical and subtropical regions of the world, posing a significant burden on numerous low and low-middle income countries [124–126]. In 2010, 251 million cases of malaria were reported globally, according to the World Malaria Report [127]. An estimate that had dropped dramatically to 227 million reported cases by 2018, illustrating the tremendous efforts undertaken to eliminate this disease over the last decade [128]. Globally, however, the decline in malaria cases had stalled in 2019, with an estimated 227 million cases in the 85 malaria-endemic countries [129]. Notably, the number of malaria cases increased to 241 million cases in 2020 during the COVID-19 pandemic, an increase of 14 million cases compared to the previous year [4]. The COVID-19 pandemic has put additional strain on healthcare systems worldwide, particularly in countries with fragile medical management systems such as sub-Saharan Africa. Such disruptions led to the suspension of key malaria control strategies during the pandemic, including reduced access to effective antimalarial drugs and a halt to all insecticide-treated net campaigns, ultimately resulting in a significant increase in malaria-related deaths (Figure 1.6) [130,131]. Despite the uncertainties in the 2020 estimates, arising from the challenges of reliably measuring COVID-19-associated disruptions, the data show that the number of reported malaria cases in 2020 was comparable to that recorded in 2000 in 108 malaria-endemic countries, suggesting a setback in the malaria eradication program [4]. The largest increase in case numbers in 2020 occurred in the African Region of the World Health Organization (WHO), which was home to 95% of all malaria cases and 96% of all related deaths [4,129]. From 2000 to 2015, malaria deaths declined steadily from 896 000 to 562 000 and in 2019 to 558 000. Nevertheless, malaria deaths increased by 12% in 2020 to an estimated 627 000. Amongst these, 69 000 malaria deaths were attributable to healthcare disruptions during the COVID-19 pandemic [4,129].

Malaria cases climbed again between 2020 and 2021, but at a slower rate than in the previous year. According to the World Malaria Report 2022, the global tally of malaria cases reached 247 million in 2021, highlighting the long road that lies ahead to meet malaria eradication goals. However, between 2020 and 2021, the number of malaria deaths fell to 619 000, suggesting that the rise in malaria deaths caused by the COVID-19 pandemic has plateaued [117]. WHO's African Region shoulders the heaviest burden of the disease, with 80% of all malaria deaths in this region occurring among children under the age of 5. Even though the percentage of malaria deaths in this age group has

declined over the past two decades - from 87% in 2000 to 76% in 2019 - mortality rates have risen to nearly 80% in 2021 [117]. According to the WHO, progress in malaria control remains at a plateau, particularly in high-burden, low-income African countries. Global malaria control initiatives, on the other hand, have averted 2 billion malaria cases and 12 million malaria-related deaths since 2000, thus representing one of the major global health victories of recent years [4].

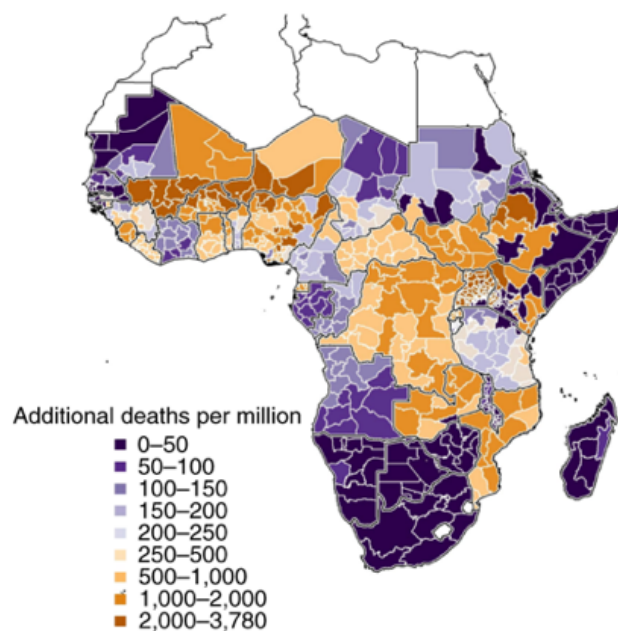


Figure 1.6 - Number of estimated additional malaria deaths per million, in different regions of sub-Saharan Africa, due to COVID-19 mitigation strategies.

Malaria interventions were halted for 6 months due to COVID19-related medical management disruptions, relative to normal service in the absence of COVID-19. Adapted from [120].

As a rule of thumb, where malaria thrives most, human societies thrive least. There is evidence that malaria has strong regressive economic effects, as poor households spend a disproportionately higher percentage of their income on treatment and prevention than high-income families [132–134]. Malaria is the disease that has the greatest impact on social growth and development in developing countries, particularly in Africa [124]. It places an enormous financial impact on households, healthcare systems and economies [135–137]. At the household level, malaria imposes direct costs, including lost work time, prevention costs and medical treatment costs. Additionally, households incur indirect costs as malaria leads to lost productive time and work redistribution within the household. Indirect consequences of malaria also include malnutrition, physical disability, and cognitive difficulties in affected children [124,135–137]. Furthermore, malaria is responsible for 5-8% of all absenteeism among African school children, which equates to 50% of all avoidable absenteeism [138,139]. Malaria costs Africa more than US\$12 billion each year, hampering Africa's economic development by 1.3% per year [18,124,135,140].

Rising to the Challenge of Malaria Eradication

Significant successes have been achieved in the fight against malaria since the beginning of the millennium. Such progress derives mainly from the policies and strategies implemented by the WHO [20,128]. The development of dichlorodiphenyltrichloroethane (DDT), the first residual insecticide, in the early 1940s radically changed malaria control strategies [74]. In 1955, WHO launched the Global Malaria Eradication Programme (GMEP), which advocated the use of CQ to prevent and/or treat malaria and DDT to control mosquitoes. This has greatly reduced the malaria burden worldwide, but no major success was observed in sub-Saharan Africa. In 1969, the GEMP was discontinued which ultimately led to the resurgence of malaria in many countries [141,142]. The goal of malaria eradication was abandoned in favor of malaria control. The next two decades were marked by a global increase in malaria incidence due to reduced investment. A Ministerial Conference in Amsterdam convened by WHO in 1992 marked a watershed moment in global efforts to combat malaria [143,144]. In the years that followed, the WHO Global Malaria Control Strategy was endorsed by the World Health Assembly (WHA) and the Roll Back Malaria Initiative was launched in 1998 with the aim of halving the global malaria burden by 2010 [145–147].

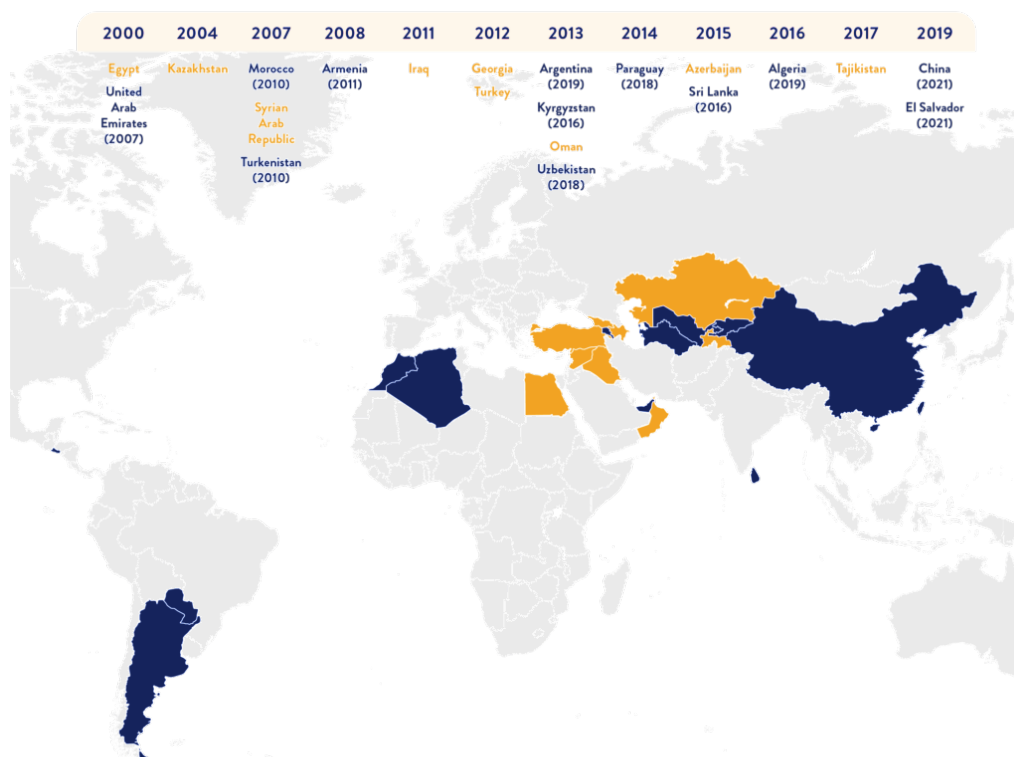


Figure 1.7 - Malaria-free countries since 2000. Countries are listed by the year in which they achieved three straight years of zero indigenous cases. Malaria-free countries are represented in navy and the year of certification is enclosed by parenthesis. From [148].

From 2000 to 2015, increasing coverage of key malaria control measures, mainly in Sub-Saharan Africa, led to a 41% reduction in global case incidence and a 62% decrease in mortality rates [149,150]. Moreover, while only 13 countries reported fewer than 1000 malaria cases in 2000, a total of 33 countries had reached this milestone by 2015, indicating that eradication was on the horizon [149–151]. Importantly, over the last 20 years, 10 countries have received WHO certification as malaria-free, and 21 have no reported cases of the disease (Figure 1.7) [148]. However, this progress has been uneven and sparse, with a plateau first seen in 2006 and continuing to this day. In this context, the Global Technical Strategy (GTS) jointly established by the WHO Global Malaria Programme (GMP) was launched in 2015 [152]. This strategy provides a comprehensive framework for the next 15 years and calls for a reduction in malaria incidence and mortality rates of at least 40% in 2020 and 90% in 2030 [129,152].

Malaria prevention

Core malaria prevention measures include: i) vector control strategies which aim at reducing transmission to and from humans; ii) chemoprevention, which prevents blood stage infection, and iii) case management strategies, which include early diagnosis and treatment of existing infections [16,153]. Insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) are currently the most effective and preferred methods for malaria vector control. ITNs confer a high level of personal protection but rely on pyrethroids, to which malaria vectors have developed resistance in most countries [16,154–157]. Notably, the non-insecticidal chemical piperonyl butoxide (PBO) can also be employed for ITNs, rendering this method highly valuable in areas where mosquito populations are heavily resistant to pyrethroids [158,159]. Conversely, the insecticides used for IRS are less restricted, with 16 WHO-approved insecticide formulations from five different classes: organochlorines (such as DDT), organophosphates, carbamates, and pyrethroids, as well as the recently added neonicotinoids [160,161]. By employing this approach, insecticide resistance can be successfully managed, thereby ensuring the long-term sustainability of vector control measures.

In endemic countries, the use of both methods alone or in combination has led to a significant reduction in malaria-associated morbidity and mortality [159,161]. The first malaria prevention strategies were implemented in Africa in the 1950s. Chemoprevention consisted of CQ or sulphadoxine-pyrimethamine (SP) treatment, but in 2004 chemoprevention was no longer recommended owing to the emergence of

parasite resistance. Malaria prevention strategies currently recommended by WHO include: intermittent preventive treatment in infants (IPTi), or in pregnancy (IPTp) and seasonal malaria chemoprevention (SMC) [153,162,163]. SMC targets children living in areas of high seasonal malaria transmission and treatment consists of monthly SP and amodiaquine (AQ) cycles during the rainy season, regardless of their infection status [86,162,163]. For infants less than 12 months of age living in African regions with moderate-to-high transmission, WHO recommends IPTi with SP as a routine infant immunization strategy [153]. For pregnant women living in malaria-endemic areas of Africa, IPTp with SP is recommended as part of prenatal care, which has been shown to markedly reduce severe maternal anemia, low birth weight and perinatal mortality [86,152,164]. For travelers from non-endemic to endemic countries with ongoing malaria transmission, chemoprophylaxis is recommended and includes atovaquone-proguanil, known as Malarone, doxycycline or MQ [165–167]. Presumptive anti-relapse therapy (PART) with PQ is also recommended for travelers with intense or prolonged exposure to recurrent malaria species, including *P. vivax* and *P. ovale* [168]. In 2018, tafenoquine, a new compound based on the same 8-aminoquinoline scaffold originally discovered by the Walter Reed Army Institute of Research (WRAIR), was approved for malaria chemoprophylaxis [169,170]. As a derivative of PQ, tafenoquine also presents toxicity when administered to G6PD patients.

Malaria vaccine

Vaccines are the most cost-effective public health tools and have been instrumental in eradicating a variety of diseases worldwide. Malaria vaccines have been under development since the 1960s, but significant advances have only become apparent in the last decade. Albeit the challenges, attempts are currently underway to achieve long-term immunity and durable protection against the disease. October 6, 2021 marks a historic day in the development of malaria vaccines with RTS,S/AS01 (RTS,S) completing Phase III clinical trial and being approved by the WHO for widespread use in children in Sub-Saharan Africa and other areas with moderate to high transmission of *P. falciparum* [171–173]. The RTS,S subunit vaccine, known as Mosquirix™, is a virus-like recombinant particle that targets the circumsporozoite surface protein (CSP) of *P. falciparum*, which is expressed by pre-erythrocytic stage parasites. The CSP sequence is co-expressed with fused and free hepatitis B antigens [172,174] and AS01 is used as an adjuvant. Development of this vaccine began in 1987 at GlaxoSmithKline (GSK), as

part of an ongoing collaboration with the WRAIR [175–177]. Coordinated by the Malaria Vaccine Implementation Programme (MVIP), a large-scale post-licensing pilot implementation program is currently underway in Ghana, Kenya and Malawi and has reached 1.5 million children with at least one vaccine dose since 2019. The MVIP aims to comprehensively evaluate the feasibility of administering the four doses of the RTS,S vaccine. Other factors under evaluation include the impact on malaria morbidity and mortality, and the safety of the vaccine for routine use. These pilot trials will continue through 2023 to assess mortality prevention and the added value of the fourth vaccine dose [178,179]. It is worth noting that the Phase III trial evaluating the effectiveness of the vaccine in African children aged 5 to 17 months revealed 55.8% efficacy with a 39% reduction in malaria infection rates and a 31% reduction in severe malaria over 2 years [174]. Vaccine's efficacy, however, appeared to wane over time, particularly in high endemic areas [172,174,175,180]. The protective efficacy of this vaccine is impacted by a variety of factors, including dosing regimen/boosting dose, age of children, parasite species and intensity of transmission in the population. To tackle such drawbacks, a next-generation RTS,S vaccine comprising additional CS antigens and known as R21 vaccine is under development [181]. Other prospects envisioning increased vaccine efficacy include alternate dosing schedules, booster doses, enhanced immunogenicity through the use of distinct adjuvants, and also the use of other vaccine candidates targeting other stages of the parasite life cycle [181,182].

Malaria case management: from diagnosis to treatment

Malaria case management consisting of early diagnosis and effective treatment is essential for the control and elimination of malaria [104,183]. Currently, the WHO recommends that all suspected cases of malaria, including patients with a history of persistent fever in a malaria transmission area, should be promptly examined for *Plasmodium* infection - either by light microscopy or rapid diagnostic test (RDT) [152,153,184]. While light microscopy entails high training and equipment costs [185–187], RDTs are immune-chromatographic tests that do not require electricity nor special equipment as they rely on the detection of two major parasite-specific antigens: the *P. falciparum* histidine-rich protein 2 (PfHRP-2) and the *Plasmodium* lactate dehydrogenase (pLDH) system [49,187]. Although all cases of symptomatic malaria can be diagnosed by microscopic examination of thick and thin blood smears, RDTs should be preferred when quality-assured microscopy is not readily available or in patients who have recently received antimalarial treatment (artemisinin).

Even though RDTs have limited sensitivity to *Plasmodium* species other than *P. falciparum* and *P. vivax*, they are quick and easy to perform [187–189]. RDTs targeting HRP2 are now a mainstay of malaria diagnosis since they are less expensive, more stable over a wider temperature range, and have lower detection thresholds than pLDH-based tests [150]. Remarkably, 3.5 billion RDTs for malaria testing were sold between 2010 and 2021, with over 82% of all sales occurring in sub-Saharan Africa [117]. Other methods for diagnosing malaria include DNA/RNA-based detection techniques, particularly the polymerase chain reaction (PCR). This offers the highest sensitivity but requires trained personnel and specialized equipment [189,190]. PCR is the recommended approach for the detection of mixed infections, particularly low parasite densities (undetectable by RDT or microscopy), and also for differentiating new infections from relapses, as it enables the distinction of morphologically similar *Plasmodium* species [11,189]. It is important to note that surveillance of submicroscopic infections, including the asymptomatic population, is critical to preventing malaria transmission. However, PCR is time- and resource-consuming and therefore not suitable for routine detection in the field. Therefore, alternative screening tools have emerged, including loop-mediated isothermal amplification (LAMP) [184,191,192]. LAMP is a molecular technique for nucleic acid amplification where target gene detection can be accomplished in one isothermal step [193]. This renders LAMP technique much simpler, faster and cheaper than PCR while still offering a high level of accuracy [194,195].

Prompt medical care is crucial for all infected individuals after diagnosis. Since the release of WHO malaria treatment guidelines in 2006 and 2010, all *P. falciparum*-endemic countries have switched from monotherapy to the currently recommended ACTs. This resulted in a global reduction in malaria-derived morbidity and mortality, yet resistance to artemisinin in *P. falciparum* has been recently reported in South-East Asia [196]. ACTs were implemented in 2013 as national policy for first-line treatment for uncomplicated *P. falciparum* malaria. The treatment guidelines include five ACTs: artemether plus lumefantrine, artesunate plus AQ, artesunate plus MQ, artesunate plus SP, and dihydroartemisinin plus PQ [66,86,104]. The choice of the ACT employed depends on the therapeutic efficacy of each combination in the country of intended use, the antimalarial drug resistance pattern, the relative efficacy and safety of the combinations, their cost and availability [16,104,167]. Untreated severe *P. falciparum* malaria, particularly cerebral malaria, has an alarming mortality rate nearing 100%. However, with prompt and effective malaria treatment along with supportive care, the mortality drops to 10-20%. Currently, WHO-recommendations for severe malaria

treatment consist of a full quinine regimen followed by a full dose of effective oral ACT [104]. *P. vivax* malaria accounts for approximately half of all malaria cases outside Africa, and is generally considered a benign malaria form [197]. Nonetheless, infection often leads to severe anemia in young children due to the parasite's preference for reticulocytes [198]. Moreover, of the six *Plasmodium spp.* affecting humans, only *P. vivax* and the two species of *P. ovale* [198] develop hypnozoites, dormant parasite forms in the liver that cause recurrence weeks to years after the primary infection. With the exception for artesunate + SP, ACTs are at the forefront of treatment for *vivax* malaria, including CQ-resistant *P. vivax*, allowing for unification of malaria treatment. Regardless of the impact of ACTs on sexual blood stages, patients carrying gametocytes in the peripheral blood are still infectious for days or weeks after ACT use, so a single dose of PQ is routinely used to reduce infectivity and transmission [81,83,167,199].

Malaria Drug Accelerator

Malaria has been a threat to global health for centuries, so historically *P. falciparum* was probably the pathogen exerting greater pressure on human evolution [200]. Despite the effect of ITNs and ACTs in reducing the global malaria burden, the spread of resistance to first-line antimalarial drugs, together with the loss of efficacy of insecticides has resulted in a rising number of malaria cases. This underscores the need to develop new treatment options as well as efficient control measures. Notably, increasingly interest in antimalarial drug development has been observed over the past decade [4]. In 2012, the Malaria Drug Accelerator (MalDA) was formed to overcome limited private investment in malaria research. Currently, MalDA comprises a consortium of 18 laboratories, funded by the Bill and Melinda Gates Foundation, whose major goal is to accelerate the development of new antimalarial therapies by investing in new target identification, screening, and hit-to-lead development (Figure 1.8) [201].

In response to resistance to ACTs, fully synthetic antimalarials such as OZ277 and OZ439 with improved pharmacokinetics have been developed. For instance, OZ439, also known as artefenomel, is a synthetic peroxide drug candidate being developed as a partner drug for ACT. Unlike artemisinin, OZ439 is highly efficient in a single dose regimen, which is attributed to its longer half-life in circulation [202]. Besides activity against ART-resistant parasites, OZ439 has also shown prophylactic potential and transmission-blocking activity [203]. While OZ277 is now marketed as Synriam (in combination with PQ) [204], OZ439 is in clinical trials [205]. Over the past 15 years, most

antimalarial drug candidates have been revealed using phenotypic screening approaches, with over seven million compounds tested against asexual blood stage parasites [206]. Among these compounds, WM382, a dual inhibitor of *P. falciparum* plasmepsin IX and X, has emerged as the most promising candidate to date [201,207]. It exhibits multi-stage activity against all developmental stages of *Plasmodium*, both in the mammalian host and the mosquito vector [207]. Even though *P. berghei* liver-stage forms are not fully eliminated, a single dose of WM382 prevented the liver-to-blood stage transition and disease's onset. In addition, WM382 inhibited asexual and sexual blood stage development of *P. falciparum* as well as oocyst development in the mosquito vector, thereby impacting both disease onset and transmission [207]. This new compound has therefore the potential to fill a critical and widening gap in efforts to control and eliminate malaria.

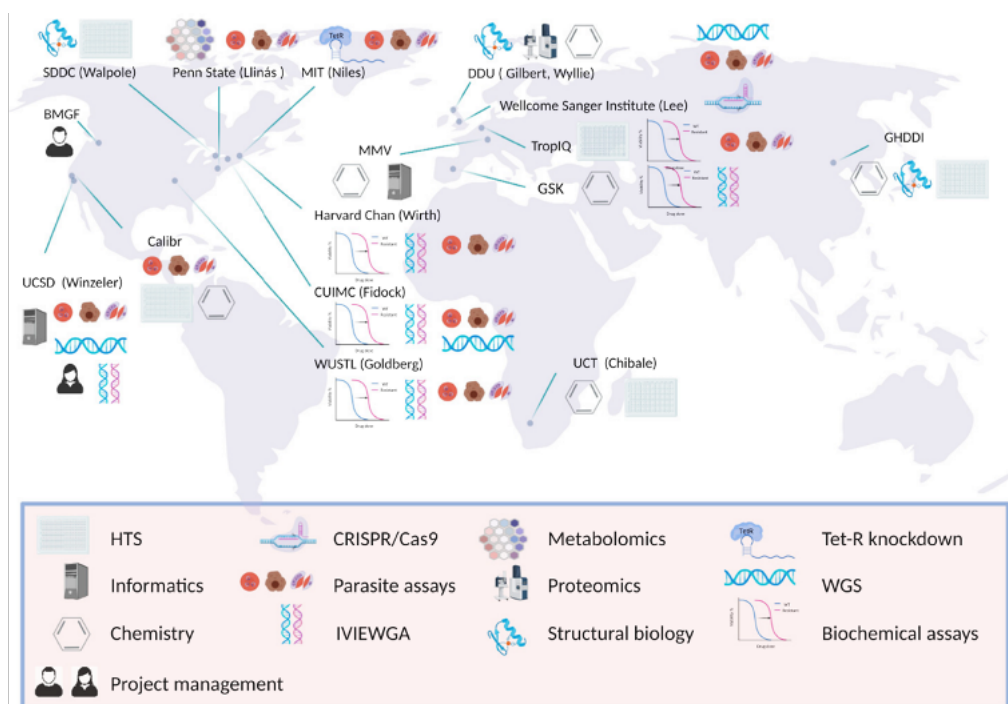


Figure 1.8 – Overview of the Malaria Drug Accelerator (MaIDA). MaIDA's goal is to improve and expedite the development of antimalarial drugs by discovering new, essential drug targets. Represented are 15 of the 18 laboratories of the MaIDA consortium. From [208].

The Complex Life Cycle of *Plasmodium* Parasites

Plasmodium parasites pursue a complex life style, cycling between a mosquito vector and a vertebrate host (Figure 1.9). Such an intricate life cycle relies on the parasite's capacity to differentiate into morphologically distinct forms, that alternate between invasive stages (sporozoite, merozoite and ookinete) and replicative stages. The parasite's capacity to overcome population bottlenecks at each stage transition and increase population numbers upon transmission is also of notable importance in maintaining such an intricate life cycle [209–211]. Malaria is transmitted by the bite of an infected female *Anopheles* mosquito, which harbors the parasite in its sporozoite form in the salivary glands. Sporozoites persist in a quiescent state [212], followed by rapid reactivation of the transcriptional machinery once the anopheline vector finds a suitable capillary to feast on, thereby injecting 15-120 motile sporozoites under the human dermis during a blood meal [213]. Real-time imaging of *P. berghei* parasites revealed that sporozoites rely on gliding motility, a substrate-dependent form of locomotion, to traverse skin cells, enter nearby blood vessels, and reach the liver [214,215]. In order to establish in the mammalian liver, the invasive sporozoites must first cross endothelial cells, Kupffer cells, or the liver-resident macrophages, and also some hepatocytes before undergoing a functional switch that allows them to settle into a suitable hepatocyte [13,216–218].

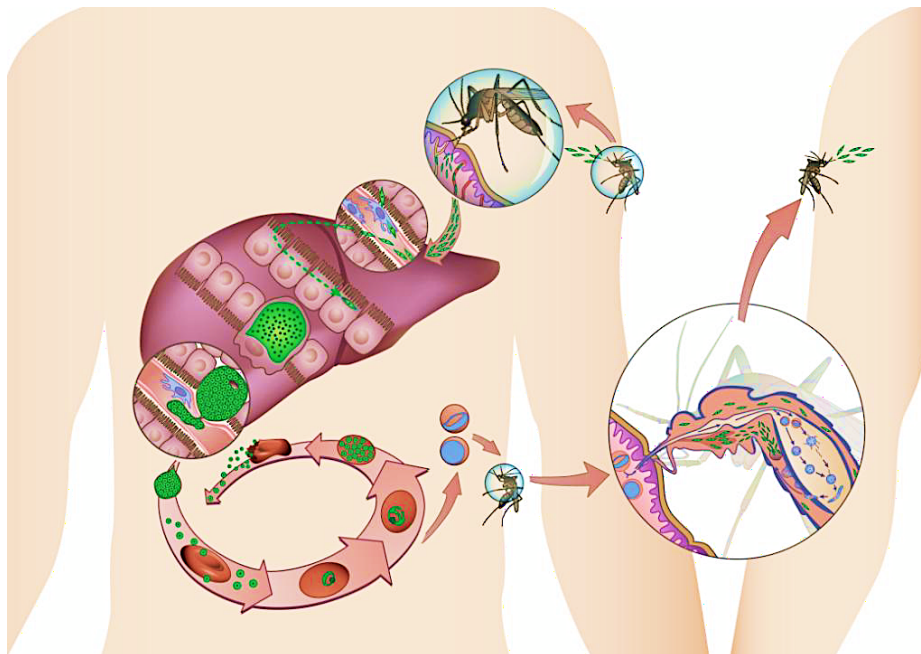


Figure 1.9 - The complex two-host life cycle of *Plasmodium* parasites. From [4].

The invasion of a final hepatic cell culminates in the formation of a parasitophorous vacuole (PV) and the concomitant establishment of the parasite within a PV membrane (PVM) [219]. The PVM is extensively modified by the developing liver stage parasite and serves as the main barrier to the host cell cytoplasm, but also as a channel for communication and nutrient uptake [220]. The liver stage represents the first obligatory site of infection, but is clinically silent, i.e., asymptomatic, and lasts 5 to 17 days in humans, depending on the infective *Plasmodium* species [221–223]. At this stage, parasites enter an intense phase of asexual replication known as exoerythrocytic schizogony, in which multiple rounds of DNA replication and nuclear division occur without cell division, prior to a final step of cellularization (Figure 1.10).

Rodent malaria sporozoites evolve into mature liver stage forms containing up to 29,000 exoerythrocytic merozoites in as few as two days, making it one of the most fast and exceptional eukaryotic replication events [224]. *P. falciparum* liver-stage schizogony, on the other hand, lasts longer, with 90,000 exoerythrocytic merozoites generated and packaged into fully individualized parasites within 7 days of infection [225]. This represents the first population bottleneck parasites overcome, as they multiply their population size ten thousand-fold during liver stage development. However, not all *Plasmodium* species have evolved the capacity to achieve such astonishing replication rates. An example are the malaria parasites of birds and reptiles, which infect macrophages rather than hepatocytes and only develop into a dozen of new parasites, which then migrate and invade red blood cells (RBCs) [226]. The remarkable ability to achieve such massive growth and the specific liver tropism of mammalian invading malaria species may be closely related; a hypothesis that has not yet been fully elucidated. Among the five *Plasmodium* species that naturally infect humans, *P. vivax* and *P. ovale* have the unique peculiarity of evolving dormant pre-erythrocytic forms known as hypnozoites, which strikingly magnifies their clinical and epidemiological complexity. These dormant forms can reactivate the parasite's life cycle and lead to disease relapses months or years after primary infection, allowing the parasite to survive periods of irregular transmission by the mosquito vector [227]. The mechanisms underlying hypnozoite activation are still unclear; the risk, timing and frequency rate of relapses is likely to be influenced by the presence of different strains of the parasite in the blood as well as the presence and feeding of uninfected mosquito vectors [8,228].

Completion of hepatic schizogony leads to disruption of the PVM and concomitant death of the infected host hepatocyte. Packaging of liver stage merozoites into vesicles (merosomes) is key to ensuring transport to the microvasculature of the lungs and subsequently to the circulating erythrocytes (Figure 1.10). While sporozoites rely on their motile and membrane-wounding capacity to evade host immune cells, immotile merozoites have evolved this membrane-wrapping strategy and migrate through the bloodstream in vesicles surrounded by the hepatocyte membrane [229]. Merosomes are thereby immunologically silent carriers that offer protection against phagocytosis by Kupffer cells [224,230,231].

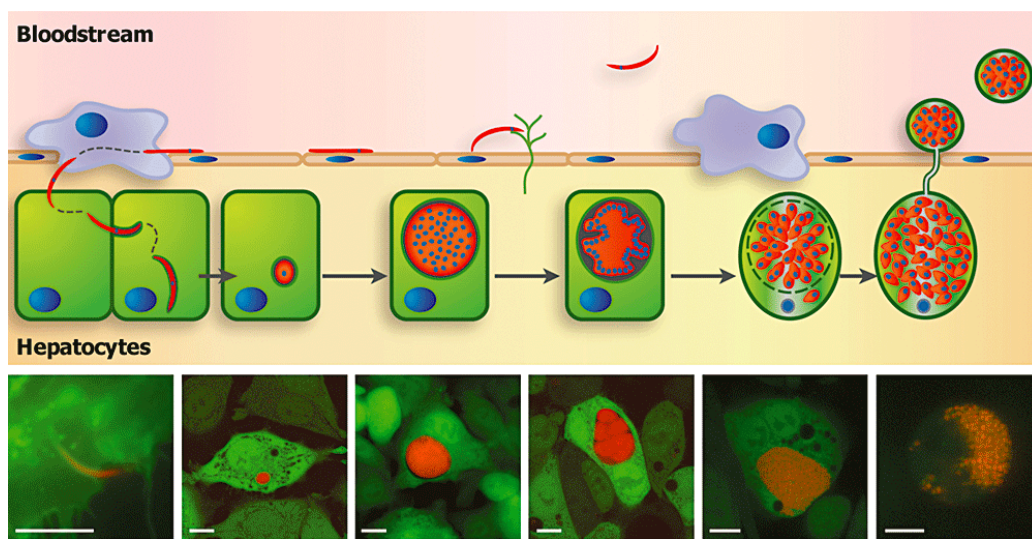


Figure 1.10 - The pre-erythrocytic stage of *Plasmodium* parasites. Parasites shown in red, hepatocytes in green, and Kupffer cells in purple. The lower panels show GFP-expressing HepG2 cells (green) infected with mCherry- or RedStar-expressing *P. berghei* parasites at different developmental stages. Scale bars= 10 μ m. From [232].

Merozoites have a short lifespan and must infect erythrocytes immediately after their release into the bloodstream. Merozoite invasion of erythrocytes is a highly complex, multistep process that requires the coordinated interaction between parasite proteins, including merozoite surface proteins (msp), and receptors on the erythrocyte surface [233,234]. During the symptomatic erythrocytic stage of infection, parasites follow replication cycles of 24-, 48-, or 72 hours, depending on the *Plasmodium* spp. [47,222]. Intra-erythrocytic schizogony is characterized by parasite development through three distinct morphological stages: ring stage, the earliest parasite form that establishes soon after merozoite invasion of the host erythrocyte; the metabolically active trophozoite stage and finally the schizont stage where consecutive rounds of DNA replication and nuclear division occur prior to cellularization [47,235]. By the end of schizogony, each

invading merozoite produces a mature schizont with 16-32 nuclei (next-generation merozoites), depending on the parasite *spp.* [236–238]. These are released into the circulation upon rupture of host's erythrocytes. Free merozoites rapidly re-invade erythrocytes, leading to the classic symptoms of the disease [13,47,222,239]. The intra-erythrocytic stage of *Plasmodium* infection is described in more detail in a section below, as it is the focus of this dissertation.

Throughout the blood stage of infection, 5 to 10% of parasites develop into sexual-stage forms called gametocytes, which are key for transmission to the mosquito vector [33,240,241]. Micro- and macrogametocytes, i.e., male and female sexual parasite forms, are eventually ingested by a female *Anopheles* mosquito during a subsequent blood meal [33]. Within the mosquito vector, ingested gametocytes rapidly mature into gametes, triggered by pH changes and exposure to xanthurenic acid [235,242]. Gametes fuse to form a diploid zygote, which grows into a motile ookinete that invades the epithelium of the midgut and destroys the host cells. The midgut wall of the mosquito is repaired by expelling the damaged cells [229] and the growing extracellular parasite now resides underneath the basal lamina. Upon invading the midgut wall of the mosquito, the ookinete develops into an oocyst that undergoes multiple rounds of endomitotic replication, culminating in the production of thousands of sporozoites, a process known as sporogony. Sporulating oocysts eventually rupture one to two weeks after maturation, releasing sporozoites into the hemolymph, which migrate to the mosquito's salivary glands [47,235,243], where they reside until transmission to the next suitable host.

During transmission of gametocytes from the blood of the mammalian host to the mosquito midgut, malaria parasites undergo significant reductions in population, experiencing dramatic losses at each developmental stage. This results in very low parasite numbers and represents the second population bottleneck. This reduction is triggered by the transition from an intracellular (erythrocytes) to an extracellular gametocyte form in the midgut of the mosquito, exposing parasites to noxious constituents of the host [210]. Of the thousands of gametocytes that an *Anopheles* mosquito ingests during a blood meal, only 50-100 develop into ookinetes, of which only five survive and develop into oocysts [244,245]. Such a population reduction is offset by a substantial rate of replication during sporozoite development, with each oocyst producing thousands of invasive sporozoites.

***Plasmodium* Schizogony: An Unusual Mode of Replication**

P. falciparum parasites grow within erythrocytes in 48-hour cycles of asexual replication and nuclear division, going through three unique intra-erythrocytic developmental stages: ring-, trophozoite- and schizont-stage (Figure 1.11). Apicomplexans like *Plasmodium* parasites deviate from the canonical mode of cell division: instead of binary fission, parasites undergo an asynchronous but syncytial mode of replication known as schizogony. Upon invasion, merozoites invaginate the erythrocyte membrane and acquire a ring-shaped appearance that can be observed under Giemsa stains. Ring-stage parasite forms consist of thick rims of cytoplasm containing important organelles such as the nucleus, mitochondrion, ribosomes and the endoplasmic reticulum [237]. At this stage, which lasts up to 26 hours, parasites have a single haploid nucleus in the interphase (G₀)/G₁ cell cycle phase and metabolic activity is minimal [236].

Notably, the parasite induces changes on the erythrocyte surface very early after invasion, extensively remodeling the host cell from within. This occurs mostly at the membrane and cytoskeleton levels and facilitates nutrient uptake and parasite sequestration in the deep vascular tissue, while conferring protection against host immune defenses [246]. The adhesive phenotype of malaria parasites causes changes in the host cell, with erythrocytes being distorted and showing knobby protrusions [247]. While developing in erythrocytes, malaria parasites grow and multiply inside a PV [246], through which they ingest small portions of host cell hemoglobin [237]. As parasite growth progresses, the PVM enlarges and ring stage parasites develop into rounded-shaped forms known as trophozoites. This stage transition is accompanied by marked changes in parasite and host cell properties and a shift to a highly metabolically activate state lasting 12 to 14 hours (36 to 38h of development time) [246–248]. As the cell cycle advances, multiple rounds of mitosis yield a multinucleated syncytium prior to synchronized budding and cytokinesis [249]. The schizont stage, lasting about 8 to 10 hours (38-48 hours of development time), is achieved through alternating asynchronous rounds of S- and M- phases. Notably, nuclei often divide in an asynchronous manner despite sharing the same cytoplasm, resulting in non-geometric growth and an odd number of daughter merozoites in each replication cycle [238]. The last round of mitosis, however, is synchronous, with multiple copies of parasite DNA being evenly split and packaged into up to 32 daughter individual merozoites - the progenitors of the ring-stage form [250,251]. The fully developed schizont now fills the entire erythrocyte compartment and becomes spherical, with a dense, rounded mass of hemozoin crystals visible in the

center [237]. Notably, 80% of the host cell's hemoglobin is endocytosed into the PV, which is later detoxified into hemozoin. Next-generation merozoites rupture the host erythrocyte and, once in the bloodstream, rapidly invade new erythrocytes, repeating multiple rounds of asexual development and increasing the parasite population exponentially [252].

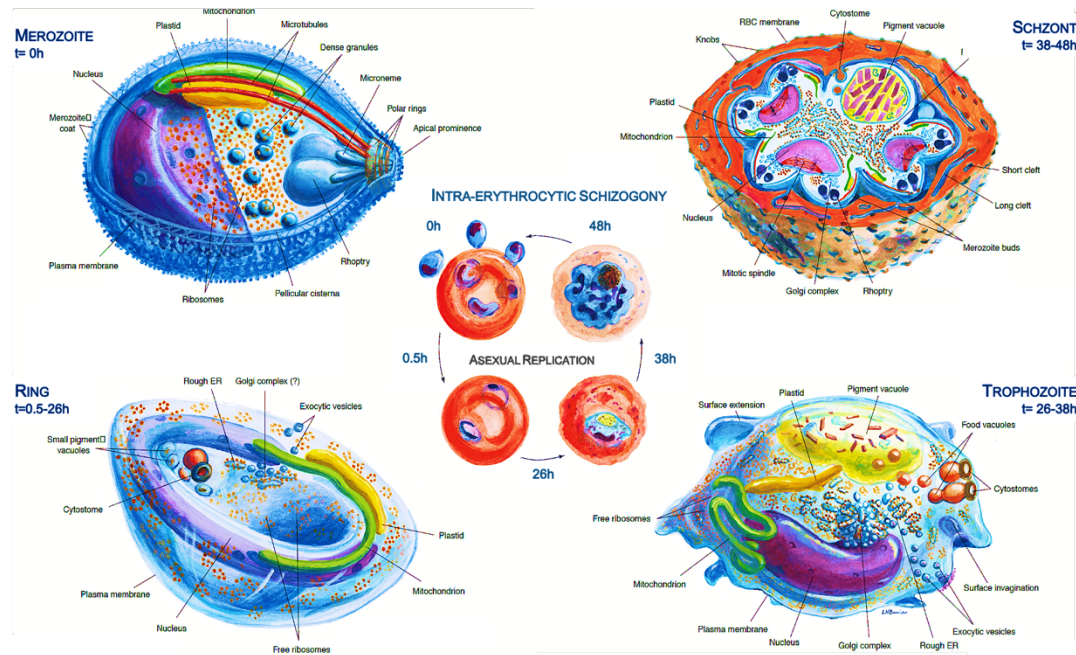


Figure 1.11 - *Plasmodium falciparum* intra-erythrocytic development cycle (IDC). The 48h IDC in *P. falciparum* can be divided in three distinct developmental stages including the ring-, the trophozoite- and the schizont-stage, which occur approximately at 26, 38 and 48h after merozoite invasion. Upon schizont rupture, the invasive merozoite form is released into the bloodstream, invading erythrocytes anew. Adapted from [253].

II. Fork In the Road: Host-Parasite Competition for Resources

Living organisms are inextricably linked, with biotrophic parasitism being a ubiquitous aspect of life as parasites are found in organisms of all species [254]. Parasitism represents a form of symbiosis in which the parasite resides within a second species (the host), utilizing resources while providing protection, illustrating an evolutionary success story. Notably, a parasitic lifestyle has profound effects on the host, thereby influencing the genetic composition of the host population. However, parasites are completely dependent on the host for nutrient supply, leading to mutual co-evolution of host and parasite, so that the parasite's genetic landscape is also heavily influenced by this lifestyle.

A Co-Evolutionary Signature: The Phenomenon of Genome Reduction

Obligate intracellular pathogens, including *Plasmodium*, often reveal genome reduction through gene loss over time due to their obligatory relationship with the host [255,256]. The transition from a free-living to an intracellular life-state is therefore associated with the loss of large segments of DNA, with gene degradation targeting overlapping subsets of potentially dispensable genes [257,258]. However, such gene deletions often comprise DNA segments encoding proteins or even entire metabolic pathways that provide nutrients which can be acquired from the host [259]. An in-depth analysis of both the genome [260] and metabolome [261] of *P. falciparum* revealed a remarkable metabolic simplification over evolution towards a parasitic lifestyle, such that the enzymatic machinery necessary for the synthesis of several biologically relevant molecules, including glucose [262], purine nucleotides [263], vitamins [264] and a wide range of amino acids [265] is lacking from the parasite's genome. This renders the parasite dependent on its host for the nutrients it requires for its own development. The transition from a free-living to an obligate intracellular state was accompanied by irreversible genome loss - an evolutionary path to niche specialization with no return.

***Plasmodium* Nutrition: A Minimalist Lifestyle**

The uptake and metabolism of nutrients is central to all living organisms as they provide the energy and biomass needed to survive. Mammalian cells have evolved multiple mechanisms to ensure the necessary nutrients that fuel their metabolic needs. While growing in metabolite-rich conditions, mammalian cells rely on transmembrane transporters as well as endolysosomal pathways to import such nutrients [266]. In addition, by using distinct nutrient sources, mammalian cells acquired metabolic plasticity, which is key for survival during periods of starvation [267]. Many protozoan parasites invade mammalian cells and live intracellularly, and *Plasmodium* is no exception. The intracellular environment offers obvious advantages as parasites can successfully evade the host's humoral immune response [268]. However, a parasitic mode of life entails several barriers to nutrient uptake as the malaria parasite must compete with its insect vector and the vertebrate host for the acquisition of many essential metabolites. Moreover, by cycling between two distinct hosts, *Plasmodium* must establish and reside in different cellular environments. To subvert the host cell and support its own metabolism, *Plasmodium* must coordinate multiple transport activities across the host cell membrane to maintain the intracellular niche. Efficient uptake and transport mechanisms that ensure nutrient import, waste outflow, effector protein export, and host cell cytosol uptake are of critical importance for parasite survival and virulence [269].

Mammals, birds, and reptiles are just a few of the many vertebrate hosts that *Plasmodium* parasites infect. The *Plasmodium* parasite, however, is quite specific about the host cell it actively invades and multiplies in. With the exception of the initial liver stage of infection, which comprises invasion of nutrient-rich hepatocytes, the malaria parasite exclusively invades erythrocytes or, in some species, reticulocytes [267]. Such a selectivity for a niche to reside in poses significant challenges for the parasite, since erythrocytes are metabolically inactive and lack the necessary resources to support active growth and division of the parasite. Furthermore, after entering the host cell, *Plasmodium* settles and grows in a vacuole contained by the PVM. This, together with the parasite's plasma membrane (PPM) and the host's plasma membrane, creates a three-layer barrier between the parasite's metabolic and biosynthetic machinery and the nutrient-rich host's blood plasma [269,270]. While the PVM shelters the parasite, it also precludes the parasite from utilizing the host's cell digestive enzymes for production or direct access to essential solutes [254]. Fusion with the host endosomal network and the

direct and free flow of proteins from the host cytosol into the vacuolar space are also prevented [254]. Therefore, blood stage parasites export a vast assortment of effector proteins via the PVM, which extensively remodels the host cell, ensuring protein trafficking, nutrient harvesting and elimination of metabolic waste [271–273].

Glucose transport

The blood is a steady and rich source of glucose (~5mM) for *Plasmodium* parasites that reside and multiply inside erythrocytes. Not surprisingly, intra-erythrocytic stages rely on glucose as the main energy source [274]. The dependence of *Plasmodium* on glucose is evidenced by a 75-fold increase in glucose consumption by iRBCs, relative to uninfected cells [275]. Despite recent evidence indicating that intra-erythrocytic parasites encounter a variety of nutritional states during natural infection, it is generally accepted that glycolysis serves as the chief pathway for the parasite's energy supply [276]. Despite possessing a mitochondrion, the *Plasmodium* mitochondrial genome is the smallest sequenced genome to date, with no clear evidence for the existence of a functional tricarboxylic acid (TCA) cycle during intra-erythrocytic development [277]. Such a rapidly multiplying lifestyle results in a significant consumption of glucose, especially for *P. falciparum* which lacks a spare energy storage [278]. Consequently, glucose must be supplied continuously, yet it must first cross the plasma membrane of the erythrocyte so that it can be used by the parasite as a source of metabolic energy. Due to the high copy number of GLUT1 - a high-capacity equilibrative hexose transporter - in the human erythrocyte membrane [269,279,280], glucose crosses the human erythrocyte membrane extremely swiftly (Figure 1.12) [281].

Facilitative transporters like GLUT1 function as passive carriers, moving solutes along concentration gradients [282]. Once inside the erythrocytes, the parasite is surrounded by the PVM, which is highly permeable to soluble macromolecules such as monosaccharides, including glucose, and amino acids (AA) up to 1,400 Da in size [283]. These molecules readily flow via highly capacitive but weakly selective channels between the erythrocyte cytoplasm and the vacuolar space [283–285]. Finally, glucose uptake into the parasite via the PPM is mediated by a facilitative, i.e., non-concentrative, hexose transporter encoded by the *Plasmodium falciparum* hexose transporter gene (*PfHT*), which is a homologue of GLUT1 and is the major hexose transporter expressed in the plasma membrane of the parasite [286].

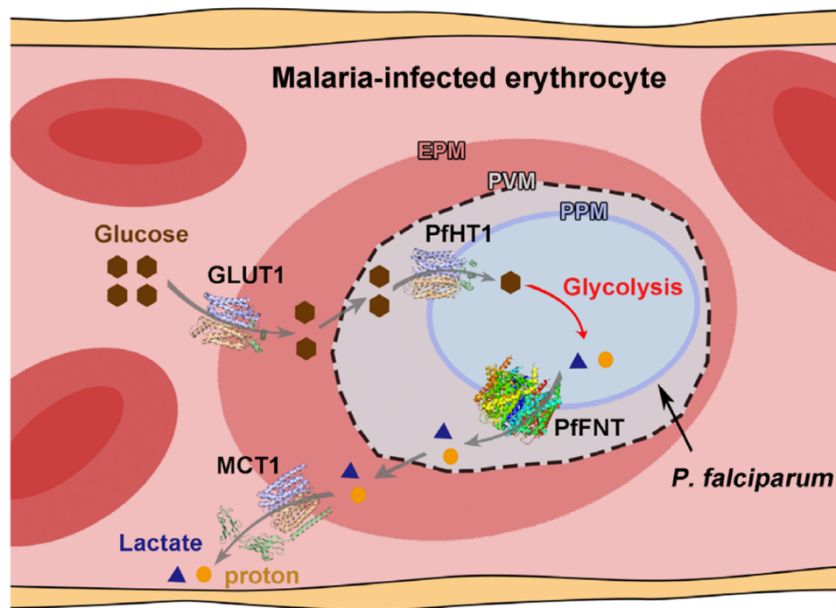


Figure 1.12 - Glucose uptake by *Plasmodium falciparum* intra-erythrocytic stages. *P. falciparum* infected erythrocyte illustrating the localization of PfHT1 transporter at the PPM and the GLUT-dependent uptake of sugars, including glucose. From [73].

Purine metabolism

Plasmodium also requires purines to synthesize important biomolecules including DNA and RNA. All parasitic protozoa studied to date are unable to synthesize purines *de novo* and must scavenge these essential nutrients from the host. Erythrocytes contain millimolar concentrations of adenosine triphosphate (ATP) in equilibrium with adenosine diphosphate (ADP) and adenosine monophosphate (AMP). Therefore, no pressure has been exerted to retain *de novo* purine synthetic pathways throughout the evolution of these parasites [287]. Genome sequencing of *P. falciparum* has failed to reveal genes encoding enzymes involved in the biosynthesis of purine nucleosides or nucleobases [288]. Malaria parasites, on the other hand, have evolved to resort to a purine-salvage route, withdrawing purines from the host RBC for cofactors and the synthesis of nucleic acids [289–291]. Purines first cross the plasma membrane of the host iRBC via host purine transporters, such as the hENT1 [292], and the human facilitative nucleobase transporter hFNT1 [293]. While the parasite-encoded NPPs, located at the host cell membrane, do not appear to play a significant role in purine uptake, the innate parasite-specific uptake system serves as the primary route by which purines pass the PPM. Hypoxanthine, the primary source of purines for the parasite, is produced from host-derived adenosine via the *Plasmodium* purine-salvage enzymes and further transported by the PfNT1 transporter [292,294]. On the other hand, the parasite can produce its own pyrimidines from bicarbonate and glutamine [295].

Polyamines metabolism

The naturally occurring polyamines, spermidine (SpD), spermine (SpM), and their precursor putrescine (Putr) are key regulators of several biological processes including cell growth, development, protein synthesis and cell cycle progression. These low molecular weight polycationic organic amines are synthesized from amino acid precursors [296,297] and are particularly abundant in rapidly proliferating cells, including cancer cells and protozoan parasites. Consequently, inhibiting their synthesis has been exploited for antiproliferative strategies [298–302]. In eukaryotes, polyamines are produced from the amino acids arginine, ornithine, and methionine. The diamine putrescine is initially synthesized from arginine, which is then converted into ornithine through the activity of the enzyme arginase. Putrescine is produced through the decarboxylation of ornithine by the action of ornithine decarboxylase (ODC) [296,303–305]. Parallel to putrescine production, methionine is converted into S-adenosylmethionine (SAM), which is subsequently decarboxylated into decarboxylated SAM (dcSAM) by the enzyme SAM decarboxylase (SDC) [296,306]. Spermidine synthesis comprises the addition of an aminopropyl group, supplied by dcSAM, to putrescine - a reaction catalyzed by spermidine synthase (SpdS) [300,307,308]. Lastly, spermine synthase (SpmS) adds a second aminopropyl group to spermidine, resulting in the production of tetraamine spermine [296,300,307]. Polyamine metabolism in *Plasmodium falciparum* is remarkable in that the two rate-limiting enzymes of the pathway, ODC and SDC, are encoded by a single gene, resulting in a unique bifunctional protein with a N-terminal ODC and a C-terminal SDC (Figure 1.13) [309–312].

The regulatory features underpinning ODC and SDC function are also unique among *Plasmodium spp.* as the mechanisms underlying their activity in other species, such as antizyme and prozyme, are lacking in the parasite [304,305]. Although the active sites of each decarboxylase operate independently, their regulation appears to be intertwined: ODC activity is stimulated by the presence of the C-terminal SDC domain, whereas SDC is inhibited by the presence of the N-terminal ODC. This enables for the equimolar synthesis of Putr and SAM [309–311]. It is interesting to note that Putr exhibits regulatory effects through feedback inhibition of *P. falciparum* ODC activity, and its potency in this regard is 10-fold greater than that observed for mammalian ODC. However, in contrast to what has been found for mammalian SDC, Putr does not enhance *P. falciparum* SDC activity [310,313].

Red blood cells are devoid of polyamine biosynthetic machinery and contain only trace amounts of polyamines (5-50 μ M) [261,314]. Serum polyamine concentrations are as low as 0.1 μ M. However, during *P. falciparum* infection, polyamine levels increase, as does the expression of key enzymes involved in polyamine metabolism (including ODC and SDC) [315,316]. Polyamines are key metabolites in *Plasmodium*, accounting for approximately 14% of the total metabolome of isolated parasites, with parasite survival and rapid proliferation critically depending on the availability of polyamines [302,317,318]. The polyamine pool in the parasite is primarily composed of spermidine (SpD), which is synthesized at all stages of parasite development and has a maximum abundance of 6 mM, followed by its precursor Putr at 3 mM [300,316]. However, since the malaria parasite lacks a SpmS, it contains only trace amounts of SpM (0.5 mM) [316].

Unlike its mammalian counterparts [319], which have strong specificity for Putr, the parasite SpdS demonstrates promiscuous activity by accepting also SpD as a substrate, albeit at a much lower extent. Therefore, *Plasmodium* SpdS has not only the capacity of synthesizing SpD but also SpM [300,308]. Contrarily to most organisms, no evidence of polyamine conversion has been found in *Plasmodium*. Thus, polyamine levels in the parasite are regulated by the coordinated expression of the bifunctional SDC/ODC enzyme. Therefore, the metabolic flux relies solely on *de novo* SpdS, which not only regulates SpD [302,320] but also SpM levels [308]. The impact of polyamine depletion in *Plasmodium* development has been extensively studied through inhibition of the bifunctional SDC/ODC enzyme with ODC inhibitors. The most widely used ODC inhibitor is α -difluoromethylornithine (DFMO), which induces a cytostatic effect (rather than cytotoxic) in *P. falciparum*. DFMO treatment significantly reduces Putr levels inside iRBCs, followed by an 80% decrease in SpD levels, while SpM levels remain relatively steady [300,316,321]. Remarkably, addition of exogenous polyamines (Putr or SpD, but not SpM) can reverse this inhibitory effect even in the presence of DFMO; However, such an inhibitory effect is not curative for *in vivo* infections with *P. berghei* [322]. On the other hand, inhibition of SDC activity with MDL 73811 resulted in a significant drop in both SpD and SpM levels, while Putr levels increased three- to four-fold. A similar effect on the growth of *P. falciparum* was observed in parasites treated with the SDC inhibitor, which halted parasite growth at the trophozoite stage [300,323]. However, MDL 73811 had no impact on the progression of parasitemia in *P. berghei*-infected mice [323]. Interestingly, the simultaneous inhibition of both decarboxylases by DFMO and MDL 73811 had an additive rather than a synergistic impact on parasite growth [321,323].

Another approach to disrupt polyamine production and function is to use naturally occurring polyamines [324] or polyamine analogues to block decarboxylase activity [325,326]. These polyamine analogues have shown an inhibitory role on both the *in vitro* development of *P. falciparum* and the *in vivo* growth of rodent malaria *spp.* For instance, treatment with the bis(benzyl)polyamine analogue, MDL 27695, had a significant impact on the course of infection in both species. Interestingly, MDL 27695 demonstrated potent antimalarial activity against both chloroquine-sensitive and resistant *P. falciparum* strains and, in combination with DFMO, was successful in treating malaria in mice [327].

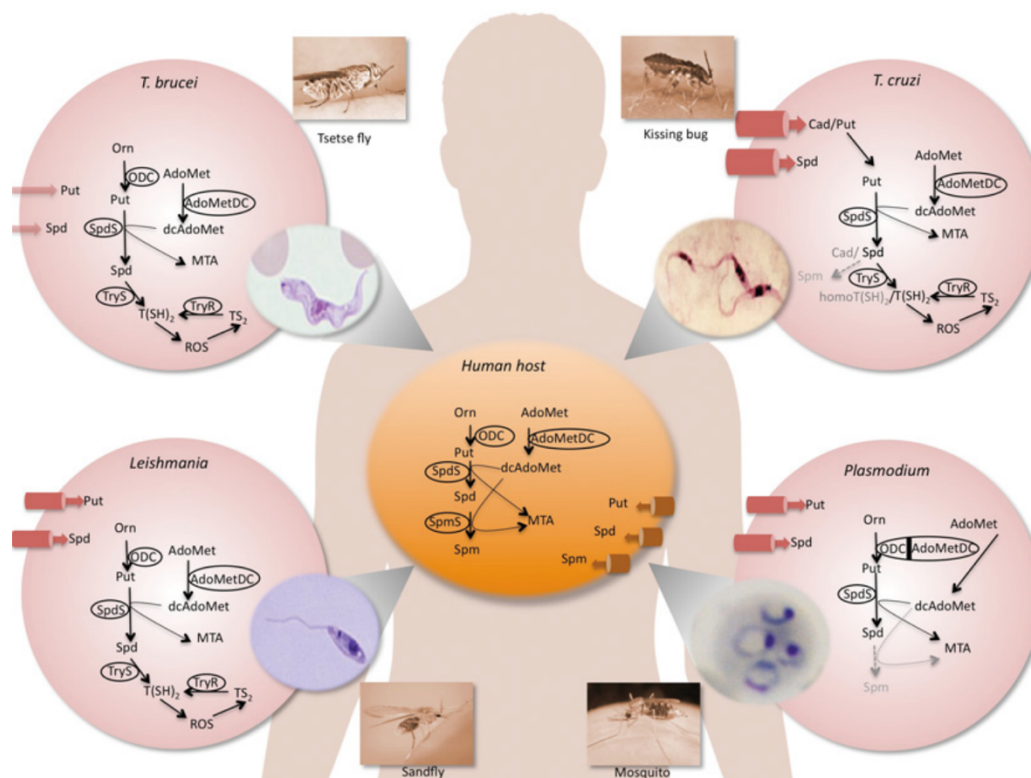


Figure 1.13 - Comparison of polyamine metabolism and transporters in trypanosomatids, apicomplexan parasites and their human host. *T. brucei* is spread by tsetse flies while *T. cruzi* is spread by kissing bugs. These trypanosomatids are responsible for sleeping sickness or Chagas disease. Sand flies transmit *Leishmania spp.*, while *Anopheles* mosquitoes transmit *Plasmodium* parasites. Among them, *Plasmodium* is the only parasite with a bifunctional SDC/ODC enzyme, a critical feature in the regulation of polyamine pools in the parasite. Cad, cadaverine; homoT(SH)₂, homotrypanothione; On, ornithine; Put, putrescine; ROS, reactive oxygen species; Spd, spermidine; Spm, spermine. Adapted from [300].

The Building Blocks of Life: Amino Acid Metabolism

Intra-erythrocytic development is accompanied by a rapid increase in parasite size and number, and therefore the rate of protein synthesis in the parasite must necessarily support such prodigious growth [328]. It is well recognized that the intra-erythrocytic malaria parasite digests the host cell cytosol to acquire amino acids (AAs), the building blocks of proteins that also serve as substrates for many other metabolic pathways [261,329]. Notably, extensive genomic [260] and biochemical [330] analyses in *P. falciparum* have revealed no evidence for a *de novo* AA biosynthetic pathway. Surprisingly, malaria parasites have lost the capacity to synthesize most AAs *de novo*, instead relying on scavenging and breaking down of hemoglobin (Hb). As an auxotroph for most AAs, *Plasmodium* relies on three strategies to secure their AA supply: degradation of host cell Hb [331], active transport of extracellular free AAs through host erythrocytes and parasite membranes [332] and *de novo* biosynthesis from carbon sources (of only a few AA) [265]. Malaria parasites fix CO₂ to synthesize the AAs alanine, aspartic acid and glutamic acid [329,333,334]. It is worth noting that the free amino acid pool of iRBCs is enriched for such AAs [329,335,336] but only a few of these are incorporated into *Plasmodium* proteins, indicating that this route may have served an anaplerotic function by providing AAs as intermediate metabolites to the parasite.

Given the restricted ability for AA biosynthesis, *Plasmodium* relies on the ingestion of plasma-free AAs and host cell Hb for protein synthesis. As the parasite develops inside erythrocytes, it commences to ingest and degrade Hb from the host cell [337]. Hb is consumed via the cytostome and stored in food vacuoles, where it undergoes auto-oxidation in a process involving several proteolytic enzymes [338]. Because unbound heme is highly toxic, the parasite quickly converts it into an inert crystal known as hemozoin (also referred to as the malaria pigment) [329]. It is noteworthy that schizont-stage parasites do not digest host erythrocyte Hb but are actively engaged in protein synthesis [339]. This indicates that protein synthesis in the parasite relies on AAs from the extracellular medium. Interestingly, despite consuming up to 75% of the host cell's hemoglobin [337], the parasite uses less than 20% of the Hb-derived AAs for protein synthesis, with the majority being rather exported to the extracellular medium [330,340]. This has led some to propose that the parasite alternatively digests Hb to make room in erythrocytes [330] or to maintain osmotic balance [341]. However, malaria parasites retain high intracellular concentrations of free AAs [318], which are mainly derived from Hb degradation, but also by selective uptake of AAs from the external medium via plasma

membrane transporters [342–344]. Thus, AA availability for protein synthesis in the parasite is determined by both dynamic contributions from Hb-derived AAs and the inflow of AAs from the extracellular media. This is supported by an inability of *P. falciparum* to sustain long-term growth in medium lacking any of the seven AAs: cystine, glutamate, glutamine, isoleucine, methionine, proline, and tyrosine; nor in medium containing only the five AAs on which is more reliant – the essential AAs cysteine, glutamate, glutamine, methionine and isoleucine [345,346].

Notably, adult human Hb is devoid of isoleucine (Ile) and parasite growth depends on the exogenous supply of this AA [346,347]. Another AA found at very low levels in human Hb is methionine (Met), for which some *P. falciparum* strains require an external source to sustain normal growth [331,347]. However, *Plasmodium* parasites are capable of incorporating all the 20 naturally occurring AAs into proteins [342,346,348,349]. In fact, parasitized erythrocytes incorporate exogenously supplied AAs at an accelerated rate compared to uninfected erythrocytes. This is consistent with the existence of AA transport pathways in erythrocytes that coordinate both AA influx and efflux [329]. The L-system serves as a saturable carrier for Ile and Met in healthy human erythrocytes [350–352]. Remarkably, the parasite modulates host cell permeability, which gradually but significantly increases as the parasites advance through the intra-erythrocytic asexual cycle [332]. Compared to uninfected cells, the transport rate of Ile and Met into human erythrocytes infected with *P. falciparum* trophozoites is dramatically enhanced, approximately 5- [343] and 15-fold [344], respectively. Such an increased rate of AA influx is attributable to the NPPs induced by the parasite in the host erythrocyte membrane [343], either by activating endogenous quiescent channels in the host cell; or by parasite-encoded proteins that are synthesized, exported, and incorporated into the erythrocyte membrane (Figure 1. 14) [353,354]. NPPs are broad specificity pathways permeable to a variety of low molecular weight organic and inorganic solutes, whose activity is blocked by various anion transport inhibitors such as furosemide [353–355]. Transport through these channels is key to ensure that the rate of protein synthesis is not limited by the AA flux across the erythrocyte membrane. Met and Ile enter the parasitized erythrocytes via a combination of the endogenous erythrocyte transporter, the L-system, and the parasite-induced NPPs, the latter accounting for approximately 80% of the total AA influx [343].

Upon entering the infected erythrocyte, both Ile and Met are uptaken by the parasite via the Neutral Amino Acid Transporter (NAAT). NAAT has broad specificity but low affinity and functions by importing neutral AAs that are poorly represented in Hb, such as Ile and Met, while exporting those that are highly represented in Hb, such as alanine and leucine [343,344,356]. The *P. falciparum* genome encodes at least six putative candidate AA transporters [357] that are expressed during blood stage development [358,359]; However, the molecular identity of such AA transporters as well as their substrate specificity and localization are yet to be explored.

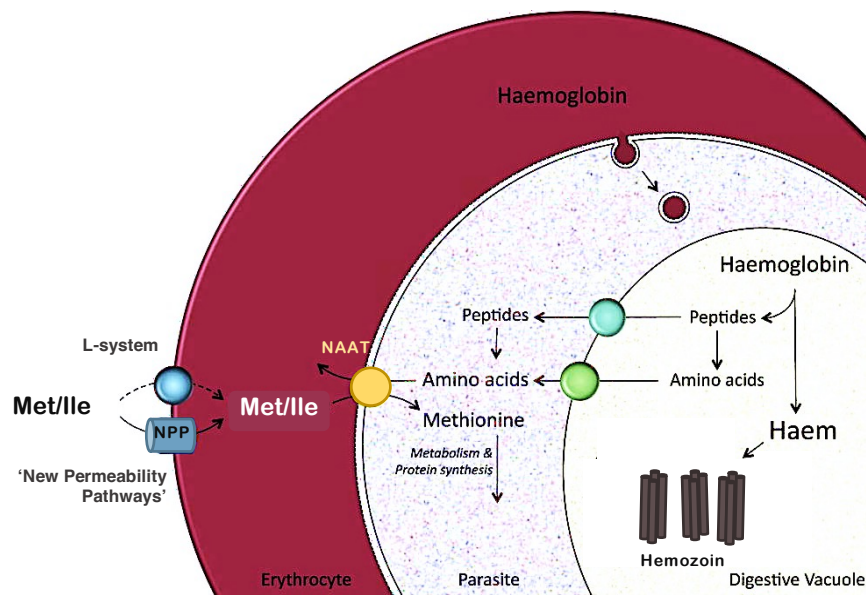


Figure 1.14 - Schematic representation of the transport of methionine and isoleucine into *Plasmodium*-infected erythrocytes. Met and Ile enter the infected erythrocyte via a combination of the endogenous L-system and the New Permeability Pathways (NPPs) induced by the parasite in the host cell membrane, the latter mediating 80% of the total AA influx. Adapted from [320].

III. Food For Thought: Nutrient Sensing & Metabolic Decisions

Infection and (mal)nutrition have always been intricately connected, with fluctuations in host nutritional status, be it in quantity or quality, having a substantial impact on the dynamics of infectious diseases. In recent decades, nutrition has been shown to be an important environmental component affecting host vulnerability to infection as well as host immunological response [360]. Malnutrition is, in fact, the most important environmental factor leading to immunosuppression and increased susceptibility to infections. In humans, for instance, insufficient protein intake is often associated with impairment of cell-mediated immunity, phagocyte function and cytokine production [361]. Dietary availability of vitamins and metals may also have a deleterious effect on immune system function and susceptibility to infection [360]. Nutrients are simple organic molecules that either fuel biochemical reactions to generate energy or are components of cellular biomass. Macronutrients include carbohydrates, amino acids, and fatty acids, whose major function is to provide energy, as well as structural components critical for growth and development. Instead, micronutrients are those dietary components that are required in small amounts, provide no significant calorie intake, but are nonetheless critical to vital cellular functions. These mostly consist of vitamins (both fat and water soluble) and minerals, each serving a specific function [362]. The intracellular lifestyle of protozoan parasites brings some benefits to the parasite, but it also poses some hurdles, especially regarding nutrient uptake due to the parasite's high replication rates. The main requirements of *P. falciparum* include metabolites such as glucose, lipids, and amino acids and so parasite growth is dependent on the external supply of macro- and micronutrients from its host cell. These include pantothenate, glucose, calcium and isoleucine, although other amino acids have been shown to enhance parasite growth rates [331,346,363]. The parasite tackles some of these challenges by inducing NPPs in the erythrocyte membrane. Furthermore, parasite growth is sensitive to changes in the availability of metabolites such as vitamins and metal ions, namely iron and zinc levels in the host [364,365].

Sensing Changes in Host Nutritional Levels

The Apicomplexa phylum includes a broad spectrum of parasitic protozoa that evolved from a free-living ancestor to obligate intracellular species. As an intracellular parasite cycling between distinct hosts (the mammalian host and the insect vector), *Plasmodium* faces a variety of nutritional and metabolic environments, particularly during host transitioning stages. Consequently, malaria parasites evolved mechanisms to withstand a myriad of stressors including pH shifts, extreme temperatures (fever), variable parasite densities, nutrient fluctuations, intracellular and extracellular lifestyles, and also drug exposure [366–368]. All these environmental pressures are known to impact virulence while also contributing to the emergence of drug-resistant *P. falciparum* strains. In addition, while inhabiting distinct environments, the parasite must fulfil diverse biological functions, including asexual multiplication, fertilization and meiosis, invasion, and gliding. This diversity emphasizes the extremely versatile metabolic capabilities of malaria parasites, which entails important morphological changes achieved through coordinated regulation of expression of the 5500-6000 genes that make up the *Plasmodium* genome [369].

Most parasite genes are selectively expressed at specific stages of intra-erythrocytic development, resulting in stage-specific transcriptional signatures [359,370,371]. The same applies for the sexual stages, where a unique set of genes is transcribed in male and female gametocytes. The transcriptional profile of mosquito-specific genes is still unexplored, but recent evidence suggests a unique gene expression profile for each developmental stage - consistent with the physiological changes and the distinct environments the parasite encounters [369]. To cope with the different environments that its life cycle entails, *Plasmodium* mainly relies on the expression of distinct transcriptomes at each developmental stage [358,359,370]. Aside from the ever-changing environment that parasites encounter during their regular life cycle, the environment within a single host is also extraordinarily dynamic, with parasites often being exposed to variable immune responses, drug pressures and metabolic fluctuations [364]. Nutritional fluctuations can be encountered during invasion of distinct tissues or cell types, as parasites evade host immune surveillance or when exposed to induced drug pressure [242,372,373]. Even throughout the blood stage of infection, when developing inside erythrocytes, *Plasmodium* parasites encounter a variety of nutritional and metabolic environments. The level of certain metabolites is not stochastic, but changes during maturation from young reticulocytes to mature erythrocytes [374].

In addition, the parasite itself affects the metabolome of the infected erythrocyte in such a way that the levels of some metabolites significantly change over the course of the intra-erythrocytic cycle [261,375]. With such a complex multi-host life cycle, the parasite must constantly be able to recognize and adapt to the different environments in which it resides. Since the parasite relies on its hosts for nutrient supply, it is not unforeseen that the mechanisms used to detect resource fluctuations have been conserved throughout evolution, allowing for adaptability to available resources in a wide range of environments.

Sensing environmental cues and responding to fluctuations in nutritional and metabolic levels are critical for virtually all living organisms to maintain homeostasis and ultimately thrive. The ability to quickly rewire its metabolism is at the heart of the parasite's adaptability to distinct environments. A prime illustration of this is the fact that asexual blood stages rely heavily on glycolytic metabolism for energy production, while mosquito stages rely on the tricarboxylic acid cycle (TCA) for energy supply [372]. The nutritional adaptability of malaria parasites is notorious when moving from the mammalian to the mosquito vector, as *Plasmodium* gametocytes undergo gametogenesis to produce male and female gametes. This differentiation step is triggered by the parasite's ability to sense and respond to a drop in temperature, a rise in pH, and xanthurenic acid, a mosquito-specific factor [242]. Even within the same host, metabolites such as glucose are known to decline during infection, leading to severe hypoglycemia (blood glucose < 50 mg/dl) [376], emphasizing the need for efficient mechanisms that detect and swiftly respond to any alterations within and across hosts. Such an adaptive response could entail modulation of either replication or a differentiation program. It is important to highlight that such sudden molecular and cellular reprogramming can only be achieved by certain parasite stages that are molecularly primed to elicit differential stress responses [377].

A prime example is the transcription of translationally repressed mRNAs originally transcribed in female gametocytes after fertilization in the mosquito midgut [378]. Similar mechanisms support the developmental transition from the mosquito vector back to the mammalian host, with sporozoites remaining viable and infective but quiescent in the mosquito's salivary glands for weeks before invading the mammalian host's liver. Sporozoite latency has been shown to be regulated by Puf2, a RNA-binding protein that prevents premature conversion of sporozoite into liver stage forms while in the mosquito's salivary glands [379]. Kinases, a class of regulatory proteins, are responsible

for orchestrating these rapid life-stage changes. Therefore, at the foundation of a mechanism that enables parasites to adapt and thrive in distinct environments, there must be a molecular sensor capable of recognizing and conveying information so that an adaptive response occurs.

Sensing low energy levels: KIN as a glucose sensor

Plasmodium asexual blood stages primarily use glucose as a carbon source to fuel aerobic glycolysis and generate ATP [380]. Recent evidence shows that malaria parasites can sense fluctuations in the nutrient levels of the mammalian host, while exploiting them to sustain growth and survival [373]. In fact, the extensive proliferation rates that the parasite achieves in both the liver- and blood stages of its development primarily rely on host glucose availability. Even though dietary glucose levels are tightly regulated through homeostatic mechanisms, blood glucose levels can drop as low as 0.4mg/mL (versus 0.63 to 1mg/mL reference values) in malnourished children or patients with severe malaria [261,365]. Caloric restriction (CR), consisting of a 30-40% reduction in calorie intake, results in a drastic reduction in blood glucose levels, but also in lipid and insulin levels [365]. At the parasite level, CR impacts intra-erythrocytic replication, with significantly fewer merozoites being produced per schizont compared to *ad libitum* conditions [373]. Interestingly, analysis of global gene expression profiles of synchronized *P. berghei* parasites revealed a transcriptional reprogramming under such a regimen. Processes such as ion transport, DNA replication, and cell cycle, which are closely related to parasite maturation and replication, were found to be suppressed. In contrast, processes involved in gene regulation and signaling (including some kinases), were markedly activated.

The CR-induced reduced growth phenotype is consistent with a halt in the mechanisms necessary for intra-erythrocytic development and an adaptive response to nutrient-limiting conditions, as reported in other species [381]. A screen of kinase mutant knockout lines encompassing the *P. berghei* non-essential kinome [382] disclosed that the parasite *PbKIN* kinase, who shares homology with the yeast and mammalian SNF1/AMPK α kinases, is a master regulator of energy levels in the parasite (Figure 1.15). In fact, when the protein kinase KIN was genetically inactivated, the reduction in merozoite yield per schizont in response to CR was rescued. Interestingly, KIN knockout mutants failed to trigger a transcriptional shift in response to CR, demonstrating that *PbKIN* plays a crucial role as a link between energy sensing and gene expression.

Furthermore, adding glucose, albeit in non-physiological levels, abolished the impact of CR on parasite growth as well as the associated transcriptional rewiring of gene expression. CR thereby induces a protective transcriptional response in the parasite mediated by the nutrient sensor KIN, which is activated under nutrient-limiting conditions, eventually leading to decreased expression of replication-related genes and attenuated proliferation and virulence [373]. Although the nutrient sensor of this energy sensing pathway has been identified, the transcription factors driving this response are still unclear.

Sensing lipid levels: The sexual commitment switch

Plasmodium heavily relies on lipid uptake and synthesis while replicating within RBCs. This is essential to support the increased membrane biogenesis and facilitate intracellular signaling. Phosphatidylcholine (Ptd-cho) is the chief phospholipid in the parasite membrane, which is synthesized from lysophosphatidylcholine (LysoPC) [383–386]. LysoPC in the host serum has been shown to be a key nutrient for the parasite, as the breakdown of this lipid or its downstream metabolites (e.g., choline) strongly impacts both the parasite's asexual growth and commitment to sexual development. While sexual commitment is repressed under high serum levels of LysoPC (and high choline levels), low levels of LysoPC trigger parasite's sexual differentiation [386]. RNA-Seq analysis of parasite gene expression upon LysoPC depletion unveiled significant changes in two key transcriptional programs: the induction of genes comprising an alternative pathway for Ptd-cho synthesis and an upregulation of *Pfap2g* gene - the master regulator of sexual commitment (Figure 1.15). Moreover, depletion of LysoPC leads to the overexpression of genes involved in the SAM-dependent phosphoethanolamine methyltransferase (PMT) pathway [386], an alternative pathway in the parasite for Ptd-cho synthesis under choline-limiting conditions [387–389]. Single cell analysis of *P. falciparum* has shown that after LysoPC depletion, PMT is upregulated in all parasite stages irrespective of sexual commitment rates; However, the LysoPC sensor as well as the transcription factor that drive such a metabolic reprogramming have not been identified [385,386].

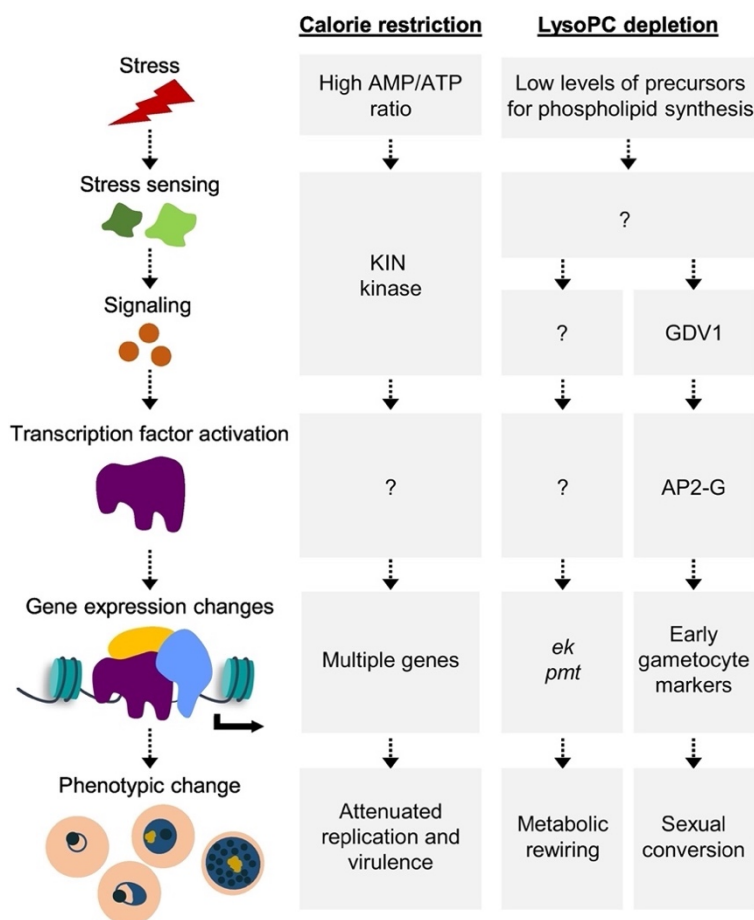


Figure 1.15 - Diagram highlighting the main players of directed transcriptional responses to nutrient fluctuations in *Plasmodium* spp. The steps of a canonically directed transcription response are seen on the left. The most important parasite elements involved in the response to calorie or LysoPC depletion are shown. A question mark indicates that the related factors were not identified [390].

Mechanisms of Growth Control and Stress Response

Cellular responses to stress encompass a range of mechanisms, including activation of survival-promoting signaling pathways and initiation of programmed cell death [391]. Stress response mechanisms allow organisms to adapt to harsh conditions and are primarily geared to ensure recovery and survival. Cell survival is critically dependent on the ability to orchestrate appropriate responses to environmental perturbations, which is why such survival mechanisms have been largely conserved throughout evolution.

Malaria parasites use a variety of strategies to evade host immune responses and assure successful infections [364]. Commitment to gametocytogenesis is a well-established stress response mechanism in the parasite that is regulated by a variety of external variables, including nutritional status, oxidative stress, parasitemia, and drug treatment [392–394]. Conversion to terminally differentiated and metabolically inactive sexual parasite forms affords protection from unfavorable host environmental conditions, such as host immune responses and drug treatment [395,396]. Asexual blood stages are also

vulnerable to host temperature fluctuations, starvation, and oxidative stress, particularly in the invasive merozoite stage when the parasite dwells extracellularly [397]. To cope with such unpredictable fluctuations within a single niche, parasites evolved alternative strategies, including efficient sensors and tight regulation of nutrient-sensing signaling pathways [365,373,386]. Therefore, responding to environmental stress and surviving in suboptimal circumstances is imperative for viability, and blood stage parasites must have evolved alternative and more stringent strategies to survive periods of AA scarcity.

TOR complex: A central controller of cell growth

The Target of Rapamycin (TOR) is an evolutionarily conserved signaling protein kinase found in virtually all eukaryotic organisms. Cell growth is a temporally and spatially regulated process, and it must be closely coordinated with nutrient availability. At the heart of this coordination is the TOR complex, which acts as the master regulator of growth and integrates a variety of extracellular and intracellular cues. The TOR complex controls both anabolism and catabolism by regulating numerous metabolic processes, including protein translation, ribosome biogenesis and assembly, mRNA stability, cell cycle, transcription and cellular architecture [398]. The TOR complex functions as a signaling network that, in response to growth-permissive conditions, promotes anabolic processes such as protein, lipid, and nucleotide synthesis while suppressing catabolic processes such as autophagy, to ultimately enhance cell growth and viability. TOR is activated by several factors including AAs, cellular energy levels and growth factors, but it is also adversely regulated by a number of stressors, particularly AA deficiencies [399,400]. AA deficiency has been shown to inhibit TOR activity by triggering rapid dephosphorylation of TOR-downstream effectors. This culminates in the downregulation of protein synthesis and the promotion of autophagy, which helps preserve cellular integrity and ensures adaptability to the constantly changing environment [399].

The phosphatidylinositol kinase-related kinase (PIKK) family, to which the highly conserved TOR protein belongs, is characterized by a carboxy-terminal serine/threonine protein kinase domain. A mutation in *Saccharomyces cerevisiae* that conferred resistance to the growth inhibitory effects of rapamycin contributed to the discovery of the TOR protein [401]. The immunosuppressive antibiotic rapamycin inhibits the growth-promoting function of TOR by interacting and binding to the cellular cofactor FK506-binding protein (FKBP) [402]. Notably, FK506-binding proteins have been conserved throughout evolution and are found across Apicomplexan parasites including *T. gondii*

[403] and *Plasmodium spp.* [404]. Even though a putative TOR complex was discovered in the *T. gondii* parasite, which is closely related to *Plasmodium*, genomic sequence data failed to identify a TOR homologue or its signaling components in *Plasmodium* parasites. The lack of such an essential growth regulator is particularly intriguing considering that rapamycin has demonstrated significant antimalarial activity, albeit via a TOR-independent mechanism [404].

eIF2 α -signaling pathway: A link between nutritional stress and adaptation

Stress-induced phosphorylation of a conserved serine residue (Ser 51) in the α -subunit of the eukaryotic initiation factor 2 (eIF2 α) serves as the central mechanism regulating translation in response to various stressors, such as nutrient limitation and particularly amino acid deficiencies [405–407]. When eIF2 α is phosphorylated (eIF2 α -P) by its cognate sensor kinase, it undergoes conversion from a substrate to a competitive inhibitor of guanine nucleotide exchange factor (eIF2B). This curbs the initiation phase of protein synthesis and leads to a global halt in translation, allowing cells to conserve resources while a new gene expression program is rewired. Simultaneously, translation of a subset of mRNAs encoding transcription factors engaged in compensatory signaling pathways, such as those involved in amino acid biosynthesis, is enhanced [408,409].

In eukaryotes, eIF2 α phosphorylation is carried out by a well-characterized family of four distinct serine-threonine kinases known as eIF2 α kinases: PKR (Protein kinase R / RNA-activated protein kinase); PERK (PKR-like endoplasmic reticulum kinase); HRI (heme-regulated inhibitor); and GCN2 (general control non-derepressible-2) [410,411]. Each primarily responds to a specific type of stress by curtailing general translation while driving programs of stress-induced gene expression [411,412]. PERK is primarily activated by the accumulation of misfolded proteins in the endoplasmic reticulum (ER). As a result, PERK phosphorylates eIF2 α , resulting in inhibition of new polypeptide synthesis and a subsequent reduction in the entry of nascent peptides into the ER [413]; PKR was first discovered as the kinase that phosphorylates eIF2 α in response to viral infection, thereby preventing viral mRNA translation [413]; However, it also responds to other signals such as oxidative and ER stress [414,415]; The HRI kinase is activated by heme deficiency and couples the synthesis of globin genes to the amount of available heme in the cell [416]; GCN2 is the only eIF2 α kinase conserved in all eukaryotes [411] and functions as a sensor for AA availability and as the primary responder to AA deficiency [407,417]. Until activated by a signal such as AA deficiency or UV radiation

[416], GCN2 is kept in its inactive state via multiple autoinhibitory interactions [418–421]. GCN2 activation occurs in AA-depleted cells by binding of uncharged transfer RNAs (tRNAs) to the histidyl-tRNA synthetase (HisRS) domain of this kinase. Uncharged tRNAs accumulate in response to a shortage of essential AAs and also when the synthesis of nonessential AAs is inhibited [422]. Dimerization is required for GCN2 activation, as it is for other eIF2 α kinases [418], while autophosphorylation appears to be required for optimal kinase activity [423]. GCN2-mediated eIF2 α phosphorylation in AA deficiency induces a global halt in mRNA translation, thereby sparing energy and nutrients, while allowing the cell time to successfully adapt to stress conditions [400,405,417,424].

In *Plasmodium* parasites, three eIF2 kinases, namely eIK1 (PF3D7_1444500), eIK2 (PF3D7_0107600), and PK4 (PF3D7_0628200) have been found to mediate eIF2 α phosphorylation at Ser59 [425]. This phosphorylation site is conserved across *Plasmodium* species and corresponds to the regulatory Ser51 found in human eIF2 α [426]. While eIK1 and PK4 are predominantly active in asexual blood stages, eIK2 primarily functions in mosquito salivary glands during sporozoite development. It is worth noting that eIK2-mediated eIF2 α phosphorylation plays a crucial role in preventing the conversion of sporozoites into liver stage forms in mosquito salivary glands [427]. Furthermore, because the genetic locus encoding for this kinase could not be disrupted, the *P. falciparum* PK4 kinase was identified as critical for blood stage development [428]. While the exact function of PK4 is still elusive, eIF2 α phosphorylation by this kinase is essential for the completion of the erythrocytic cycle during schizont and gametocyte development [426]. On the other hand, eIK1, the third eIF2 α kinase orthologue discovered in *Plasmodium*, closely cluster with the mammalian nutrient sensor GCN2 kinase and promotes eIF2 α phosphorylation in response to AA starvation [429].

Artemisinin resistance: an active stress response mechanism

Artemisinin and its derivatives (ARTs) stand at the forefront of malaria treatment, rapidly eliminating parasitemia; yet, ART monotherapy often leads to relapses. Reduced sensitivity to this antimalarial has been identified as a drug tolerance mechanism orchestrated by the parasite to withstand drug pressure. At the heart of parasite resistance to ART lies a metabolic shift, with a portion of the drug-exposed parasite population entering a dormant state (also referred to as hibernation) as persistent forms. During this dormancy-like state, the parasite halts in the ring stage [430]. However,

parasites quickly resume growth when drug therapy is discontinued. The parasite re-enters the cell cycle and continues its asexual developmental cycle, leading to recurrent malaria infections [431]. This concept of stress-induced dormancy implies that *P. falciparum* has the capacity to sense external environmental cues and actively fine-tune its growth accordingly.

Transitioning to a quiescent state is a multifaceted process that involves the regulation and coordination of virtually all aspects of metabolism and often affects critical cellular functions, including energy production, cell cycle progression, replication, transcription and translation [432]. Even though the ART-induced quiescence mechanism of *P. falciparum* is not fully understood, stress-induced dormancy has been found in other species, including the related parasite *T. gondii*, the causative agent of toxoplasmosis [433]. Notably, the ability of *T. gondii* to enter a quiescent state allows for the establishment of persistent and chronic infections. After exposure to stressors, the actively growing tachyzoite undergoes a metabolic reprogramming that leads to the conversion into a quiescent bradyzoite form. This shift is accompanied by a reduction in cell cycle activity, upregulation of heat shock proteins, and translational repression. Among the various environmental factors shown to trigger this conversion from tachyzoites to bradyzoites, amino acid deficiency has a well-established impact on cell growth regulation in higher eukaryotes [397,433].

The stress-induced dormancy model: adaptation to low AA levels

Malnutrition and infection are closely linked and have a synergistic relationship, each promoting the other. This connection is particularly evident in the context of human malaria infections, with malaria being more prevalent in regions where malnutrition prevails [434]. It is worth noting that the nutritional status of malaria-infected individuals influences the severity and onset of the disease [435]. Malnutrition encompasses various nutritional deficiencies, including iron, vitamins, zinc, folate, and proteins, all of which impact parasite burden. This occurs either through direct competition with the host for limited resources or indirectly by impacting the host's immune response [436–438]. Although human clinical studies of malaria-infected patients have yielded conflicting results regarding the effects of protein malnutrition on mortality [439,440], research in model organisms has shown that low-protein diets significantly reduce parasite burden and virtually eliminate cerebral malaria [441–443].

Interestingly, the addition of just four essential amino acids: threonine, valine, methionine, and isoleucine, to mice fed on a low-protein diet was sufficient to elicit an increase in parasitemia, significantly heightening the severity and morbidity of the disease. Such an effect could not be observed with any of the other amino acids [444]. Importantly, rodent hemoglobin (Hb) contains all of the amino acids, including methionine and isoleucine; however, these are the two least represented AAs [445]. Adult human Hb, on the other hand, contains no Ile, and parasite development has been shown to depend on an external supply of this AA at concentrations above 20 μ M [331,346]. In fact, Babbitt *et al.* demonstrated that Ile deficiency causes trophozoite parasites to cease the cell cycle before DNA synthesis begins [446]. The failure to discover any molecular factors influencing parasite recovery after Ile starvation has led to the assumption that malaria parasites cannot adapt to Ile starvation. Instead, malaria parasites enter a state of hibernation, exhibiting low metabolic activity until Ile levels are restored (Figure 1.16A).

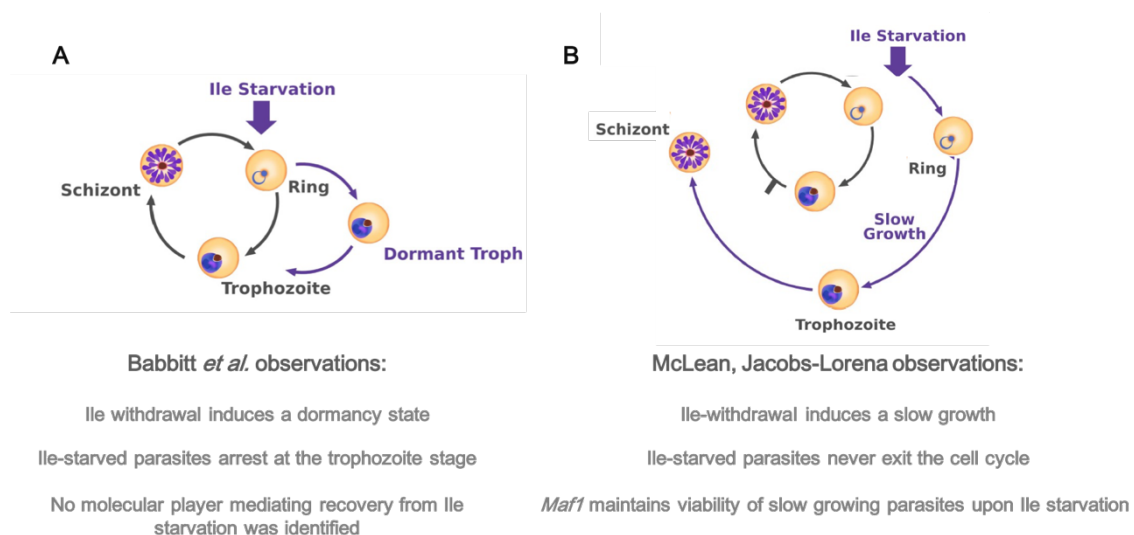


Figure 1.16 - *P. falciparum* response to Ile starvation. **A.** Following Ile removal, *P. falciparum* exits the cell cycle and arrests at the trophozoite stage. Ile-arrested parasites remain viable for several days and resume growth upon Ile supplementation. **B.** Ile withdrawal from young rings results in delayed development. Parasites successfully complete the cell cycle, albeit at a reduced pace. Adapted from [447].

While this response does not completely recapitulate the dormancy model established for artemisinin resistance, it represents the first clear evidence that parasite growth is modulated in response to environmental fluctuations. However, in a parallel line of research, the Ile-induced hibernation phenotype was re-examined, revealing that Ile-starved parasites do not actually exit the cell cycle but instead continue to grow at a slower pace [448]. Remarkably, *Maf1*, a downstream effector of TOR, was identified as a key player in maintaining parasite viability after Ile withdrawal (Figure 1.16B).

Met, the least abundant AA in adult human Hb, has also been shown to be necessary for the normal development of certain, but not all, *P. falciparum* strains [331,347]. Malnutrition often leads to a significant drop in human plasma AA levels, with Ile levels falling below 10 μ M in malnourished children [449], as opposed to 100 μ M in healthy individuals [450]. Additionally, Met levels can decrease to as low as 5-10 μ M in malnourished children [449,451] compared to 45-90 μ M in healthy individuals [450]. Since these AAs are essential for mammals, their decreased availability is closely related to protein restriction [265]. Additionally, since malaria-infected individuals are likely to experience other nutritional deficiencies, the impact of malnutrition on parasite development and illness progression is yet to be fully understood. However, previous studies on rodents and primates under protein-restricted conditions suggest that malaria parasites may have evolved AA-sensitive growth control mechanism which implies the existence of a conserved stress response mechanism in *Plasmodium*.

THESIS AIMS

Thesis Aims & Scope

The rate and efficiency of parasite replication and growth play a critical role in the pathogenesis of malaria, as high parasite burdens are closely associated with the most severe disease outcomes. Although the life cycle of malaria parasites has been known for over a century, its intricacies remain a mystery - a knowledge gap that may prove crucial in tackling the disease. The life cycle of *Plasmodium* is complex and includes invasive, trophic, and replicative forms in two distinct hosts. Nutrients, the most fundamental source of energy in virtually all living organisms, seem to hold the key to such a complex life cycle. Malaria parasites develop extremely rapidly in the liver stage of infection, attaining replication rates of up to 10^4 to 10^5 -fold and generating thousands of new parasites by the end of hepatic schizogony [452,453]. Nonetheless, the underlying factors that enable such outstanding growth are still unknown, as is the relationship between such a rapid rate of proliferation and the exclusive tropism of the mammalian infective *Plasmodium* species to the liver. Subsequent development in the blood involves a remarkable series of morphological changes as the parasite matures through the ring, trophozoite and schizont stages and finally ruptures to release up to 32 new daughter merozoites.

To support its rapid rate of multiplication and meet its metabolic needs, *Plasmodium* has evolved a remarkable array of strategies to scavenge nutrients from the host hepatocytes [279,452–455], erythrocytes, and in some cases the serum [330,331,446,456]. *Plasmodium* parasites are entirely dependent on the host for resource acquisition as they are auxotrophs for most nutrients, including amino acids, the building blocks of proteins. A landmark study published in 2012 by Babbitt *et al.* [446] disclosed a dormancy-like state of low metabolic activity in *Plasmodium* parasites starved for the essential amino acid isoleucine (the sole AA lacking in adult human hemoglobin), suggesting that malaria parasites cannot actively adjust their growth in response to nutrient fluctuations. The inability to uncover a genetic factor or biochemical pathway coordinating such a response prompted several researchers to adopt a hibernation model to explain *Plasmodium falciparum*'s ability to withstand nutrient scarcity.

However, a divergent line of research has shown that after isoleucine withdrawal, *Plasmodium* parasites do not exit the cell cycle but instead continue to grow at a drastically reduced pace, with parasite viability relying on *Maf1* activity - a protein whose regulation is TOR-dependent [457]. It is not yet known whether malaria parasites

recognize and adapt to changes in AA levels in the absence of a TOR signaling pathway (the master regulator of growth in eukaryotes) and the downstream transcription factors underlying GCN2 action (the amino acid sensing kinase).

At the inception of this thesis, we established several goals. First, we aimed at establishing the role of AA metabolism throughout the parasite life cycle, focusing on isoleucine and methionine, the only AA missing and the least abundant, respectively, in adult human hemoglobin. To achieve this, we employed a transgenic parasite line knockdown for SAMS and a genetically engineered mouse line knockout for MAT, each corresponding to the first enzyme in the methionine cycle in *Plasmodium* and mammals, respectively; as well as several growth media conditions lacking methionine or isoleucine. Second, we sought to unveil whether malaria parasites evolved efficient sensing mechanisms to detect and adapt to AA fluctuations, and if so, to uncover the molecular determinants underlying such an active response. Finally, we wanted to determine whether methionine and lipid metabolism are linked in *Plasmodium* parasites, specifically through the SAM-provided methyl groups, and whether the rate of sexual commitment is affected by metabolic competition for these methyl groups. These objectives were achieved by using a variety of methodological approaches and experimental tools, including established *in vitro*, *ex vivo*, and *in vivo* infection models, rodent transgenic models, real-time quantitative PCR techniques, immunofluorescence microscopy, flow cytometry, immunoblotting analysis, thin layer chromatography analysis, and LC-MS based metabolomics to name a few.

Chapter II focuses on establishing how *Plasmodium* intra-erythrocytic stages respond to AA deficiency, particularly isoleucine or methionine, with a particular emphasis on cell cycle progression and replication rates. To elucidate the molecular mechanisms and the key players underpinning the parasite's active response to low AA levels, a kinome screen of 15 *P. berghei* lines with loss-of-function mutations in protein kinase genes was performed. Comprehensive phenotypic analysis of these mutants provided pioneering evidence for an AA-sensing pathway in the parasite that is critical for a prompt and suitable adaptation to AA deficiency during intra-erythrocytic development.

Chapter III examines the role of AA metabolism during the elusive and asymptomatic pre-erythrocytic stage of *Plasmodium* development, with a particular focus on methionine - which is primarily metabolized by the liver and converted to SAM. This chapter provides new insight into one of the most vexing questions in the malaria field: how does the

exclusive hepatic tropism of mammalian malaria parasites relate to their astonishing ability to generate thousands of new parasites in the liver? By extensive examination of parasite and host responses to methionine depletion, as well as its downstream products, we concluded that host-derived methionine and SAM are an important driving force underlying the parasite's ability to achieve such remarkable proliferation rates in the liver.

Chapter IV takes a slight detour and examines how methionine and lipid metabolism overlap during sexual commitment and differentiation. Using LC-MS-based metabolomic approaches, we explore how changes in intracellular SAM levels, specifically variations in methyl group ratio, influence histone methylation levels and, subsequently, the parasite's sexual commitment rate.

These findings provide new mechanistic insights into how metabolic pathways are regulated and interconnected in malaria parasites, which could thereby represent suitable targets for malaria prophylaxis, transmission-blocking strategies, or even as preventive or therapeutic options for ongoing blood stage infections. This opens new avenues for the development of new interventions strategies by converting highly replicating and virulent parasites into slow-growing and non-replicating forms, previously shown to be protective in humans [458].

CHAPTER II

CHAPTER II

A non-canonical sensing pathway
mediates *Plasmodium* adaptation to AA deficiency

The Chapter II: “A non-canonical sensing pathway mediates parasite adaptation to amino acid deficiency” consists of a first authorship publication. Here is clarified the contribution of each of the authors:

Publication:

Inês M. Marreiros^{1,2}, Sofia Marques¹, Ana Parreira¹, Vincent Mastrodomenico³, Bryan C. Mounce^{3,4}, Chantal T. Harris^{5,6}, Björn F. Kafsack⁵, Oliver Billker⁷, Vanessa Zuzarte-Luís¹ and Maria M. Mota^{1*}

¹ Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal.

² Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal.

³ Department of Microbiology and Immunology, Stritch School of Medicine , Loyola University Chicago, Maywood, Illinois 60153, United States.

⁴ Infectious Disease and Immunology Research Institute, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153, United States.

⁵ Department of Microbiology and Immunology, Weill Cornell Medical College, New York, USA.

⁶ Immunology & Microbial Pathogenesis Graduate Program, Weill Cornell Medicine, USA.

⁷ Molecular Infection Medicine Sweden, Molecular Biology Department, Umeå University, Umeå, Sweden

Inês M. Marreiros, the candidate for the PhD degree at the University of Porto contributed to the paper by first designing the project and developing a transgenic parasite line critical for the project. Inês also established continuous *in vitro* *P. falciparum* cultures in the laboratory, which enabled the functional characterization of key metabolites and enzymes/kinases during intra-erythrocytic development. Inês first observed that depletion of specific amino acids leads to reduced parasitemia – an outcome of decreased parasite replication and delayed development, as observed by light and fluorescence microscopy as well as by flow cytometry analysis. She screened 15 *P. berghei* lines with loss-of-function mutations in protein kinase genes and identified several protein kinases involved in an active parasite response to amino acid scarcity. Inês also prepared parasite samples for thin layer chromatography (TLC) analysis in order to measure endogenous polyamines.

The contribution of each author to the publication is clarified in the section “Authors contribution” as follows: “**I.M.M.**, V.Z.L. and M.M.M. conceived and led the study. **I.M.M.** conducted all the experiments with the assistance of V.Z.L, S.M. and A.P. V.M. and B.C.M performed the TLC analysis. C.T.H. and B.F.K generated the *P. falciparum* sams-glmS transgenic parasite line. O.B. contributed with conceptual input, manuscript reviewing and interpretation. **I.M.M.** and M.M.M. wrote the manuscript”.

Published in *Communications Biology*. First published online on Feb 21st 2023.
<https://doi.org/10.1038/s42003-023-04566-y>

Abstract

Eukaryotes have canonical pathways for responding to amino acid (AA) availability. Under AA-limiting conditions, the TOR complex is repressed, whereas the sensor kinase GCN2 is activated. While these pathways have been highly conserved throughout evolution, malaria parasites are a rare exception. Despite auxotrophic for most AA, *Plasmodium* does not have either a TOR complex nor the GCN2-downstream transcription factors. While Ile starvation has been shown to trigger eIF2 α phosphorylation and a hibernation-like response, the overall mechanisms mediating detection and response to AA fluctuation in the absence of such pathways has remained elusive. Here we show that *Plasmodium* parasites rely on an efficient sensing pathway to respond to AA fluctuations. A phenotypic screen of kinase knockout mutant parasites identified nek4, eIK1 and eIK2 - the last two clustering with the eukaryotic eIF2 α kinases - as critical for *Plasmodium* to sense and respond to distinct AA-limiting conditions. Such AA-sensing pathway is temporally regulated at distinct life cycle stages, allowing parasites to actively fine-tune replication and development in response to AA availability. Collectively, our data disclose a set of heterogeneous responses to AA depletion in malaria parasites, mediated by a complex mechanism that is critical for modulating parasite growth and survival.

Introduction

Malaria, a disease caused by *Plasmodium* parasites, kills a child every minute. While there have been significant reductions in the number of infection and mortality rates since 2000 (51% and 27% reduction, respectively), the plateau in progress, first observed in 2017, still persists. Notably, during the past two years, the COVID-19 pandemic has resulted in unprecedented challenges to health systems worldwide, having disrupted malaria services, leading to a marked increase in cases and deaths. In 2020, there were an estimated 241 million malaria cases and 627 000 malaria deaths worldwide, which represents about 14 million more cases and 69 000 more deaths compared to 2019 [4].

Both parasite and host genetics, as well as host immune responses, contribute to the outcome of infection [260,459–461]. Still, the most consistent indicator of disease severity is parasite biomass [462]. *Plasmodium* is a rapidly multiplying unicellular organism undergoing a complex developmental cycle that takes place in the mammalian and mosquito hosts; a lifestyle that requires rapid adaptation to distinct environments. In the mammalian host, *Plasmodium* initially infects hepatocytes to generate thousands of merozoites, which are later released into the bloodstream where they invade and replicate within red blood cells (RBCs). Each merozoite replicates by schizogony to generate 10-30 new merozoites every cycle, which occurs at 24, 48 or 72 hours, depending on the *Plasmodium* species. As a rapidly multiplying obligatory intracellular parasite, *Plasmodium* has high nutritional demands relying on the nutrients provided by its host to survive. To cope with the different environments and conditions its life cycle entails, *Plasmodium* mainly relies on the expression of distinct transcriptomes at each developmental stage [358,359,370]. However, besides the ever-changing environment accompanying its normal life cycle progression, the environment within a single host is also extremely dynamic, with parasites constantly facing variable immune responses, drug pressures and metabolic fluctuations [364]. We and others have provided evidence that parasite adaptation to unpredictable fluctuations within each niche requires alternative strategies, such as efficient sensors, and a tight regulation of nutrient-sensing signaling pathways [365,373,386]. *Plasmodium* parasites are auxotrophic for most amino acids (AA), and the blood stages acquire the majority of them through the digestion of host erythrocyte hemoglobin [331,337]. However, some AA are either absent (isoleucine) or rare (methionine) within human hemoglobin and must be acquired from extracellular sources [463,464]

In eukaryotes, from yeast to mammals as well as plants, the target of the rapamycin (TOR) complex and the general control nonderepressible 2 (GCN2)/eIF2 α signaling cascades are two well-characterized mechanisms to sense AA fluctuations [465,466]. Notably, no homologue of the TOR complex has been identified to date in *Plasmodium*; however, a downstream effector, *Maf1*, is conserved in *P. falciparum* and has been described as essential for the parasite's asexual blood stage [457]. On the other hand, although the *Plasmodium* eIK1 kinase [429] closely clusters with the GCN2 AA-sensing kinase, the parasite lacks some key components of this signaling pathway, including the downstream transcription factors as well as the biosynthetic pathways that mediate GCN2 action, namely the orthologues for starvation-response regulators such as GCN4 in yeast and ATF4 in mammals [408,467]. This has led some to propose that *Plasmodium* evolved a stripped-down starvation-response pathway [446], suggesting a reduced capacity to adapt to AA depletion, rather than actively responding to alterations in AA levels. The present paradigm is that *P. falciparum*, in the absence of canonical eukaryotic nutrient stress-response pathways, can cope with an inconsistent AA supply by hibernating until more nutrients are provided [446]. We now challenge this paradigm by showing that both *P. berghei* and *P. falciparum* parasites can actively mount a response to a decrease in two distinct AAs (methionine and isoleucine) by activating, in each life cycle stage, a different protein kinase, ultimately leading to a reprogramming of replication and parasite development.

Results

AA depletion impacts *Plasmodium* development and replication

To assess the effect of AA depletion during parasite intra-erythrocytic development, we monitored the growth of both *P. falciparum* and *P. berghei* parasites cultured in media with or without methionine (Met) or isoleucine (Ile) (Figure 2.1). Sorbitol-synchronized *P. falciparum* NF54 wild-type (WT) parasites cultured in medium containing methionine (MetS) or isoleucine (IleS) (0.100 mM Met; 0.38 mM Ile) develop and replicate every 48h, resulting in a marked increase in parasitemia with each generation. However, when cultured in either Met deficient (MetD) or Ile deficient (IleD) medium, parasites undergo impaired growth, as evidenced by a marked reduction in parasitemia (Figure 2.1).

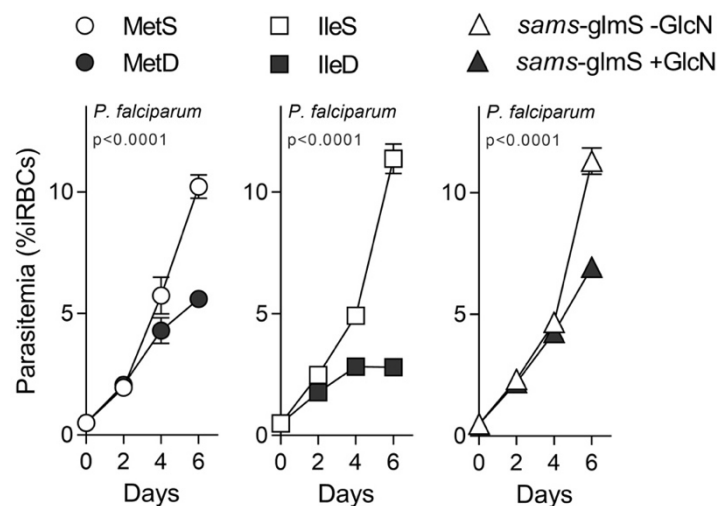


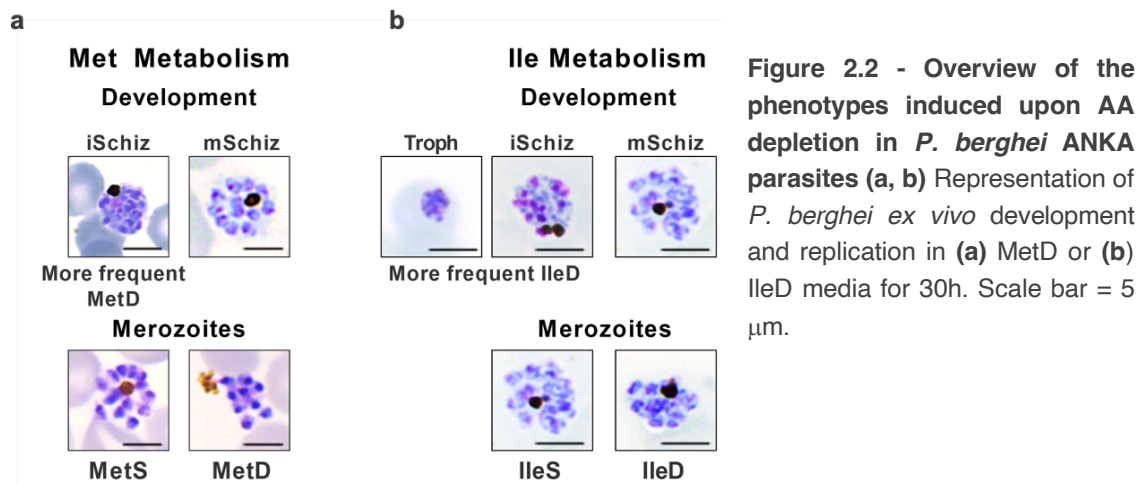
Figure 2.1 - AA availability impacts parasite intra-erythrocytic development and replication. Parasitemia of synchronized *P. falciparum* wt parasites cultured in AA deficient media (MetD or IleD), or *P. falciparum* parasites knockdown for the SAMS enzyme (*Pfsams-glmS* + GlcN), by flow cytometry analysis after SYBR Green staining. Data represents the mean percentage of iRBCs $\pm$ SEM (2-way ANOVA); $n=2$ independent experiments.

Notably, while the effect of IleD on parasitemia is evident from day 2 onwards, parasitemia in MetD only becomes significantly different from day 4 onwards. Such difference may be explained by the fact that Ile is totally absent from human hemoglobin, while Met is present, although at low levels [468]. Neither IleD [446] nor MetD impact parasite viability for a 48h period, as synchronized ring-stage forms quickly resume growth upon AA supplementation (Supplementary Figure 2.1a). Methionine, among the twenty amino acids, plays an integral role in protein biosynthesis and in regulation of

translation and global gene expression - as it is coded by the translation initiation codon. Met is metabolized into S-adenosylmethionine (SAM), the cellular methylation currency, via an ATP-dependent process which is catalyzed by the *Plasmodium* SAMS enzyme. Therefore, besides MetD we have also focused on the SAMS enzyme, since SAM is the major biological methyl donor and the precursor of polyamines via the aminopropylation pathway, or glutathione (GSH), upon entry into the transsulfuration pathway, where homocysteine (Hcy) is metabolized to GSH (Figure 2.5). Addressing both MetD and parasites knockdown for the SAMS enzyme allowed us to establish the role of Met in protein incorporation but also to disentangle the role of methylation, GSH and polyamines in parasite growth. To do so, we employed a previously generated [469] glucosamine (GlcN)-regulatable parasite line in which the *P. falciparum* SAMS, the first and rate-limiting enzyme of the methionine cycle, was conditionally knocked down (*Pfsams-glmS* parasite line). Interestingly, the knockdown of the SAMS enzyme, achieved by GlcN addition to the culture media (*Pfsams-glmS* +GlcN), phenocopies WT parasites growing in MetD media, as evidenced by a marked reduction in parasitemia from day 4 onwards (Figure 2.1).

We next sought to determine whether reduced parasitemia observed upon AA limitation was an outcome of defective parasite development. We employed the *P. falciparum* 3D7 strain which exhibited a shorter life cycle length, replicating every 36h to 42h in control conditions, in accordance to previous studies estimating a 38.8h cell cycle length for this particular strain [426,428]. Time course analysis of synchronized *P. falciparum* 3D7 ring-stages growing in MetD or IleD media reveals impaired parasite development in both conditions, yet with some particularities unique to each condition (Supplementary Figures 2.1b, c and 2.2). Both MetD- and IleD- media growing parasites fail to complete maturation in a timely and coordinated manner, arresting at the immature schizont-stage, as evidenced by an increase in the time spent at this stage. Ile depletion, however, has a more pronounced impact on cell cycle progression, affecting also the trophozoite stage, with parasites arresting for one complete developmental cycle at this particular stage (Supplementary Figure 2.1c). In fact, such a remarkable delay in progression of isoleucine-starved parasites through the trophozoite stage was first documented by Babbitt *et al.*, who proposed a dormancy-like model for AA starvation [446]. This immediate and pronounced effect of Ile depletion on parasite growth, compared to methionine depletion, could result from a faster depletion of Ile stocks, as it is totally absent from human Hb. However, our results show that even though the appearance of next generation merozoites is delayed both in MetD and IleD conditions, parasites can

invade erythrocytes efficiently, as evidenced by the emergence of second-generation ring-stage forms that peak at ~72h-84h of development (Supplementary Figure 2.1b, c). Notably, when employing a malaria parasite of rodents, *P. berghei* ANKA, a similar impact was observed in MetD and IleD conditions (Figure 2.2).



Blood stages of rodent malaria parasites can only be maintained in culture for one developmental cycle as newly developed merozoites cannot reinvade erythrocytes anew. Even though synchronization cannot be achieved in these short-term *ex vivo* systems, parasites fully mature from rings/young trophozoites to segmented schizonts that do not rupture, even when fully mature [470] - allowing for a thorough characterization of schizont development.

Microscopy analysis show that *P. berghei* parasites either growing in MetD or IleD media exhibit impaired development *ex vivo*, with only 30% of the parasites successfully developing into mature schizonts (Figure 2.3a, b; Supplementary Figure 2.3a, b). As for *P. falciparum*, while IleD leads to a significant delay in both trophozoite- and immature-schizont stages, MetD mainly impacts the immature-to-mature schizont transition. Surprisingly, providing *P. berghei* parasites an additional 6h of maturation time (30h timepoint) did not reflect in increased maturation rates under AA deficiency (Figure 2.3). This suggests that ~70% of parasites growing under AA-limiting conditions exhibit growth arrest, resembling the hibernation phenotype previously described by Babbitt *et al.* [446].

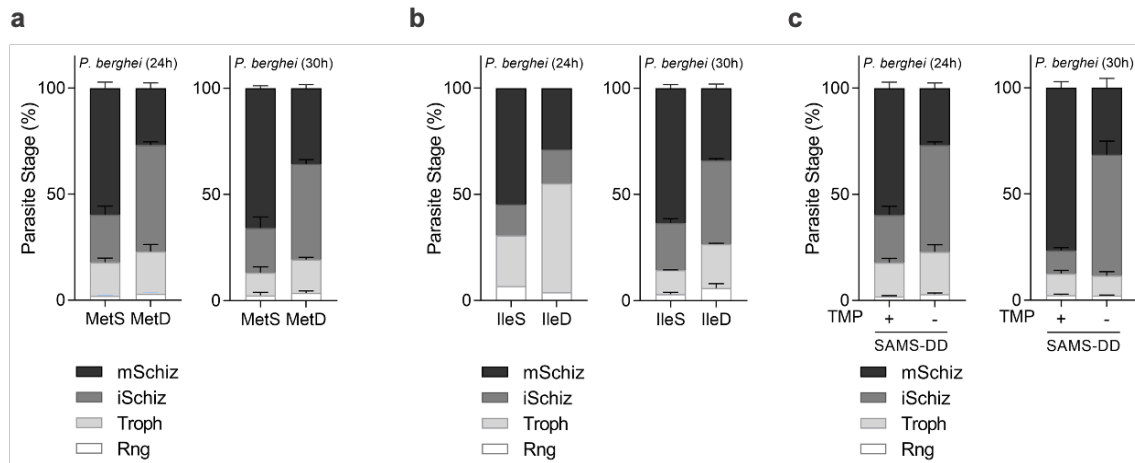


Figure 2.3 - AA depletion impacts parasite progression through the cell cycle during intra-erythrocytic development *ex vivo*. (a-c) Quantification of *P. berghei* intra-erythrocytic developmental stages in (a) MetD or (b) IleD media and in (c) parasites knockdown for the SAMS enzyme (*PbSAMS-DD* -TMP) after *ex vivo* cultivation for 24h or 30h. Data is shown as mean percentage of parasites at each developmental stage with error bars representing SEM; $n=3$ independent experiments, except for Ile at 24h ($n=2$). Individual data points are provided in Supplementary Figure 2.3. Rng, ring; troph, trophozoite; iSchiz, immature schizont; mSchiz, mature schizont.

Again, a *P. berghei* transgenic parasite line (*PbSAMS-DD* parasite line) in which the first enzyme of the methionine cycle was knocked down (*PbSAMS-DD* -TMP) phenocopied WT parasites cultured in MetD conditions (Figure 2.3c; Supplementary Figure 2.3c). In this species, SAMS knockdown was achieved by employing the destabilizing domain (DD) system [471]. The construct containing the *sams* gene fused to the DD and the hemagglutinin (HA) tag was introduced into *P. berghei* genome using double crossover homologous recombination (Supplementary Figure 2.1d, e). Protein stabilization *in vivo* was achieved by providing trimethoprim (TMP) in drinking water for 2 days prior to infection (*PbSAMS-DD* +TMP), while SAMS conditional knockdown (*PbSAMS-DD* -TMP) was achieved in non-TMP treated mice, as evidenced by a significant decrease in SAMS-HA-DD protein levels, measured by immunoblotting analysis (Supplementary Figure 2.1f). Similar results were observed by immunofluorescence analysis of *P. berghei* trophozoites and schizonts in which the SAMS enzyme was knocked down (*PbSAMS-DD* -TMP; Troph, Schiz), as evidenced by a significant decrease in SAMS-HA-DD protein levels, relative to control parasites (Supplementary Figure 2.1g). Notably, *PbSAMS-DD* -TMP merozoites exhibit SAMS expression levels comparable to those observed in control parasites (Supplementary Figure 2.1h).

Plasmodium schizogony culminates in the production of individualized merozoites that quickly re-invade new erythrocytes. We observed that under AA deficient conditions, ~30% of parasites successfully complete the cell cycle in a timely manner, developing into mature schizonts containing individualized next generation merozoites. Yet, the number of merozoites each schizont contained was dependent on the growth conditions. Microscopy and flow cytometry analysis in *P. falciparum* and *P. berghei* showed significantly fewer merozoites in schizonts grown in MetD or IleD media, as well as in transgenic parasites knockdown for the SAMS enzyme (*Pfsams-glmS* +GlcN; *PbSAMS-DD* -TMP) (Figure 2.4; Supplementary Figure 2.4a-d). Allowing *P. berghei* parasites an additional 6h maturation time (30h of culture) did not restore merozoite numbers under either condition (Supplementary Figure 2.4e), suggesting that lower replication rates result neither from the asynchronous nature of *Plasmodium* schizogony nor from delayed maturation, but rather from the ability of parasites to adapt to low AA levels.

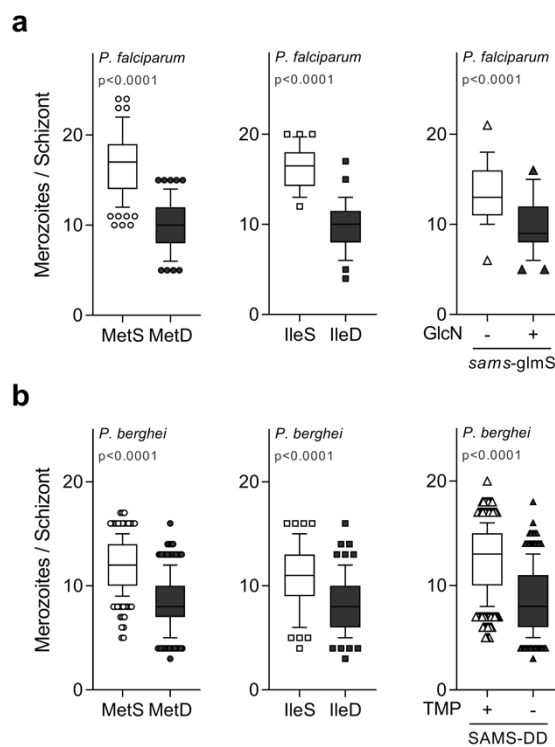


Figure 2.4 - AA depletion impacts parasite intra-erythrocytic replication rates. (a,b) Mean merozoite number per schizont in (a) *P. falciparum* or (b) *P. berghei* parasites, cultured in MetD or IleD media or in parasites knockdown for the SAMS enzyme. Data is represented as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (Mann-Whitney) in 2 independent experiments, except in b. *Pb* MetS, MetD ($n=3$) and *PbSAMS-DD* +/- TMP ($n=5$).

To disclose the role of AA metabolism in the dynamics of an *in vivo* malaria infection, we provided a short-term, methionine-deprived (0% methionine, MetD) regimen to BALB/c mice. Body weight analysis shows that, in accordance to previous findings [472], MetD regimen leads to weight loss relative to the methionine-sufficient (MetS) regimen (Supplementary Figure 2.4f). To exclude severe weight loss as a confounder, the MetD regimen was initiated 3 weeks before *P. berghei* ANKA infection and mice were only infected when their body weight had stabilized. The 3-week time point was chosen based on previous studies showing steady body weight and an improved metabolic profile after a 2- to 3-week regimen switch from *ad libitum* to calorie restriction [373]. Flow cytometry analysis of MetD-fed BALB/c mice infected with WT parasites shows a significant reduction in peripheral parasitemia relative to the MetS-fed group (Supplementary Figure 2.4g). Interestingly, the same was observed in mice fed on a MetS diet but infected with *PbSAMS-DD -TMP* parasites (Supplementary Figure 2.4h). Moreover, the reduced number of merozoites per schizont we observed after conditional knockdown of *PbSAMS* *ex vivo* was also confirmed *in vivo* (Supplementary Figure 2.4i). Altogether, these findings show that fluctuations in AA availability have an effect on parasite growth, by either stalling progression through the intra-erythrocytic asexual cell cycle, via a mechanism resembling hibernation, or by reducing parasite replication rates.

Polyamines regulate *Plasmodium* response to MetD

Amino acids are not only the building blocks of proteins but also serve as precursors for the synthesis of many metabolites with diverse functions in growth and other biological processes of a living organism. It is therefore likely that disruption of AA metabolism will profoundly affect the levels of many other AA-dependent metabolites. While Ile shares the first enzymatic steps of its metabolism with two other AAs (leucine and valine) [473], the initial steps of Met metabolism are unique among all AAs, rendering the products of its metabolism ideal candidates. To find out which Met-downstream metabolites could rescue attenuated replication and impaired development in *P. berghei* parasites knockdown for the SAMS enzyme, we supplemented media with SAM, Hcy, GSH or the polyamines putrescine (Putr), spermidine (SpD) and spermine (SpM) (Figure 2.5).

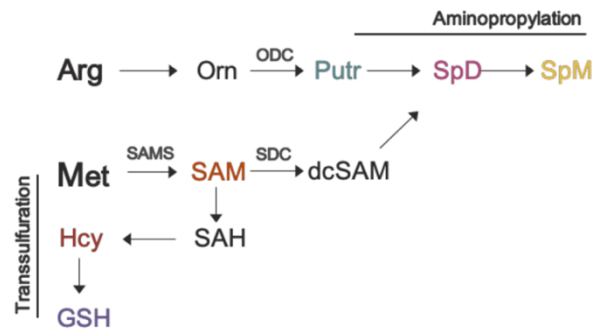


Figure 2.5 - Schematic representation of the methionine cycle, the aminopropylation and the transsulfuration pathways in malaria parasites. In *Plasmodium*, polyamines and methionine metabolism are closely connected as both rely on the *Plasmodium* bifunctional SDC/ODC enzyme. The biosynthesis of polyamines depends on the decarboxylation of ornithine to Putr, through ODC activity, and SAM to decarboxylated SAM (dcSAM), a role filled by SDC. Subsequent attachment of aminopropyl groups, provided by dcSAM, to Putr forms SpD and SpM.

Microscopic analysis shows that SAM rescues neither impaired maturation nor reduced numbers of merozoites produced by *PbSAMS-DD* -TMP schizonts, relative to the control group (Figure 2.6a, b; Supplementary Figure 2.5). This is not surprising since SAM transport across the plasma membrane occurs to a minimal extent in mammalian cells [474]. However, the results show that among the SAM-downstream metabolites, the polyamines Putr and SpM are the only metabolites governing parasite's response to SAMS deficiency (Figure 2.6, Supplementary Figure 2.6a). In schizonts lacking the SAMS enzyme (*PbSAMS-DD* -TMP), while SpM reverses impaired maturation, Putr fully restores merozoite numbers (Figure 2.6c, d). Furthermore, the simultaneous addition of SpM and Putr (Putr & SpM) rescues not only the impaired development of *PbSAMS-DD* -TMP parasites but also the reduced replication rates, which is reflected in a higher percentage of mature schizonts and an increased number of merozoites per schizont (Figure 2.6c, d). These data support the existence of two distinct parasite populations, one that stalls development and hibernates and other that, while completing the cycle at a normal pace, develops into schizonts containing fewer offspring. These two parasite populations are independently regulated by two distinct metabolites (SpM or Putr) and are likely governed by different mechanisms. In an attempt to unveil how parasite replication and development are independently regulated by distinct polyamines, we assessed the impact of MetD on polyamine biosynthesis throughout parasite intra-erythrocytic development. Thin layer chromatography (TLC) analysis of *P. falciparum* NF54 parasites shows an overall reduction in polyamine synthesis throughout development in MetD (Figure 2.7).

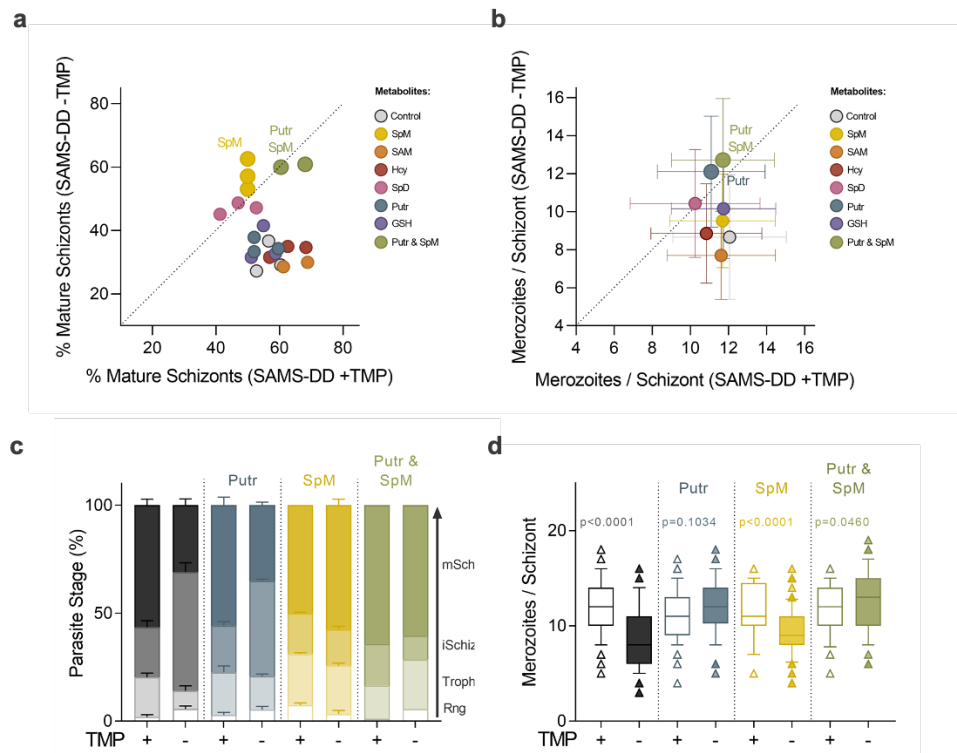


Figure 2.6 - Polyamines regulate parasite response to methionine depletion. (a, b) Percentage of mature segmented schizonts and **(b)** merozoite numbers per schizont in *PbSAMS-DD* +TMP vs *PbSAMS-DD* -TMP (knockdown) parasites cultured with the Met-downstream metabolites. **(c, d)** Quantification of **(c)** *P. berghei* intra-erythrocytic developmental stages and **(d)** merozoite numbers in *P. berghei* parasites knockdown for the SAMS enzyme after supplementation with the polyamines putrescine (Putr), spermine (SpM) or both (Putr & SpM). **a, b.** Data represents the **a.** percentage of mature schizonts and the **b.** mean merozoite numbers produced *ex vivo* by *PbSAMS-DD* -TMP (knockdown) parasites after supplementation with the Met-downstream metabolites. Polyamine supplementation was examined in $n = 3$ independent experiments, except for Putr + SpM and SAM ($n = 2$). Error bars represent the SD. **c.** Data is shown as mean percentage of parasites at each developmental stage, with error bars representing SEM. Parasite staging was performed in triplicate, averaged and repeated in $n = 3$, except for Putr + SpM ($n = 2$). Individual data points are represented in Supplementary Figure 2.6a,b. Rng: ring; troph: trophozoite; iSchiz: immature schizont; mSchiz: mature schizont. **d.** Data is represented as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (2-way ANOVA). Boxplots show the data of $n = 2$ independent experiments and for the following number of schizonts (N): **d.** SAMS-DD +TMP, $N = 122$, -TMP, $N = 117$; +TMP +Putr, $N = 50$; -TMP +Putr, $N = 48$; +TMP +SpM, $N = 37$; -TMP +SpM, $N = 102$; +TMP +Putr & SpM, $N = 47$; -TMP +Putr & SpM, $N = 47$.

Notably, the effect of MetD on Putr production is more prominent in early stages of parasite development (trophozoite, 24h) whereas the production of SpD (and likely that of its downstream product, SpM) is mainly reduced during late schizogony (36h) (Figure 2.7a, b). Due to its very low abundance in the parasite, SpM levels in TLC analysis were significantly lower than those observed for Putr and SpD (Figure 2.7a). This supports our previous findings in the rodent malaria species, *P. berghei* ANKA, showing a role for Putr during replication (Figure 2.6b,d), and for SpM at later stages of parasite development, particularly during schizont segmentation (Figure 2.6a,c).

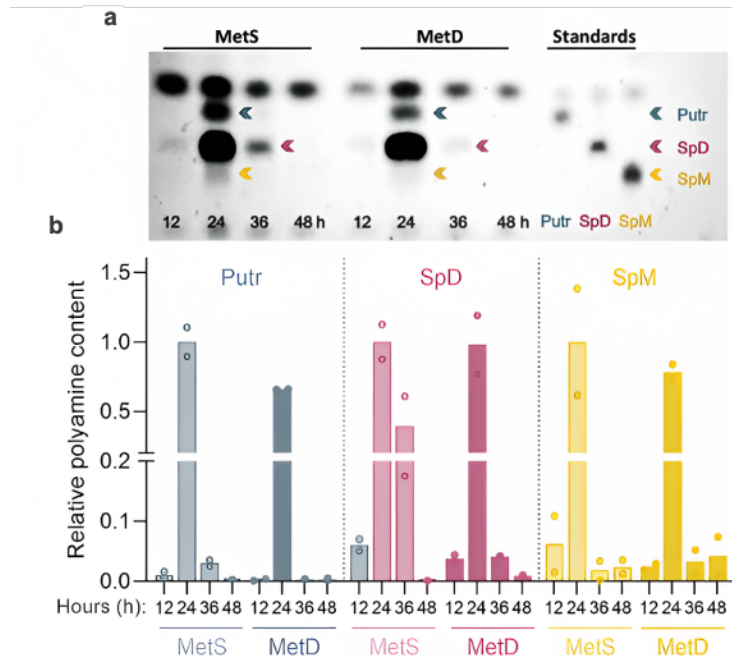


Figure 2.7 - Polyamine biosynthesis is downregulated in *P. falciparum* upon methionine withdrawal. (a, b) (a) Polyamine visualization by thin layer chromatography (TLC) analysis, representative of $n=2$ independent experiments, in synchronized *P. falciparum* NF54 WT parasites cultured in MetD media. Each lane represents a different timepoint. **(b)** Quantification of relative polyamine levels at 12h (ring-stage), 24h (trophozoite-stage), 36h (schizont-stage) and 48h (next generation ring-stages) after sorbitol synchronization in MetS and MetD conditions. The same number of parasites was loaded per condition and timepoint. Due to its very low abundance in the parasite, SpM could only be detected by TLC analysis 24h after sorbitol synchronization. Blue, pink and yellow arrows represent Putr, spermidine (SpD) and SpM, respectively, in standards and samples.

These findings were recapitulated in the human malaria parasite, *P. falciparum*, in MetD conditions, as evidenced by a sharp increase in parasitemia upon polyamine supplementation (Supplementary Figure 2.6c). Indeed, our data are consistent with previous findings showing that inhibition of *P. falciparum* ODC enzyme (critical for Putr synthesis) induces cytostatic arrest at the trophozoite stage and halts parasite development at G1/S transition, a phenotype that is rescued by the addition of exogenous polyamines [475–477]. So far, our data show that the developmental and replication responses to methionine depletion are independent and each rely on a different polyamine. Not surprisingly, the effects of Met-downstream metabolites, namely polyamines, did not extend to conditions of Ile depletion, showing that these were specific to Met depletion signaling (Figure 2.8; Supplementary Figure 2.6b).

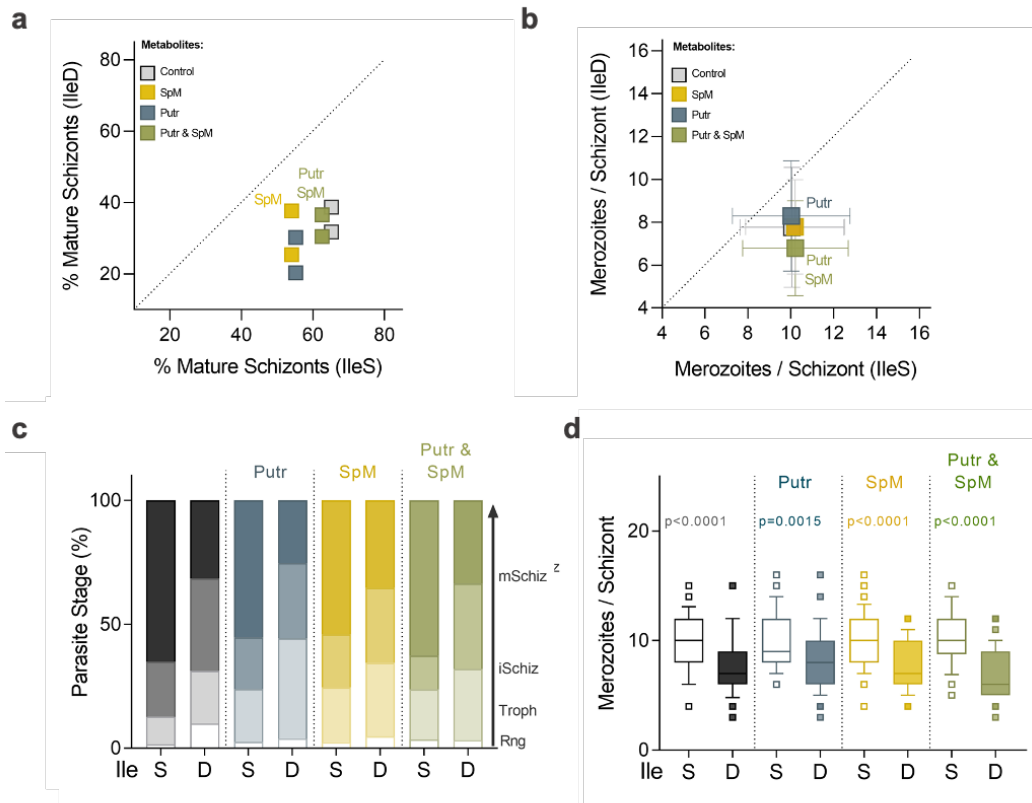


Figure 2.8 - Polyamines have no impact in IleD-induced phenotypes, as it regards to development and replication. (a) Percentage of mature segmented schizonts and (b) mean merozoite numbers in *P. berghei* WT parasites cultured in IleS vs IleD media supplemented with the polyamines Putr, SpM or both. (c, d) Quantification of (c) *P. berghei* intra-erythrocytic developmental stages and (d) mean merozoite numbers in *P. berghei* WT parasites cultured in IleS or IleD media supplemented with polyamines. a, b. Data represents the a. percentage of mature schizonts and the b. mean merozoite numbers produced *ex vivo* by *Pb* wt parasites growing in IleD media supplemented with polyamines. Polyamine supplementation was examined in $n=2$ independent experiments. Error bars represent the SD. c. Data is shown as mean percentage of parasites at each developmental stage, with error bars representing SEM. Individual data points are represented in Supplementary Figure 2.6a. b. Rng, ring; troph, trophozoite; iSchiz, immature schizont; mSchiz, mature schizont. d. Data is represented as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (2-way ANOVA). Boxplots show the data for the following number of schizonts (N): IleS, $N=38$; IleD, $N=47$; IleS + Putr, $N=59$; IleD + Putr, $N=66$; IleS + SpM, $N=56$; IleD + SpM, $N=57$; IleS + Putr & SpM, $N=38$; IleD + Putr & SpM, $N=53$.

The data show that when each amino acid (Met or Ile) is depleted, separate mechanisms are orchestrated, with the downstream metabolites triggering these responses being specific to the metabolic pathway that is affected. It is important to note that the different responses observed at the phenotypic level likely translate to the different roles each AA plays in the cell. Our findings so far led us to hypothesize that *Plasmodium* parasites have the ability to sense fluctuations in AA levels—as well as in the downstream metabolites—which independently regulate parasite development or replication, functioning as key molecular players during distinct stages of the parasite's life cycle.

Nek4, eIK1 and eIK2 are key in the response to AA limitation

Cells respond to environmental stress conditions by adjusting gene expression and protein synthesis, a role mainly filled by protein kinases signaling pathways. However, the key molecular players involved in AA-sensing regulation in other organisms are absent from the genome of the malaria parasite. To identify potential regulators, we examined a panel of 15 eukaryotic protein kinases (ePKs) which were previously shown to be non-essential for *P. berghei* asexual erythrocytic development (Figures 2.9, 2.10) [382].

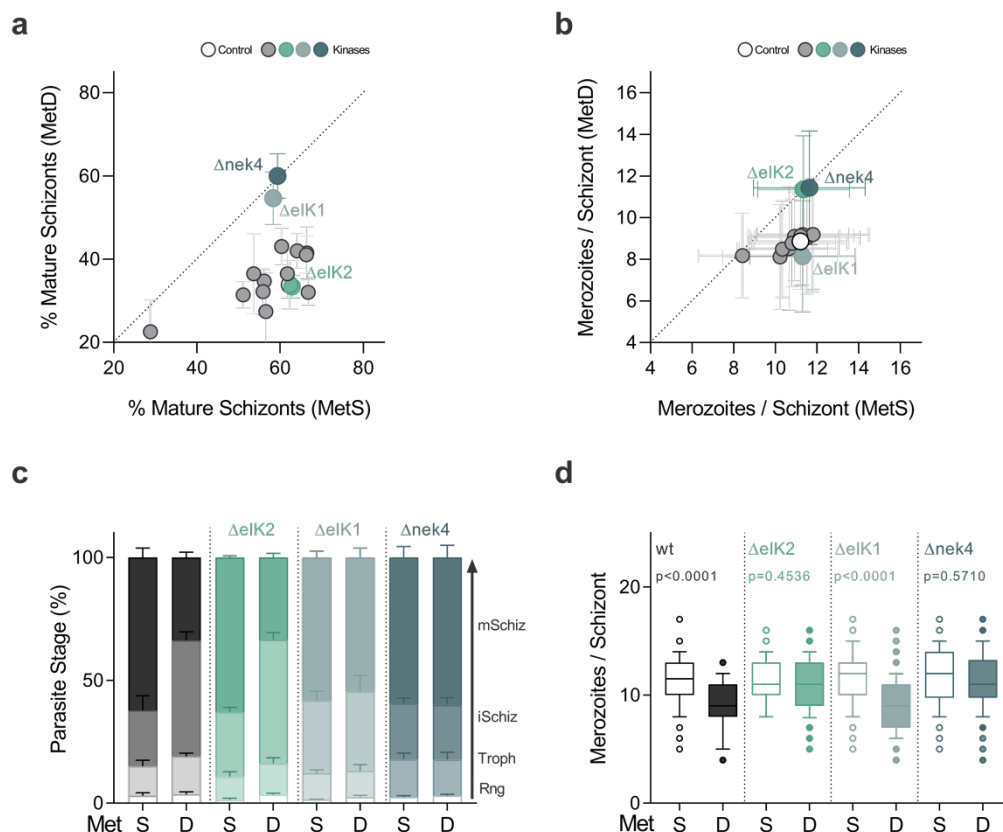


Figure 2.9 - A signaling pathway comprising 3 distinct parasite kinases regulates the stress response to AA depletion (a, b) Screen of 15 non-essential *P. berghei* kinases in loss-of-function mutants using the *ex vivo* maturation assay. **(a)** Percentage of mature segmented schizonts and **(b)** merozoite numbers per schizont in wild-type and kinase knockout lines ($\Delta nek4$: PBANKA_061670; $\Delta eIK1$: PBANKA_1308400; $\Delta eIK2$: PBANKA_0205800) cultured in MetS vs MetD media. **(c, d)** Quantification of **(c)** *P. berghei* intra-erythrocytic developmental stages and **(d)** merozoite numbers of *P. berghei* wild-type and kinase mutant $\Delta eIK2$, $\Delta eIK1$ and $\Delta nek4$ parasites cultured in MetS or MetD media. **a, b.** Kinase knockout mutants were analyzed in triplicate and averaged, with each data point representing the **a.** mean percentage of mature schizonts or **b.** the mean merozoite number produced per schizont. Error bars represent the SE Diff or SD between replicates, respectively. $n=3$ independent experiments for wild-type, $\Delta eIK2$, $\Delta eIK1$ and $\Delta nek4$ or $n=2$ for the other knockout lines. **c.** Parasite staging was performed in triplicate, averaged and repeated in $n=3$ independent experiments. Data is shown as mean percentage of parasites at each developmental stage, with error bars representing SEM. Individual data points are represented in Supplementary Figure 2.9a, **b., d.** Data is represented

as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (2-way ANOVA). Boxplots show the data of $n=3$ independent experiments and for the following number of schizonts (N), wild-type: MetS, $N=148$; MetD, $N=148$; Δ eIK2: MetS, $N=85$; MetD, $N=158$; Δ eIK1: MetS, $N=220$; MetD, $N=238$; and Δ nek4: MetS, $N=106$; MetD, $N=106$.

By growing *P. berghei* lines with loss-of-function mutations in protein kinase genes [382] in AA depleted media (Figures 2.9 and 2.10; Supplementary Figures 2.7 and 2.8) we have identified Nek4, eIK1 and eIK2 mutants as unresponsive to both MetD and IleD, suggesting a role for these kinases in AA sensing, albeit with some relevant particularities at the phenotypic level. In MetD or IleD conditions, Δ nek4 parasites mature and replicate similarly to wild-type parasites in MetS or IleS.

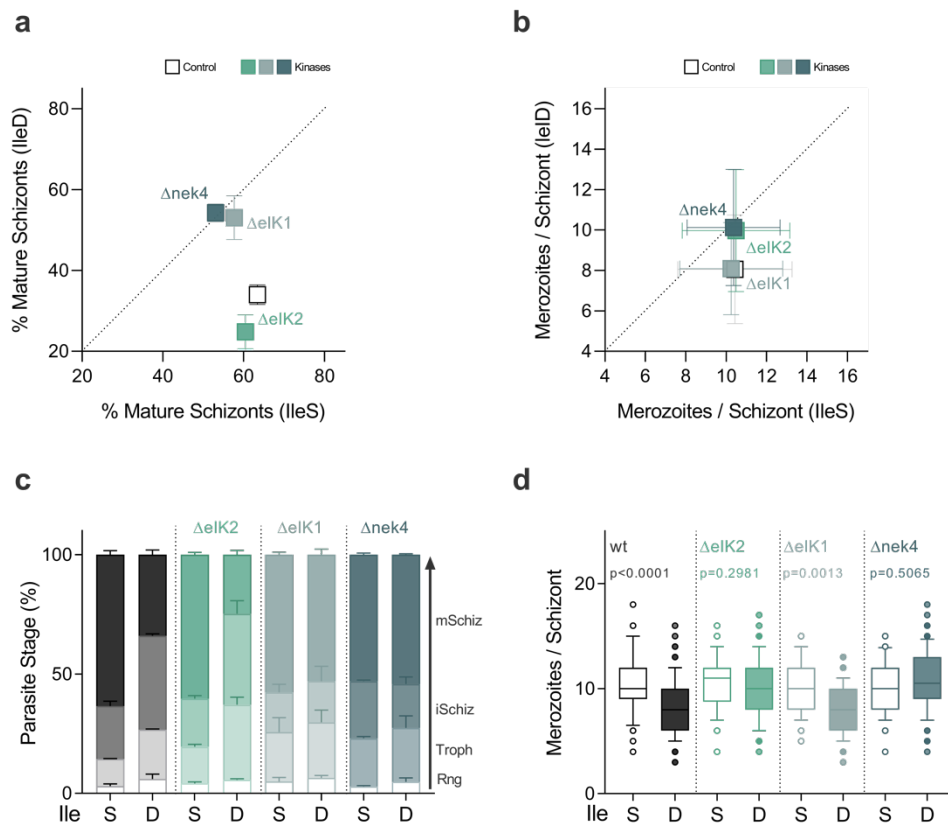


Figure 2.10 – Adaptation to isoleucine depletion also relies on eIK1, eIK2 and nek4. (a, b) (a) Percentage of mature segmented schizonts and (b) merozoite numbers in wild-type and kinase knockout parasite lines cultured in IleS vs IleD media. (c, d) Quantification of (c) *P. berghei* intra-erythrocytic developmental stages and (d) merozoite numbers of *P. berghei* wild-type and kinase mutant Δ eIK2, Δ eIK1 and Δ nek4 parasites cultured in IleS or IleD media. a, b. Kinase knockout mutants were analyzed in triplicate and averaged, with each data point representing the a. mean percentage of mature schizonts or b. the mean merozoite number produced per schizont. Error bars represent the SE Diff or SD between replicates, respectively. $n=2$ independent experiments. c. Data is shown as mean percentage of parasites at each developmental stage, with error bars representing SEM. Individual data points are represented in Supplementary Figure 2.9. d. Data is represented as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (2-way ANOVA). Boxplots show the data for the following number of schizonts (N), wild-type: IleS, $N=114$; IleD, $N=133$; Δ eIK2: IleS, $N=70$; IleD, $N=68$; Δ eIK1: IleS, $N=75$; IleD, $N=94$ and Δ nek4: IleS, $N=90$; IleD, $N=102$.

When assessing parasite growth, replication rates and intra-erythrocytic development (Figures 2.11 and 2.12; Supplementary Figure 2.10), *P. falciparum* kinase mutant knockout lines (elK1 [429], elK2 [427] and nek4 [478]) showed a similar response to MetD or IleD conditions. Interestingly, the inability of kinase mutant knockout parasites to control growth in response to AA depletion leads to parasite death after several rounds of replication, as illustrated by a stalling (Δ elK2, Δ elK1) or even decline (Δ nek4) in parasitemia from 96h onwards (Figure 2.11).

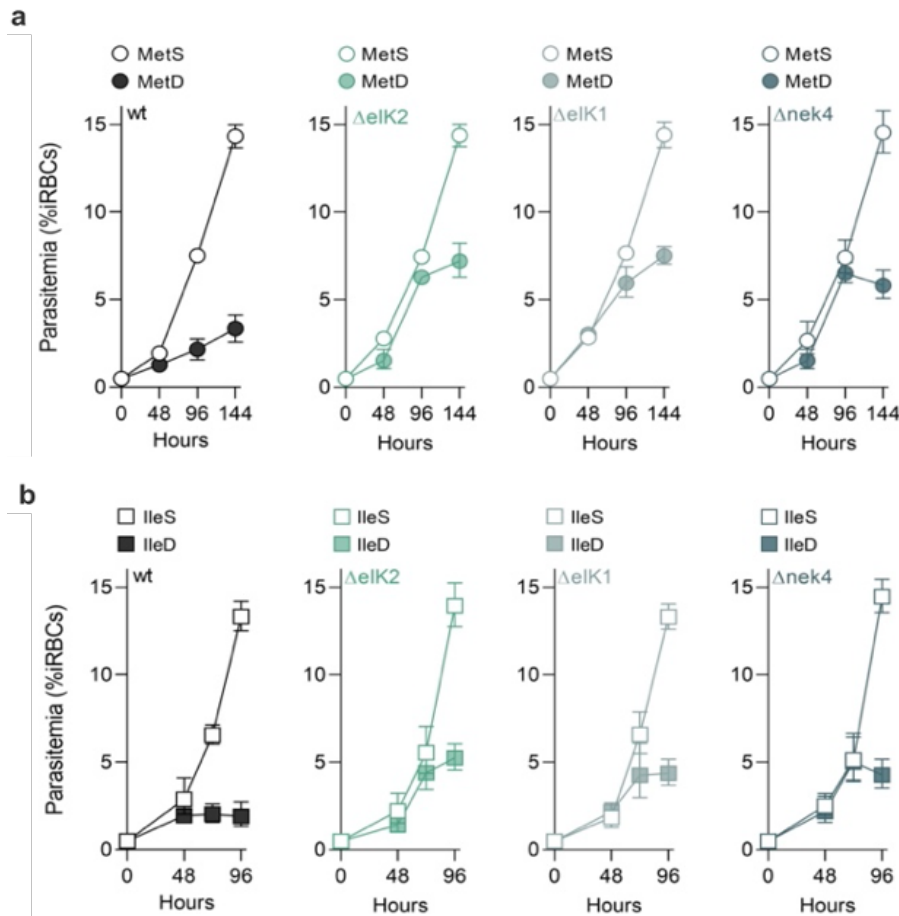


Figure 2.11 - Intra-erythrocytic development of *P. falciparum* elK1, elK2 and nek4 kinases knockout mutants upon AA withdrawal. (a, b) Parasitemia of synchronized *P. falciparum* 3D7 WT, Δ elK2, Δ elK1 and Δ nek4 parasites cultured in (a) MetS or MetD media and (b) IleS or IleD media. Data represents the mean percentage of iRBCs. Parasitemia was performed in triplicate, averaged and repeated in $n=2$ independent experiments.

These findings show that the previously described kinases function as a coping strategy for Met or Ile shortage, as parasites lacking these specific kinases cannot fine-tune growth to nutrient availability and thus fail to enter hibernation and to adjust replication rates. The involvement of Nek4, elK1 and elK2 in parasite adaptation to AA deficiency strongly suggests that these kinases regulate an active parasite response to AA

starvation, whose modulation occurs in a stage-specific and temporally-regulated manner, a mechanism that, to the best of our knowledge, is hitherto unknown. Moreover, our data strongly suggests that Nek4 acts upstream to eIK1 and eIK2 in the pathway, regulating both replication (via eIK2) or development (via eIK1).

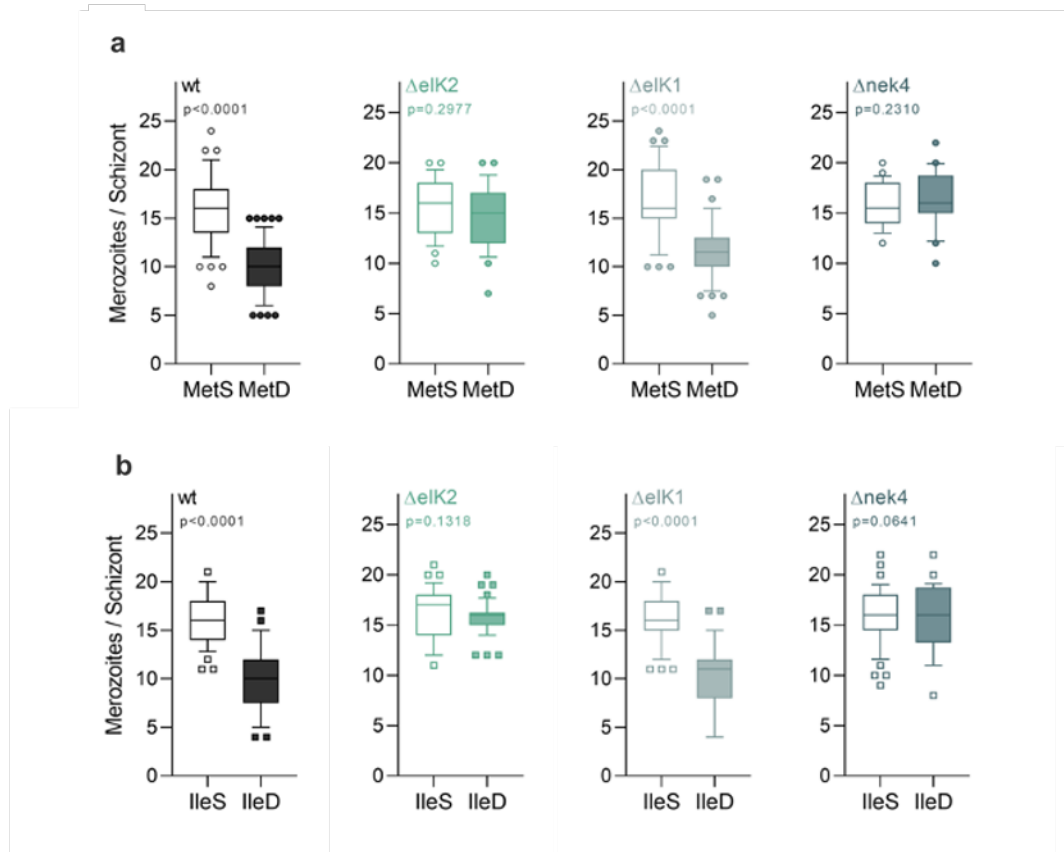


Figure 2.12 - Replication rates of *P. falciparum* eIK1, eIK2 and nek4 kinases knockout mutants upon AA withdrawal. (a, b) Merozoite numbers per schizont in *P. falciparum* parasites cultured in (a) MetS or MetD media and (b) IleS or IleD media. **a, b.** Data is represented as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (Mann-Whitney). Boxplots show the data of $n=2$ independent experiments and for the following number of schizonts (N): wild-type, MetS, $N=57$; MetD, $N=58$; IleS, $N=37$; IleD, $N=33$; Δ eIK2, MetS, $N=26$; MetD, $N=25$; IleS, $N=37$; IleD, $N=42$; Δ eIK1, MetS, $N=35$; MetD, $N=44$; IleS, $N=41$; IleD, $N=36$; and Δ nek4, MetS, $N=22$; MetD, $N=20$; IleS, $N=45$; IleD, $N=28$.

Parasite response to low AA implies eIF2 α phosphorylation at different stages of the cell cycle

P. falciparum parasites growing in AA-free media exhibit increased levels of phosphorylation of the eukaryotic initiation factor 2 α (eIF2 α) [429], which is a well characterized mechanism in eukaryotic model organisms that blocks translation under AA depletion [405,406,479]. From yeast [480] to mammals [417], such a mechanism is coordinated by the eIF2 α kinase GCN2, which recognizes the increased accumulation of uncharged tRNAs induced by AA depletion [405,479]. Three eIF2 α kinases have been identified in *Plasmodium*: eIK1, eIK2 and PK4 [425] whose only described function is to phosphorylate the *Plasmodium* eIF2 α orthologue under different stresses [426]. Since our screening data revealed a key role for eIK1 and eIK2 (two of the three eIF2 α kinases) in parasite response to AA depletion, we next sought to establish whether these kinases coordinate an adaptive response mechanism and thereby modulate the phosphorylation of their downstream target eIF2 α .

Immunoblotting analysis of synchronized *P. falciparum* WT parasites reveals that under control conditions (MetS, IleS conditions) eIF2 α phosphorylation is lower at the ring stage, increasing later during schizont development (Figure 2.13) a process required for successful schizont maturation, through reduction of protein synthesis [481]. By contrast, MetD and IleD conditions impact on eIF2 α phosphorylation status (Figure 2.13), suggestive of an early and active parasite response to low AA levels, yet with some particularities - as previously observed at the phenotypic level (Figures 2.1 and 2.2). Under both MetD and IleD conditions, eIF2 α phosphorylation is strongly induced early in development, at the ring stage. However, while enhanced phosphorylation is maintained throughout schizont development in MetD conditions (Figure 2.13a); under IleD, eIF2 α phosphorylation is rapidly lost and restored to basal levels as schizont development progresses (Figure 2.13b).

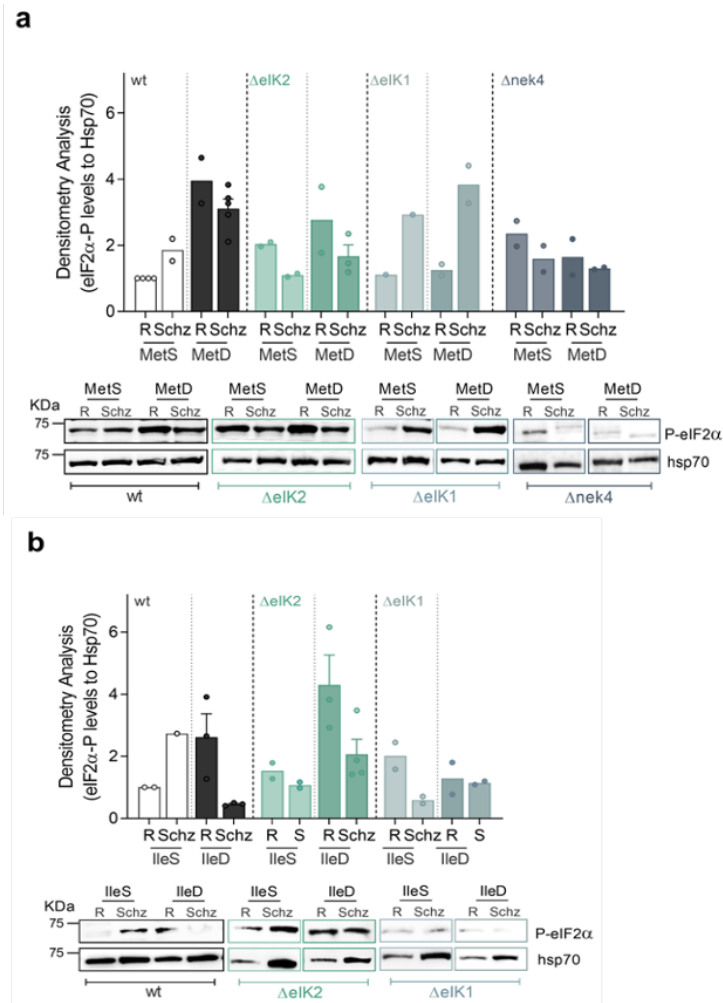


Figure 2.13 - Parasite response to AA depletion results in enhanced phosphorylation of the *Plasmodium falciparum* eIF2 α orthologue. (a, b) Time course analysis of eIF2 α phosphorylation status upon AA starvation of sorbitol-synchronized *Pf*3D7 WT, eIK2, eIK1 and nek4 knockout mutants (ring-stage window of $\pm$ 8h). Parasites were cultured in (a) MetS and MetD media or (b) IleS and IleD media. Starvation pulses were performed for 6 hours at the ring- (R) or schizont- (Schz) stage. Parasite proteins were extracted and probed for the phosphorylation of the translation initiation factor 2 α (P-eIF2 α) at the serine residue 51. Representative phospho-western blots and bar graphs quantifying phosphorylation levels of eIF2 α , in ring- and schizont-stage parasites, relative to levels in ring-stage parasites growing under control conditions (MetS or IleS, respectively). Bars represent the mean of eIF2 α -P levels relative to hsp70 levels $\pm$ SEM.

This is consistent with our previous findings that IleD primarily affects early developmental stages, while MetD has the greatest impact during schizont development (Supplementary Figure 2.1b, c). Our results show that Δ eIK1 parasites can only respond to AA depletion during the schizont stage, as evidenced by increased eIF2 α phosphorylation levels at this particular stage and an apparent loss of eIF2 α phosphorylation during ring-stage development. By contrast, while Δ eIK2 schizonts are incapable of phosphorylating eIF2 α , ring-stage parasites successfully respond and adapt

to AA scarcity. Remarkably, $\Delta nek4$ parasites are completely unable to respond to MetD, with negligible eIF2 α phosphorylation levels in both the ring and schizont stages (Figure 2.13a). Therefore, while eIK1 seems to be critical for parasite's response during early-stages of development, eIK2 activity emerges as only relevant towards late stages of development - at least in MetD. Remarkably, Nek4 activity appears to be critical in the parasite's response to AA deficiency throughout asexual intraerythrocytic development (Figure 2.13a) – a kinase previously known to function during gametocytogenesis [482].

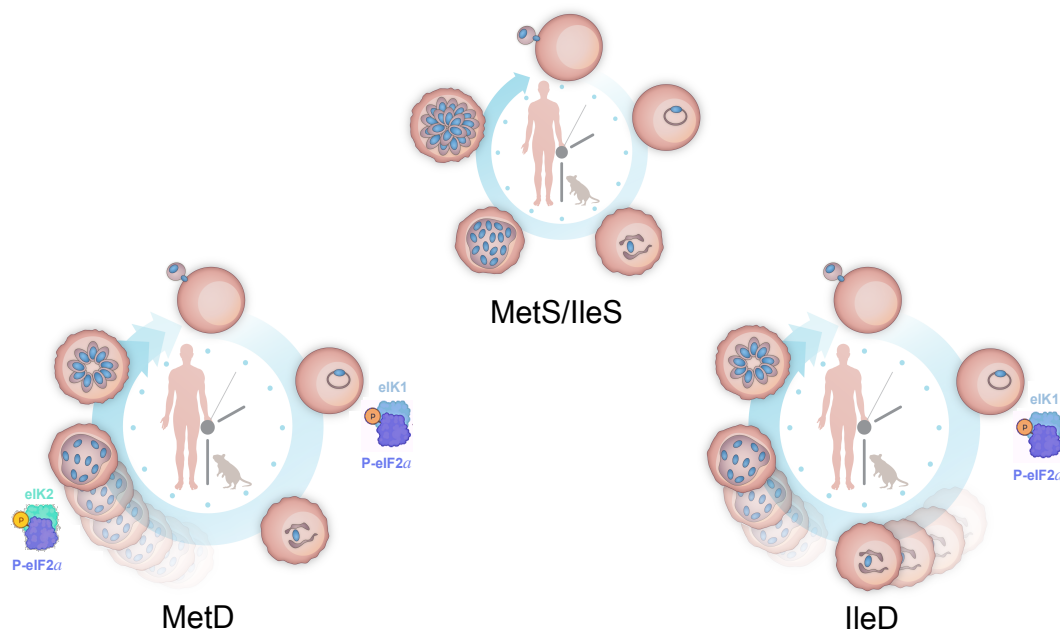


Figure 2.14 - Schematic model for parasite's adaptive response to AA depletion during intraerythrocytic development. *Plasmodium* parasites sense and actively respond to fluctuations in the availability of two distinct AAs: Ile and Met. Such response is heterogeneous as it depends on the AA that is depleted yet, it implies a common pathway comprising the phosphorylation of eIF2 α by two distinct kinases, eIK1 and eIK2, which are temporally regulated at distinct stages of the life cycle, allowing parasites to fine-tune replication and development accordingly. This response leads to decreased replication, entry into a hibernation-like state and ultimately, extended survival.

While it has previously been shown that ring-stage parasites lacking eIK1 do not phosphorylate eIF2 α in response to AA depletion, the ability of schizonts to actively respond to AA fluctuations, as well as the role of eIK2 (whose only known function is in the sporozoite stage [429]) and Nek4 in response to AA depletion remained unknown. Overall, the data show that *Plasmodium* parasites can detect AA fluctuations and mount active responses to individual AAs. This occurs via a common mechanism comprising the phosphorylation of eIF2 α by two different kinases - eIK1 and eIK2 – whose function is likely coordinated by Nek4 in a temporally-regulated manner, with each kinase affecting a specific, but sequential, stage of development (Figure 2.14).

Discussion

Nutrient scarcity is an important selective pressure that has shaped the evolution of many organisms and most cellular processes. While nutrient sensing pathways have been established and studied in model systems, malaria parasites' ability to sense nutrients has only recently been uncovered. This owes to the fact that *Plasmodium* parasites do not appear to possess any of the canonical eukaryotic nutrient-sensing pathways, including the SNF/AMPK, TOR and GCN2 biosynthetic pathways. Notably, while the canonical SNF/AMPK energy-sensing pathway was previously thought to be absent, we have recently shown that a parasite kinase, KIN, is a putative functional AMPK homologue, working as a broad metabolic/energy sensor that drives an active and coordinated parasite response to energy fluctuations [373]. On the other hand, the master regulator of cell growth in eukaryotes (the TOR complex) is completely absent in *Plasmodium*, which has been shown to possess only a rudimentary AA starvation-sensing eukaryotic initiation factor 2 α (eIF2 α), that does not directly promote parasite survival upon AA starvation [429,446].

In many model organisms, GCN2 has been shown to sense the uncharged tRNAs that accumulate upon AA deprivation. GCN2 attenuates translation, which not only consumes AA, but is also one of the most energy-demanding cellular processes [405,407,417]. Our extensive phenotypic analysis in both MetD or IleD media and in the kinase knockout mutant parasite lines has allowed the identification of 3 distinct parasite kinases that function as key regulators of parasite growth under AA scarcity: Nek4, a kinase so far only described to function during gametocyte development [463], eIK1 and eIK2. The inability to reconcile growth with AA availability in the absence of such kinases suggests that asexual blood stage parasites can actively adapt to a decrease in AAs via these respective kinases. Additionally, our data suggests that these two distinct eIF2 α kinases function in a temporally-regulated manner, with eIK1 regulating developmental stage transition but not replication and eIK2 showing the opposite behavior as it regulates replication but not development.

While the distinct dynamics of eIF2 α phosphorylation in each kinase mutant during development under AA-deprived conditions supports this, additional research into kinase activity levels and activation status at each parasite stage would corroborate such a complex model. Indeed, our data suggest that in order to survive under AA deficiency, malaria parasites must be able to modulate replication levels or enter hibernation. Thus,

decreased replication and halted cell cycle progression in wild-type parasites mirrors the ability of malaria parasites to successfully respond to AA depletion; an adaptive mechanism that requires enhanced phosphorylation of eIF2 α , which must either be maintained throughout development (MetD) or not (IleD), depending on the AA that is lacking. Despite the cost to offspring, such an adaptive response ensures that parasites can balance nutrient availability with growth, ultimately extending survival. In contrast, nek4 parasites' inability to control replication and growth under AA deficiency, which is evidenced by the complete loss of eIF2 α phosphorylation, results in high growth rates at first but quickly leads to parasite death. Interestingly, Δ eIK1 and Δ eIK2 parasites exhibit an intermediate phenotype under AA deficiency, with each kinase regulating either replication or growth and phosphorylating eIF2 α in a stage-specific manner. Despite the ability of eIK2 ring-stage parasites to adapt, schizonts fail to phosphorylate eIF2 α in response to AA deficiency, suggesting that parasite replicative rates might be pre-determined at this stage. While eIK1 schizonts can adapt, ring-stages fail to phosphorylate eIF2 α in response to AA deficiency. This most likely explains Δ eIK1 parasites' ability to complete the cell cycle and develop into mature schizonts, as eIF2 α phosphorylation at the schizont stage has been shown to be critical for schizont maturation under basal conditions [482].

Interestingly, eIF2 α kinases' stage-specific and sequential activation constitutes a major hallmark of the integrated stress response in other eukaryotes [426] including the closely related apicomplexan parasite *Toxoplasma gondii* [483]. Our data show that adaptation to low AA levels entails a common AA-sensing pathway, comprising Nek4 and the phosphorylation of eIF2 α by eIK1 or eIK2. However, the overall parasite response to the two AAs tested, Met and Ile, exhibits some peculiarities, with each AA affecting parasite progression through the cell cycle as well as the dynamics of eIF2 α phosphorylation in a unique manner. This is not surprising when taking into account current evidence in other eukaryotes showing that GCN2-mediated response to AA starvation differs according to the specific AA that is depleted [484] - with Met starvation leading to the most striking decrease in protein translation in mammalian cells [485–488]. Further studies may be needed to fully appreciate the metabolic rewiring induced by AA starvation and how this triggers the signaling cascade driving the adaptive processes described herein. Still to be revealed is whether Nek4 acts upstream of eIK1 and eIK2, which would support our phenotypic analysis showing a role for Nek4 in both replication and development. Additional studies addressing whether, under AA depleting conditions,

eIK1 and eIK2 activation is modulated by Nek4 activity in asexual blood stages would provide further insight on how this AA sensing pathway is regulated in the parasite. Additional studies addressing the role of PK4 (already shown to phosphorylate eIF2 α during schizont development [489] in parasite response to AA starvation may also provide additional insight into the dynamics of eIF2 α phosphorylation and the function of these kinases as critical components of a GCN2-like sensing pathway in the parasite. Moreover, future experiments investigating the phosphorylation status and activity of *Maf1* under AA deficiency, the sole downstream component of the TOR pathway identified to date in *Plasmodium*, may disclose whether a TOR pathway operates in *Plasmodium* and how it interacts with the GCN2 pathway to ensure a suitable cellular adaptation to nutritional fluctuations.

While eradicating malaria is highly desirable, it appears to be rather challenging to achieve. This is primarily due to the emergence of parasite resistance to artemisinin-based combination therapies (ACTs), the forefront treatment for *Plasmodium falciparum* malaria. While the mechanism of action of artemisinin (ART) is not fully understood, ART treatment has been shown to block host cell hemoglobin uptake while markedly increasing general protein damage, resulting in delayed parasite clearance and the persistence of hemoglobin non-degrading ring-stage forms⁵⁵. Therefore, ART treatment is mostly effective at later stages of development when parasites exhibit high metabolic activity [426,428]. Moreover, in addition to a transient reduction in cell cycle pace, ART treatment also enhances eIF2 α phosphorylation, thereby resembling the amino acid withdrawal-induced phenotypes. Importantly, increased eIF2 α phosphorylation has been shown to be essential for ART-induced latency and parasite recrudescence [447,490,491]. Such a programmed state of low metabolic activity, both upon ART-treatment or AA withdrawal, could render parasites insensitive to a broad range of inhibitors, as already shown for the exoerythrocytic stages of other human malaria parasites [492]. By blocking the molecular players identified as part of a *Plasmodium* AA sensing pathway, namely Nek4 or the two eIF2 kinases, eIK1 and eIK2, these findings could represent an alternative and promising strategy to prevent the parasite's ability to enter this latent phase, thereby precluding malaria recrudescence following ART treatment. Furthermore, interventions targeting these kinases would transform highly replicating and as such virulent parasites into attenuated parasite forms, leading to low parasitemia, previously shown to be protective in humans [48].

Materials & Methods

Chemicals and Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, no glutamine (21870076), HEPES (15630), Gentamicin (15750), L-glutamine (25030-024), Albumax II (11021-029) and SYBR green I (S7567) were purchased from Gibco, Thermo Fisher Scientific. RPMI 1640 Medium, no isoleucine (R9014) was purchased from US Biologicals. Bovine Serum Albumin (BSA) Fraction V (MB046) was purchased from NZYTech. RPMI 1640 no methionine, no cysteine (R7513), Glucose (G6152), Saponin (47036), Spermine (S4264), Spermidine (S0266), Putrescine (D13208), L-Glutathione reduced (G4251), L-Homocysteine (69453) and Trimethoprim (TMP) were purchased from Sigma-Aldrich. S-Adenosylmethione-1,4-butanedisulfonate (NA58198) was purchased from Biosynth Carbosynth. Paraformaldehyde was purchased from Santa Cruz Biotechnology.

Mice, diets and treatments

Male C57BL/6J and BALB/c wild-type mice, aged 5 to 8 weeks, were purchased from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France). All mice used in this study were housed in the Rodent Facility of Instituto de Medicina Molecular João Lobo Antunes (Lisboa, Portugal), five per cage and kept in specific-pathogen-free conditions. Mice were randomly assigned to different experimental conditions and allowed free access to water and food. Blinding was not possible in MetD-fed mice as mice exhibit a clear difference in body weight. All experimental procedures involving mice were approved by iMM's Animal Welfare Body (ORBEA-iMM), licensed by the national regulator, Direcção Geral de Alimentação e Veterinária (DGAV), and performed in strict compliance with national and European regulations. Methionine- sufficient (MetS) and deficient (MetD) diets were manufactured and purchased from SSniff (Soest, Germany). TMP was provided in drinking water at 0.25 mg per mL starting two days prior to infection and changed every 48 hours.

Parasite lines

The following parasite lines were employed in this study: GFP-expressing *P. berghei* ANKA (GFPcon, clone 259cl2), obtained from the Leiden Malaria Research Group; *P. berghei* ANKA kinase deficient parasite lines, were previously generated [458]; *P. falciparum* NF54 and *P. falciparum* 3D7 were obtained through MR4 (www.mr4.org); *P. falciparum* *sams-glmS* transgenic parasite line was kindly provided by Björn Kafsack

(Weill Cornell Medical College, New York, USA) and the *P. falciparum* 3D7 kinase deficient parasite lines were kindly provided by Mathieu Brochet (University of Geneva, Geneva, Switzerland) and Christian Doerig (Monash Biomedicine Discovery Institute, Clayton, Australia).

The *P. berghei* SAMS-DD parasite line was generated by double crossover homologous recombination of the *pPbsams*.DD.HA construct at the *sams* locus in a GFP expressing parental line (line-507cl1) [382]. The *pPbsams*.DD.HA construct contains a truncated *Pbsams* ORF in fusion with a destabilizing domain (DD) – a mutant form of the *E. coli* dihydrofolate reductase (ecDHFR) protein, engineered to be degraded [493] - an HA tag and also a cassette for transgenic expression of the human *dhfr* - conferring resistance to pyrimethamine -which is flanked by the *sams* 3'UTR (Supplementary Figure 2.1d). Transfection was performed using a protocol described elsewhere [471]. Briefly, blood from a BALB/c mouse infected with the parental line was collected and cultured for 16 hours *in vitro*. Mature schizonts were purified by a Nycodenz gradient and transfected using Amaxa electroporation system (Lonza). Transfected merozoites were injected into the tail vein of a BALB/c mouse, positively selected by providing pyrimethamine in drinking water (70µg/ml) and harvested on day 7-10 post transfection. Pyrimethamine resistant, transgenic parasites were dilution cloned before further experimentation and genotyped by PCR (Supplementary Figure 2.1e). using the primers listed in Table 2.1.

Table 2. 1 - List of primer sequences used

PCR reaction	Primer pairs (Forward; Reverse)
<i>Pt</i> SAMS-HA-DD wt locus	p1: 5' TAGGTACCGAGGAAATTTTCTATTTACTTCG 3' p3: 3' ATGCGGCCGCGCAACTAATAAAATCCAGGAAATA 5'
<i>Pt</i> SAMS-HA-DD modified locus	p1: 5' TAGGTACCGAGGAAATTTTCTATTTACTTCG 3' p4: 3' ATGCGGCCGCTCATCGCCGCTCCAGAATCTC 5'
DNA quality control	p1: 5' TAGGTACCGAGGAAATTTTCTATTTACTTCG 3' p2: 3' TAGGGCCCATTTTTTAAACATTTTTTTCGTG 5'

***In vivo* mice infections and parasitemia determination**

Infection of experimental mice was performed by intraperitoneal (*i.p.*) inoculation of 1×10⁶ infected RBCs (iRBCs) into C57BL/6J or BALB/c mice, obtained by passage in the correspondent genetic background mice. For GFP-expressing parasites, peripheral blood parasitemia was determined by flow cytometry analysis of one drop of tail blood collected in 200µL PBS. A total number of 1 million events (day 1-2 of infection) or 100-200 thousand events (onwards) were acquired on a BD LSR Fortessa using the BD

FACSDiva™ Software (v6.2) and the parasitemia expressed as the percentage of GFP+ RBCs. For non-GFP expressing parasite lines, parasitemia was monitored by Giemsa-stained thin blood smears [493]. A total of 5-10 thousand RBCs per slide, was randomly acquired and the percentage of infected RBCs (iRBCs), or parasitemia, was semi-automatically counted using Fiji software (version 2.1.0) (<https://imagej.net/software/fiji/>).

***P. falciparum* culturing and parasitemia determination**

P. falciparum NF54, 3D7 and derived knockout clones were maintained in culture [494] at 5% hematocrit in human O+ erythrocytes (Instituto Português do Sangue e Transplantação). Of note, whereas the *P. falciparum* NF54 parasite line exhibits a 48h cell cycle length, the *P. falciparum* 3D7 line shows an accelerated asexual blood stage cycle of 36-42h. Parasites were cultured in Malaria Complete parasite Media (MCM) prepared by supplementing RPMI 1640 no glutamine, with 2mM L-glutamine, 2g/L NaHCO₃, 5 mM glucose, 25 mM Hepes, 100 μM hypoxanthine, 10μg/mL gentamicin, and 10% Albumax II - except for the AA starvation assays. For such experiments, MCM media was prepared by adding all compounds to RPMI lacking either methionine (MetD) or isoleucine (IleD). Respective control media, containing methionine (MetS) or isoleucine (IleS) were prepared by supplementing deficient media with the appropriate AAs, at the concentrations found in regular RPMI 1640 (100 μM Met, 382 μM Ile).

Synchronous *P. falciparum* cultures were obtained according to previously described protocols [495]. To achieve synchronization, *P. falciparum* parasites were treated twice with 5% sorbitol to achieve a ring-stage window of $\pm$ 8h interval in the previous cycle. To do so, cultures were allowed to grow up to 5-10% parasitemia and harvested when parasites were mostly at the ring stage. Parasite pellets were treated with 5% sorbitol for 10 minutes at RT, centrifuged at 600 x g and washed thrice in MCM. Parasitemia was assessed as well as parasite morphology and parasites were sub cultured at 5% hematocrit for one additional cycle. The sorbitol treatment was repeated after one cycle – approximately 48h for NF54 and 40h for 3D7 – as previously described [494,496]. Ring-stages were then cultured at 2% hematocrit and the starting parasitemia adjusted to 0.5% in 96 well plates containing 200 μL of the respective media.

Parasitemia in each developmental cycle was analyzed by flow cytometry analysis after SYBR Green staining [496]. Briefly, 50 μL of *P. falciparum* cultured samples were mixed with the same volume of SYBR Green I solution, previously diluted in water (1:1000). Samples were incubated in the dark for 20 min at room temperature and washed in 500

μL PBS. Samples were acquired on a BD LSR Fortessa using the BD FACSDiva™ Software (v6.2). A total of 100 000-200 000 events was analysed per condition. The data was further processed on FlowJo X TreeStar) using the gating strategy detailed in Supplementary Figure 2.4.

***P. berghei* ex vivo maturation assays**

P. berghei blood-stage parasites were collected by cardiac puncture of BALB/c-infected mice (1-2% parasitemia), washed in pre-warmed incomplete RPMI and cultured in 96-well plates containing complete RPMI culture medium, prepared as follows: RPMI 1640, no glutamine (21870076), supplemented with 5 mM glucose, 2 mM L-glutamine, 25 mM HEPES, 2g/L NaHCO₃, 50μg/L Gentamicin and 20% of Fetal Bovine Sera (FBS). Homemade complete medium sufficient for methionine (MetS) was prepared by supplementing RPMI 1640 no methionine, no cysteine (R7513) with the respective amino acids at the concentration found in regular RPMI (208μM and 100 μM, respectively); whereas methionine deficient media (MetD) consisted of RPMI 1640 no methionine, no cysteine (R7513) medium supplemented only with cysteine. Isoleucine-sufficient (IleS) media was prepared by supplementing RPMI 1640 Medium, no isoleucine (IleD; R9014) with the concentration of the respective amino acid found in complete RPMI media (382 μM). Ninety-six-well plates were incubated at 37°C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂ for 18-30h, allowing parasites to mature from rings/young trophozoites into segmented schizonts.

***In vitro* quantification of stage progression and merozoite number**

Parasite cell cycle progression was assessed at distinct time points - 18h (*data not shown*), 24h and 30h for the rodent malaria parasite - or every 12h for the human malaria parasite. Parasite staging was performed by microscopic analysis of 1-10 thousand, randomly acquired, Giemsa-stained RBCs. Mature schizonts were assessed by microscopic analysis of Giemsa-stained smears and manually quantified using ImageJ software. Fifty to one hundred segmented schizonts with clearly individualized merozoites containing a single hemozoin crystal were quantified per condition. Additionally, rodent blood stage nuclei were stained with DAPI and merozoite number was determined by fluorescence microscopy.

Ex vivo screen of Met-downstream metabolites

To screen for methionine-downstream metabolites, wild-type parasites were cultured in MetS or MetD media and IleS or IleD media and further supplemented with each metabolite - and the appropriate vehicle (as control). The final concentration used for each metabolite was set according to the physiological ranges described during a malaria infection or as previously described elsewhere, as follows: 1.5 mM S-adenosylmethionine (SAM) [497], 1 mM glutathione (GSH) [498], 20 μ M homocysteine (Hcy) [499–501], 1 mM putrescine (Putr) [502], 1 mM spermine (SpM)[316], and 1 mM spermidine (SpD) [503]. For *P. berghei*, an *in vitro* maturation assay was employed with each metabolite being added to the media and incubated for 24- or 30 hours. For *P. falciparum*, each metabolite was added to the culture media at day 4 of *in vitro* culture and incubated throughout one complete intra-erythrocytic developmental cycle. Ninety-six-well plates were incubated at 37°C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. The effect of metabolite supplementation on parasite stage progression and schizont development was assessed by microscopy of Giemsa-stained smears as described above.

Thin Layer Chromatography of *Plasmodium* polyamines

Polyamines were separated by thin-layer chromatography as previously described [503]. For all samples, cells were collected and centrifuged. Pellets were washed with Phosphate Buffered Saline (PBS) and then resuspended in 100 μ L of 2% perchloric acid. Samples were then incubated overnight at 4°C. One hundred μ L of supernatant was combined with 200 μ L of 5 mg/ml dansyl chloride (Sigma Aldrich) in acetone and 100 μ L of saturated sodium bicarbonate. Samples were incubated in the dark overnight at room temperature (RT). Excess dansyl chloride was cleared by incubating the reaction with 100 μ L 150mg/mL proline (Sigma Aldrich). Dansylated polyamines were extracted with 50 μ L toluene (Sigma Aldrich) and centrifuged. Five μ L of sample was added in small spots to the TLC plate (Sigma Aldrich) and exposed to ascending chromatography with 1:1 cyclohexane: ethylacetate. Plate was dried and visualized via exposure to UV.

Amino acid starvation assays for SDS-PAGE analysis

P. falciparum 3D7 parasites and knockout parasite lines, namely the two eIF2 α kinases [504,505], *Pf* Δ eIFK1 (PF3D7_1444500) - previously described to closely cluster with mammalian GCN2 - and *Pf* Δ eIFK2 (PF3D7_0107600) - were maintained as described above in MCM media supplemented with human O+ erythrocytes at 2% haematocrit.

Parasites were sorbitol-synchronized to the ring-stage and allowed to grow in culture for up to 5% parasitemia. Parasites were collected at two distinct stages of the intra-erythrocytic developmental cycle: ring (12h) or schizont (38h). Ring- or schizont-stages were pelleted by centrifugation, washed twice in PBS, pH 7.5, equally partitioned and cultured at 37°C with 5% CO₂ for a 6h pulse in media deficient for methionine- or isoleucine- (MetD or IleD) and the respective control media (MetS or IleS) - prepared as previously described. Cultures were then harvested by centrifugation at 1300 x g for 5 minutes at RT and further processed for parasite pellet isolation.

Parasite pellet harvesting and isolation for SDS-PAGE

Pelleted erythrocytes were washed in equal volume of PBS, harvested by centrifugation at 1300 x g for 2 minutes at 4°C and further lysed on ice by treatment with 0.15% saponin in PBS. Parasite pellets were isolated by centrifugation at 5500 x g for 10min, 4°C and parasites were washed twice in PBS. Blood stage parasite proteins were isolated by lysis in 50-200 µL of radioimmunoprecipitation assay (RIPA) buffer and washed in PBS with cOmplete™ protease inhibitor cocktail (Roche). Samples were resuspended in 5x SDS-Laemmli buffer and denatured at 95°C.

Immunoblotting Analysis

Denatured parasite proteins were resolved on 10% SDS-polyacrylamide gel and transferred to 0.2 µm nitrocellulose membranes (Bio-Rad) using wet-transfer in Towbin buffer (25 mM Tris, 192 mM Glycine (Sigma) and 20% ethanol in ddH₂O). Membranes were blocked in 5% BSA in PBS-0.1% Tween 20 (PBST) at RT for 1h, with rocking. Membranes were then incubated with the appropriate primary antibodies, diluted 1:1000 in 5% BSA in PBST, for overnight at 4°C. Membranes were washed thrice in 0.1% PBST for 10 minutes (RT) followed by incubation with appropriate secondary antibodies (conjugated to Horseradish peroxidase) diluted 1:10 000 in 5% BSA in 0.1% PBST, for 60 min at RT. Membranes were washed thrice and bound antibodies were detected by using Immobilon ECL Ultra Western HRP Substrate (Merck Millipore) on the ChemiDoc XRS+ system. The primary antibodies used for probing membranes were rabbit α HA (clone C29F4, Cell Signaling Technology, Danvers, MA, USA), rabbit α phospho-eIF2α (S51) (119A11, Cell Signaling Technology) and mouse α *Pbhsp70* (produced in house). The secondary antibodies used were: goat α mouse IgG, HRP conjugate (BML-SA204-0100, Enzo Life Sciences, Lausen, Switzerland) and goat α rabbit IgG, HRP-linked Antibody (7074, Cell Signaling Technology).

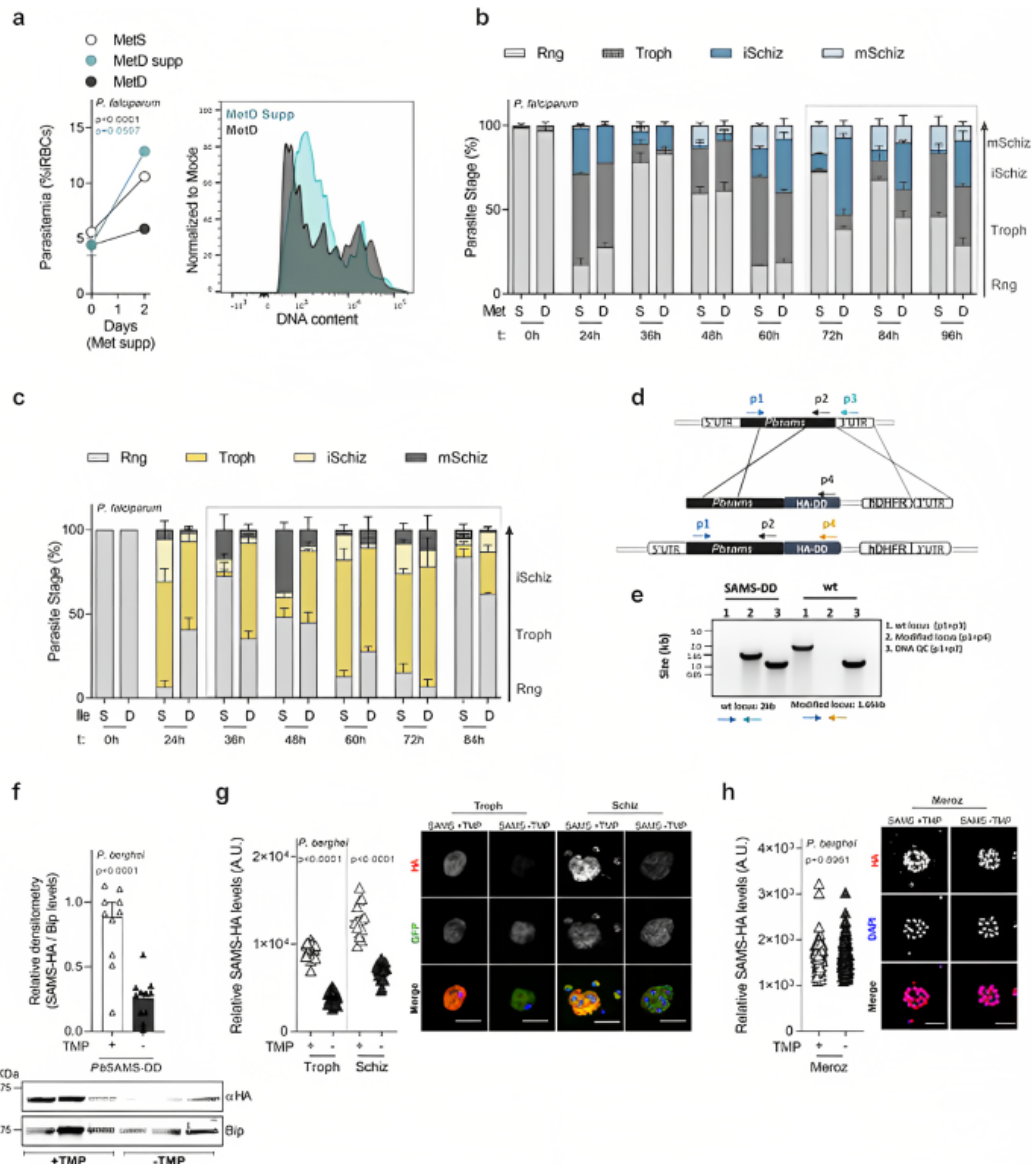
Immunofluorescence Assay

Immunofluorescence analysis of SAMS-DD-HA protein levels was performed in blood smears of *P. berghei* parasites cultured *in vitro* for 24h. MetS-fed BALB/c mice infected with *P. berghei* SAMS-DD parasites were either treated, or not, with TMP in drinking water two days prior to infection to achieve SAMS stabilization or knockdown, respectively. Parasites were cultured for 24h as previously described for the *in vitro* maturation assays and blood smears were fixed in 4% PFA-PBS solution for 10 min at RT. Fixed parasites were washed in PBS and permeabilized in 0.1% PBS-Triton X-100 solution for 10 min at RT. Cells were then blocked in 3% BSA for 1h at RT. Primary antibodies were diluted 1:400 in blocking solution and incubated at 4°C, overnight. Slides were rinsed thrice in PBS and incubated in secondary antibodies or dyes, diluted 1:400 or 1:1000, respectively, in blocking solution. Stained samples were rinsed in PBS, mounted in Fluoromount G and allowed to dry overnight. Primary antibodies used for fluorescence microscopy include: rabbit α HA (C29F4, Cell Signaling Technology) and mouse α *Pb*hsp70 (produced in house). Secondary antibodies and dyes used for fluorescence microscopy include Alexa-488 conjugated donkey α mouse GFP (A21311, Thermo Fischer Scientific), Alexa-647 conjugated donkey α rabbit IgG (A32795, Thermo Fischer Scientific) and DAPI for nuclear staining (D1306, Invitrogen, Thermo Fischer Scientific). Slides were imaged in Zeiss LSM 710 confocal laser point-scanning fluorescence microscope (ZEN 2 software, Blue edition) and merozoite counting was performed by the use of x63 oil objective.

Statistics and Reproducibility

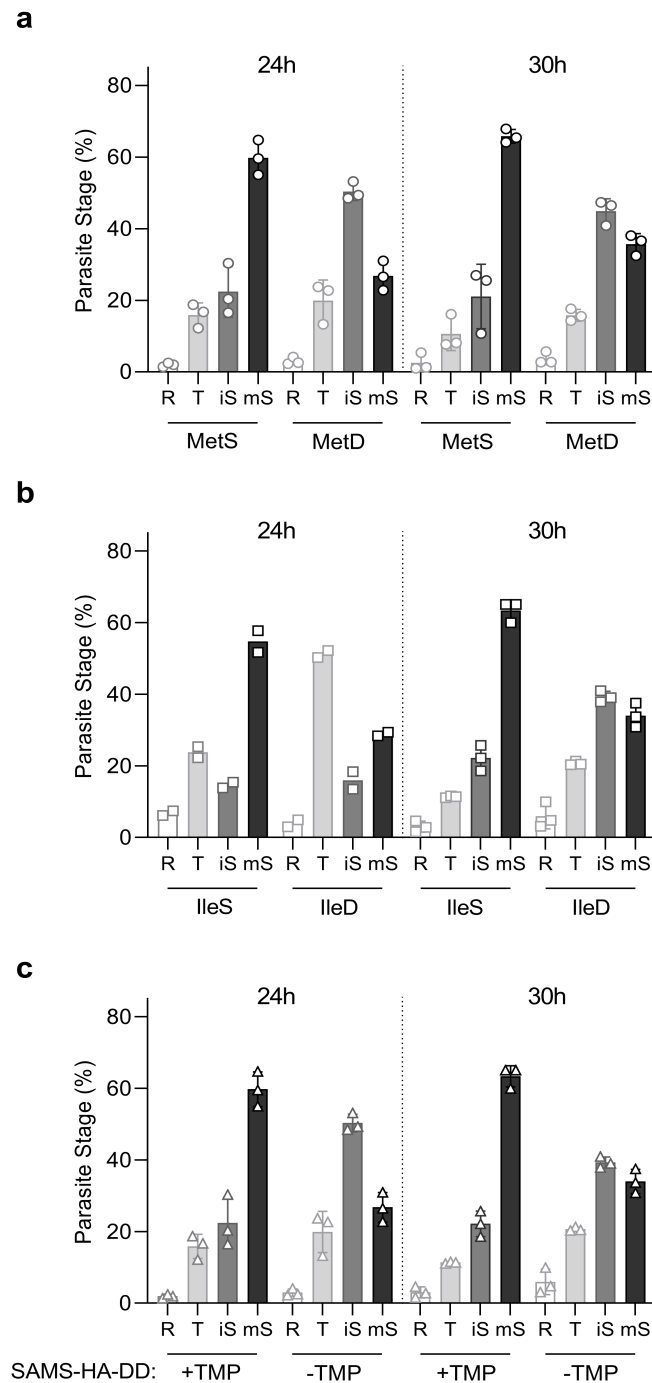
Significance was determined by different statistical tests on the GraphPad (Prism, version 8.4.3) software, as follows: Mann-Whitney U tests was used for comparisons between two different groups or conditions, two-way ANOVA was used to compare parasitemia, development and merozoite numbers between the control counterparts and the following experimental conditions: MetD-, IleD-, kinase mutants or metabolite supplementation assays. The log-rank (Mantel-Cox) test was used to compare the survival distributions of two groups. Significance was considered for p values below 0.05. The outliers in the boxplots represent 10% of data points. Biological replicates (*N*) indicated in figure legends refer to the number of parasites. The number of independent experiments is referred to as *n*. Sample sizes were chosen on the basis of historical data. No statistical method was employed to predetermine sample size.

Supplementary Information

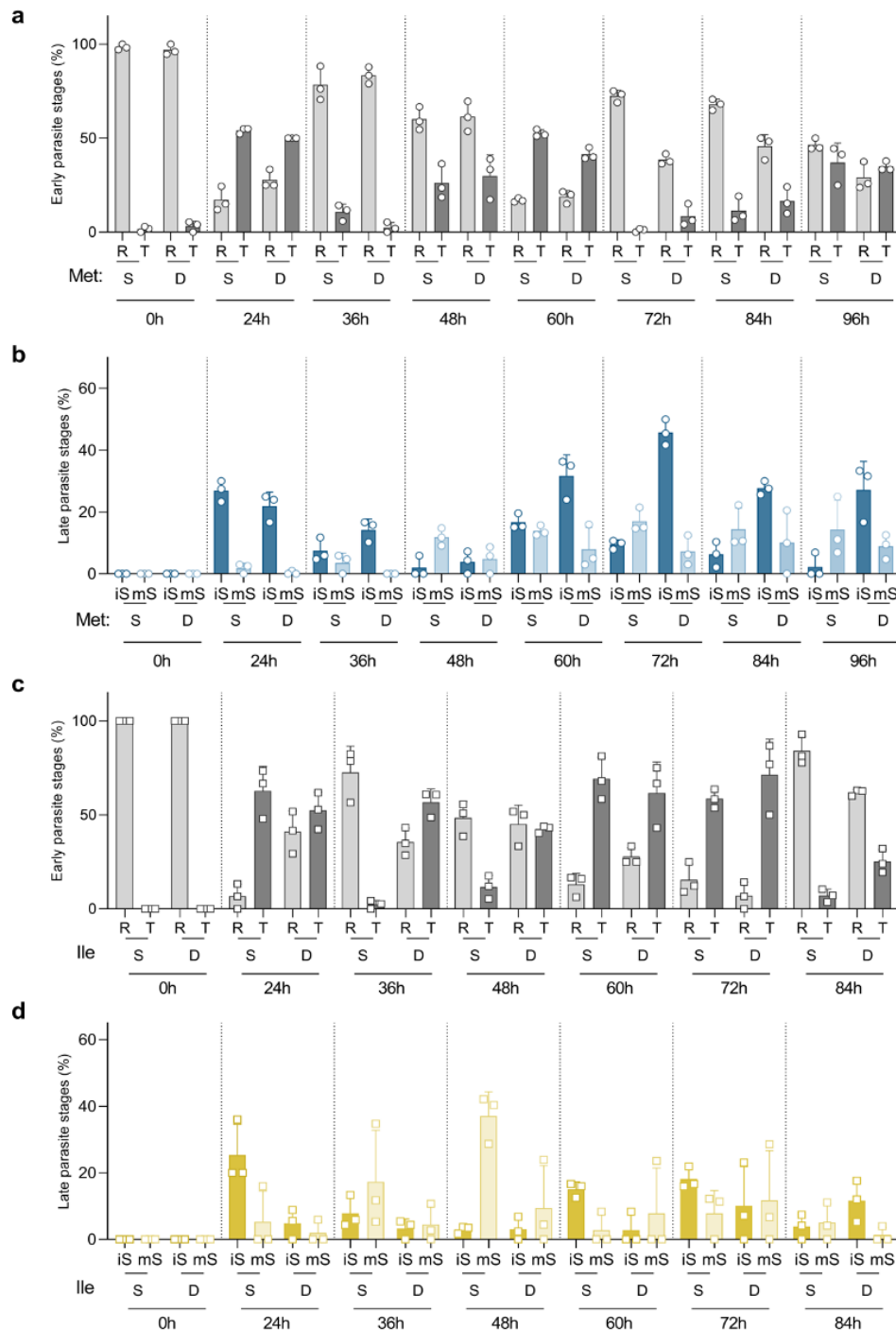


Supplementary Figure 2.1 - AA depletion impacts parasite intra-erythrocytic development but not viability. (a) Parasitemia of synchronized *P. falciparum* WT parasites cultured in MetS, MetD or MetD media supplemented with methionine (MetD supp). Methionine was provided to the media at the concentration found in RPMI (100 μ M) from day 4 to day 6 after sorbitol synchronization. Parasitemia was measured by flow cytometry after SYBR Green staining. (b, c) Full course analysis of *Pf*3D7 WT developmental stage progression in (b) MetS or MetD media and (c) IleS or IleD media for 2 developmental cycles. Parasite staging was performed by microscopy analysis of Giemsa-stained blood smears. Individual data points, for each timepoint and condition, are provided in Supplementary Figure 2.2. (d) Double crossover homologous recombination at the *Pbsams* locus and the 3'UTR was employed to introduce a fusion of *Pbsams* ORF with the destabilizing domain system (DD) and an HA tag (*PbSAMS-DD* line). Annealing sites for genotyping primers are illustrated and primer sequences are given in Table 2.1. (e) Agarose gel image, showing diagnostic PCR products from *P. berghei* SAMS-DD and *P. berghei* WT genomic DNA, after dilution cloning of the *PbSAMS-DD* transgenic parasite line. (f) Densitometry analysis of SAMS-HA-DD protein levels in BALB/c mice infected by intraperitoneal inoculation of 1×10^6 *PbSAMS-DD* infected RBCs (iRBCs). SAMS protein levels were

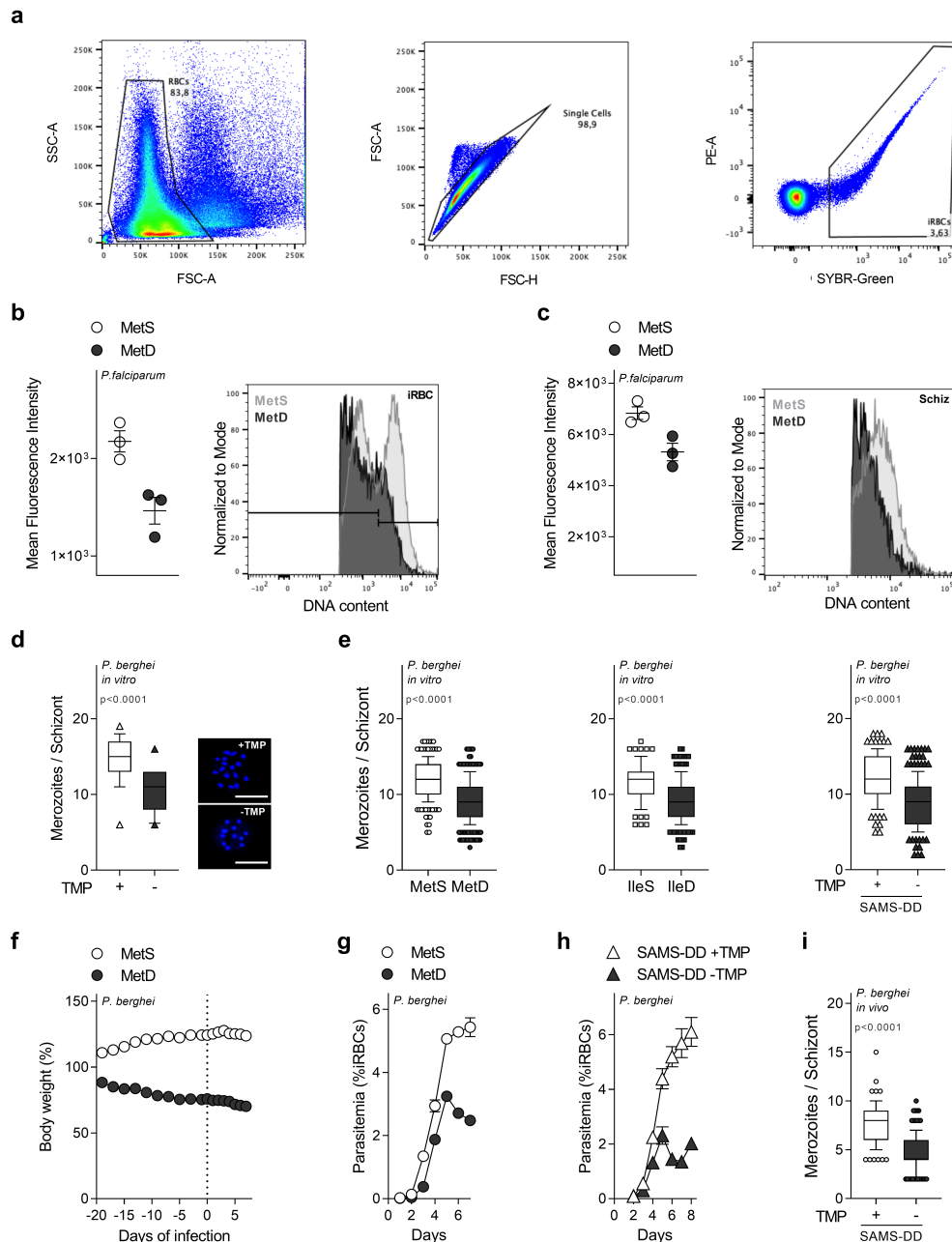
probed for the HA-tag (*PbSAMS*-HA-DD line; αHA, 70KDa). Bip was employed as loading control. **(g, h)** Representative image and quantification of SAMS protein levels at the **(g)** trophozoite (Troph), schizont (Schiz) and **(h)** merozoite (Meroz) stages in *PbSAMS*DD parasites developing in BALB/c mice treated or not with TMP, measured by fluorescence microscopy after probing for the HA-tag. **a.** Data represents the mean percentage of iRBCs in $n=2$ independent experiments, each performed at least in triplicate. **b, c.** Data is shown as mean percentage of parasites at each developmental stage with error bars representing SEM. $n=3$ independent experiments. The dashed squares represent the temporal window during which amino acid deprivation has the greatest impact on parasite development. **f.** Representative western blot image, showing SAMS stabilization after TMP treatment. Data is shown as mean $\pm$ SEM of SAMS-HA-DD levels normalized to Bip levels (Mann-Whitney). Data represents $n=3$ independent experiments and each lane represents an individual mouse (10 mice per condition). **g, h.** Scatter dot plots represent SAMS intensity levels in $n=2$ independent experiments (2 mice /condition). Data is shown as SAMS mean intensity values inside *PbSAMS*-DD +TMP and *PbSAMS*-DD -TMP parasites, normalized to parasite area (Mann-Whitney). Scatter dot plots show the data for the following number of parasites (N): Trophozoite stage: SAMS-DD: +TMP, $N=20$; -TMP, $N=20$; Schizont-stage: SAMS-DD: +TMP, $N=15$; -TMP, $N=21$ and Merozoite-stage: SAMS-DD: +TMP, $N=102$; -TMP $N=108$. Scale bars = $5\mu\text{m}$.



Supplementary Figure 2.2 - Impact of AA depletion throughout *P. falciparum* intra-erythrocytic development. (a,b) Quantification of (a) ring- and trophozoite- parasite stages (early parasite stages) or (b) immature- and mature- schizont stages (late parasite stages) in MetS or MetD media. (c,d) Quantification of (c) ring- and trophozoite- stages or (d) immature- and mature- schizont stages in IleS or IleD media. Data represents the mean percentage of parasites at each developmental stage with individual datapoints representing independent experiments ($n=3$). Error bars represent SD. R= ring; T= trophozoite; iS = immature schizont; mS= mature schizont.

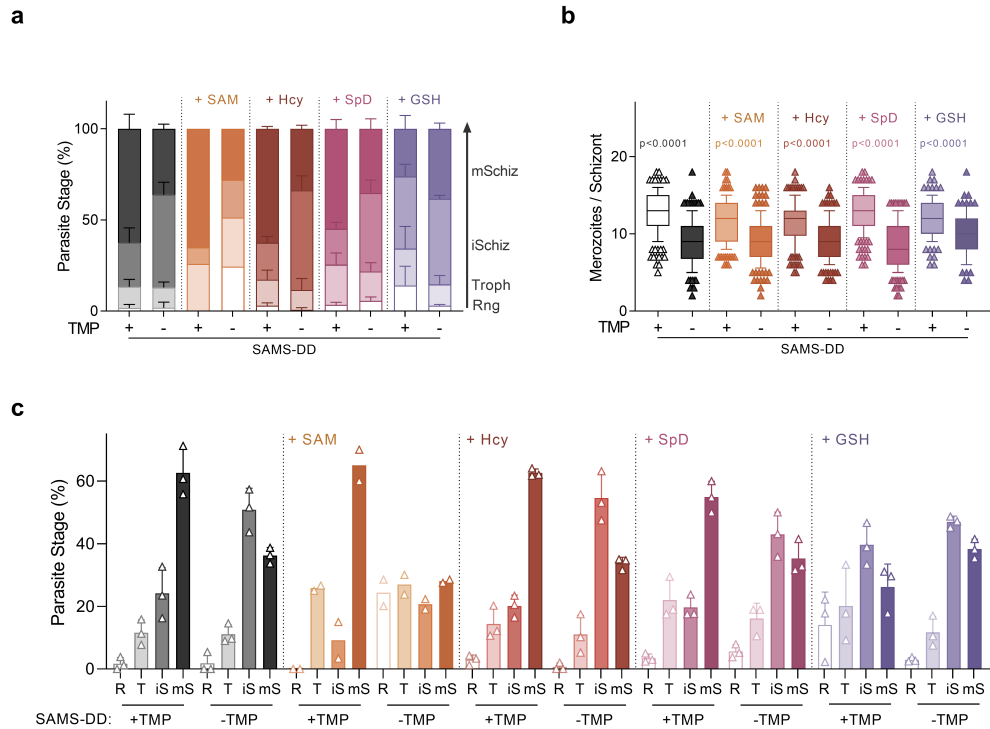


Supplementary Figure 2.3 - Impact of AA depletion in *P. berghei* intra-erythrocytic development using the *ex vivo* maturation assay. (a-c) Quantification of *P. berghei* developmental stage progression under (a) MetS and MetD or (b) IleS and IleD media and in (c) SAMS knockdown parasites when cultured *ex vivo* for 24 or 30 hours. Data represents the mean percentage of parasites at each developmental stage with individual datapoints representing independent experiments. $n=3$ independent experiments, except for IleS, IleD at 24h ($n=2$). Error bars represent SD. R= ring; T= trophozoite; iS = immature schizont; mS= mature schizont.

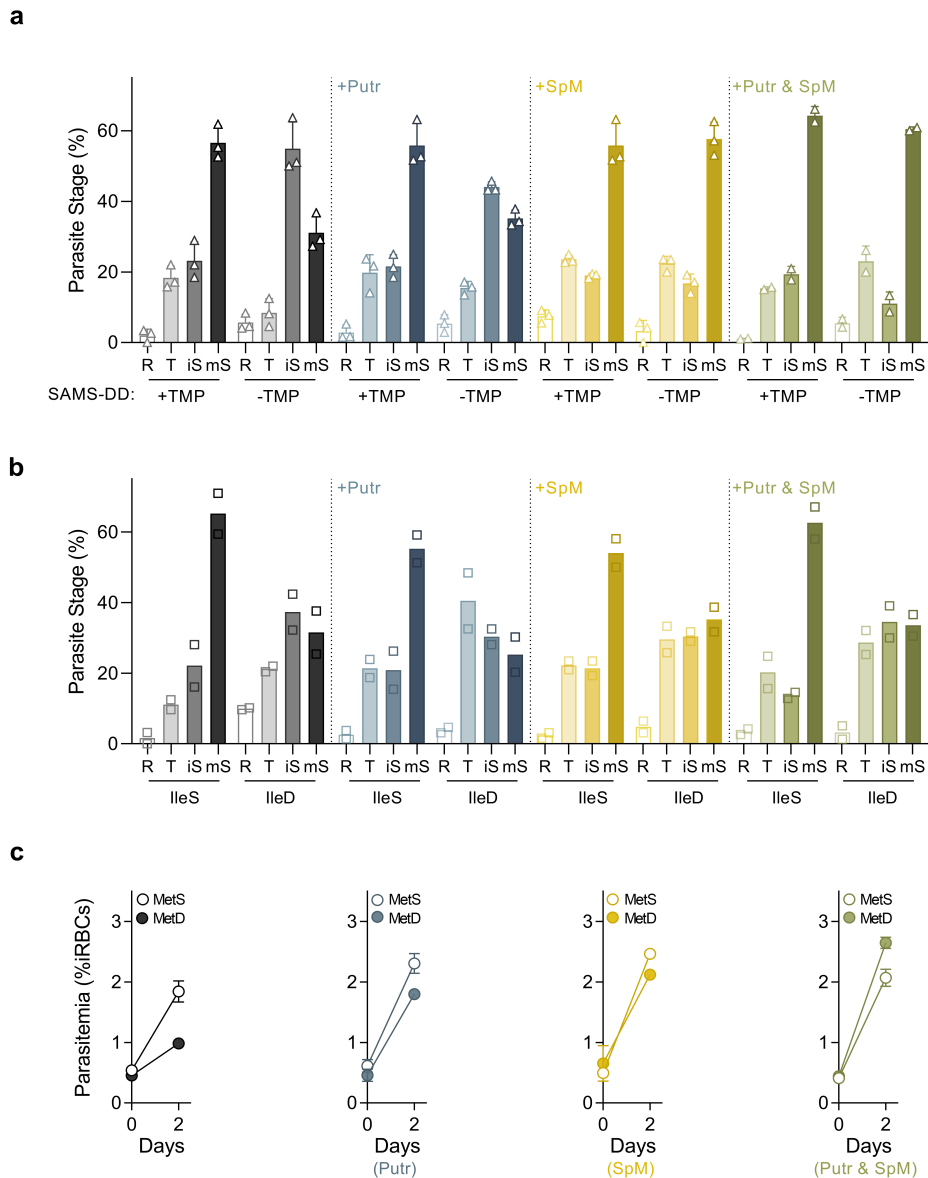


Supplementary Figure 2.4 - Effect of AA depletion *in vitro*, *ex vivo* and *in vivo*. (a) Representative image of flow cytometry plots and gating strategy for analysis of synchronized *P. falciparum* parasites cultured in MetS or MetD media after SYBR-Green staining. Samples were analyzed on BD LSRFortessa and the number of total acquired events ranged from 50 000 to 10 000/condition. The data was further processed on FlowJoTM 10 Software (Tree Star Inc.). Single RBCs were selected on the basis of their size (left and middle panel) and, subsequently, on the DNA content, excluding false positives associated to RBC autofluorescence (right panel). (b, c) Mean Fluorescence Intensity (MFI) in (b) RBCs infected with synchronized *P. falciparum* WT parasites or (c) *P. falciparum* WT schizonts cultured in MetS or MetD media. Histogram plot showing the fluorescent intensity comparison between MetS and MetD. (d) Mean merozoite numbers in *P. berghei* SAMS-DD parasites, treated or not with TMP, using the *ex vivo* maturation assay. Mature schizonts were visualized by microscopy after DNA staining with DAPI and quantified using the ImageJ counter tools. Representative fluorescent microscopy image shows the mean merozoite number comparison between SAMS-DD +TMP and SAMS-DD -TMP (knockdown) parasites. Scale bar = 5 μ m. (e) Box plot of mean merozoites number per schizont of *P. berghei* parasites after *ex vivo* culture for 30h, in MetS- or MetD-media, IleS- or IleD-media and in SAMS-DD media. (f) Body weight (%) of *P. berghei* parasites over time. The plot shows body weight (%) for MetS (open circles) and MetD (filled circles) parasites over days of infection. (g) Parasitemia (%iRBCs) of *P. berghei* parasites over time. The plot shows parasitemia (%iRBCs) for MetS (open circles) and MetD (filled circles) parasites over days. (h) Parasitemia (%iRBCs) of *P. berghei* parasites over time. The plot shows parasitemia (%iRBCs) for SAMS-DD +TMP (open triangles) and SAMS-DD -TMP (filled triangles) parasites over days. (i) Mean merozoite numbers in *P. berghei* SAMS-DD parasites, treated or not with TMP, using the *ex vivo* maturation assay. The plot shows Merozoites / Schizont for TMP + and - conditions.

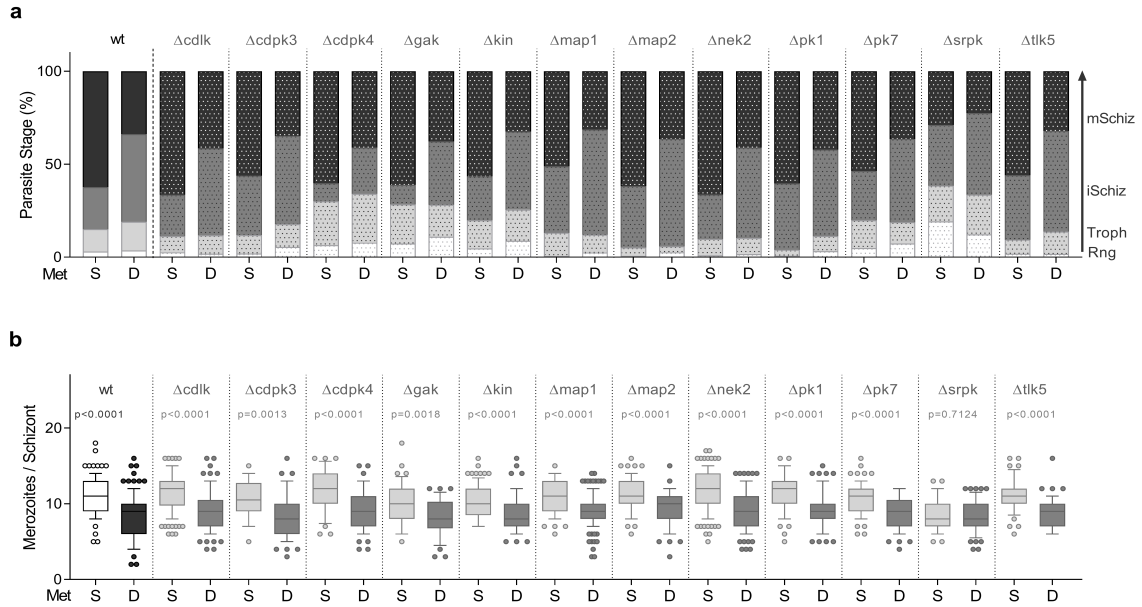
SAMS knockdown parasites. **(f)** Body weight change in BALB/c male mice under a methionine restriction-regimen (MetD) or in regular diet, sufficient for Met (MetS). **(g)** Parasitemia of BALB/c mice (15mice/group) under a MetS or MetD regimen, infected by *i.p.* injection of 1×10^6 GFP-expressing *P. berghei* ANKA WT iRBCs. **(h)** Parasitemia of MetS-fed BALB/c mice infected with *PbSAMS-DD* parasites and treated, or not, with TMP in drinking water. **(i)** Mean merozoite number per schizont of *P. berghei* SAMS-DD parasites developing *in vivo* in MetS-fed BALB/c mice treated or not with TMP. **b, c.** Data is shown as scatter dot plot and represents 1 of $n=2$ independent experiments (3 mice/condition). **d, e.** Data is represented as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (Mann-Whitney). Boxplots show the data of **d.** $n=2$ or **e.** $n=3$ independent experiments and for the following number of schizonts (N): **d.** MetS, $N=20$; MetD, $N=20$; **e.** MetS, $N=261$, MetD, $N=340$; IleS, $N=113$, IleD, $N=192$; SAMS-DD +TMP, $N=163$, SAMS-DD -TMP, $N=200$. **f.** Data is represented as mean percentage of body weight change. Body weight data was normalized to the initial weight of each animal and examined in $n=2$ independent experiments. **g, h.** Data is represented as mean percentage of iRBCs $\pm$ SEM, determined in $n=2$ (MetS, MetD) or $n=3$ (SAMS-DD-HA) independent experiments. **i.** Data is represented as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (Mann-Whitney). Boxplots show the data of $n=3$ independent experiments and for the following number of schizonts (N): SAMS-DD +TMP, $N=152$; SAMS-DD -TMP, $N=131$. Scale bars= $5 \mu\text{m}$.



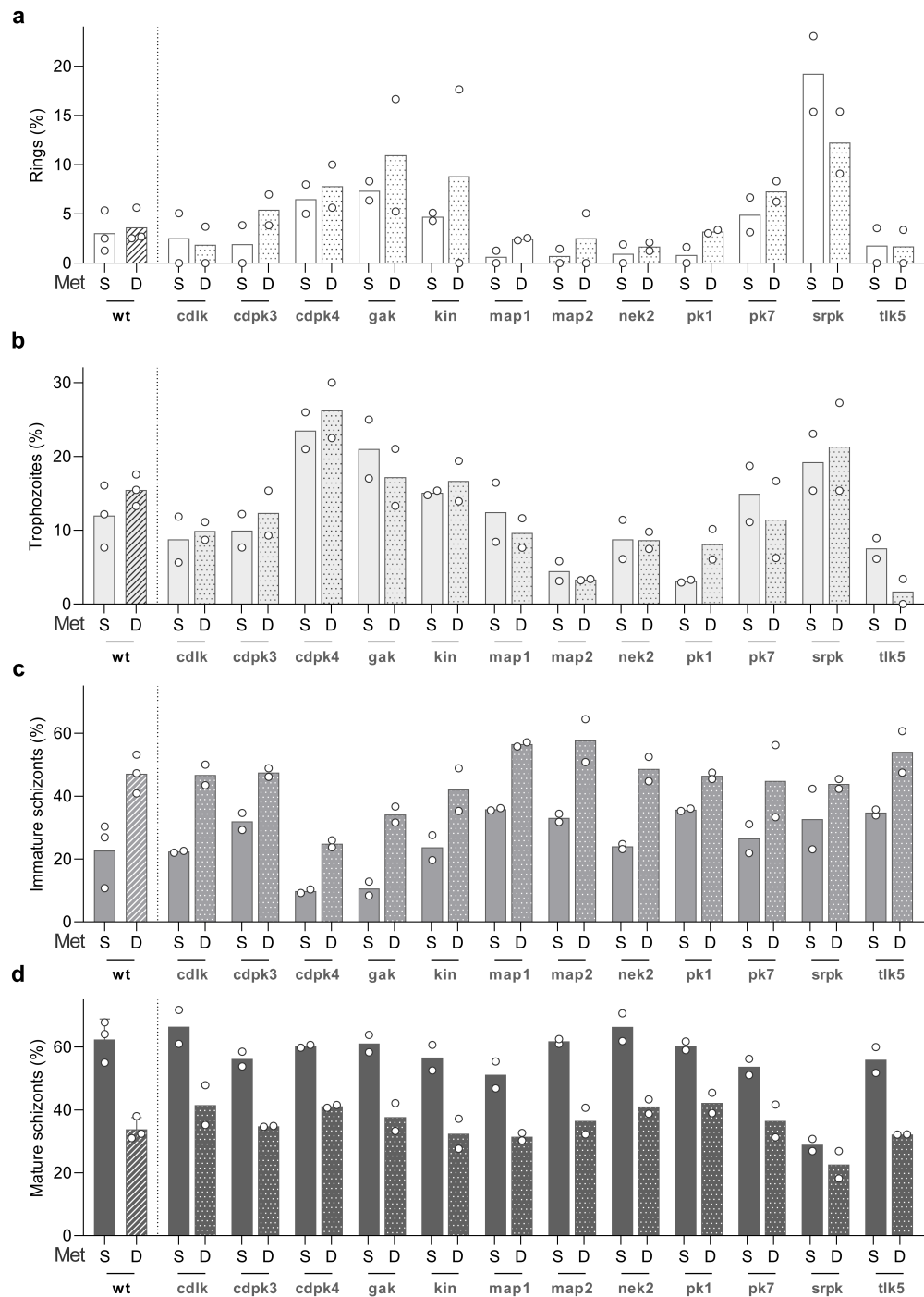
Supplementary Figure 2.5 - Effect of Met-downstream metabolites in *Plasmodium* development and replication. (a, b) Quantification of (a) intra-erythrocytic developmental stages and (b) mean merozoite numbers in *P. berghei* parasites knockdown for the SAMS enzyme after supplementation with S-adenosylmethionine (SAM), homocysteine (Hcy), spermidine (SpD) and glutathione (GSH). (c) Percentage of rings, trophozoites, immature and mature schizonts in *P. berghei* SAMS-DD knockdown parasites growing in media supplemented with the Met-downstream metabolites. **a.** Parasite staging was performed in triplicate, averaged and repeated in $n=3$ independent experiments, except for the SAM metabolite ($n=2$). Data is shown as mean percentage of parasites at each developmental stage, with error bars representing SEM. Individual data points are represented in **c**. Rng: ring; troph: trophozoite; iSchiz: immature schizont; mSchiz: mature schizont. **b.** Data is represented as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (2-way ANOVA). Boxplots show the data of $n=3$ independent experiments, except for the SAM metabolite ($n=2$) and for the following number of schizonts (N): SAMS-DD: +TMP, $N=200$; -TMP, $N=210$; +TMP +SAM, $N=141$; -TMP +SAM, $N=135$; +TMP +Hcy, $N=130$; -TMP +Hcy, $N=129$; +TMP +GSH, $N=102$; -TMP +GSH, $N=83$; +TMP +SpD, $N=154$; -TMP +SpD $N=154$. **c.** Bars represent the mean percentage of parasites at each developmental stage with individual data points representing each independent experiment. $n=3$ independent experiments, except for the SAM metabolite ($n=2$). Error bars represent the SD.



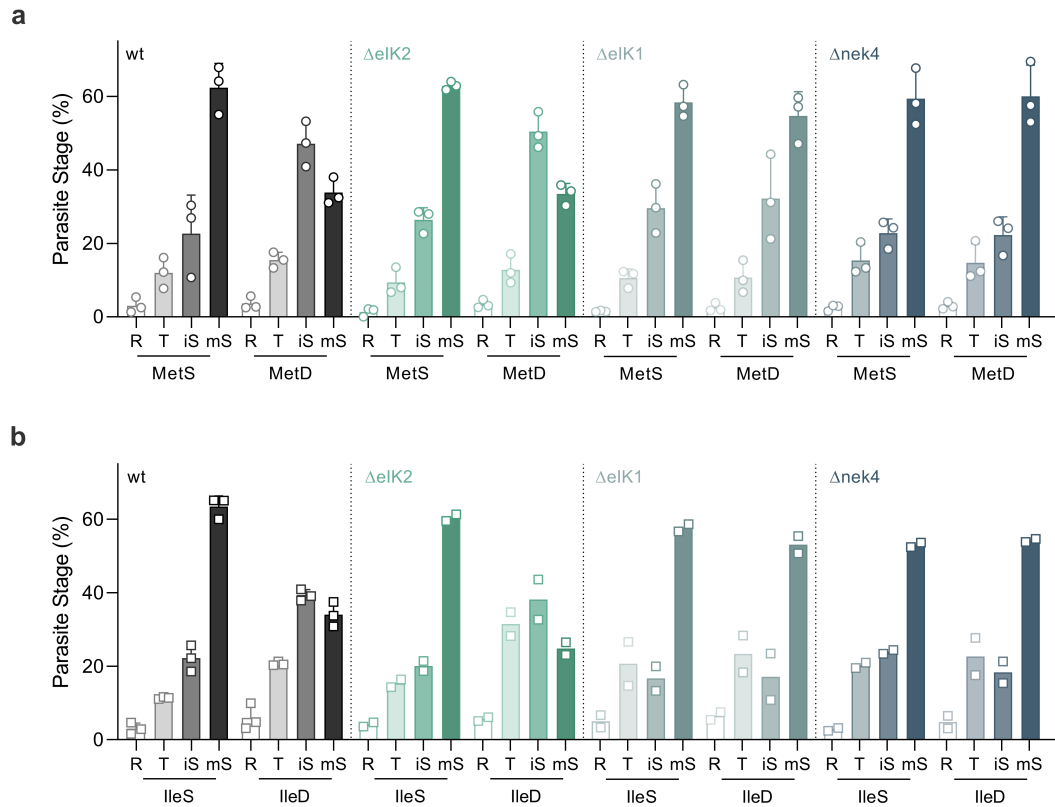
Supplementary Figure 2.6 - Spermine but not putrescine rescues methionine depletion-induced growth impairment. (a, b) Percentage of rings, trophozoites, immature and mature schizonts in *P. berghei* parasites growing in **(a)** MetS or MetD media and **(b)** IleS or IleD media supplemented with the polyamines SpM, Putr or both (Putr & SpM). **(c)** Parasitemia in synchronized *P. falciparum* 3D7 WT parasites cultured in MetD media supplemented with Putr, SpM or both. Parasitemia was measured by flow cytometry after SYBR Green staining. **a.** $n=3$ independent experiments, except for Putr + SpM ($n=2$) or **b.** $n=2$ independent experiments. **a, b.** Bars represent the mean percentage of parasites at each developmental stage with individual data points representing an independent experiment. Error bars represent the SD. **c.** Data represents the mean percentage of iRBCs $\pm$ SEM in $n=2$ independent experiments, each performed at least in triplicate.



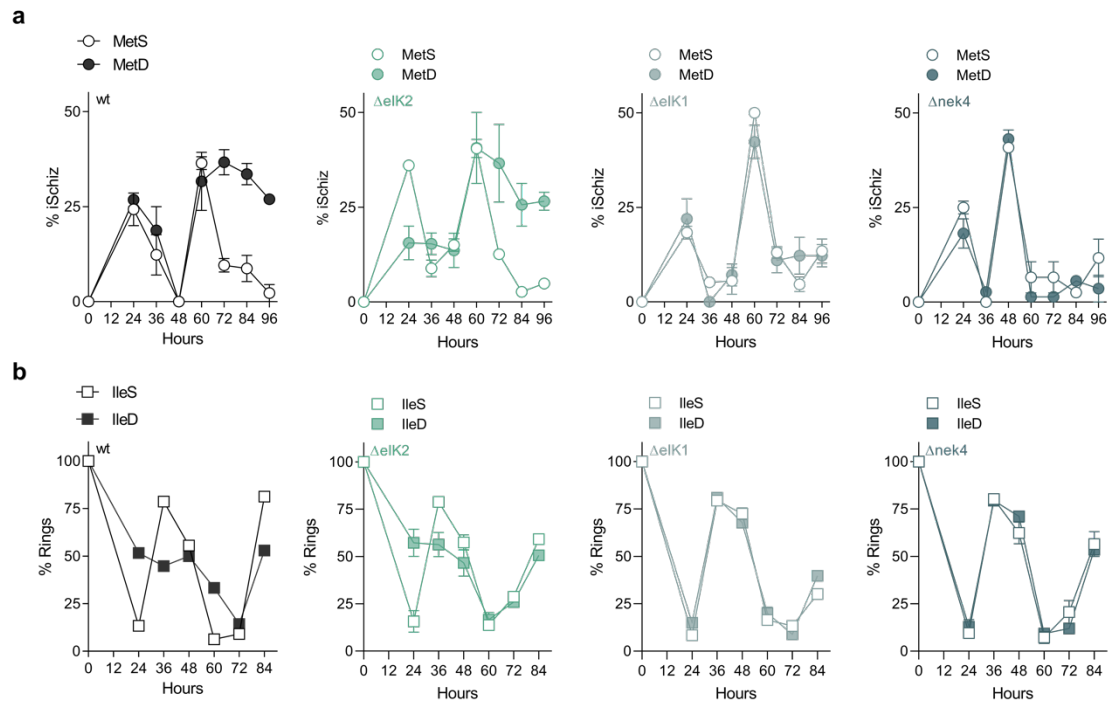
Supplementary Figure 2.7 - Phenotypic screen of *P. berghei* kinase knockout mutant parasites cultured *ex vivo* in AA deficient media. (a, b) Quantification of (a) intra-erythrocytic developmental stages and (b) mean merozoite numbers in *P. berghei* wild-type and kinase knockout mutant parasite lines using the *ex vivo* maturation assay. a. Parasite staging was performed in triplicate, averaged and repeated in $n=2$ independent experiments. Data is shown as mean percentage of parasites at each developmental stage. Individual data points are represented in Supplementary Figure 2.8. Rng= ring; troph= trophozoite; iSchiz= immature schizont; mSchiz= mature schizont. b. Data is represented as box-whisker plot (10-90 percentile) of mean merozoite number per schizont $\pm$ SD (2-way ANOVA). Boxplots show the data of $n= 2$ independent experiments and for the following number of schizonts (N): *P. berghei* wt: MetS, $N= 92$; MetD, $N= 99$; Δ cdlk: MetS, $N=110$; MetD, $N= 81$; Δ cdpk3: MetS, $N= 28$; MetD, $N= 55$; Δ cdpk4: MetS, $N= 33$; MetD, $N= 56$; Δ gak: MetS, $N= 43$; MetD, $N= 34$; Δ kin: MetS, $N= 85$; MetD, $N= 80$; Δ map1: MetS, $N= 82$; MetD, $N= 133$; Δ map2: MetS, $N= 64$; MetD, $N= 58$; Δ nek2: MetS, $N= 112$; MetD, $N= 96$; Δ pk1: MetS, $N= 49$; MetD, $N= 64$; Δ pk7: MetS, $N= 69$; MetD, $N= 73$; Δ srpk: MetS, $N= 37$; MetD, $N= 54$; Δ tlk5: MetS, $N= 44$; MetD, $N= 51$.



Supplementary Figure 2.8 - Parasite stage progression in wt and kinase mutant knockout lines culture in MetD media, using the *ex vivo* maturation assay. (a, d) Percentage of (a) ring- , (b) trophozoite-, (c) immature schizont- and (d) mature schizont-stage parasites cultured in MetS or MetD media. Bars represent the mean percentage of parasites at each developmental stage with individual data points representing an independent experiment. $n=2$ independent experiments



Supplementary Figure 2.9 - *elK1* and *nek4*, but not *elK2*, regulate parasite development under AA deficiency. (a, b) Percentage of ring-, trophozoite-, immature schizont- and mature schizont-stage parasites cultured in **(a)** MetS or MetD media and **(b)** IleS or IleD media. Bars represent the mean percentage of parasites at each developmental stage with individual data points representing an independent experiment. $n=3$ independent experiments, except for kinase knockout mutants growing under IleS or IleD conditions ($n=2$).



Supplementary Figure 2.10 - Impact of AA depletion in *P. falciparum* kinase knockout mutant parasites. (a) Time course analysis of immature schizont-stage *P. falciparum* 3D7 WT and kinase mutant parasites cultured in MetS or MetD media. (b) Time course analysis of ring-stage *P. falciparum* 3D7 WT and kinase mutant parasites cultured in IleS or IleD media. a, b. Data represents the mean percentage of a. immature schizonts or b. ring-stages $\pm$ SEM, each performed in triplicate, averaged and determined in $n=2$ independent experiments.

Acknowledgments

From IMM-JLA, Lisbon, Portugal, we would like to acknowledge Inês Bento for discussions, critical input and reading of the manuscript, Ângelo Ferreira Chora for guidance with flow cytometry analysis and for critical reading of the manuscript, Yash G. Pandya for critically reviewing this manuscript, the Bioimaging Facility for assistance with microscopy experiments, the Rodent Facility for overall animal husbandry and support with mice handling and the Flow Cytometry facility for assistance with equipment handling. We would like to thank Mathieu Brochet (University of Geneva Department of Microbiology and Molecular Medicine, Genève, Switzerland) and Christian Doerig (Monash Biomedicine Discovery Institute, Clayton, Australia) for kindly gifting the *P. falciparum* kinase mutant parasite lines: Δ elK1, Δ elK2 and Δ nek4. We would like also to express our gratitude to Arthur Scherf (Biology of Host-Parasite Interaction, Institut Pasteur, Paris, France) for discussions and for critically reviewing this manuscript.

Work at IMM-JLA was supported by Fundação para a Ciência e Tecnologia (PTDC/BIA-MOL/30112/2017) and the “La caixa” Banking Foundation (HR17-00264-PoEMM) grants attributed to V.Z.L. and M.M.M., respectively. Work at the Weill Cornell Medicine (BK. and CT) was funded by NIH 1R01 AI141965 (BK), NIH 1R01 AI138499 (BK), Alice Bohmfalk Charitable Trust Research Grant (B.K.), NIH 5F31AI136405-03 (C.H.). Work at the Loyola University Chicago (B.M. and V.C.) was supported by funds from NIH NIGMS R35GM138199. M.I.M., S.M. and V.Z.L. were supported by Fundação para a Ciência e Tecnologia, Portugal (PD/BD/135454/2017, DL57/2016/CP1451/CT0010 and DL 57/2016/CP1451/CT0022 respectively).

CHAPTER III

CHAPTER III

Plasmodium parasites exploit liver-specific methionine metabolism to drive high replication rates

The Chapter III: “*Plasmodium parasites exploit liver-specific methionine metabolism to drive high replication rates*” consists of a second authorship publication. Here is clarified the contribution of each of the authors:

Publication:

Vanessa Zuzarte-Luis¹, Inês M. Marreiros^{1,2}, Catarina Pedro¹, Ksenija Slavic¹, Sofia Marques¹, Aparajita Lahree¹, João Mello-Vieira¹, Maurice Itoe¹, Miguel Prudêncio¹, José M. Mato³, Maria M. Mota¹

¹ Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina Universidade de Lisboa, 1649-028, Lisbon, Portugal

² Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal.

³ Precision Medicine and Metabolism Laboratory, CIC bioGUNE, Basque Research and Technology Alliance, Derio, Spain.

Inês M. Marreiros, the candidate for the PhD degree at the University of Porto contributed to the paper by thoroughly characterizing the previously generated SAMS-deficient transgenic parasite line (*see Chapter II*) critical for this project. Under the supervision of Dr. Vanessa Zuzarte-Luís, Inês has generated and characterized the *PbSAMS-DD* parasite line during liver stage development, disclosing novel insight on how *Plasmodium* exploits the host methionine metabolism to sustain growth. The PhD candidate has first determined the extent of SAMS protein knockdown *in vivo* throughout the entire lifecycle, by performing immunoblotting analysis of blood stage parasites or immunofluorescence analysis for liver- and mosquito-stage developing parasites. Inês has also conducted experiments addressing the role of the SAMS enzyme during blood stage development in mice.

By assessing parasitemia and survival in BALB/c and C57BL/6 rodent malaria models, Inês has disclosed a key role for this enzyme in parasite intra-erythrocytic growth as well as virulence. She further assessed the impact of SAMS depletion during liver stage development by performing qPCR analysis to measure parasite liver load. Together, these approaches have revealed that whilst essential during the blood stage of development, *Plasmodium* does not rely on its own SAMS enzyme to successfully develop inside host hepatocytes.

Inês also participated in the characterization of the MAT KO mouse model, by performing *in vivo* experiments as well as qPCR analysis to determine liver load. Additionally, Inês has participated in the writing of the manuscript for peer-reviewed publication and as an integral part of the thesis. Dr. Vanessa Zuzarte-Luís designed and supervised the project and Dr. Maria M. Mota revised the manuscript.

The contribution of each author to the publication is clarified in the section “Authors contribution” as follows: Conceptualization: V.Z.-L and M.M.M; Methodology: V.Z.-L; Investigation: **I.M.M**; C.P.; K.S., S.M., I.B., A.L., J.M.-V., M.I. Writing – Original draft: V.Z.-L and M.M.M.; Writing – Review and Editing: **I.M.M.**, S.M., I.B., M.P. and M.M.M.; Funding Acquisition: V.Z.-L and M.M.M.

Manuscript *in preparation*.

Abstract

Plasmodium parasites have evolved to live within the mammalian red blood cells of their vertebrate host and cause malaria. However, the very first round of *Plasmodium* replication in the mammalian host does not occur inside those cells. Instead, mammalian malaria parasites home to the liver, where each parasite replicates into tens of thousands of erythrocyte-infectious parasites, termed merozoites. In stark contrast, avian and reptile malaria parasites first infect macrophages at the mosquito bite site and differentiate into only dozens of merozoites. However, the reason for this markedly distinct behavior remains utterly unexplored. Here, we show that *Plasmodium*'s remarkable replication rate in the mammalian liver depends on the uniqueness of the mammalian hepatic methionine (Met) metabolism, conferred by the liver-specific methionine adenosyltransferase (MAT1) enzyme and its capacity to generate unlimited amounts of S-adenosylmethionine (SAM). Our results demonstrate that *Plasmodium*'s intensive replication inside hepatocytes occurs independently of its intrinsic SAMS enzyme (the orthologue of mammalian MAT1), but relies on the host's MAT1 enzyme activity. We further show that the impairment of infection observed upon Met depletion is reversible both *in vitro* and *in vivo* by exogenous administration of SAM. By revealing the crucial role of liver-specific Met metabolism in the malaria parasite's hepatic replication, our results provide new insight on the evolutionary selection of the liver as the exoerythrocytic site of *Plasmodium* development in mammals, prior to infection of red blood cells.

Introduction

With an estimated yearly toll of 200 million cases and more than 600 thousand deaths, malaria continues to present a substantial challenge to both global health and economies [4]. The disease is caused by *Plasmodium* parasites, transmitted to their vertebrate hosts as sporozoites injected by the bites of infected *Anopheles* mosquitoes. *Plasmodium* parasites have evolved to reside within the red blood cells of their vertebrate hosts and cause malaria. However, prior to the onset of symptomatic disease, injected sporozoites undergo an obligatory pre-erythrocytic stage, transforming into blood-infectious merozoites. In avian and reptile malaria, this initial, pre-erythrocytic phase of *Plasmodium* development occurs near the bite site inside macrophages, where each sporozoite replicates through schizogony into dozens of erythrocyte-infectious merozoites [226]. Conversely, in mammals, mosquito-transmitted parasites travel to the liver and infect hepatocytes, where they undergo extensive replication, generating tens of thousands of merozoites that are subsequently released into the bloodstream, initiating the blood stage of the infection [13]. Despite the central role of the liver in this process, the underlying reason for the remarkable difference in the extent of pre-erythrocytic *Plasmodium* replication between mammals and other vertebrates is still poorly understood.

Methionine (Met) is an essential amino acid used in protein synthesis and in the generation of S-adenosylmethionine (SAM), through the action of methionine adenosyltransferase (MAT) enzymes. MAT activity in mammals is the product of two genes, *MAT1A* and *MAT2A*, encoding the MAT1 and MAT2 isoenzymes, respectively, which display a tissue-specific pattern of expression [506,507]. While *MAT2A* is ubiquitously expressed and is responsible for SAM synthesis in extrahepatic tissues, *MAT1A* is expressed only in the adult liver (reviewed in [506]). Remarkably, although all mammalian cells are capable of synthesizing and use SAM, these MAT isoenzymes differ radically in their kinetic and regulatory properties, resulting in distinct cellular SAM levels in different tissues. While the extra-hepatic MAT2A is strongly inhibited by SAM, the liver-specific MAT1A is not inhibited by its product. As a result, about 50% of all dietary Met is metabolized in the liver and up to 85% of all SAM-dependent transmethylation reactions take place in hepatocytes [508,509].

SAM is the most important methyl donor in cellular metabolism, and the source of the propylamine groups used in polyamine biosynthesis [507]. Under normal physiological conditions, most available SAM is used in transmethylation reactions, during which its

methyl group is transferred to a large variety of methyl acceptors, such as DNA, RNA, proteins, and lipids. Among other metabolic pathways, SAM-dependent transmethylation reactions have been implicated in the generation of S-adenosylhomocysteine (SAH), which is hydrolyzed into homocysteine (Hcy) and adenosine [507]. Hcy is an important metabolic hub that can be remethylated to re-generate methionine or enter the unidirectional transsulfuration cycle, ultimately leading to the synthesis of glutathione (GSH), a major cellular antioxidant [507]. SAM is also a source of propylamine groups, used in the aminopropylation cycle for the synthesis of polyamines (PA), involved in the control of cell growth [510]. Thus, disruption of the liver-specific MAT1A results in hypermethioninemia (776%), as well as in a marked reduction in hepatic levels of glutathione (40%) and SAM content (74%) [511], which occur in spite of the induction of MAT2A expression and activation [511].

The *Plasmodium* genome includes a gene that encodes a MAT orthologue (SAMS enzyme), as well as all the necessary enzymes to complete methionine (Met) metabolism [512–514]. Ample evidence indicates a pivotal role for Met during the erythrocytic phase of the malaria parasite life cycle [344,477,515–517]. Furthermore, a recent study demonstrated the essentiality of the *Plasmodium* SAMS enzyme during the blood stage of infection [518]. However, except for an association between histone methylation and the activation of dormant parasite forms in the liver, termed hypnozoites [519], the importance of *Plasmodium*'s Met metabolism during liver stage development remains largely elusive.

Given the critical role of the liver in methionine metabolism, we sought to investigate how liver-stage *Plasmodium* parasites exploit this metabolic resource. Our results unveil a pivotal role for the hepatocyte-specific MAT1 enzyme in generating the large amounts of SAM required by hepatic parasites to sustain their developmental process. These findings provide a long-sought explanation for *Plasmodium* sporozoites' tropism for the mammalian liver and for their remarkably high intra-hepatic replication capacity, and may pave the way for the development of novel strategies aimed at blocking the initial stages of malaria infection in humans by targeting the hepatic methionine metabolism.

Results

SAM is the critical methionine metabolite for parasite replication

To assess the importance of Met for *Plasmodium*'s intra-hepatic development, C57BL/6 mice were kept on a Met-deficient (MD) diet prior to injection of *P. berghei* sporozoites, and the ensuing liver infection was compared to that of control mice fed on a Met-sufficient (MS) diet. The results show that the administration of a MD diet for 4 days, starting 2 days prior to sporozoite injection, led to a significant reduction in liver parasite load relative to that observed in control mice (Figure 3.1A). Further analysis of the liver tissue revealed that, while no significant differences in parasite numbers were observed between the two groups of mice (Figure 3.1B), a clear decrease in the size of the parasite's exoerythrocytic forms (EEFs) could be observed in mice under MD diet (Figure 3.1C). Accordingly, a detailed analysis of developing EEFs by super resolution microscopy followed by 3D reconstruction showed that the number of nuclei inside EEFs developing under Met deficiency was significantly lower than that observed under MS conditions, suggesting that the lack of Met strongly impacts parasite replication (Figure 3.1D, E).

We then assessed the effect of Met restriction on hepatic *Plasmodium* infection *in vitro* employing HepG2 cells grown in either MS or MD medium. Bioluminescence and flow cytometry analyses of cells infected by luciferase- and GFP-expressing *P. berghei* (*Pb_{LUC}-GFP*) or GFP-expressing *P. berghei* (*Pb_{GFP}*) parasites, respectively, revealed that, similarly to what we observed *in vivo*, the infection load in HepG2 cells cultured under MD conditions was lower than that of cells grown in MS medium (Figure 3.1F). In agreement with the data obtained using rodent models, while a similar number of infected cells was observed in HepG2 cells grown in MD or MS medium (Figure 3.1G), EEFs exhibited impaired development under MD conditions, relative to MS conditions (Figure 3.1H). Importantly, our data also show that host cell viability is not affected by Met deficiency throughout the duration of the experiment, suggesting that the observed effects on the parasite are not due to impaired host cell fitness (Supplementary Figure 3.1A). Additionally, our *in vitro* data were further validated employing mouse primary hepatocytes (mPH) as host cells. Our results show that, similar to HepG2 cells, the overall infection load is lower in mPH cultured on MD medium compared to MS medium (Supplementary Figure 3.1B). This decrease in infection load is not attributed to a

reduced number of infected cells (Supplementary Figure 3.1C), but rather to a defect in parasite development (Supplementary Figure 3.1D). Furthermore, Met deficiency did not affect mPH viability under the experimental conditions, consistent with the findings obtained for HepG2 cells (Supplementary Figure 3.1E).

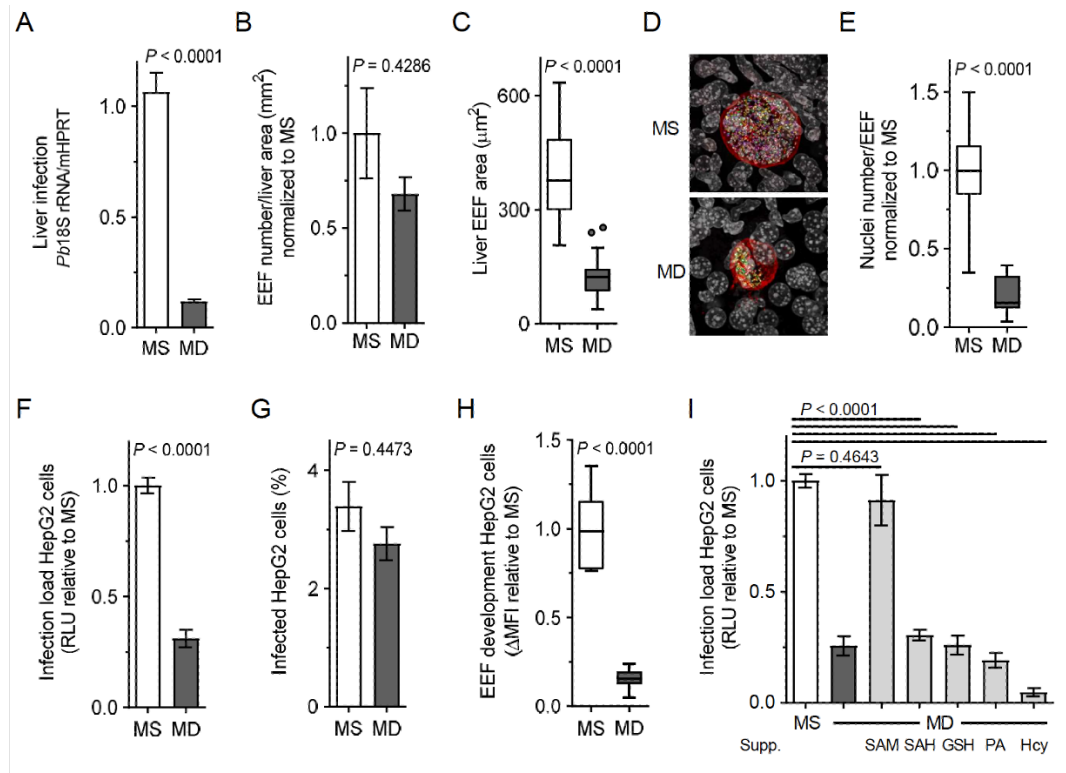


Figure 3.1 - The impact of methionine depletion in *Plasmodium* liver-stage development is strongly linked to decreased SAM levels. (A) Parasite liver load (mean±SEM, Mann-Withney) in MS- or MD-fed mice at 48h after sporozoite infection, measured by qRT-PCR of *P. berghei* 18S rRNA. Values are normalized to hypoxanthine-guanine phosphorubosyltransferase (*Hprt*). (MS, $n=18$; MD, $n=20$) (B) Parasite density (mean±SEM, Mann-Withney) and (C) EEF size (Mann-Withney) in liver sections of MS- and MD-fed mice at 48h after infection. EEFs numbers and size were visualized and quantified by fluorescence microscopy. (For parasite numbers: MS, $n=5$; MD, $n=6$; for EEF size, boxplots show the data for the following number of parasites: MS, $n=57$; MD, $n=39$.) (D) Representative images of EEFs and (E) quantification of nuclei number per EEF (Mann-Withney) in liver sections of MS- and MD-fed mice at 48h after infection with *P. berghei* sporozoites. Nuclei were visualized and quantified by super-resolution microscopy, followed by 3D reconstruction. PVM stained with anti-uis4 (red) and parasite DNA with hoechst (artificially coloured in multiple colors) to score the number of nuclei per EEF. Boxplots show the data for the following number of parasites: MS, $n=15$; MD, $n=15$. (F) Quantification of *P. berghei* infection load (mean±SEM, Mann-Withney) in HepG2 cells cultured in MS- or MD-media at 48h after sporozoite infection, assessed by relative luminescence units of luciferase expressing parasites. Bars represent the data for the following number of replicates: MS, $n=9$ and MD, $n=9$. (G) Percentage of infected cells and (H) EEF development (Mann-Withney) in HepG2 cells cultured in MS- and MD-media, 48h after sporozoite addition to the culture media. Infected hepatocytes were analysed and quantified by flow cytometry. $n=14$ for all experimental conditions. (I) Quantification of parasite load in HepG2 cells cultured in MS- or MD-media supplemented with S-adenosylmethionine (SAM), adenosylhomocysteine (SAH), glutathione (GSH), the polyamine putrescine (PA) and homocysteine (Hcy), analyzed by relative luminescence units of luciferase expressing parasites at 48h after sporozoite infection.

Met is an important precursor of the aminopropylation and transsulfuration pathways, and a crucial substrate for the methylation cycle [520]. Therefore, we assessed whether exogenous administration of any of the products of Met metabolism could revert the effect of the absence of this amino acid on *Plasmodium* liver infection. To this end, we compared the infection load of *Pb_{Luc-GFP}*-infected HepG2 cells or mPH cultured in MD medium supplemented with S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), glutathione (GSH), polyamines (PA) or homocysteine (Hcy) to infected cells cultured in MS medium. Our data show that administration of SAM, but not of SAH, GSH, PA or Hcy, compensates for the lack of Met. Notably, cells grown in MD medium supplemented with SAM exhibit an infection load comparable to that in cells grown in MS medium (Figure 3.1I and Supplementary Figure 3.1F).

After establishing the crucial role of Met and its downstream metabolite, SAM, during *Plasmodium* hepatic infection, we next sought to determine the exact timeframe during the parasite's developmental process at which they are essential. We accomplished this by supplementing Met or SAM to infected cells grown in MD medium at different stages of liver infection. These phases included the initial stage of sporozoite differentiation into a round EEF (0-16h) and the parasite's replicative stage (from 16h onwards). Our results show that Met/SAM are only required during the replicative phase of *Plasmodium* development (Figure 3.2A). Consistent with this observation, EEFs developed in HepG2 cells cultured in SAM-supplemented MD medium to a similar extent as in cells cultured in MS medium (Figure 3.2B), indicating a role for SAM in hepatic *Plasmodium* replication.

Next, we sought to characterize the parasite's replication dynamics during liver stage development, under both the presence or absence of Met. This was achieved by analysing the temporal pattern of DNA replication throughout infection. Because *Plasmodium* lacks the capacity to use thymidine analogues (BrdU/EdU) for nucleotide conversion and DNA synthesis due to the absence of the thymidine kinase (TK) enzyme in its genome, we engineered a *P. berghei*-GFP-TK transgenic parasite line. The TK sequence was integrated into the parasite's non-essential P230p gene locus via a double-crossover process. *Plasmodium*'s uptake of EdU throughout liver-stage infection was monitored by infecting HepG2 cells. Our findings clearly indicate that although *Plasmodium* DNA replication (EdU incorporation) remains comparable between MD and MS conditions up to 24hpi, there is a marked decline in DNA replication in parasites under MD conditions at 36 and 48hpi. This pattern is in stark contrast to parasites developing under MS conditions (Figure 3.2C).

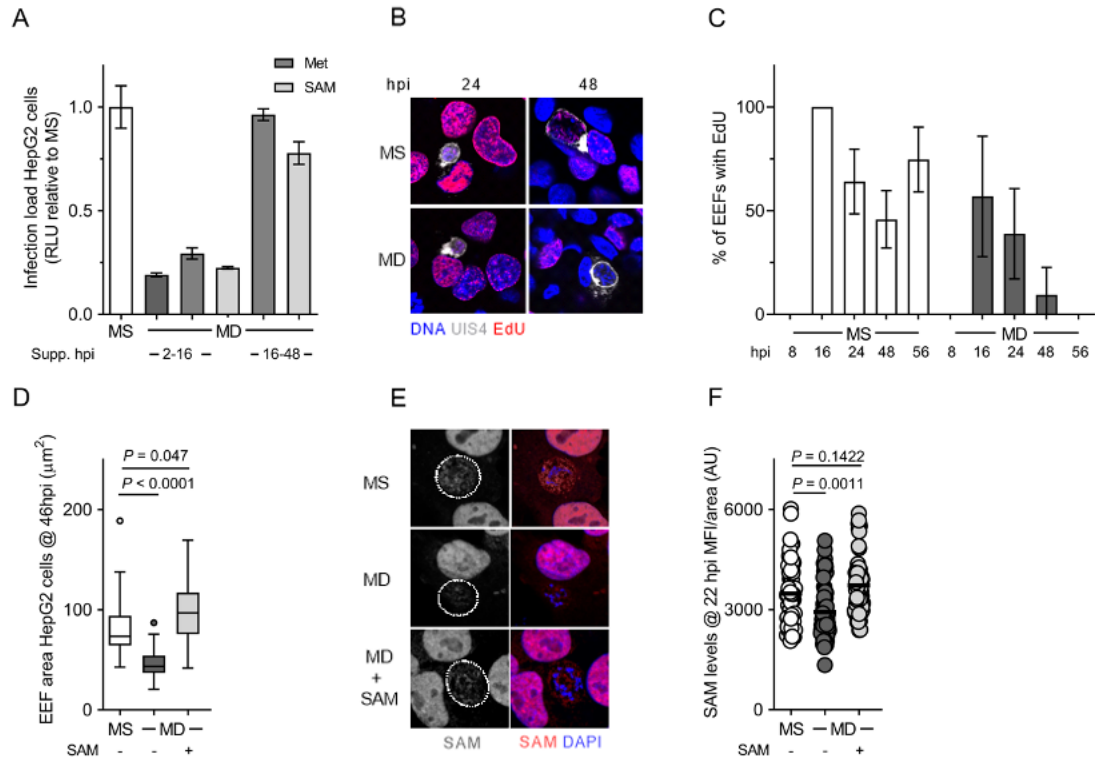


Figure 3.2 - Met/SAM are only required during the replicative phase of *Plasmodium* development. (A) Time course analysis of parasite infection load (mean±SEM, Mann-Whitney) in HepG2 cells cultured in MS-, MD- or MD-media supplemented with Met or SAM. Methionine and SAM were provided to the media during sporozoite to EEF transition (2-16h timepoint) or later in development, during liver-stage schizogony (16-48h timepoint). Infection load was assessed by relative luminescence units of luciferase expressing parasites at 46h after sporozoite infection. Bars represent the mean of 3 independent experiments for all experimental conditions. (B) Representative images of EEFs in HepG2 cells cultured in MS- (top) and MD- (bottom) media at 24 and 48h after infection with *P. berghei* sporozoites expressing a transgene for the *Herpes simplex* thymidine kinase that allows for EdU incorporation during replication; EdU was supplied 4h prior to fixation for analysis to score the number of EEFs undergoing replication at that point. EdU labelled by click chemistry (red), PVM stained with anti-uis4 (white) and parasite DNA with hoechst (blue). (C) Quantification of EEFs showing one or more replication event as identified by EdU staining inside, Bars represent the mean of 3 independent experiments for all experimental conditions. (D) Quantification of EEF size (Mann-Whitney) in HepG2 cells cultured in MS-, MD- and MD-media supplemented with SAM, at 46h after sporozoite infection. EEFs were visualized and quantified by fluorescence microscopy. (MS, $n=45$; MD, $n=59$; MD+SAM, $n=46$). (E) Representative immunofluorescence images for quantification of SAM levels. HepG2 cells were cultured in MS-, MD- and MD media supplemented with SAM, at 22h after sporozoite infection. SAM levels were visualized and measured by immunofluorescence microscopy using a SAM-specific antibody (magenta). (F) Quantification of SAM levels (mean; Mann-Whitney) inside EEFs. Scatter dot plots show the data for the following number of EEFs: MS, $n=54$; MD, $n=73$; MD+SAM, $n=70$.

Subsequently, a SAM-specific antibody was employed to monitor SAM levels within developing intra-hepatic parasites at 22 hours post-infection (hpi), shortly after the replication process had started, when growth impairment caused by Met deficiency was

not yet evident (Supplementary Figure 3.1G). Our results show that, at this specific timepoint, SAM levels in parasites developing under MD conditions are notably lower than those observed in control cells growing under Met sufficient conditions (Figure 3.2D, E). Importantly, our data also show that the supplementation of SAM to MD medium effectively restores intra-parasitic SAM levels to levels comparable to those in control parasites, suggesting the ability of developing parasites to uptake exogenous SAM to meet their developmental demands (Figure 3.2E, F). Collectively, our results demonstrate that the massive replication rate achieved by *Plasmodium* parasites within hepatic cells is reliant on the availability of Met, especially the SAM metabolite.

***Plasmodium* MAT enzyme (SAMS) is dispensable for parasite replication inside hepatocytes**

Having confirmed the critical role of SAM in *Plasmodium* liver-stage replication, we then sought to identify the source of the SAM metabolite that the parasite utilizes to sustain its development. While our results suggest that *Plasmodium* is able to take up SAM from the host cell, *P. berghei* also encodes a MAT enzyme, referred to as SAM synthetase (SAMS), capable of metabolizing Met to produce SAM [513]. The *Pbsams* gene is ubiquitously expressed at all stages of the parasite life cycle, but its expression levels vary significantly. Interestingly, SAMS mRNA levels peak before each replicative phase in the mosquito-, liver- and blood-stages (Supplementary Figure 3.2A-C). During the liver stage of infection, SAMS mRNA levels decrease throughout intra-hepatic development (Supplementary Figure 3.2B), while protein levels increase over the same period (Supplementary Figure 3.2D, E).

To address the role of *Plasmodium* SAMS enzyme during liver-stage development, we employed a previously generated SAMS-deficient *P. berghei* line [521]. Since *sams* is refractory to gene deletion, a destabilizing domain (DD) system employing trimethoprim (TMP) as a stabilizer was used to generate a SAMS-conditional knockdown (*PbSAMS*-cKD, - TMP) line [518]. This enabled the selective depletion of SAMS at specific stages of the parasite's life cycle (Figure 3.3).

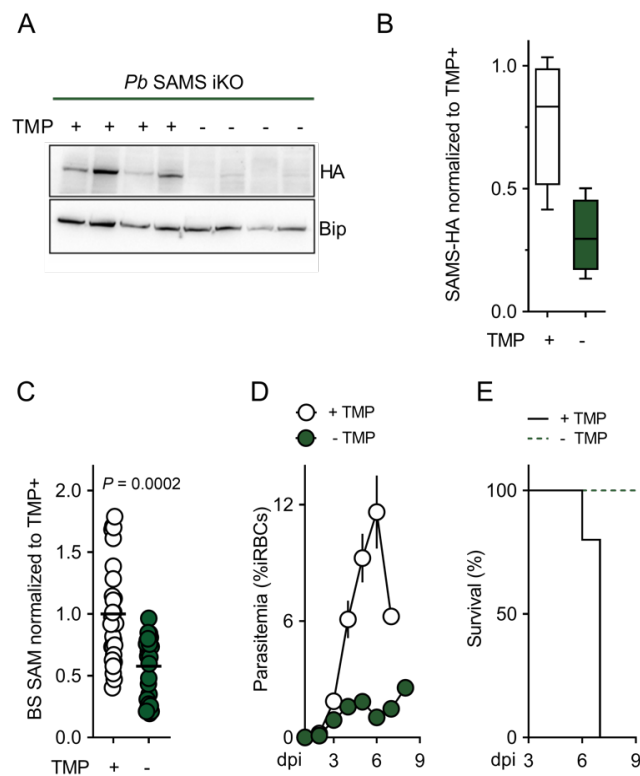


Figure 3.3 – *Plasmodium* SAMS enzyme is essential for parasite development inside erythrocytes (A)

Representative image of the immunoblot analysis and (B) quantification of SAMS protein levels (Mann-Whitney) in *P. berghei* blood-stage parasites. C57BL/6J mice were infected with *PbSAMS*-HA-DD iRBCs and treated or not with TMP, for 2 days prior to infection, to achieve the SAMS conditional knockdown. Parasite pellets were saponin-lysed and probed for HA-tag (*PbSAMS*-HA-DD, 70kDa). *PbBip* was used as loading control to normalize SAMS expression levels. Boxplots show the mean values of 4 independent experiments (C) SAM levels quantification (mean; Mann-Whitney) in blood stage *PbSAMS*-HA-DD parasites developing in mice treated or not with TMP, measured by immunofluorescence

microscopy. Parasites were probed with the Alexa Fluor 647 anti-SAM antibody. SAM mean intensity values inside *PbSAMS*-HA-DD parasites developing in +TMP or -TMP conditions were normalized to parasite area. Scatter dot plots represent 3 independent experiments and show the data for the following number of parasites: SAMS +TMP, $n=28$; SAMS -TMP, $n=25$. (D) Parasitemia (mean $\pm$ SEM; two-way ANOVA) and (E) survival of C57BL/6 mice infected by intraperitoneal (*i.p.*) inoculation of 1×10^6 *PbSAMS*-HA-DD infected RBCs (iRBC). Mice were treated with TMP in drinking water to achieve SAMS stabilization; SAMS cKD was achieved in non TMP-treated mice (-TMP; $n=5$ mice/group).

To confirm the success of the cKD approach we started by performing immunoblot analysis to determine SAMS protein levels in blood stage parasites, in the presence or absence of TMP (Figure 3.3A, B). To determine the impact of SAMS depletion on the blood stage of infection *in vivo*, C57BL/6 mice were injected with *PbSAMS*-cKD-infected red blood cells (iRBC) in the presence or absence of TMP, administered in the drinking water. Fluorescence microscopy analysis showed that SAMS cKD (-TMP) resulted in decreased SAM levels in blood stage parasites, relative to the control counterpart (+TMP) (Figure 3.3C). Moreover, blood-stage parasite growth was severely impaired in the absence of SAMS, as demonstrated by a marked decrease in parasitemia (Figure 3.3D) and a marked increase in survival (Figure 3.3E) of mice maintained in the absence of the stabilizing ligand. We then investigated the impact of SAMS abrogation during the hepatic stage of infection by injecting C57BL/6 mice with *PbSAMS* cKD sporozoites, in the presence or absence of TMP, and monitoring the ensuing infection in the liver of these mice. Our results show that while hepatic parasites have a dramatic decrease in

SAMS protein levels in the absence of TMP (Figure 3.4A, B), the amount of intra-parasitic SAM (Figure 3.4C), the parasite load (Figure 3.4D) and development (Figure 3.4E) in the liver are completely unaffected by the absence of the parasite's enzyme.

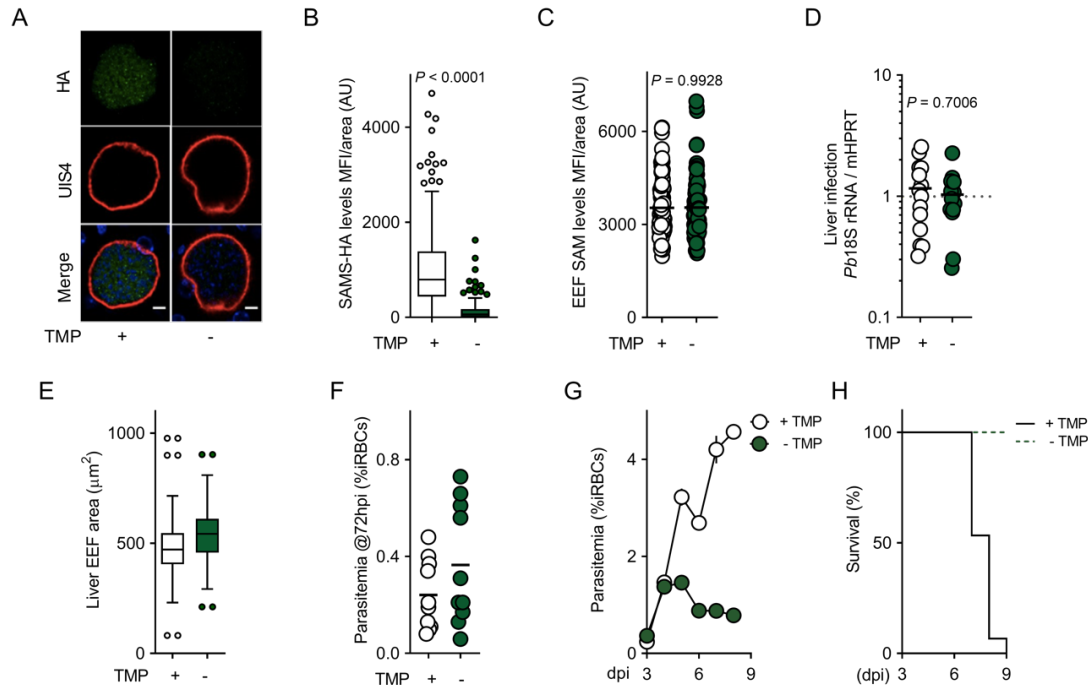


Figure 3.4 - *Plasmodium* SAMS enzyme is dispensable for parasite replication inside hepatocytes (A) Representative image and (B) quantification of SAMS-HA protein levels (Mann-Whitney) in EEF's from liver sections of mice treated or not (SAMS cKD) with TMP. To achieve SAMS-HA-DD stabilization during liver stage development, mice were TMP-treated for 3 weeks prior to infection. Infected liver sections were probed for the HA-tag (*PbSAMS-HA-DD*) and for UIS4, a protein from the PVM. SAMS protein levels were measured by immunofluorescence microscopy and normalized to parasite area. Boxplots show the data for the following number of EEFs: SAMS +TMP, $n=168$; SAMS -TMP, $n=174$. (C) Quantification of SAM levels (mean; Mann-Whitney) inside liver stage EEFs in C57BL/6 mice infected with *PbSAMS-HA-DD* sporozoites, treated or not with TMP, measured by immunofluorescence microscopy. Parasites were probed with the Alexa Fluor 647 anti-SAM antibody. SAM mean intensity values inside *PbSAMS-HA-DD* parasites developing in +TMP or -TMP conditions were normalized to parasite area. Scatter dot plots represent $n=3$ independent experiments and show the data for the following number of parasites: SAMS +TMP, $n=91$; SAMS -TMP, $n=85$. (D) Parasite liver load (mean $\pm$ SEM, Mann-Whitney) in *PbSAMS-HA-DD* infected mice, treated or not with TMP, quantified by qRT-PCR of *P. berghei* 18S rRNA at 48h after sporozoite infection ($n=14$ /group). Values are normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) and plotted in logarithmic scale. (E) Parasite development in BALB/c mice infected with 50 000 *PbSAMS-HA-DD* sporozoites. EEFs size was measured at 48h after sporozoite infection by immunofluorescence microscopy analysis. Boxplots show the data for the following number of parasites: SAMS +TMP, $n=230$; SAMS -TMP, $n=242$. (F) Parasitemia (mean; two-way ANOVA) of C75BL/6 mice ($n=10$ /group), measured by flow cytometry at 72h post infection with 50 000 *PbSAMS-HA-DD* sporozoites. (G) Full course parasitemia (mean $\pm$ SEM; two-way ANOVA) and (H) survival of C75BL/6 mice ($n=15$ /group) infected with 50 000 *PbSAMS-HA-DD* sporozoites and treated or not with TMP in drinking water.

Finally, to comprehensively assess the essentiality of SAMS during both the liver and blood stages of the *Plasmodium* life cycle *in vivo*, *PbSAMS*-cKD sporozoites were injected into C57BL/6 mice maintained either in the presence or absence of TMP, throughout the duration of the hepatic infection and the ensuing erythrocytic infection. Our data unequivocally demonstrates that SAMS cKD does not affect the development of liver stage parasites, as indicated by the same number of parasites in the blood at 72 hpi in the two groups of mice (Figure 3.4F). However, the blood stage is severely affected by SAMS depletion, as evidenced by the marked differences in blood parasitemia (Figure 3.4G) and survival rate (Figure 3.4H) of these mice. In summary, our results show that while the parasite relies on SAMS to develop in the blood, this protein is entirely dispensable for parasite development within the liver cells of its mammalian host.

***Plasmodium's* high replication capacity in hepatocytes depends on the liver-specific MAT1A enzyme**

Our results so far indicate that while *Plasmodium* strictly relies on SAM to proliferate in liver cells, it does not rely on its own Met metabolizing enzyme (SAMS) to make this metabolite. These data, combined with the parasite's ability to uptake exogenously supplied SAM (Figure 3.2E, F), led us to hypothesize that the parasite utilizes SAM generated by the host's hepatocyte during Met metabolism to meet its high metabolic requirements. Hepatocytes are unique in that they are the only mammalian cells expressing the *mat1a* gene, whereas the ubiquitous *mat2a* gene is only expressed in the liver when *mat1a* is absent [522]. Importantly, MAT1 activity, unlike MAT2, is not inhibited by its products, implying that MAT1-mediated SAM synthesis in hepatocytes is virtually unhindered [523]. We started by quantifying EEF size in three types of hepatic host cells that express varying levels of *mat1a*. Remarkably, a clear correlation was observed between *mat1a* gene expression and parasite development in liver hepatocytes, mPH and HepG2 cells (Figure 3.5A), suggesting a dependence of parasite growth on MAT1A expression.

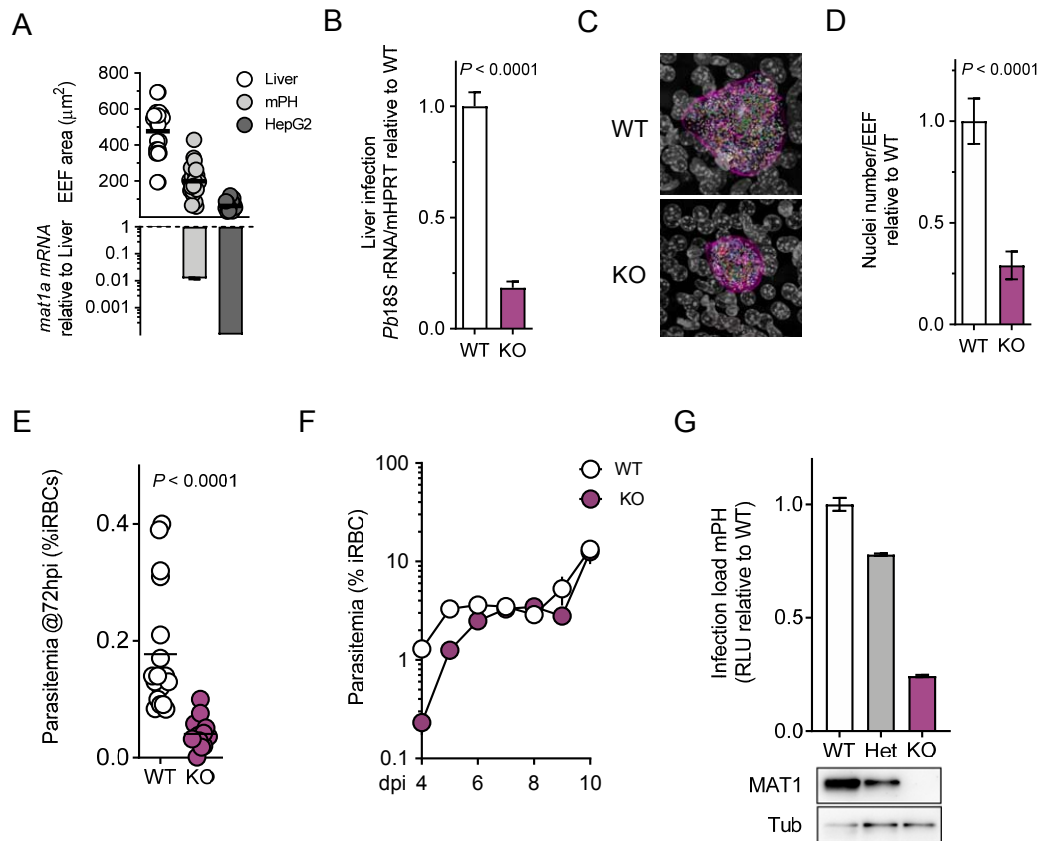


Figure 3.5 - Liver-specific MAT1A sustains parasite high multiplication rates. **(A)** EEF size (top graph) and *mat1a* mRNA levels (bottom graph) in liver sections, cultured mouse primary hepatocytes (mPH) and in HepG2 cells at 48h after infection with *P. berghei* sporozoites; EEFs sizes quantified by fluorescence microscopy and *mat1a* expression determined by qRT-PCR, values were first normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) and then to values obtained for liver expression. **(B)** Parasite liver load (mean $\pm$ SEM, Mann-Whitney) in C57BL/6J MAT1-WT and MAT1-KO mice, measured at 48h post sporozoite infection by qRT-PCR of *P. berghei* 18S rRNA. Values are normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). Bars show the mean of MAT1-WT, $n=23$; MAT1-KO, $n=19$. **(C)** Representative image and **(D)** quantification of relative nuclei frequency (mean $\pm$ SEM, Mann-Whitney) in liver sections of MAT1-WT and MAT1-KO mice, 48h after infection with *P. berghei* sporozoites. Nuclei were visualized and quantified by super-resolution microscopy, followed by 3D reconstruction. PVM stained with anti-uis4 (magenta) and parasite DNA with Hoechst (artificially colored in multiple colors) to score the number of nuclei per EEF. (MAT1-WT, $n=15$; MAT1-KO, $n=17$). **(E)** Parasitemia (mean; two-way ANOVA) of MAT1-WT and MAT1-KO C57BL/6 mice (MAT1-WT, $n=18$; MAT1-KO, $n=14$), measured by flow cytometry at 72h post infection with 35 000 *P. berghei* GFP-expressing parasites. **(F)** Full course parasitemia of MAT1-WT and MAT1-KO mice after infection with *P. berghei* sporozoites (MAT1-WT, $n=18$; MAT1-KO, $n=14$). Parasitemia was determined as in **D**. Values are shown in logarithmic scale and represent 2 independent experiments. **(G)** Parasite load (mean $\pm$ SEM, Mann-Whitney) in mouse primary hepatocytes infected with luciferase-expressing *P. berghei* WT sporozoites. Hepatocytes were isolated from MAT1A- WT, heterozygous (Het) and KO mice and liver load was assessed at 48h after sporozoite infection, by relative luminescence units of luciferase expressing parasites ($n=2$ for all conditions). Representative image of MAT1A protein expression levels in mouse primary hepatocytes WT, heterozygous or KO for the *mat1a* gene. MAT1A expression levels were assessed by immunoblot analysis using an anti-MAT1 antibody and tubulin as loading control.

To further ascertain the role of MAT1 during *Plasmodium* liver stage development, homozygous *mat1a*^{-/-} (MAT1-KO) mice and *mat1a*^{+/+} (MAT1-WT) littermate control mice maintained under a MS diet were infected with *Pb*_{Luc-GFP} sporozoites, and liver infection was assessed 48 hours later by qPCR. Our findings reveal that hepatic parasite burden in MAT1-KO mice is much lower than that of MAT1-WT mice (Figure 3.5B). Of note, such a difference was maintained when mice were infected with *Pb*SAMS-cKD sporozoites, both in the presence or absence of TMP, demonstrating that parasite development in the liver is entirely reliant on MAT1 availability, but not on SAMS activity (Supplementary Figure 3.3A).

Microcopy analysis of *Pb*_{Luci-GFP}-infected MAT1-KO and MAT1-WT mouse livers showed that EEFs contain significantly fewer nuclei in the former (KO) than in the latter (WT), consistent with severe impairment of parasite growth in the absence of MAT1A (Figure 3.5C, D). Consistent with the reduced liver parasite load observed for MAT1-KO mice relative to their MAT1-WT counterparts, parasitemia is also significantly lower in MAT1-KO mice at 72 h after sporozoite injection, when hepatic merozoites are emerging from the liver (Figure 3.5E). Noteworthy, the parasitemia in the two groups of mice becomes indistinguishable after the initial rounds of replication in the blood, showing that the absence of MAT1 impairs parasite development in the liver but not in the blood (Figure 3.5F). Additionally, our *ex vivo* results show that although Met uptake by primary hepatocytes is unaffected by the depletion of MAT1 (Supplementary Figure 3.3B), parasite development in MAT1-KO mPH was significantly lower than in MAT1-WT mPH (Figure 3.5G). In fact, a clear correlation between parasite load and MAT1 protein levels was observed *ex vivo* in *Pb*_{Luc-GFP}-infected MAT1-WT, heterozygous *mat1*^{+/-} and MAT1-KO mPH (Figure 3.5G), and also *in vivo* in mice (Supplementary Figure 3.3C).

Collectively, our results demonstrate that the availability of the host's hepatocyte-specific MAT1A enzyme, and thus the availability of host-produced SAM, lies at the heart of *Plasmodium*'s ability to sustain such high rates of replication in the liver. Moreover, our data suggests that *Plasmodium* is capable of taking up host-derived SAM, likely via a, yet to be identified, plasma membrane-specific transporter.

Discussion

The development of novel strategies to combat malaria is hindered by our currently limited understanding of the complex interactions between *Plasmodium* parasites and their human hosts [524]. During mammalian infection by malaria parasites, while a few sporozoites may infect the skin cells near the bite site [525,526], most target hepatocytes by interacting with heparan sulfate proteoglycans (HSPGs) to cross the endothelial barrier and infect liver cells [527]. What follows is a phase of remarkable replication inside hepatocytes, during which *Plasmodium* multiplies by schizogony at an intriguingly high rate to generate tens of thousands of new parasite forms [13]. We and others have identified hepatocyte molecules that influence the outcome of *Plasmodium* infection during the liver stage [279,383,528–531]. Furthermore, it has been demonstrated that not only the parasite itself but also host factors influence the onset and severity of malaria [532–535]. Recent research has also highlighted the impact of dietary alterations and amino acid supplementation on host and parasite pathways, affecting the establishment and progression of *Plasmodium* hepatic infection [536–538]. However, despite these insights, the reasons behind sporozoites' striking preference for hepatocytes, and the exceptionally high multiplication rates of *Plasmodium* during liver stages remain incompletely understood.

Like most eukaryotes, *Plasmodium* cannot synthesize Met, despite its essentiality. During the erythrocytic stage, infected red blood cells exhibit a 15-fold increase in Met influx compared to non-infected cells [539]. Met is then utilized by the parasite for protein synthesis and as a substrate in various metabolic pathways. The *Plasmodium* genome has a single-copy gene that encodes for a MAT enzyme (SAMS) [512,513], as well as all other necessary enzymes to complete Met metabolism, namely DNA and histone methyltransferases, and enzymes involved in polyamine and glutathione synthesis [540–542]. Disruption of Met-related pathways impairs parasite development and viability in the blood stage, emphasizing the importance of Met metabolism for *Plasmodium* replication [539,543–546]. However, the liver stage of infection is characterized by even higher multiplication rates, approximately 1000-fold higher than those observed in red blood cells, raising questions about the mechanisms underlying the acquisition of metabolites needed to maintain such exceptional replicative capacity.

In this study, we demonstrate that hepatocytes provide *Plasmodium* with significant amounts of SAM, a metabolite derived from Met, which plays a crucial role in parasite's intra-hepatic growth [547]. SAM serves as a source of propylamine groups for the aminopropylation cycle, essential for the synthesis of polyamines [520]. In contrast to the parasite's intrinsic SAMS enzyme, which exhibits a K_m for Met of 32 μM , hepatocytes express a liver-specific MAT1 enzyme that has a higher K_m for Met. Interestingly, this MAT1 enzyme is not inhibited by physiological concentrations of SAM, unlike the extrahepatic MAT2 enzyme [522,548,549]. Our findings indicate that *Plasmodium* relies on the host MAT1 enzyme rather than its own SAMS enzyme during liver stage replication. Furthermore, supplementation with exogenous SAM, but not its downstream metabolites, fully rescues compromised parasite replication caused by Met deprivation. These results highlight the critical role of SAM uptake from the host cell by liver stage *Plasmodium* parasites to meet their extensive replication needs.

The hypothesis that *Plasmodium* imports SAM from the host aligns with the observation that various organisms transport SAM through plasma membrane-specific transporters, even though they have the capacity for *de novo* synthesis. For instance, *Rickettsia*, *Pneumocystis carinii*, fungi, and protozoa such as *Leishmania infantum*, *Trypanosoma brucei*, and *Toxoplasma gondii* either lack or inactivate their MAT genes and import SAM to fulfill their metabolic requirements [550–555].

In summary, our study establishes the pivotal roles of MAT1-mediated SAM production and SAM uptake by hepatic *Plasmodium* parasites in replication, shedding light on the unique replicative niche of liver cells for malaria parasites. These findings provide a molecular basis for the evolutionary leap that occurred when malaria parasites crossed the species barrier into mammalian hosts and the subsequent co-evolution. Moreover, these results open avenues for the development of new strategies targeting the liver stage of malaria infection, with the potential for prophylaxis against disease or radical cure of infections caused by hypnozoite-forming *Plasmodium* species.

Materials & Methods

Mice, diets and treatments

All mice used in this study were housed in the facilities of the Instituto de Medicina Molecular, four to five per cage, and allowed free access to water and food. C57BL/6J and BALB/c wild-type mice, aged 6-8 weeks, were purchased from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France). C57BL6/J MAT1a^{-/-} were a kind gift from José M. Mato (CIC bioGUNE, Basque Research and Technology Alliance (BRTA), Bilbao, Bizkaia, Spain) and were bred in specific pathogen-free facilities at the Instituto de Medicina Molecular João Lobo Antunes. In the experiments employing the genetically deficient MAT1a^{-/-} mouse line, due to the limited availability of animals, both male and female mice 6 to 8 weeks of age were used. In such experiments, wild-type C57BL/6J MAT1a^{+/+} littermates, male and/ or female mice, 6 to 8 weeks of age, were used to match gender and age of the genetically deficient mice. To address methionine requirements mice were fed a methionine deficient diet for 2 days prior to infection, control mice were fed with a methionine sufficient (MS) control diet for a similar period, both purchased from SSnif (Soest, Germany). When experimentally possible mice were randomly assigned to different experimental groups yet, blinding was not possible when using MD diet as mice fed on a MD regimen exhibit a clear difference in body weight. All *in vivo* protocols were approved by the ORBEA committee of the IMM JLA and were performed according to national and European regulations.

Genotyping of litters from in-house breeding were performed with toe samples using the following primers:

MmMAT1a_WT_F: CTT TTT GTT GGC ACA GCT ATT CT

MmMAT1a_WT_R: ATG GAC ACA GCA GAG ACC TAC AT

MmMAT1a_KO_R: CTG AAT GAA CTG CAG GAC GAG

Parasite lines, liver infection and blood infection

The following parasite lines were used in this study: GFP-expressing *P. berghei* ANKA (259cl2) and luciferase-expressing *P. berghei* ANKA (676m1cl1) kindly provided by Chris J. Janse (Leiden University Medical Center from Leiden, The Netherlands); a *P. berghei* line knockdown for the *PbSAMS* enzyme (*PbSAMS-DD-HA*) previously generated in the laboratory [521]; and a *P. berghei* line where the *SAMS* ORF is expressed in fusion with GFP (*PbSAMS-GFP*). For *in vivo* liver-stage experiments, mice infections were

performed by intravenous injection of 2×10^4 *P. berghei* sporozoites. Liver parasite load was quantified by RT-qPCR in total liver extracts or by luminescence measurement at the indicated time after infection. In specific experiments, liver infection was allowed to progress to the blood stage of infection. For blood stage experiments, mice were infected through intraperitoneal (*i.p.*) inoculation of 1×10^6 infected RBCs (iRBCs) obtained by passage in the correspondent background mice. Parasitemia was assessed daily, either by microscopy analysis of Giemsa-stained blood smears or flow cytometry analysis of one drop of tail blood for GFP-expressing parasites, as described below.

Mosquitoes and sporozoite production

Anopheles stephensi mosquitoes were obtained from the breeding facility at IMM JLA. Mosquitoes were kept at 20°C, 80% relative humidity and fed ad libitum with 10% glucose supplemented with 2% paraminobenzoic acid (PABA). For SAMS protein stabilization, the small ligand trimethoprim (TMP) was provided in the mosquito food for 3 weeks prior to dissection. Parasites were stored in frozen blood vials, containing 10^7 blood stage parasites at -80°C . For generation of sporozoites, BALB/c wild-type mice were intraperitoneally injected with 10^7 infected red blood cells (iRBCs). After 5 days of infection, exflagellation of male gametes in the blood of infected mice was assessed by light microscopy. If more than 5 events per field of view were observed, the infected mouse was used to feed naïve *Anopheles stephensi* mosquitos. 22-35 days post blood meal, salivary glands containing *P. berghei* sporozoites were dissected into basal Dulbecco's Modified Eagles Medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and collected into an Eppendorf tube (Eppendorf, Hamburg, Germany). Salivary glands were ground with a plastic pestle and filtered through a 40 μm Falcon cell-strainer (Thermo Fisher Scientific) to release sporozoites. Sporozoites were counted using a hemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany) and were used for *in vivo* or *in vitro* infections.

Hepatoma cells, *in vitro* infections, and treatments

HepG2 cells were obtained from the American Type Culture Collection (ATCC): The Global Bioresources Center (ATCC HB-8065). General information about this cell line, including the results of authentication by short tandem repeat profiling, can be found here: https://www.lgcstandards-atcc.org/Products/All/HB-8065.aspx?geo_country=ro. HepG2 cells were maintained in DMEM medium supplemented with 10% FBS, 50 μg per mL penicillin/streptomycin and 2 mM glutamine (all Gibco) at 37°C with a 5% CO_2 .

atmosphere. Twenty-four hours prior to infection, cells were seeded on glass coverslips in 24-well culture plates at a density of 65 000 cells per well. Cells were then infected with freshly dissected *P. berghei* sporozoites (3×10^4 per well) in medium supplemented with Fungizone (1 µg per mL, Gibco) and plates were centrifuged. In specified experimental conditions, 2 hours after infection, medium was replaced to RPMI without methionine, containing 1 % PenStrep, 1 % Glutamine, 10 % FBS, 1 µg/mL Fungizone, 1:1000 Gentamicin and 200 µM cysteine (MD media). MS media was obtained by supplementing MD media with 100 µM methionine – the concentration found at RPMI. For rescue assays, SAM, homocysteine, glutathione or polyamines were added to the media. For EdU assays, 40 µM of EdU was added to infected cells in pulses of 4 hours.

Murine primary hepatocyte extraction and culturing

Mouse primary hepatocytes were isolated using a modified two-step perfusion protocol followed by a Percoll purification step [556]. C57BL6/J mice were sacrificed through CO₂ narcosis and immediately processed for cannulation of the portal vein using a 26-gauge needle. The inferior vena cava was cut to allow fluid to drain. Livers were perfused at 37°C for 10min using liver perfusion medium (Gibco) at a rate of 8-9 mL/min, followed by liver digestion medium for 10min at the same flow rate and intermittent clamping of the inferior vena cava (3s clamp, every 30s). Upon digestion, livers were dissected in PBS and cells were released by mild agitation using forceps. Lysates were sequentially filtered through 100 µm and 70 µm cell strainers and centrifuged at 50 g for 3 min. Pellets were resuspended in William's medium E (Gibco) supplemented with 10% FBS and hepatocytes were isolated by density gradient, using a 60% Percoll solution (1:1). Viable hepatocytes were washed and resuspended in William's medium E supplemented with 4% FBS, 2 mM glutamine and 100 U/mL penicillin-streptomycin. Cellular viability and yield were quantified using Trypan blue exclusion staining. Hepatocytes were seeded on collagen-coated glass coverslips in either 24- or 96-well plates.

RNA, cDNA and RT-qPCR

Whole livers were homogenized in 3ml denaturing solution (4M guanidine thiocyanate; 25 mM sodium citrate pH 7; 0.5% N-lauroylsarcosine and 0.7% β mercaptoethanol, in DEPC-treated water). RNA was extracted using the RNeasy Mini kit (Qiagen) followed by cDNA synthesis using Transcriptor First Strand cDNA Synthesis kit (Roche). Gene expression analysis was performed using kits from Applied Biosystems and expression levels of all target genes were normalized against the hypoxanthine guanine

phosphoribosyltransferase (*Hprt*) housekeeping gene (ΔCt). Gene expression values were calculated based on the $\Delta\Delta Ct$ method, using the mean of the control group as the calibrator to which all other samples were compared.

The oligonucleotide primers used in qPCR experiments were: Primers used for real-time qPCR were as follow:

Pb18s – forward primer: AAGCATTAAATAAAGCGAATACATCCTTAC

Pb18s – reverse primer: GGAGATTGGTTTTGACGTTTATGTG

Mmhprt/Hshprt– forward primer: TTTGCTGACCTGCTGGATTAC

Mmhprt/Hshprt– reverse primer: CAAGACATTCTTTCCAGTTAAAGTTG

PbSAMS-DD forward primer: TAGGTACCGAGGAAATTTTCTATTTACTTCG

PbSAMS-DD reverse primer: ATGCGGCCGCTCATCGCCGCTCCAGAATCTC

To measure *sams* mRNA levels in the mosquito vector, midguts and salivary glands from infected mosquitoes were dissected in 1x PBS supplemented with 1x Ribonuclease Inhibitors (NZY Tech). Tissue lysis was performed by adding 1mL of PureZOL RNA isolation reagent (BioRad®). To determine *sams* mRNA levels in the blood, mice were infected with previously synchronized parasites and samples were collected at 6 hpi and 10 hpi (ring-stage), 14 hpi and 18 hpi (trophozoite) and 22hpi (schizont). Infected blood was lysed by adding 1mL of TRIzol® LS Reagent (ThermoFisher scientific). HepG2 cells were lysed at developmentally relevant time points post infection, through addition of 1mL of PureZOL RNA isolation reagent. RNA extraction was performed using the NZYtech Total RNA Isolation Kit followed by cDNA synthesis using Transcriptor First Strand cDNA Synthesis kit (Roche). Gene expression values were calculated as described above and for relative quantification of *Pbsams* expression levels, adenylosuccinate lyase (PBANKA_0304300) and serine-tRNA ligase (PBANKA_0615400) expression levels were used for normalization.

The oligonucleotide primers used in qPCR experiments were:

Pb serine-tRNA ligase – forward primer: ATTGCTCAACCTTATCAAACCTG

Pb serine-tRNA ligase – reverse primer: AGCCACATCTGAACAACCG

Pb adenylosuccinate lyase – forward primer: TGCTCATAAAATAAACCCA

Pb adenylosuccinate lyase – reverse primer: TCGTAAACTGTTGAATCGG

Luminescence measurement

Luminescence analysis was employed to assess infection load in HepG2 cells when using luciferase-expressing *P. berghei* parasites. The day prior to infection, cells were seeded onto black glass-bottom 96-well at a density of 50 000 cells per well in DMEM. At the day of infection, freshly dissected firefly luciferase-expressing *P. berghei* sporozoites were added to cells. Complete medium was changed 2 hours after infection for the appropriate MS or MD medium - obtained as described above. Cells were maintained in a 5% CO₂ atmosphere at 37 °C. Forty-eight hours after infection, parasite infection load was measured by bioluminescence assay using a multiplate reader Infinite M200 Tecan. Cell viability was measured by Cell Titer-Blue assay (Promega®).

Flow cytometry

Flow cytometry analysis was performed to assess infection load in HepG2 cells when using GFP-expressing *P. berghei* parasites. HepG2 cells were seeded in 24-well plates at a density of 65 000 cells per well and infected with freshly dissected *PbGFP* sporozoites. Complete medium was changed 2 hours after infection for the appropriate medium: MS or MD medium - obtained as described above – and cells were maintained in a 5% CO₂ humidified incubator at 37 °C. Forty-eight hours after infection, HepG2 cells were trypsinized and collected for flow cytometry analysis on BD Accuri C6. Infection rate and intracellular parasite development were quantified by determining the number of GFP positive cells and the mean fluorescence intensity (MFI) of the GFP signal. The number of total acquired events ranged from 100 000 to 200 000. Hepatic cells were first selected based on their size, by gating first on FSC and SSC and, subsequently, on FITC/FL1 to detect those infected with GFP-expressing parasites.

Immunofluorescence assays

HepG2 cells were infected with GFP-expressing parasites or GFP-TK parasites and seeded on glass coverslips in either 96-well - SAM quantification analysis - or 24-well imaging plates – *P. berghei* infection analysis. At different timepoints, the cell culture media was removed, followed by gentle rinsing in 1x PBS twice. Cells were fixed at distinct timepoints with 4% paraformaldehyde (PFA) for 10min, washed thrice in 1x PBS. HepG2 cells were permeabilized with 0.1% v/v Triton X-100 in PBS for 10 min and blocked with 2% w/v bovine serum albumin (BSA) in 1x PBS for 30 minutes. For SAM quantification assays, cells were incubated for 2 hours with the following conjugated antibodies: Alexa Fluor 647 anti-SAM antibody (2 µL/mL) and Alexa Fluor 488 anti-GFP

(1:400) and DNA staining was performed by employing DAPI. For *P. berghei* infection analysis, HepG2 cells were stained with the anti-UIS4 antibody (1:1000) for 1 hour and rinsed thrice in 1x PBS. Secondary antibodies used are as following: Donkey anti-Goat Alexa Fluor 56 (1:1000), anti-GFP Alexa Fluor 488 (1:1000) and DAPI for nuclei staining. All antibodies were diluted in blocking solution and all steps were carried out at room temperature (RT). Following 3 washes with PBS, coverslips were mounted on microscope slides with Fluoromount-GTM (from Southern Biotech) and allowed to dry in the dark for 12-16 h. Images were obtained by fluorescence microscopy using LSM-710 Zeiss Microscope.

Parasite pellet purification for SDS-PAGE

To prepare protein extracts of *Plasmodium* blood-stage parasites for immunoblotting analysis, RBCs from infected-BALB/c mice were collected by cardiac puncture and pelleted by centrifugation at 450g for 10 min at RT. RBCs were saponin-lysed, by repeated pipetting, in 0.2% saponin (w/v) in PBS for 15 min on ice. After lysis, parasite pellets were washed thrice in cold PBS supplemented with Complete™ protease inhibitor cocktail (Roche®). To extract blood stage proteins, parasite pellets were lysed in 50-200µL of radioimmunoprecipitation assay (RIPA) buffer supplemented with 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (Sigma) and Complete™ protease inhibitor cocktail for 30min on ice. Samples were centrifuged at 2500g for 15 min at 4°C, supernatants were resuspended in 5x SDS-Laemmli buffer (NZYtech) and denatured at 95°C for 10min.

Immunoblotting assay

Samples were resolved in 8% polyacrylamide gels and proteins were transferred to 0.2 µm nitrocellulose membranes (Bio-Rad) using wet-transfer in Towbin buffer (25 mM Tris, 192 mM Glycine (Sigma) and 20% methanol in ddH₂O) at 200mA for 2h on ice. Membranes were blocked in 5% BSA in PBS-0.1% Tween 20 (PBS-T) for 1h at RT and incubated overnight at 4°C with appropriate primary antibodies diluted in blocking solution. Membranes were washed thrice in PBS-T and then incubated with the appropriate secondary antibodies, conjugated to Horseradish peroxidase (ThermoFisher Scientific). Secondary antibodies were diluted in blocking solution and incubated for 1h at RT. After three washing steps in PBST, membranes were revealed using Amersham ECL Prime Blotting Detection Reagent (GE Healthcare) on the ChemiDoc XRS + system (Bio-Rad) using ImageLab software (v5.2.1). The primary antibodies used were rabbit

α HA tag (1:500 dilution, clone C29F4, Cell Signaling Technology, Danvers, MA, USA) and rabbit α PbBiP (1:1000 dilution, GenScript Biotech, New Jersey, USA). The secondary antibody used was the goat anti-rabbit IgG, HRP-linked antibody (1:3000 dilution, Cell Signaling Technology). Western blots were developed using Immobilon ECL Ultra Western HRP Substrate (Merck Millipore) on either ChemiDoc XRS+ system (Bio-Rad).

[3H]-Methionine uptake assay

Transport assays in homozygous *mat1a*^{-/-} (MAT1-KO), *mat1a*^{+/+} (MAT1-WT) and heterozygous *mat1a*^{+/-} (MAT1-HET) primary hepatocytes cultured in MS or MD media were performed 24 hours post sporozoite infection. The [3H]-Met (Perkin Elmer®) transport was initiated by incubating primary hepatocyte cultures in 2 uCi/mL (24nM) of [3H] Met for 30 minutes, at 37°C. Transport was stopped by washing cells twice with ice cold Stop buffer (137 mM NaCl and 10 mM HEPES, pH 7,4). Cells were lysed with lysis buffer (100 mM NaOH and 0,1% SDS) at 37°C for 15 minutes and radioactivity was assayed by liquid scintillation upon addition of scintillant solution to the cell lysate. Intracellular accumulation of [3H]-Met was measured using MicroBet Trilux, PerkinElmer®. Protein concentration was determined using Bio-Rad Bradford Reagent, and the amount of intracellular [3H]-Met was normalized to total protein content.

Flow Cytometry Analysis

Data collection was performed using BD Accuri C6 cytometer (Franklin Lakes, New Jersey, USA) and Accuri C6 software (v1.0.264.21) software was used. Data analysis was performed using FlowJo X software (FlowJo LLC, Ashland, OR, USA).

Immunofluorescence Analysis

All immunofluorescence images were processed using FIJI software (version 1.52i). Image quantification was automatically performed by employing macros generated for each analysis.

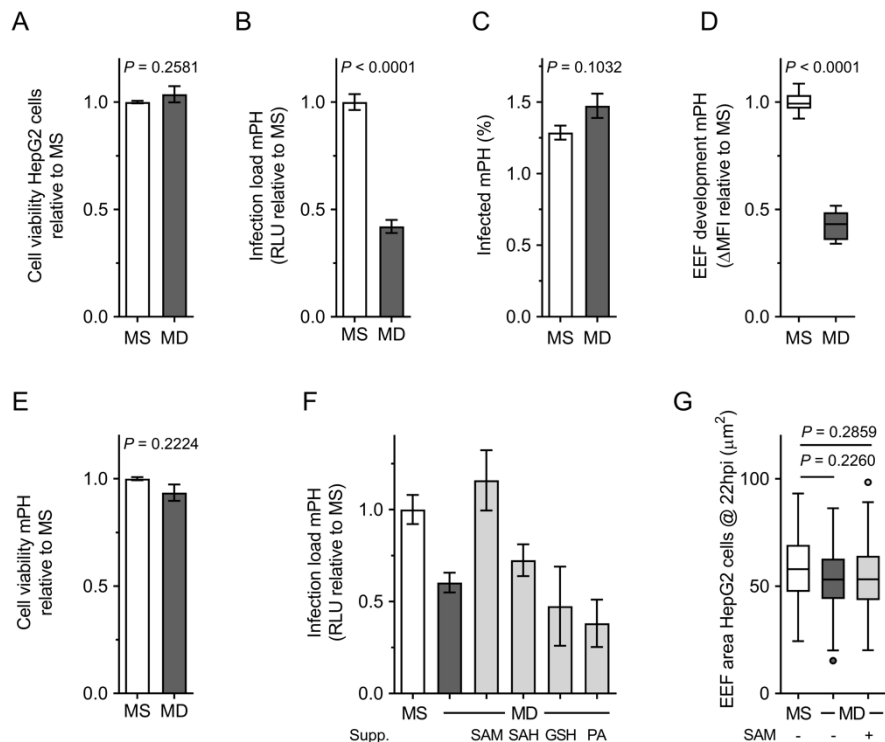
Immunoblotting Analysis

All immunoblotting images were processed using ImageLab software (v5.2.1). To assess the extent of SAMS stabilization or SAMS cKD, the intensity of the HA tag band – representing SAMS protein levels - was normalized to the intensity of the Bip band in each sample. These values were further normalized to the ratio of HA/Bip in the SAMS +TMP control group.

Statistical Analysis

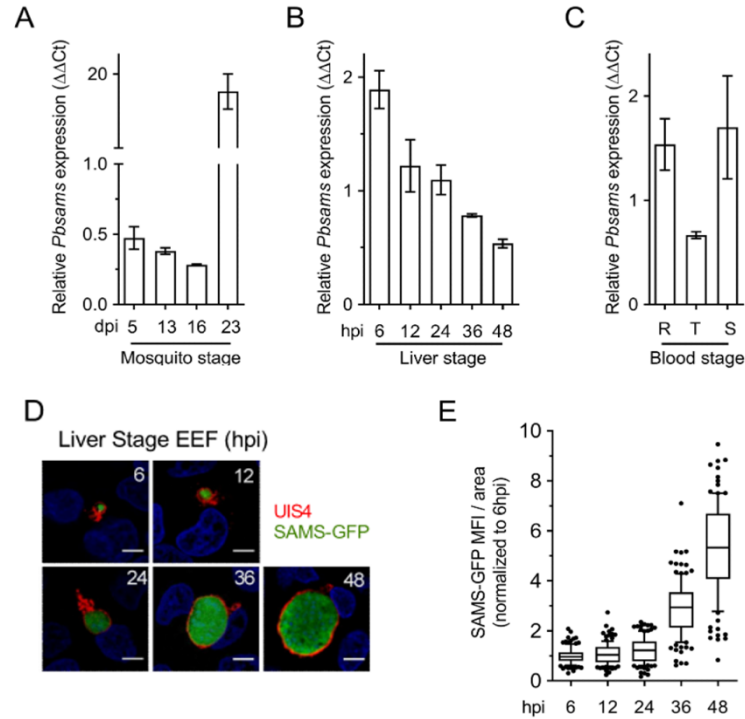
Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA). Significance of differences observed between two conditions was determined using Mann–Whitney U test, whereas two-way ANOVA was used to compare parasitemia between control and treated conditions. The log-rank (Matel-Cox) test was used to compare the survival distributions of two groups. Significance was considered for p values below 0.05. Sample sizes were chosen on the basis of historical data. Raw data and a description of statistical tests used is provided in Supplementary Information. Plots and figures were prepared using Adobe Illustrator (version CS4).

Supplementary Information

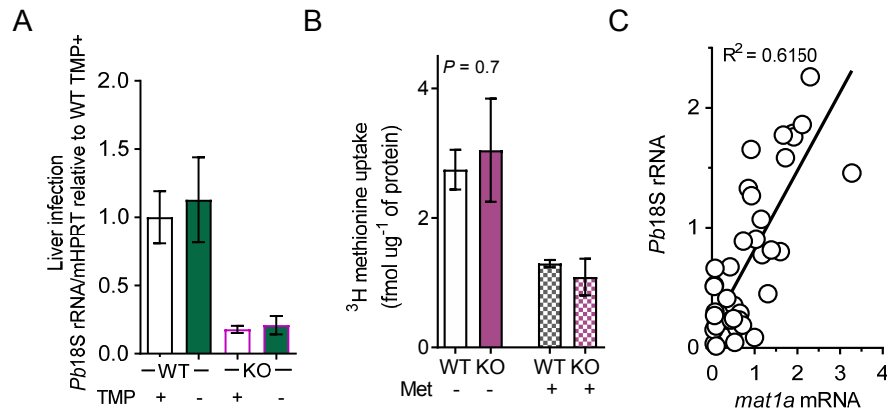


Supplementary Figure 3.1 - Methionine depletion impacts *Plasmodium* liver-stage development. (A) Cell viability in HepG2 cells infected with luciferase-expressing *P. berghei* parasites.

HepG2 cells were seeded (5×10^4 /well) in DMEM and maintained in a 5% CO₂ humidified incubator at 37°C. After sporozoite infection, cells were cultivated in MS or MD media and viability was measured by luminescence using the CellTiter-Blue assay at 48h after infection (MS, $n=9$; MD, $n=9$). (B) Mouse primary hepatocytes (mPH) infected with luciferase-expressing *P. berghei* parasites were cultured in MS- or MD-media. *P. berghei* infection load and assessed by relative luminescence units of luciferase expressing parasites. Bars represent the data for the following number of replicates: MS, $n=9$ and MD, $n=9$. (C) Percentage of infected cells and (D) EEF development (Mann-Whitney) in mPH cultured in MS- and MD-media, 48h after sporozoite addition to the culture media. Infected hepatocytes were analysed and quantified by flow cytometry. (E) Cell viability in mPH infected with luciferase-expressing *P. berghei* parasites. mPH cells were resuspended in William's medium E, seeded on collagen-coated coverslips and maintained in a 5% CO₂ humidified incubator at 37°C. After sporozoite infection, cells were cultivated in MS or MD media and viability was measured by Trypan blue exclusion (TBE) assay (MS, $n=9$; MD, $n=9$). (F) Quantification of EEF size and of host liver-specific *mat1a* mRNA expression levels in hepatocytes, mPH and HepG2 cells at 48h after sporozoite infection. mPH and HepG2 cells were cultured in MS media. EEF area was measured by immunofluorescence microscopy ($n=25$) and *mat1a* mRNA expression levels by qRT-PCR and values are normalized to *mat1a* expression levels in the liver (liver, $n=4$; mPH, $n=3$; HepG2, $n=2$). (G) Quantification of parasite load in mPH cultured in MS- or MD-media supplemented with S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), glutathione (GSH) and the polyamine putrescine (PA), analysed by relative luminescence units of luciferase expressing parasites at 48h after sporozoite infection. Bars represent the data of 2 independent experiments (MS, $n=6$; MD, $n=6$; MD+SAM, $n=6$; MD+SAH, $n=3$; MD+GSH, $n=3$; MD+PA, $n=3$). (H) Quantification of EEF size (Mann-Whitney) at 22h after sporozoite infection in HepG2 cells cultured in MS-, MD- or MD-media supplemented with SAM. Parasite development was quantified by fluorescence microscopy using the Alexa Fluor 488 anti-GFP and an anti-UIS4 antibody. Scatter dot plots represent the data of 3 independent experiments and show the data for the following number of EEFs: MS, $n=45$; MD, $n=59$; MD+SAM, $n=46$.



Supplementary Figure 3.2 - SAMS is expressed throughout all stages of *P. berghei* life cycle. (A-C) Quantification of *P. berghei sams* mRNA expression levels by RT-qPCR in (A) mosquito midguts and salivary glands measured at the indicated time points post mosquito infection; (B) HepG2-infected cells, measured at indicated time points post sporozoite infection and (C) in *P. berghei* blood-stage parasites: ring- (R), trophozoite- (T) and schizont- (S) stages. *Pbsams* expression levels were normalized against two *P. berghei* housekeeping genes, adenylosuccinate lyase and serine-tRNA ligase. Error bars represent SEM in 3 independent experiments (D) Representative image and (E) Quantification of SAMS levels in HepG2 cells infected with the *PbSAMS*-GFP transgenic line that expresses the SAMS protein in fusion with the green fluorescence protein (GFP). GFP fluorescence inside EEFs was measured by immunofluorescence microscopy at the indicated time points. SAMS fusion protein was probed with Alexa Fluor 488 anti-GFP (green), the PVM with anti-UIS4 (red) and DNA with DAPI for nuclear staining (blue). Values are normalized to the 6hpi time point and scatter dot plots show the data for the following number of EEFs: 6h, $n=127$; 12h, $n=126$; 24h, $n=130$; 36h, $n=126$; 48h, $n=121$.



Supplementary Figure 3. 3 - Host MAT1 expression levels modulate *Plasmodium* liver-stage development. **(A)** Parasite liver load (mean, Mann-Withney) in MAT1 WT and MAT1 KO mice, infected with 50 000 *PbSAMS*-DD sporozoites. To achieve stabilization, infected female *Anopheles* mosquitoes were treated (*SAMS*-DD +TMP) or not (*SAMS* -TMP, cKD) with TMP in the mosquito food for 3 weeks prior to dissection. Mice liver load was measured by qRT-PCR of *Pb18S* rRNA. Values are normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). Bars show the mean of 2 independent experiments (MAT1A WT: *SAMS* +TMP, $n=14$; *SAMS* -TMP, $n=7$; MAT1A KO: *SAMS* +TMP, $n=6$; *SAMS* -TMP, $n=4$). **(B)** Methionine uptake by MAT1A WT and MAT1A KO mouse primary hepatocytes (mPH). mPH were incubated with media containing L-[methyl-³H]-Met (³H-Met) in the absence (filled bars) or presence (checkered bars) of cold methionine. ³H-Met uptake was assed by determining ³H radioactivity levels in the cell fraction and expressed as relative to protein amount. **(C)** Linear regression of parasite liver load and *mat1a* gene expression in female C57BL/6 mice. Plotted is the correlation analysis of *mat1a*-WT, *mat1a*-Het and *mat1a*-KO mRNA expression levels and parasite liver load at 48h after sporozoite infection, determined by qRT-PCR of *Pb18S* rRNA. R^2 is the coefficient of determination ($n=45$).

Acknowledgments

From IMM-JLA, Lisbon, Portugal, we would like to acknowledge Ana Parreira for mosquito rearing and infection with *Plasmodium* parasites and the Bioimaging, Flow Cytometry and Rodent Facilities teams for their assistance. This work was supported by grants from the Fundação para a Ciência e Tecnologia (DRIVER-LISBOA-01-0145-FEDER-030751) and “laCaixa” Foundation (under the agreement HR17/52150010) to M.M.M. MIM was sponsored by FCT (PD/BD/114036/2015).

CHAPTER IV

CHAPTER IV

Sexual differentiation in human malaria parasites is regulated by competition between phospholipid metabolism and histone methylation

The Chapter IV: “Sexual differentiation in human malaria parasites is regulated by competition between phospholipid metabolism and histone methylation” consists of a third manuscript, prepared in collaboration with the Weill Cornell Medical College which is published in *Nature Microbiology*. Here is clarified the contribution of each of the authors:

Publication:

Chantal T. Harris^{1,2}, Xinran Tong^{1,3}, Riward Campelo¹, Inês M. Marreiros^{4,5}, Leen N. Vanheer¹, Navid Nahiyaan⁶, Vanessa A. Zuzarte-Luís⁴, Kirk W. Deitsch¹, Maria M. Mota⁴, Kyu Y. Rhee^{1,6}, Björn F.C. Kafsack^{1*}

¹ Department of Microbiology & Immunology, Weill Cornell Medicine, New York, USA.

² Immunology & Microbial Pathogenesis Graduate Program, Weill Cornell Medicine, New York, USA.

³ BCMB Allied Graduate Program, Weill Cornell Medicine, New York, NY 10065, USA.

⁴ Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina Universidade de Lisboa, Lisbon, Portugal.

⁵ Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal.

⁶ Division of Infectious Diseases, Weill Department of Medicine, Weill Cornell Medicine, New York, USA.

* Please send correspondence to bjk2007@med.cornell.edu

Inês M. Marreiros, the candidate for the PhD degree at the University of Porto contributed to the paper by performing all the experiments using the rodent malaria species, *Plasmodium berghei* ANKA. Inês performed mice infections using the *PbSAMS-DD* transgenic parasite line – that she had previously generated to produce the data described in chapters II and III. As part of this manuscript and of the thesis, Inês further validated this transgenic line by western blot analysis. She also performed microscopy analysis on Giemsa-stained blood smears and quantified sexual commitment rate in parasites lacking the SAMS enzyme. In addition, Inês revised the manuscript providing critical input prior to submission for peer-review.

The contribution of each author to the publication is clarified in the section “Authors contribution” as follows: Conceptualization: BFCK, KWD, MMM; Methodology: BFCK, CTH, MMM; Investigation: CTH, XT, LNV, **IMM**; Software, Formal Analysis, Data Curation: BFCK, CTH, XT; Writing – Original Draft: CTH; Writing – Review & Editing: BFCK, CTH, MMM; Visualization: BFCK, CTH; Supervision: BFCK, KYR, MMM; Project Administration: B.F.C.K; Funding Acquisition: BFCK, KWD, MMM

Published in *Nature Microbiology*. First published online on June 5th, 2023.
<https://doi.org/10.1038/s41564-023-01396-w>

Abstract

For *Plasmodium falciparum*, the most widespread and virulent malaria parasite that infects humans, persistence depends on continuous asexual replication in red blood cells, while transmission to their mosquito vector requires asexual blood stage parasites to differentiate into non-replicating gametocytes. This decision is controlled by stochastic derepression of a heterochromatin-silenced locus encoding AP2-G, the master transcription factor of sexual differentiation. The frequency of *ap2-g* derepression was shown to be responsive to extracellular phospholipid precursors but the mechanism linking these metabolites to epigenetic regulation of *ap2-g* was unknown. Through a combination of molecular genetics, metabolomics and chromatin profiling, we show that this response is mediated by metabolic competition for the methyl donor S-adenosylmethionine between histone methyltransferases and phosphoethanolamine methyltransferase, a critical enzyme in the parasite's pathway for *de novo* phosphatidylcholine synthesis. When phosphatidylcholine precursors are scarce, increased consumption of SAM for *de novo* phosphatidylcholine synthesis impairs maintenance of the histone methylation responsible for silencing *ap2-g*, increasing the frequency of derepression and sexual differentiation. This provides a key mechanistic link that explains how LysoPC and choline availability can alter the chromatin status of the *ap2-g* locus controlling sexual differentiation.

KEYWORDS

malaria; *Plasmodium falciparum*; differentiation; metabolism; chromatin; metabolic epigenome; methylation.

ABBREVIATIONS

3-DZA: 3-deaza-adenosine; cho: choline; CDP-cho: cytidine diphosphate-choline; GlcN-NAC: N-acetylglucosamine; GlcN: glucosamine; Hcy: homocysteine; iRBC: infected RBC; LysoPC: lyso-phosphatidylcholine; met: methionine; P-cho: phospho-choline; P-etn: phospho-ethanolamine; P-etn-me1/2: mono / dimethylphosphoethanolamine; PMT: phospho ethanolamine methyltransferase; PtdCho: phosphatidylcholine; RBC: red blood cell; SAH: S-adenosylhomocysteine; SAHH: S-adenosylhomocysteine hydrolase; SAM: S-adenosylmethionine; SAMS: S-adenosylmethionine synthetase; ser: serine; TMP: trimethoprim; uRBC: uninfected RBC.

Introduction

Transmission of malaria parasites requires differentiation from replicating asexual blood-stream forms that are responsible for pathogenesis into non-replicating sexual stages, called gametocytes, that can infect the mosquito vector. Parasite success depends on a careful balance between asexual replication to maintain human infection and gametocyte formation to infect the mosquito vector for further transmission [557]. The regulation of this balance is key to understanding the dynamics of malaria pathogenesis and transmission. Developmental commitment to sexual differentiation requires expression of the transcription factor AP2-G [558,559]. This requires the transcriptional activation of the *ap2-g* locus, which is actively silenced in a heterochromatin-dependent manner during asexual replication [559–562].

The frequency of sexual differentiation relies on effective heterochromatin maintenance at the *ap2-g* locus, which depends on the efficient trimethylation of lysine 9 on histone H3 of newly placed nucleosomes and recognition of this mark by heterochromatin protein 1 [559,560,563,564]. So long as *ap2-g* remains efficiently silenced, parasites continue to replicate asexually. However, AP2-G has several binding sites within its own promoter that creates a positive transcriptional feedback loop once AP2-G expression rises beyond a threshold. When heterochromatin maintenance is impaired, leaky silencing allows AP2-G expression to exceed that threshold in a greater proportion of cells, leading to the activation of the feedback loop. This drives AP2-G expression to high levels, resulting in sexual commitment and activation of the transcriptional program underlying gametocytogenesis [559,565–568].

In the laboratory, the frequency of sexual commitment of *Plasmodium falciparum*, the most widespread and virulent human malaria parasite, varies from less than 1% to greater than 40% in response to culture conditions, including parasite density and media composition [569]. Recent work showed that lysophosphatidylcholine (LysoPC) and choline, which are precursors of phosphocholine (P-cho; the headgroup required for phosphatidylcholine (PtdCho) synthesis), act as potent suppressors of sexual commitment. This ability of parasites to vary their investment into gametocyte production illustrates the parasites' ability to sense their in-host environment and respond adaptively to resource abundance [570] or anatomical niche [571,572]. However, the underlying mechanisms that link the availability of these metabolites to *ap2-g* activation remain unknown.

Here we show that the availability of P-cho precursors regulates sexual commitment by shifting the metabolic competition for the methyl donor S-adenosylmethionine (SAM) between the methyltransferase required for *de novo* synthesis of P-cho and the histone methyltransferases maintaining heterochromatin-mediated silencing, including at the *ap2-g* locus.

Results

P-cho precursors alter SAM, S-adenosylhomocysteine (SAH) and sexual commitment

As the parasite grows during its 48-hour asexual replication cycle, the membrane content of infected red blood cells (iRBCs) increases 8-fold, with PtdCho accounting for more than half of this increase in membrane biomass [573,574]. In *P. falciparum*, PtdCho is exclusively derived from P-cho, which can be generated *de novo* from serine via phosphoethanolamine (P-ethn) or scavenged from extracellular sources, including choline and choline-containing phospholipids such as LysoPC [498] (Figure 4.1a, Supplementary Figure 4.1a). Consistent with earlier reports [386], we found that supplementation of standard malaria growth media with exogenous P-cho precursors (see Supplementary Table 4.1 for media composition) during a single replication cycle substantially suppressed sexual commitment (Figure 4.1b,c) and increased the levels of P-cho and CDP-choline within iRBCs compared with conditions when these precursors were scarce (Figure 4.1d,e, Supplementary Figure 4.1b). As we had previously described [559], the change in *ap2-g* expression at the end of this cycle tracked closely with the observed changes in sexual commitment (Supplementary Figure 4.2).

When exogenous P-cho precursors like choline or LysoPC are available for scavenging, parasite levels of SAH decreased significantly compared with conditions requiring greater *de novo* synthesis of P-cho from Petn. SAM levels increased in response to LysoPC but remained unchanged upon choline supplementation (Figure 4.1d, e). A possible explanation for this difference is the additional energetic cost associated with scavenging choline, which must be phosphorylated using ATP, while LysoPC can be cleaved directly into P-cho by phospholipase C. This is supported by the observation that the suppressive effects of choline supplementation can be reversed by reducing the availability of glucose within the growth medium [386]. This metabolic response to P-cho precursors is dose dependent (Supplementary Figure 4.1), indicating that P-cho precursor availability regulates intracellular levels of SAM and SAH. These changes in intracellular levels of P-cho, SAM, and SAH were limited to iRBCs and did not occur in uninfected RBCs (Supplementary Figure 4.3).

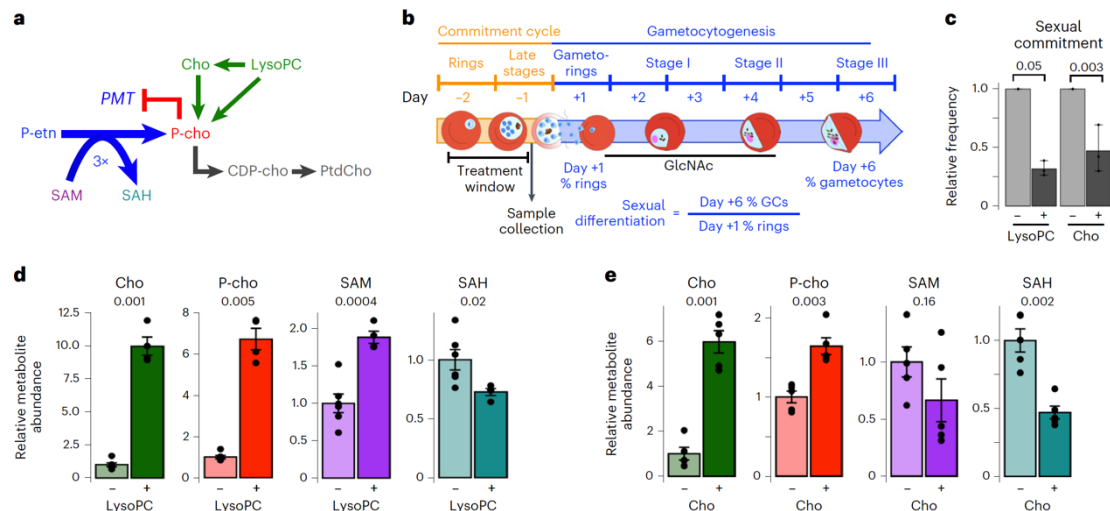


Figure 4.1 - Phosphocholine precursor availability alters parasite SAM and SAH levels. **a.** PtdCho (PC) is generated exclusively from P-cho, which can be scavenged from extracellular choline or LysoPC or synthesized *de novo* via triple methylation of P-ethn by PMT, consuming 3 equivalents of SAM and producing 3 equivalents of SAH per P-cho. **b.** Synchronous blood stages were grown for a single cycle (commitment cycle) under various nutrient conditions and samples were collected 36 hours post-invasion. Treated parasites were allowed to re-invade and 50 mM *N*-acetylglucosamine was added on Day +1 to block asexual replication. The sexual differentiation rate is defined as the percentage of Day +1 ring stages that differentiate into stage III gametocytes by Day +6. **c.** P-cho precursors inhibit the frequency of sexual differentiation ($n=3$ biologically independent samples; numbers are P values from two-sided paired *t*-tests). **d.** Intracellular metabolite levels are altered in response to LysoPC supplementation ($n=4-6$ biologically independent samples; bar graphs indicate the mean $\pm$ s.e.m. relative to the reference condition; numbers are P values from two-sided *t*-tests). **e.** Intracellular metabolite levels are altered in response to choline supplementation ($n=5$ biologically independent samples; bar graphs indicate the mean $\pm$ s.e.m. relative to the reference condition; numbers are P values from two-sided *t*-tests).

Phosphoethanolamine N-methyltransferase (PMT) activity drives the changes in SAM and SAH

Earlier work showed that in malaria parasites, *de novo* synthesis of PtdCho's choline headgroup is carried out by PMT, which generates P-cho by transferring three consecutive methyl-groups from the methyl-donor S-adenosylmethionine (SAM) onto P-ethn [498,575], and that malaria blood stages substantially down-regulate *pmt* in response to supplementation with P-cho precursors [576] (Figure 4.2a). Since *de novo* synthesis of the choline headgroup is a major consumer of SAM and source of SAH in other eukaryotes [577], we wanted to assess whether the observed changes in SAM and SAH were due to a decrease in PMT activity. Consistent with the earlier reports, we observed a significant decrease in the abundance of the mono- and di-methylated P-ethn reaction intermediates generated by PMT when cultures were supplemented with choline, and found that PMT transcript abundance was substantially lower under this condition (Figure

4.2b, c). The effect of PMT activity on overall intracellular SAM and SAH levels was unclear but if P-cho synthesis by PMT is a major metabolic sink of SAM and source of SAH, their levels should correlate with PMT activity. To test this connection between PMT activity, SAM metabolism and sexual commitment, we generated inducible PMT knockdown parasites by inserting the glucosamine-responsive autocatalytic *glnS* ribozyme [578] into the endogenous PMT 3'UTR (Supplementary Figure 4.4). Treatment of PMT-*glnS* cultures with glucosamine during the commitment cycle reduced PMT protein levels by 55% (Figure 4.2d), resulting in a 2-fold increase in intracellular SAM and significantly suppressed sexual commitment, even in the absence of P-cho precursors (Figure 4.2e, f). This fits with earlier reports that found a near complete suppression of gametocytogenesis in *pmt* knockout parasites that was restored to parental levels upon genetic complementation [578,579]

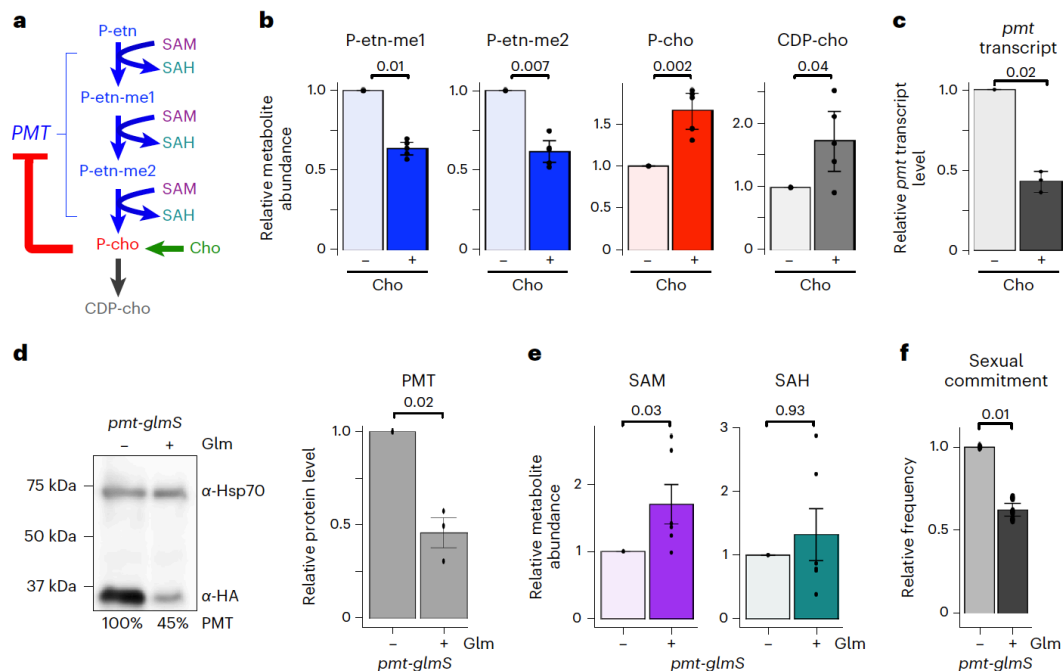


Figure 4.2 – De novo synthesis of P-cho by PMT is a major sink of SAM and source of SAH. (a-c) Increases in P-choline from choline scavenging downregulates PMT activity (a,b) as indicated by decreases in the monomethyl- and dimethyl-phospho-ethanolamine (P-ethn-me1/2) reaction intermediates) and (c) *pmt* transcript levels. **b**, $n=4$ biologically independent samples and **c**, $n=3$ biologically independent samples. Transcript abundance was normalized to seryl-tRNA synthetase transcript and shown relative to no choline supplementation. **(d)** Left panel is a representative western blot (WB) showing that inducible knockdown of PMT upon addition of 2.5 mM glucosamine (Glm) in PMT-*glnS* parasites significantly reduces PMT protein abundance relative to HSP70 loading control. Right panel shows WB quantifications ($n=3$ biologically independent samples). **(e)** Knockdown of PMT increases intracellular SAM levels ($n=6$ biologically independent samples). **(f)** Knockdown of PMT reduces sexual commitment even in the absence of P-cho precursors. ($n=3$ biologically independent samples) Bar graphs indicate the mean $\pm$ s.e.m. relative to the reference condition; numbers are P values from two-sided paired t -tests.

Intracellular SAM levels regulate sexual commitment

To determine whether these changes in SAM and SAH abundance merely correlate or directly regulate sexual commitment, we aimed to manipulate the intracellular concentrations of SAM and SAH independently of PMT activity and P-cho precursor abundance. We reasoned that if the availability of P-cho precursors suppresses sexual commitment by reducing PMT's consumption of SAM, then this suppression should depend on the overall availability of SAM, which in malaria parasites is solely generated from methionine by SAM synthetase (SAMS). Malaria parasites are methionine auxotrophs and, unlike most eukaryotes, lack orthologues to enzymes able to regenerate methionine from homocysteine [579], making parasite SAM synthesis fully dependent on methionine derived from extracellular pools and the break-down of host cell proteins (Figure 4.3a).

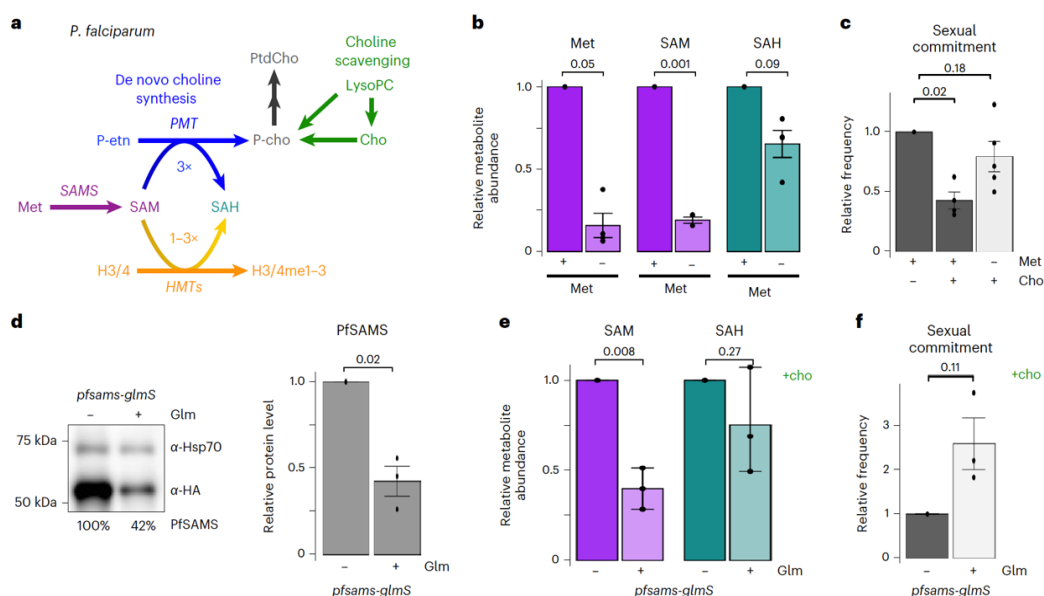


Figure 4.3 - Intracellular SAM abundance regulates the frequency of sexual differentiation in human malaria parasites. (a) SAM and SAH are substrate and product, respectively, of both de novo P-cho synthesis and histone methylation. (b) Intracellular levels of methionine, SAM and SAH in growth medium containing standard (100 μ M) or no methionine ($n=3$ biologically independent samples). (c) Removal of extracellular methionine reverses the suppressive effects of choline supplementation on sexual commitment ($n=5$ biologically independent samples). (d) Left panel is a representative WB showing that knockdown of *PfSAMS* in *pfsams-HA-glmS* parasites by treatment with 2.5 mM glucosamine (Glm) resulted in a 58% reduction in SAMS protein levels. Right panel depicts western blot quantifications ($n=3$ biologically independent samples). (e, f) Knockdown of SAMS (e) reduced intracellular SAM levels by 59% and (f) resulted in a 2.5-fold increase in sexual differentiation even in the presence of choline ($n=3$ biologically independent samples).

While removing extracellular methionine substantially decreased intracellular methionine and SAM (Figure 4.3b), it had no discernable effect on parasite replication over a two-week period (Supplementary Figure 4.5). However, while in standard growth medium containing 100 μ M methionine, choline significantly suppressed commitment; this suppressive effect was greatly reduced when methionine was removed from the growth media for even a single commitment cycle (Figure 4.3c). Since methionine is critical for a variety of cellular functions including translation, we wanted to test whether the effect of methionine on commitment levels was specific to changes in SAM availability. To this end we generated glucosamine-regulatable *sams-glmS* knockdown parasites (Supplementary Figure 4.6). Upon addition of glucosamine, protein levels of *PfSAMS* were reduced by 58% (Figure 4.3d), which, even in the presence of choline, resulted in a 2-fold reduction in SAM levels (Figure 4.3e) along with a more than >2.5-fold increase in sexual commitment (Figure 4.3f). Together, these findings demonstrate that sexual commitment is directly regulated by SAM availability.

SAM regulates sexual commitment in rodent parasites

Earlier studies found that, unlike in *P. falciparum*, supplementation with P-cho precursors had little to no effect on sexual commitment in the rodent malaria parasite *Plasmodium berghei* [386]. In the context of our proposed model, where the consumption of SAM by PMT provides the link between P-cho precursors and histone methylation, this observation is readily explained by the fact that the PMT orthologue was lost in the rodent malaria parasite lineage [580], thereby decoupling P-cho availability from SAM and SAH abundance in rodent parasites (Figure 4.4a). However, since heterochromatin-mediated silencing of the *ap2-g* locus also controls sexual commitment in rodent parasites [558,561], we hypothesized that commitment in *P. berghei* would nevertheless remain sensitive to changes in SAM availability. To test this, we generated SAMS knockdown parasites in *P. berghei* by creating a C-terminal fusion of the endogenous coding sequence with the ecDHFR-based destabilization domain (*pbsams-dd-ha*, Supplementary Figure 4.7a, b). Upon removal of the stabilizing ligand trimethoprim (TMP), *PbSAMS* expression was reduced by 60% in *pbsams-dd-ha* blood stages (Figure 4.4b, Supplementary Figure 4.7c) and resulted in a 2-fold increase in sexual commitment (Figure 4.4c). This demonstrates that SAM availability regulates the rate of sexual commitment even when decoupled from PtdCho metabolism.

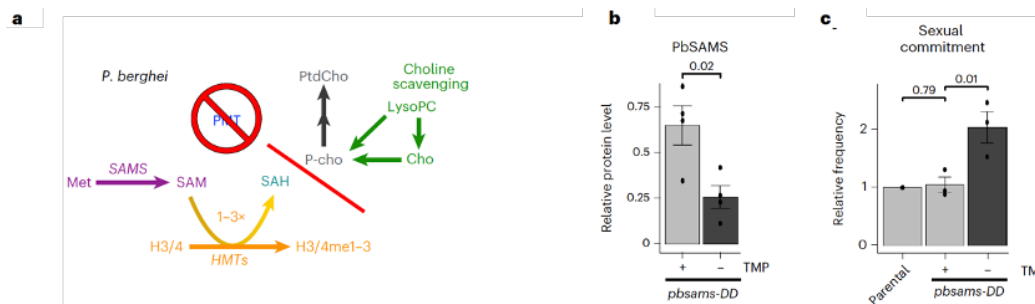


Figure 4.4 - *P. berghei* SAMS cKD parasites exhibit increased rate of sexual commitment. (a) Loss of PMT in rodent malaria parasites, such as *P. berghei*, decouples SAM and SAH from PtdCho synthesis. (b, c) Removal of the stabilizing ligand TMP from *P. berghei* *pbsams-DD* knockdown parasites (b) reduced PbSAMS by 61% and (c) resulted in a 2-fold increase in sexual differentiation ($n=4$ biologically independent samples; bar graphs show mean $\pm$ s.e.m. relative to the reference condition; numbers are P values based on two-sided paired t -tests).

Heterochromatin is responsive to changes in SAM and SAH

In higher eukaryotes, changes in metabolism that alter intracellular SAM and SAH can have major effects on the methylation states of histone and associated regulation of gene expression [577,581–585]. Methionine depletion and reduction in SAM abundance led to decreased methylation of specific histone modifications including H3K4me3, H3K9me3, H3K4me2, and H3K36me3, with H3K4me3 exhibiting the most profound changes, leading to a change in gene expression [577,582,585]. Since the frequency of sexual commitment in malaria parasites is determined by the efficiency of heterochromatin-mediated silencing of AP2-G expression and changes in intracellular SAM directly alter commitment rates (Figures 4.3 and 4.4), we used CUT&RUN chromatin profiling [586] to examine whether these changes could substantially alter the abundance or distribution of the main histone methylation marks associated with both gene silencing (H3K9me3) and active transcription (H3K4me3) (Figure 4.5) [587,588]. To evaluate the effect of SAM on histone methylation patterns we compared the distribution of these marks in the presence of abundant extracellular choline and methionine when SAM levels are high to conditions when the absence of these metabolites in the growth medium results in low SAM levels (Figure 4.5a), and elevated rates of sexual commitment (Figure 4.5b).

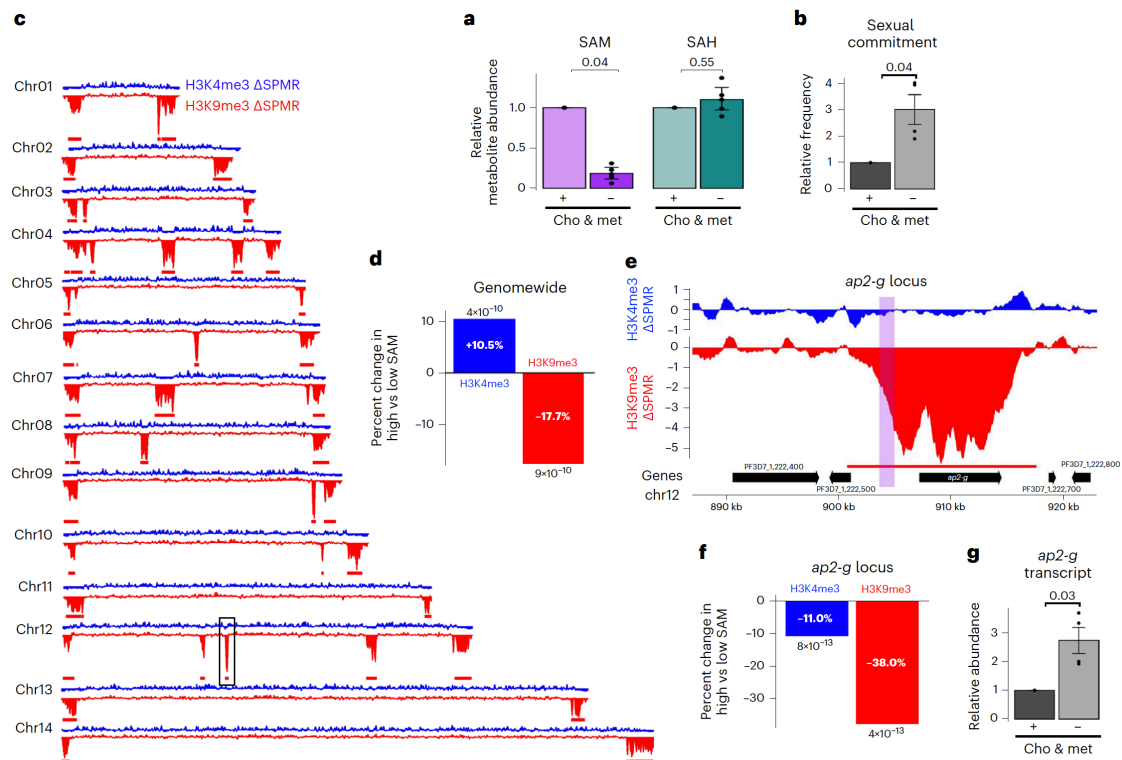


Figure 4.5 – Reducing intracellular SAM levels impairs heterochromatin maintenance and increases both AP2-G expression and sexual commitment. (a, b) Relative (a) intracellular abundances of SAM and SAH ($n=5$ biologically independent samples; numbers indicate P values based on paired two-sided t -tests) and (b) sexual commitment ($n=4$; numbers indicate P values based on paired two-sided t -tests) under high SAM (+cho & met) vs. low (-cho & met) SAM conditions. (c) Differences in H3K4me3 (blue) and H3K9me3 (red) abundance between parasites grown in low SAM vs high SAM conditions. The box on chromosome 12 indicates the *ap2-g* locus. Red bars indicate regions of heterochromatin under high SAM conditions (representative of $n=2$ biologically independent samples). SPMR, signal per million reads. (d) Genome wide change in H3K4me3 (blue) and H3K9me3 (red) coverage under low SAM vs high SAM conditions. (e) Changes in the distribution of H3K4me3 (blue) and H3K9me3 (red) at the *ap2-g* locus between parasites grown under low SAM vs high SAM conditions. The purple shaded region contains the AP2-G binding sites within the *ap2-g* promoter that drives the transcriptional feedback loop. Representative of $n=2$ biologically independent samples. (f) Change in coverage across the *ap2-g* heterochromatin peak (red bar in e) of H3K4me3 (blue) and H3K9me3 (red) between parasites grown under low SAM vs high SAM conditions. ($n=2$ biologically independent experiments; numbers are P values based on comparison of coverage across the heterochromatin region by macs2). (g) Relative abundance of *ap2-g* transcript levels between parasites grown under low SAM vs high SAM conditions ($n=4$ biologically independent samples; bar graphs show mean $\pm$ s.e.m. relative to the reference condition; numbers indicate P values based on paired two-sided t -tests).

In contrast to H3K4me3, which was somewhat elevated when intracellular SAM was low, we observed substantial genome wide reductions in the abundance of the repressive H3K9me3 mark (Figure 4.5c, d) under low SAM conditions when compared with high SAM conditions. This reduction in H3K9me3 was observed in both regions of subtelomeric heterochromatin domains and non-subtelomeric heterochromatin islands (Figure 4.5c; Supplementary Figure 4.8). Closer examination of the region containing

pfap2-g on chromosome 12 found that H3K9me3 occupancy across the locus was reduced by 38.0% under low SAM conditions (Figure 4.5e, f). At the leading edge of the heterochromatin island, which overlaps the promoter region containing the AP2-G binding sites that drive the transcriptional feedback loop, this reduction was even more profound (-42.0% under low SAM, $P < 1 \times 10^{-12}$). These reductions in the H3K9me3 silencing mark were accompanied by a 2.8-fold increase in transcript levels of *ap2-g* demonstrating that H3K9me3 occupancy and expression of the locus is responsive to changes in SAM (Figure 4.5g).

Each transfer of a methyl group from SAM generates one equivalent of SAH, which can act as a potent feedback inhibitor for many SAM-dependent methyltransferases [589], although histone methyltransferases differ in their susceptibility to feedback inhibition by SAH [590]. Since *de novo* P-cho synthesis by PMT therefore represents not only a major sink of SAM but also an equivalently large source of intracellular SAH (Figure 4.2), we also evaluated whether direct changes to the intracellular levels of SAH can alter sexual commitment. To this end, we treated parasites during a single commitment cycle with 3-deaza-adenosine (3-DZA), a potent inhibitor of SAH hydrolase (SAHH), the enzyme that converts SAH into adenine and homocysteine (Figure 4.6a) [591,592]. We found that treatment with 3-DZA increased commitment levels in a dose-dependent manner while having only a minor effect on parasite growth (Supplementary Figure 4.9). Treatment with 100 nM 3-DZA in the presence of abundant methionine and choline greatly increased both SAH and SAM levels. The latter is probably the result of reduced consumption of SAM by methyltransferases as they are feedback inhibited by the increasing SAH levels (Figure 4.6b). SAHH inhibition by 3-DZA more than doubled sexual commitment, fully reversing the suppressive effects of choline supplementation (Figure 4.6c). These results demonstrate that elevating SAH levels is sufficient to increase sexual commitment rates, even when SAM is abundant. These data are in alignment with a previous study which found that homocysteine, the metabolic product and feedback inhibitor of SAHH that accumulates in culture and in the serum of infected patients, induces gametocytogenesis in culture [593].

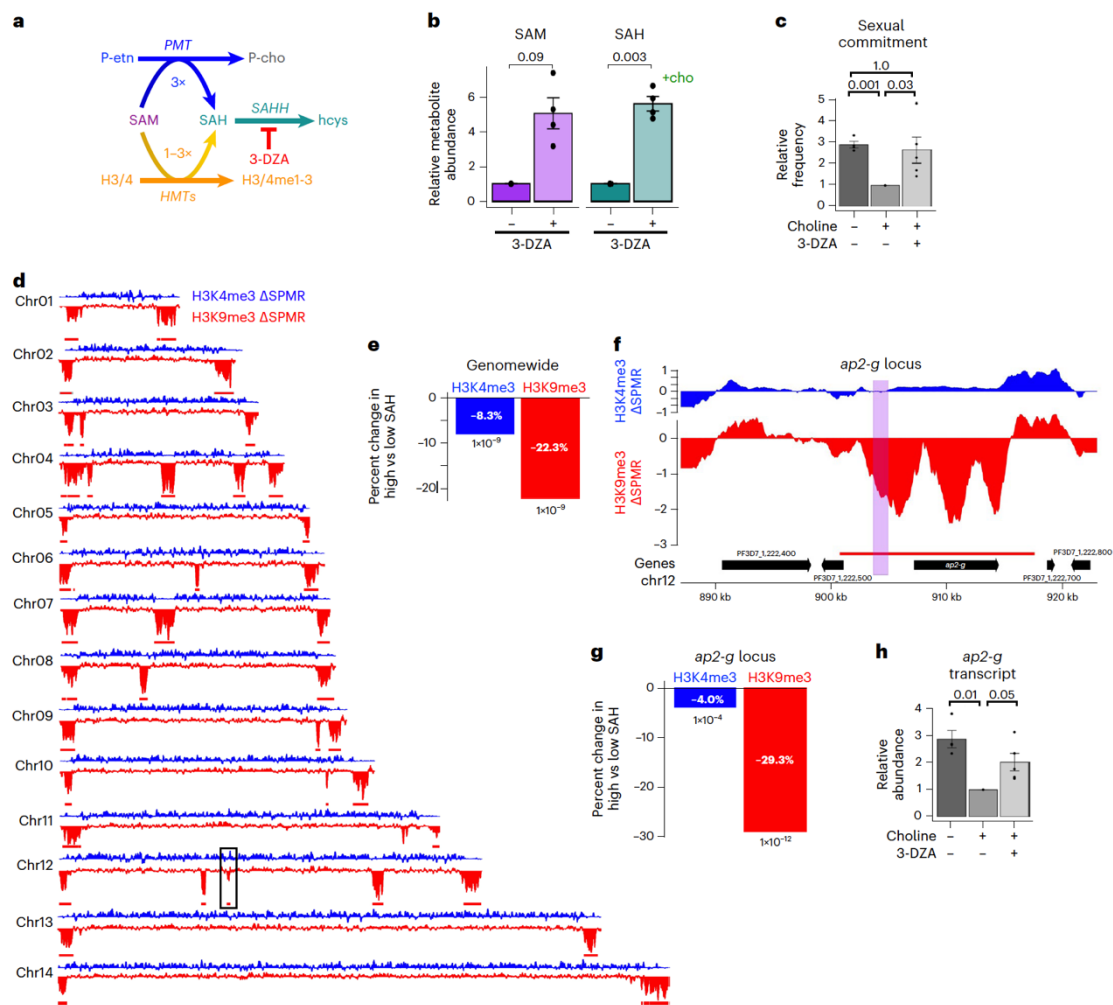


Figure 4.6 - Increasing intracellular SAH impairs heterochromatin maintenance and increases both AP2-G expression and sexual commitment. (a–c) Inhibition of SAHH with 3-DZA (a, b) increases intracellular SAM and SAH ($n = 5$ biologically independent samples) and (c) sexual commitment ($n = 4$ biologically independent samples). (d) Genome wide differences in the distribution of H3K4me3 (blue) and H3K9me3 (red) between parasites grown in high SAH (+cho, +met, +3-DZA) vs low SAH (+cho, +met) condition as determined by CUT& RUN. The box on chromosome 12 indicates the location of the *ap2-g* locus. Red bars indicate regions of heterochromatin ($n = 2$ biologically independent samples). (e) Mean genome wide change in H3K4me3 (blue) and H3K9me3 (red) coverage in high SAH vs low SAH conditions. (f) Differences in the distribution of H3K4me3 (blue) and H3K9me3 (red) between parasites grown in high SAH vs low SAH conditions at the *ap2-g* locus on chromosome 12. The purple shaded region contains key AP2-G binding sites that drive the transcriptional feedback loop. Representative of $n = 2$ biologically independent samples. (g) Mean change in coverage across the *ap2-g* heterochromatin peak (red bar in f) of H3K4me3 (blue) and H3K9me3 (red) between parasites grown in high SAH vs low SAH conditions. Numbers are P values based on two-sided paired t -tests for metabolite abundance or based on HOMER annotatePeaks and DESeq2 for histone modification abundance. (h) Relative abundance of *ap2-g* transcript levels between parasites grown under high SAH vs low SAH conditions ($n = 4$ biologically independent samples; bar graphs show mean $\pm$ s.e.m. relative to the reference condition; numbers are P values based on two-sided paired t -tests).

Since suppression of commitment with choline reduced intracellular SAH levels but left SAM levels largely unchanged, we wanted to also evaluate whether high SAH was sufficient to impair heterochromatin maintenance even when SAM is abundantly available. While treatment with 3-DZA reduced genome wide H3K4me3 abundance by 8.3%, it had even more profound effects on the H3K9me3 silencing mark, which was reduced 22.3% genome wide (Figure 4.6d, e). This reduction in H3K9me3 was again observed in both regions of subtelomeric heterochromatin domains and non-subtelomeric heterochromatin islands (Figure 4.5c and Supplementary Figure 4.10). H3K9me3 occupancy across the *ap2-g* locus on chromosome 12 was reduced by 29.3% under high SAH conditions (Figure 4.6f, g) and at the region containing the AP2-G binding sites that drive the transcriptional feedback loop; this reduction was more profound at -39.2% ($P < 1 \times 10^{-12}$) compared with low SAH conditions. These reductions in the H3K9me3 silencing mark were accompanied by a doubling of *ap2-g* transcript abundance (Figure 4.6h), demonstrating that H3K9me3 occupancy and expression of the locus are responsive to changes in SAH as well as in SAM. Even when SAM is abundant, increasing SAH also impairs heterochromatin maintenance at the *ap2-g* locus, increasing sexual commitment.

Discussion

Malaria parasites have evolved sophisticated mechanisms to sense and adapt to the diverse physiological niches they occupy during their life cycle. The switch from asexually replicating blood stage parasites to male and female gametocytes requires balancing a trade-off between maintaining in-host persistence and maximizing transmission between hosts. Several metabolites have been implicated in affecting this switch. Serine and homocysteine both increase frequencies of sexual differentiation [573,593], while LysoPC and its metabolic product, choline, both act as potent suppressors of sexual commitment [386]. Additionally, the concentrations of these precursors are especially low in the bone marrow, the primary site of *P. falciparum* gametocytogenesis [386,571]. Maturation in the bone marrow protects immature gametocytes from splenic clearance until they mature into highly deformable stage V gametocytes that are able to pass through the spleen [594,595]. Intriguingly, increased sexual commitment of parasite infecting immature RBCs has been noted repeatedly [596–598]. Unlike in mature RBCs, P-cho metabolism is highly active in immature RBCs and essential for terminal erythropoiesis [599,600]. This would reduce the availability of P-cho precursors to parasites infecting immature RBCs and make them more reliant on *de novo* PtdCho synthesis via PMT.

Here we show that a drop in P-cho precursors leads to a decrease in intracellular SAM and rise in SAH as the result of increased *de novo* P-cho synthesis by PMT. These changes in SAM and SAH reduce the efficiency of H3K9me3 maintenance as the parasite replicates its genome 20-30 times during schizogony. This reduction in heterochromatin is not limited to the heterochromatin island on chromosome 12 that contains the *ap2-g* locus but occurs genome wide. However, because AP2-G is the only transcription factor regulated by heterochromatin and because of its ability to bind the regulatory region of its own promoter, these reductions in heterochromatin can result in the activation of AP2-G and commit parasites to sexual development (Figure 5.3).

While we cannot exclude the possibility of other, unknown methylation reactions also regulating sexual commitment, H3K9 methylation has repeatedly been shown to strongly regulate AP2-G and sexual commitment in malaria parasites. Intriguingly, in malaria parasites methylation of H3K9 is more sensitive to changes in SAM and SAH than H3K4 methylation, unlike what has been reported for higher eukaryotes. This suggests a need for future examination of whether the K_m for SAM and K_i and SAH of *Pf*SET3, the

parasite's only Su(var)3-9 methyltransferase, are in the range of the intracellular concentrations of these two metabolites, which would allow their concentrations to directly regulate the deposition of the H3K9me3 silencing mark. This model also explains the previously described observation that in rodent parasites sexual commitment is not responsive to P-cho precursors, as the loss of PMT in rodent malaria parasites decouples PtdCho synthesis from SAM, SAH, and histone methylation. Strikingly, this loss of PMT in rodent malaria parasites was accompanied by a lineage-specific expansion of the fam-a gene family from a single copy in the primate lineage to 42-215 copies [601]. Members of this family contain the steroidogenic acute regulatory-related lipid transfer (START) domain and have been shown to transport PtdCho [602]. Without the ability to generate this key phospholipid *de novo*, this expansion presumably allows rodent malaria parasites to scavenge a full complement of PtdCho precursors.

In summary, changing availability of host LysoPC results in a shift in intracellular SAM/SAH which leads to changes in histone methylation, in particular the H3K9me3 silencing mark. Inefficient silencing at the *pfap2-g* locus increases the frequency of activation of the transcriptional feedback loop that commits parasites to sexual differentiation in *P. falciparum*. This allows parasites to increase sexual differentiation in the bone marrow where LysoPC is low and where gametocytes can develop without the risk of splenic clearance.

Materials & Methods

***P. falciparum* cell culture**

The parasite strains used for this study were NF54 obtained from BEI Resources and the *Pf-peg4*-tdTomato [602]. Cultures were maintained using established culturing techniques [603]. Standard complete media was RPMI-1640 supplemented with 25 mM HEPES, 368 μ M hypoxanthine, 1 mM sodium hydroxide, 24 mM sodium bicarbonate, 21 μ M gentamycin (Millipore Sigma), with Albumax II Lipid-Rich BSA (Gibco), unless otherwise stated. Concentrations for choline and LysoPC supplementation were used according to ref. [494] (see Supplementary Table 4.1 for changes to media compositions used throughout this paper). RBCs or cultures were washed three times with respective experimental media conditions at the start of each change in media condition.

Generation of *PfSAMS-glmS* and *PfPMT-glmS* knockdown lines

The 3' portion of the coding sequence for *pfsams* (577-1206bp) and *PMT* (651-1217bp) was amplified from gDNA and cloned by Gibson assembly into the pSLI-HAx3-*glmS* plasmid (gift from Professor R. Dzikowski) [578] following NotI and XmaI double digest. Following transfection of NF54 parasites, cultures were selected for the presence of the plasmid with 4nM WR99210 (gift of Jacobus Pharmaceuticals), follow by selection for integrants with G418 (Millipore Sigma). Single-crossover integration of the plasmid was confirmed via PCR (Supplementary Figures 4.4, 4.6, Supplementary Table 4.2), and parasites were cloned to isolate a population with single cross-over lacking any remaining WT loci. Integrated parasite lines were maintained under G418 drug pressure.

Animal Maintenance

Animal research was conducted at the Instituto de Medicina Molecular (Lisboa, Portugal). All protocols were approved by the animal ethics committee (ORBEA committee) at the institute and performed according to national and European regulations. BALB/c mice (age 5-8 weeks; males) were purchased from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France), kept in specific-pathogen-free conditions, and subjected to regular pathogen monitoring by sentinel screening. Experimental animals were randomly assigned and allowed free access to water and food.

Generation of *PbSAMS-DD* conditional knockdown parasites

Wild-type *P. berghei* ANKA strain was obtained from the MR4 repository (Manassas, Virginia). *P. berghei pbsams-DD* parasite line was obtained by double crossover homologous recombination. To do so, parasites were transfected by electroporation of purified schizonts, harvested on day 7-10 post transfection and genotyped by PCR. Transgenic parasites were then dilution cloned and further stored at -80 °C in frozen blood vials, containing 10⁷ blood stage parasites. Recombinant parasites carrying the human dihydrofolate reductase (*hdhfr*) gene cassette were positively selected by treatment of mice with pyrimethamine and trimethoprim to stabilize *PbSAMS*. Confirmation of transgenic parasite genotype, construct integration at the desired genomic loci and elimination of WT locus were assessed by PCR. Blood from the tail vein of infected mice was collected in 200 µL of 1x PBS and genomic DNA was isolated using the NZY Blood gDNA Isolation Kit (NZYTech), according to manufacturer's guidelines (Supplementary Figures 4.7, Supplementary Table 4.2). Stabilization of *PbSAMS-DD* fusion protein throughout infection was achieved *in vivo*, by administration of trimethoprim (TMP) to mice (0.25 mg/ml of TMP in drinking water), 2 days prior to infection.

Quantification of *PbSAMS* knockdown

All steps of parasite pellet extraction protocol were performed at 4°C to minimize protein degradation and all centrifugations were executed at 1000 x g for 10 min. Mice were sacrificed at day 4 post infection, 1 mL of blood was collected by cardiac puncture, washed in 10 mL of 1x PBS and centrifuged. Packed erythrocytes were saponin-lysed in 0.15% saponin and centrifuged. Parasite pellet was washed twice in PBS containing 1x Proteinase inhibitor cocktail (Roche cOmplete Protease inhibitor tablets, EDTA free). Parasite pellet was then resuspended in lysis buffer (4 % SDS; 0.5 % Triton X-114 in 1x PBS), incubated on ice for 10 min, centrifuged at 21 000 x g for 10 min and the supernatant was collected. Total protein content was determined using the Bio- Rad protein assay kit according to manufacturer's instructions. Protein samples diluted in 5x SDS sample buffer (NZYTech) were denatured at 95°C for 10 min and resolved in an 8% polyacrylamide gel (SDS-PAGE). Proteins were blotted into a nitrocellulose membrane by wet transfer at 200 mA for 2 hours. Primary antibodies, mouse anti-HA antibody (1:1000, from Covance) and rabbit anti-Bip (1:2000, GeneScript) were incubated overnight at 4°C. Secondary antibodies, anti-mouse horseradish peroxidase (HRP)-conjugated and anti-rabbit HRP (1:10000, Jackson ImmunoResearch

Laboratories) were incubated at RT for 1 hour. Signal detection was obtained using Luminata Crescendo Western HRP substrate (Merck Milipore.) and the ChemiDoc XRS+ Gel Imaging System (BioRad.). Protein band quantification was performed on Image Lab software (version 5.0) using BIP levels for normalization.

Quantification of gametocytes in *PbSAMS-DD-HA* parasites

Mice infections were performed by intraperitoneal inoculation of 1×10^6 infected red blood cells (iRBCs) obtained by passage in the correspondent BALB/c background mice. Parasitemia (% of iRBCs) was monitored daily and gametocytemia (% of mature gametocytes) was determined on day 3 after infection, by microscopic analysis of Giemsa-stained blood smears. A total of 5-10 thousand RBCs were analysed in randomly acquired images and semi-automatically quantified using Image J software (<http://rsbweb.nih.gov/ij>).

Induction and quantification of *P. falciparum* sexual commitment

Synchronous gametocyte induction was performed as previously described [578]. Briefly, parasites were double-synchronized with 5% sorbitol to achieve a synchrony of ± 6 h in the previous cycle. Synchronized ring-stage parasites were set up at 1.5% parasitemia (1% haematocrit) in 96-well, flat bottom plates under specific nutrient conditions to induce sexual commitment. Following reinvasion, on the first day of gametocyte development (D+1), ring-stage parasitemia was determined using flow cytometry and 50mM N-acetyl-D-glucosamine was added for 5 consecutive days. Gametocytes were then counted on day 6 (D+6) and commitment rate was determined by dividing the D+6 gametocytemia by the D+1 parasitemia assessed prior to N-acetyl-D-glucosamine addition. Gametocytes induced using the *Pf-peg4*-tdTomato fluorescent gametocyte reporter line were counted using flow cytometry [603].

RNA Extraction, cDNA synthesis, and quantitative RT-PCR

Total RNA from saponin-lysed parasites was extracted using Trizol (Invitrogen) or Direct-Zol RNA MiniPrep Plus kit (Zymo Research). 500 ng total RNA was pre-treated with 2U amplification grade DNase I (Invitrogen) and reverse transcribed using SuperScript III Reverse Transcriptase kit (Invitrogen) and random hexamers (Invitrogen). Quantitative PCR was performed on the Quant Studio 6 Flex (Thermo Fisher) using iTaq Sybr Green (Bio-Rad) with specific primers for selected target genes (Supplementary Table 4.2) and normalized to seryl-tRNA synthetase or ubiquitin-conjugating enzyme transcript

abundance. To quantify AP2-G transcript levels at the very end of the commitment cycle, 2.5 μ M Compound 1 (a generous gift of Dr. Jeffrey Dvorin) was added at 36 hpi to prevent egress and RNA was extracted 12 hours later.

Metabolite Analysis

Synchronous *P. falciparum* parasites were grown from 8hpi to 34hpi $\pm$ 6 hpi (100 mL, 3% hematocrit, 5-6% parasitemia) under the indicated growth conditions. Infected red blood cells (iRBC) were then purified to >90% purity using a 70/40% percoll/sorbitol density gradient and centrifugation (4700 g for 15 minutes at room temperature), then washed three times with minimal RPMI. Following isolation, equal numbers of isolated iRBC, as determined by the Beckman Coulter Z1 Coulter Particle Counter, were then re-incubated in their respective treatment conditions for four more hours to allow parasites to recover from percoll/sorbitol isolation. Following recovery, the purity of iRBCs was assessed using flow cytometry, cells were then pelleted, washed once with 1 mL of 1x PBS, and quickly lysed with 500 μ L of 90% ultrapure HPLC Grade methanol (VWR), followed by exactly 10 seconds of vortexing before being stored in dry ice.

Lysed samples were then centrifuged at top speed at 4°C and supernatant collected for metabolite analysis. Uninfected red blood cells (uRBC) were incubated under the same treatment conditions as iRBC, counted, and methanol extracted to distinguish iRBC metabolite signal from uRBC signal. Extracted metabolites were stored at -80°C. LC-MS based metabolomic analysis was performed as previously described [565]. 10 μ L of extract was separated on an Agilent 1290 Infinity LC system containing a Cogent Diamond Hydride Type C silica column (150 mm x 2.1 mm; Microsolv Technologies). Acquisition was performed on an Agilent 6230 TOF mass spectrometer (Agilent Technologies, Santa Clara, CA) employing an Agilent Jet Stream electrospray ionization source (Agilent Technologies, Santa Clara, CA) operated in high resolution, positive mode. Metabolite identification was verified by exact mass (to 35 ppm) and co-elution with authentic standards purchased from Millipore Sigma. Batch feature extraction and chromatographic alignment across multiple data files was performed using the Agilent MassHunter Profinder software and extracted metabolite data was exported for further statistical analysis using R.

Western Blotting

PfPMT-glmS and *PfSAMS*-glmS knockdown western blots were performed using whole cell lysate. Ring-stage parasites (12hpi $\pm$ 6 hpi) were treated with or without 2.5mM glucosamine (VWR) to induce protein knockdown as previously described. Parasites were saponin lysed at 36hpi $\pm$ 6 hpi and whole cell extract collected in SDS sample buffer, then boiled for 10 minutes. Proteins were then separated on 12% SDS PAGE and transferred to a PVDF membrane (Millipore Sigma, 0.2 μ M). Membranes blocked with 5% milk were then probed with anti-HA (1:2000, Abcam, ab9110), and anti-hsp70 (1:2000, StressMarq SPtdCho-186) primary antibody solutions, followed by anti-rabbit IgG (1:5000, Millipore Sigma 12-348) secondary. Chemiluminescence was measured using the Azure c-Series imaging systems (Azure Biosystems) and quantified using the Fiji open-source image processing package based on ImageJ.

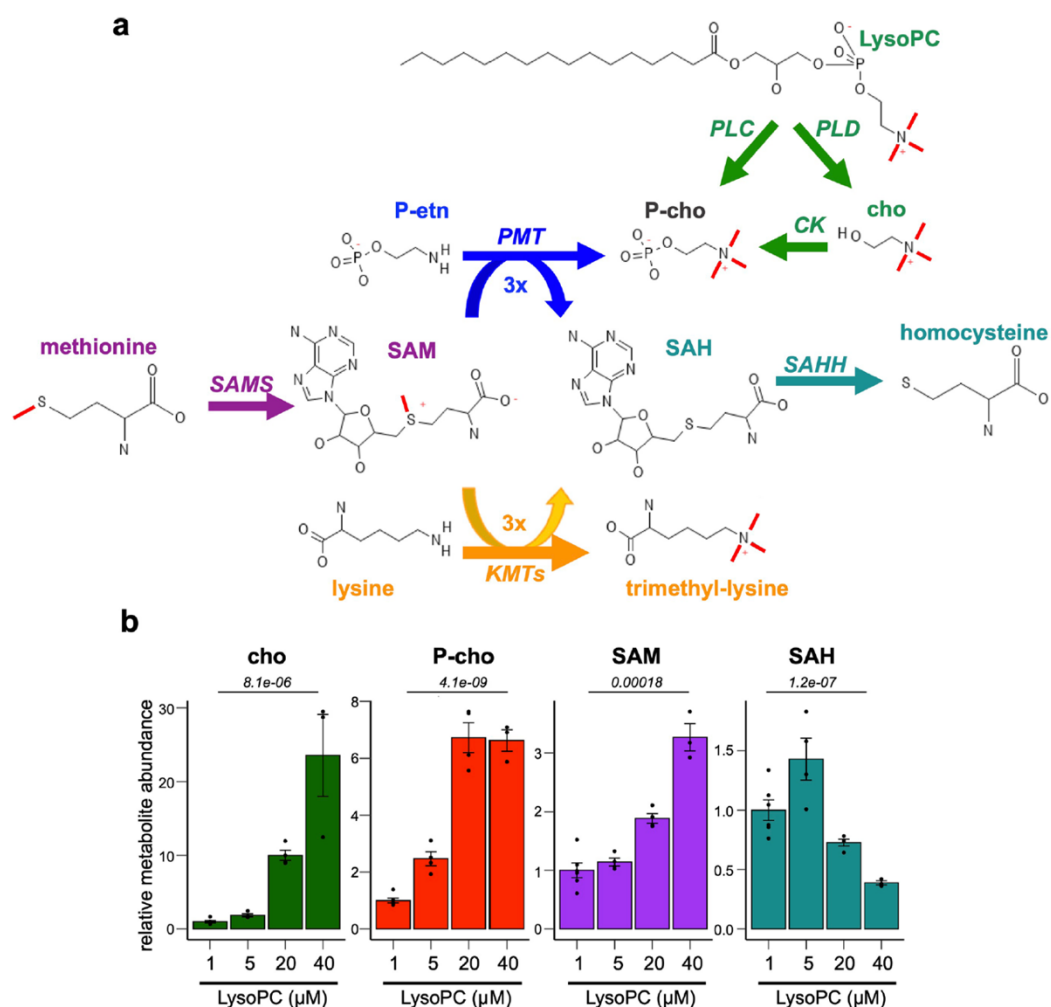
Quantification of changes in H3K9me3 and H3K4me3 abundance

H3K9me3 and H3K4me3 abundances were measured by Cleavage Under Targets & Release Using Nuclease (CUT & RUN) [604] adapted for *P. falciparum*. Approximately 1×10^7 percoll/sorbitol isolated schizonts (34-38 hpi) per sample were washed three times with 1 mL wash buffer (20 mM HEPES at pH 7.5, 150 mM NaCl, 0.5 mM Spermidine, and 1x Roche complete protease inhibitor) at room temperature and resuspended in 225 μ L of wash buffer. 25 μ L of Concanavalin A-coated beads were washed and resuspended in binding buffer (20 mM HEPES-KOH at pH 7.5, 10mM KCl, 1mM CaCl₂, 1 mM MnCl₂) before being added to each sample. Samples were then rotated for 10 minutes at room temperature. Then, samples were placed on a magnetic stand to clear (30s to 2 minutes) and remove all the liquid. Samples were washed 3x's with DIG wash buffer (wash buffer with 0.025% digitonin). For each sample, 150 μ L of antibody wash buffer was added (wash buffer, 0.025% digitonin, and 2 mM EDTA at pH 8.0) with H3K4me3 (0.005 μ g/ μ L, C15410003-50, Diagenode), H3K9me3 (0.005 μ g/ μ L, ab8898, abcam), or IgG isotype control (0.005 μ g/ μ L, 02-6102, ThermoFisher) was added to the sample tube and incubated at 4°C, rotating, overnight. Following antibody incubation, samples were placed on a magnetic stand to clear and pull off the liquid, then washed 3 x's with DIG wash buffer, 150 μ L of ProteinA/G-MNASE fusion protein (dilution 1:60, 15-1016, EpiCypher) in DIG wash buffer and rotated at 4°C for 1 hour. After washing samples 3x's with DIG wash buffer, samples were then washed 3x's with Low Salt Rinse Buffer (20 mM HEPES-NaOH at pH 7.5, 0.025% digitonin, 0.5 mM spermidine). 200 μ L of ice-cold incubation buffer (3.5 mM HEPES-NaOH at pH 7.5,

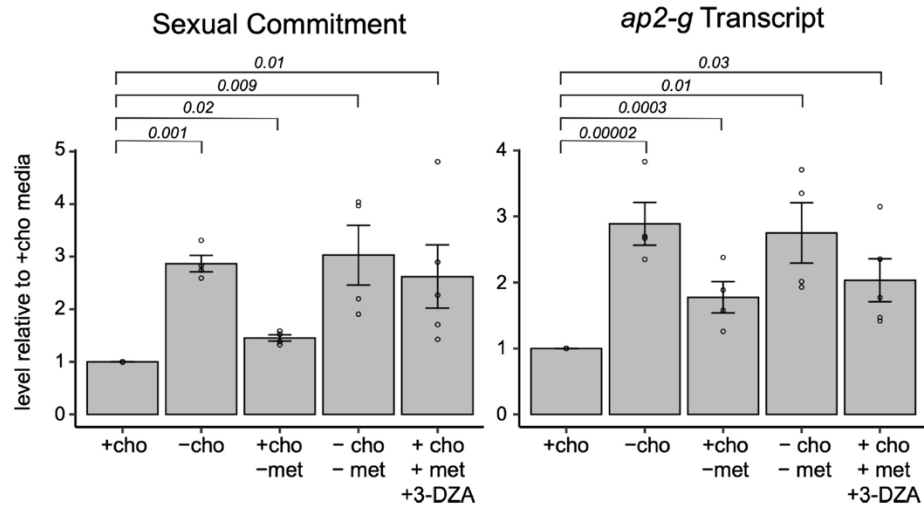
100mM CaCl₂, 0.025% DIG) was added and samples were equilibrated at 0°C for 30 minutes to achieve targeted digestion. Digestion was then stopped by adding 200µl of stop buffer (170 mM NaCl, 20 mM EGTA at pH 8.0, 20 mM EGTA, 0.0.25% digitonin, 25 µg/mL glycogen, 50 µg/mL RNase A). After incubation at 37°C for 30 minutes to release soluble fragments, samples were digested by adding 2.5 µL proteinase K (20 mg/ml) and 2 µL 10% SDS, and then incubated at 50°C for 1 hour. DNA fragments were purified with phenol–chloroform–isoamyl alcohol and washed by ethanol precipitation, and finally dissolved in 30 µL TE buffer (1mM Tris-HCl pH 8, 0.1 mM EDTA). Library construction was carried out using NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645S, NEB). 4ng of fragmented DNA was used for end-repair/A-tailing, ligation, and post ligation cleanup with 1.7x volumes of AMPure XP beads (Catalog number, company). Following cleanup, PCR amplification was performed using 2x KAPA HotStart ready mix (Catalog number, company) and NETFLEX primer mix (Catalog number, Bio Scientific) with PCR program: 1 min @ 98°C/15 cycles, 10 sec @ 98°C, 1 min @ 65°C, 5min @ 65°C and hold 4°C. PCR products were size selected with 0.8x volumes, then 1.2x volumes of AMPure XP beads.

Beads were then washed twice with 80% ethanol and DNA eluted with 0.1x TE and used for sequencing in a Nextseq2000 system as paired-end reads, following quality control. After sequencing, raw reads were trimmed using Trimmomatic v0.38 [604] to remove residual adapter sequences and low quality leading and trailing bases. Both paired and unpaired reads were retained for read lengths that were at least 30 bases after trimming. Trimmed paired reads were aligned to the PlasmoDB version 46 *P. falciparum* 3D7 reference genome [604] using BWA v.0.7.1 [579]. SAMtools v.1.10 [605] was used to remove low quality alignments as well as sort and index sample files. Normalized fold enrichment tracks were generated by using the MACS2 v.2.2.7.1 [606] callpeak function with settings: -f BAMPE -B -g 2.3e7 -q 0.05 –nomodel –broad –keep-dup auto –max gap 500. Bedgraph outputs were then passed into the bdgcmp function with the setting -m FE (fold enrichment) to generate signal tracks to profile histone modification enrichment levels compared to whole genome. Peak sets from replicates were compared with Bedtools intersect v2.26.0 [607], and peaks that overlapped by at least 1 bp were considered shared. Fold enrichment bedgraphs and peak sets were then output to RStudio Server (v1.4.1717) for further analysis using the GenomicRanges Package v.1.44.0 [608] and visualized with the GViz v1.38.1 package [609] within the Bioconductor project (release 3.13) [610]. The full analysis pipeline can be found at <https://github.com/KafsackLab/MetChoH3K9me3>.

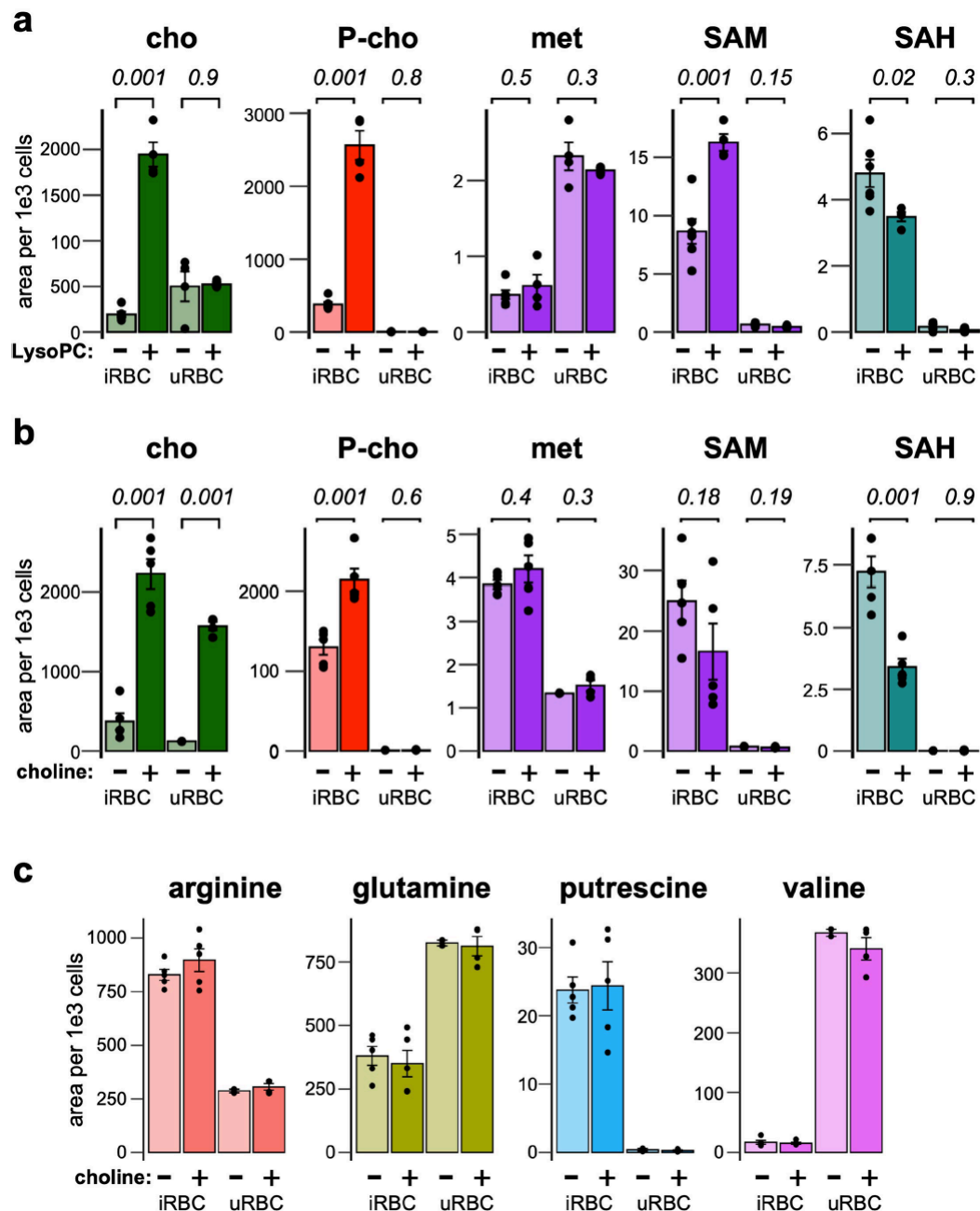
Supplementary Information



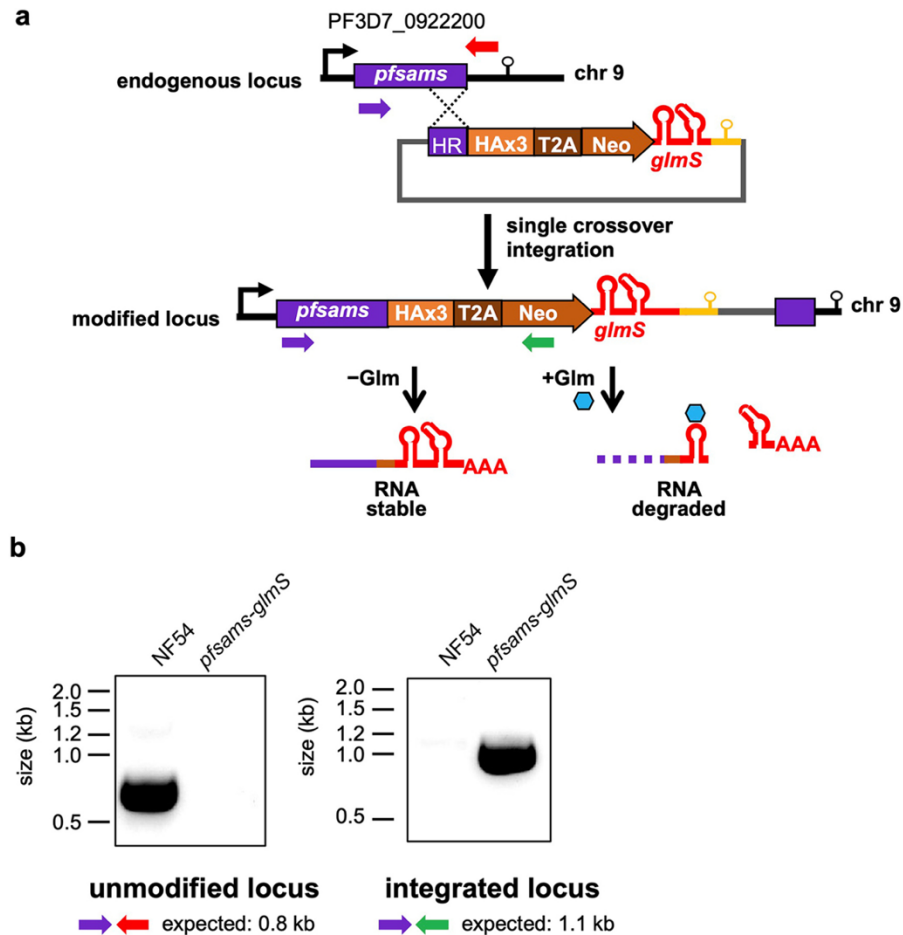
Supplementary Figure 4.1 - Dose-dependent metabolic response to LysoPC. (a) Molecular structures of the central metabolites in this study. Methyl groups being transferred are highlighted in red and names of enzymes involved in their interconversion are noted in italics. (b) Parasites were cultured in media spiked with increasing concentrations of LysoPC. Bar graphs show the mean intracellular metabolite abundances per thousand parasites $\pm$ s.e.m ($n=5$ biologically independent samples). Italicized numbers are p-values based on two-sided ANOVA tests.



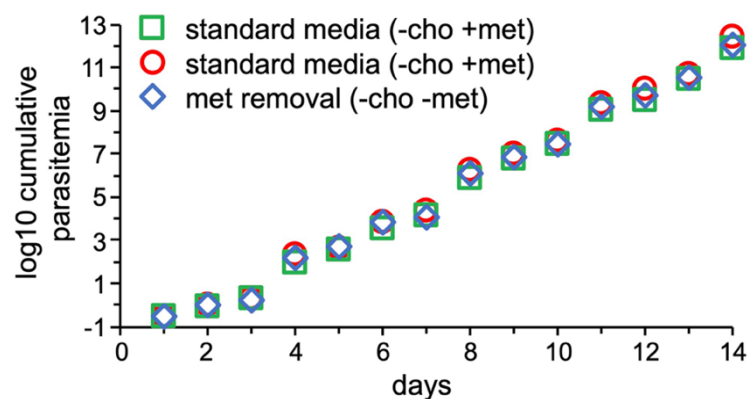
Supplementary Figure 4.2 - Schizont *ap2-g* transcript abundances closely track sexual commitment under various nutrient conditions. Bars indicate the mean sexual commitment (left) and *ap2-g* transcript abundance (right) in schizonts relative to conditions of abundant choline and methionine (+cho) when parasites were exposed to different growth media during the commitment cycle. Error bars and p-values indicate the standard error of the mean and the significance of the mean difference relative to those under conditions of abundant choline and methionine (+cho), respectively. (n= 4-5 biologically independent samples).



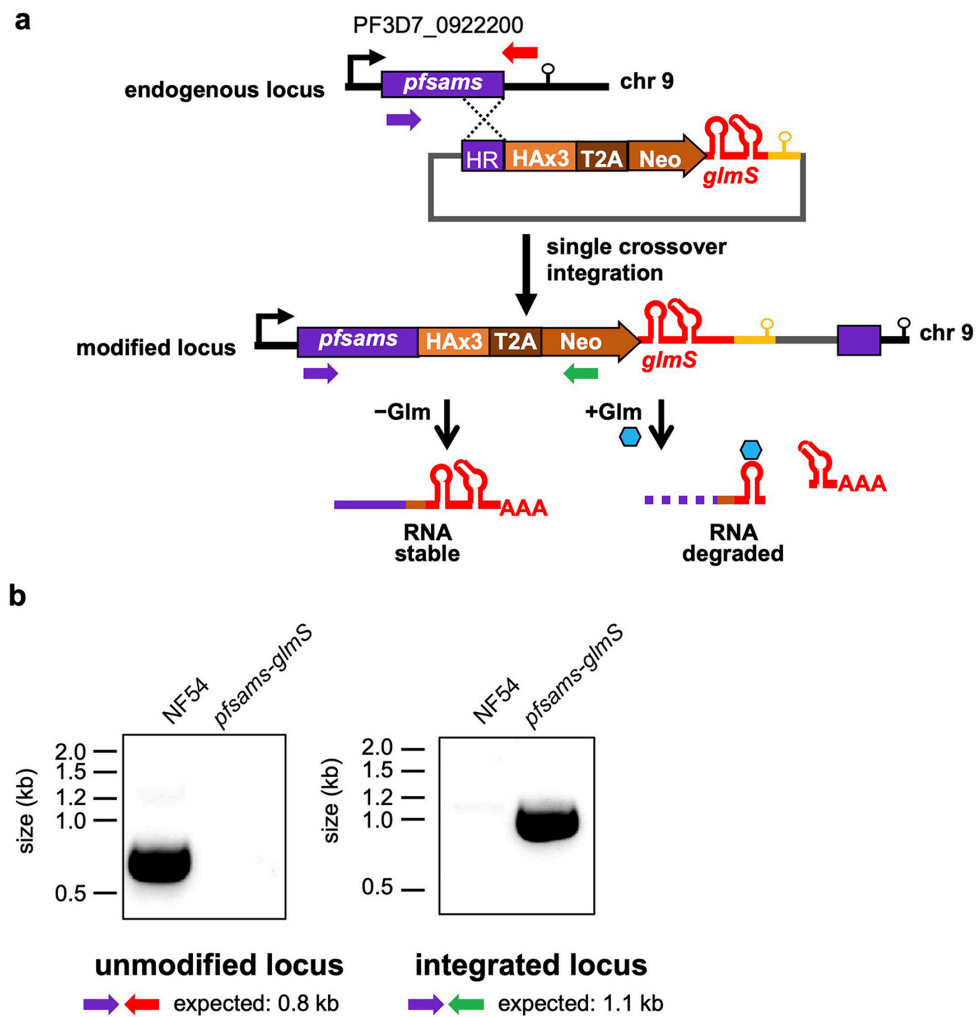
Supplementary Figure 4.3 - Changes in SAM/SAH metabolism are specific to parasite metabolism. LC-MS quantification of indicated metabolites. (a,b) Infected and uninfected cultures were grown in the presence or absence of **(a)** 20 μ M LysoPC or **(b)** 420 μ M choline for ~36 hpi during the commitment cycle. Infected (iRBC) and uninfected (uRBC) erythrocytes were then extracted, and metabolite abundances were quantified by LC-MS. **(c)** Abundances of four additional metabolites unrelated to this study are included to illustrate reproducibility of metabolite extraction and quantification by LC-MS. Bar graphs show the mean intracellular metabolite abundances per thousand cells $\pm$ s.e.m ($n = 4$ biologically independent samples). Italicized numbers are p-values based on two-sided paired t -tests.



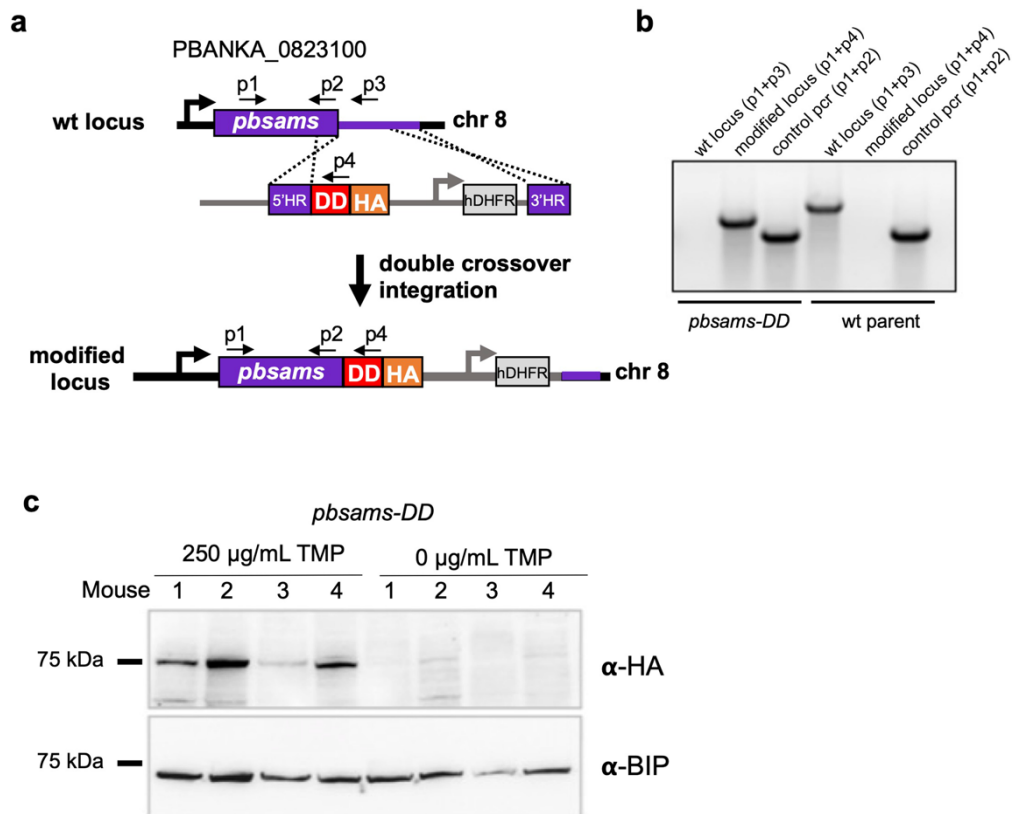
Supplementary Figure 4.4 – Validation of PMT-*glmS* knockdown parasite line. (a) Generation of PMT-*glmS* knockdown parasites by selection-linked integration. (b) Validation PCR demonstrating tagging of the endogenous PMT locus.



Supplementary Figure 4.5 - Methionine depletion does not alter parasite growth. Removal of methionine (blue diamond) or supplementation with choline (red circles) had no observable effect on growth of NF54 compared to growth in standard malaria medium (green squares). $n=1$.



Supplementary Figure 4.6 - Validation of *pfsams-glmS* knockdown parasite line. (a) Generation of *pfsams-glmS* knockdown parasites by selection-linked integration. **(b)** PCR Validation demonstrating tagging of the endogenous *pfsams* locus.



Supplementary Figure 4.7 - Validation of *pbsams*-DD knockdown parasite line. (a) The endogenous *pbsams* locus in the *P. berghei* ANKA strain background was modified by homologous integration to add the ecDHFR destabilization domain (DD) and hemagglutinin epitope tag (HA) at the 3' end of the *pbsams* coding sequence. Simultaneous integration of a hDHFR expression cassette allows for selection of integrants. (b) PCR validation of successful tagging in *PbSAMS*-DD-HA parasites. (c) Successful knockdown of *PbSAMS* upon removal of trimethoprim (TMP) from the drinking water in mice infected with *pbsams*-DD parasites. Parasite lysates were assayed for the abundance of *PbSAMS*-DD with antibodies against the HA epitope tag and *PbBIP*, which served as a loading control and was used for normalization.

Supplementary Table 4.1 – Compositions of the cell culture media used.

Label	Differences from standard malaria complete media
-cho	0 μ M choline
+cho	420 μ M choline
-LysoPC	30 μ M palmitate, 30 μ M oleate, 1.25 μ M LysoPC, 4 g l ⁻¹ fatty acid free BSA, no Albumax II,
+LysoPC	30 μ M Palmitate, 30 μ M oleate, 40 μ M LysoPC, 4 g l ⁻¹ fatty acid free BSA, no Albumax II,
-Glm	none
+Glm	2.5 mM glucosamine
-met	0 μ M methionine
+met	none (100 μ M methionine)
-3-DZA	420 μ M choline
+3-DZA	420 μ M choline, 100 nM 3-DZA

Supplementary Table 4.2 – Primers used in this study.

PrimerID	Sequence	Strand	Purpose
BKO-1424	TATGCTGATATCTTACTGCTTGC	F	RT-qPCR for <i>pfpm</i> t transcript
BKO-1425	GGACCAGCCATCATCAAGAC	R	RT-qPCR for <i>pfpm</i> t transcript
BKO-1626	AAGTAGCAGGTCATCGTGGTT	F	RT-qPCR for serine t-RNA ligase
BKO-1627	TTCCGGCACATTCTTCCATAA	R	RT-qPCR for serine t-RNA ligase
BKO-1004	ctatagaataactcaagctcgccgcGGAGCACATACTCACGGTATAGAT	F	for cloning <i>pfpm</i> t homology block for <i>pfpm</i> t- <i>glm</i> S tagged line
BKO-1005	ccgggacgtcgtagcggtaccgggATTTTGGTGGCCTTAAATAACC	R	for cloning <i>pfpm</i> t homology block for <i>pfpm</i> t- <i>glm</i> S tagged line
BKO-1002	ctatagaataactcaagctcgccgcCCTTTACGTGTTCTACTGTTCTTATT	F	for cloning <i>psams</i> homology block for <i>psams</i> - <i>glm</i> S tagged line
BKO-1003	ccgggacgtcgtagcggtaccgggATTTTAAATGCATTTTTCGTG	R	for cloning <i>psams</i> homology block for <i>psams</i> - <i>glm</i> S tagged line
BKO-0750	TGACTTTGATTGAAACTTAACTCTG	F	PCR verification of <i>pfpm</i> t- <i>glm</i> S line; binds within <i>pfpm</i> t CDS upstream of homology region
BKO-1661	CTGCATATTATGCATCGGGATAC	R	PCR verification of <i>pfpm</i> t- <i>glm</i> S line; binds within <i>pfpm</i> t 3' UTR
BKO-1023	GTATCTTACCTATTATTAGGACCTGATG	F	PCR verification of <i>psams</i> - <i>glm</i> S; binds in <i>psams</i> CDS upstream of homology region
BKO-1009	ACATTCTCTTCTCTCATTCCTAAAT	R	PCR verification of genome editing; binds within <i>psams</i> 3' UTR
BKO-0217	AGTGACAACGTCGAGCACAG	R	PCR verification of <i>pfpm</i> t- <i>glm</i> S and <i>psams</i> - <i>glm</i> S lines; binds within the neomycin resistance gene
P1	taggtaccGAGGAAATTTTCTATTACTTCG	F	Validation of <i>psams</i> - <i>dd</i> - <i>ha</i> parasites, binds within <i>psams</i> CDS upstream of homology region
P2	tagggcccATTTTTTAAACATTTTTTTCGTG	R	Validation of <i>psams</i> - <i>dd</i> - <i>ha</i> parasites, binds at 3' end of <i>psams</i> CDS, used as a PCR positive control
P3	atgcggccgcCAACTAATAAAATCCAGGAAATA	R	Validation of <i>psams</i> - <i>dd</i> - <i>ha</i> parasites, binds within <i>psams</i> 3'UTR, downstream of the 3' homology region
P4	taGCGGCCGCTCATCGCCGCTC	R	Validation of <i>psams</i> - <i>dd</i> - <i>ha</i> parasites, binds within the DD CDS sequence
BKO-1394	TGGTGGAATAAGAACAACAGAGGT	F	RT-qPCR for <i>pfap2-g</i> transcript
BKO-1395	CCATCATAATCTTCTTCTCGTCG	R	RT-qPCR for <i>pfap2-g</i> transcript
BKO-2322	GGTGTAGTGCTCACCAATAGGA	F	RT-qPCR for ubiquitin-conjugating enzyme (<i>pfuce</i>) transcript
BKO-2323	GTACCACCTCCCATGAGTA	R	RT-qPCR for ubiquitin-conjugating enzyme (<i>pfuce</i>) transcript

Upper case letters indicate the annealing region matching the template

Acknowledgments

General: We wish to thank Dr. Choukri Ben Mamoun (Yale School of Medicine) for the gift of the *pfpm* knockout and complemented lines and the Weill Cornell Medicine genomics core for technical support.

Funding: This work was supported by funds from Weill Cornell Medicine (BK), NIH 1R01 AI141965 (BK), NIH1R01 AI138499 (KWD), NIH 5F31AI136405-03 (CH), NIH R25 AI140472 (KYR), the Fundação para a Ciência e Tecnologia (MMM, DRIVER-LISBOA-01-0145-FEDER-030751) and “laCaixa” Foundation (MMM, under the agreement HR17/52150010).

Author contributions: Conceptualization: BFCK, KWD, MMM; Methodology: BFCK, CTH, MMM; Investigation: CTH, XT, LNV, IMM; Software, Formal Analysis, Data Curation: BFCK, CTH, XT; Writing – Original Draft: CTH Writing – Review & Editing: BFCK, CTH, MMM; Visualization: BFCK, CTH; Supervision: BFCK, KYR, MMM; Project Administration: B.F.C.K; Funding Acquisition: BFCK, KWD, MMM

CHAPTER V

CHAPTER V

General Discussion

The main findings of this thesis are summarized below:

Plasmodium blood-stage parasites sense and actively adapt to fluctuations in host nutritional levels (Figure 5.1):

- i. Isoleucine and methionine depletion result in impaired parasite intra-erythrocytic growth;
- ii. SAMS-deficient parasites phenocopy parasites growing in MetD conditions (SAMS is essential during blood stage development);
- iii. *Plasmodium* actively responds to AA-limiting conditions by readjusting development and reducing replication;
- iv. Distinct metabolites (polyamines) regulate parasite replication and development;
- v. Three parasite kinases coordinate development or replication under AA scarcity in a temporally-regulated manner;
- vi. Adaptation to low AA levels is mediated by eIF2 α phosphorylation via a GCN2/eIF2 α signaling pair.

Liver-specific methionine metabolism plays a key role in sustaining *Plasmodium* high replication rates within hepatocytes (Figure 5.2):

- i. Methionine depletion impacts parasite intra-hepatic development, resulting in fewer nuclei;
- ii. Parasite's SAMS enzyme is dispensable during the liver stage of infection;
- iii. The host's liver-specific MAT1A is essential for parasite intra-hepatocyte replication;
- iv. SAM is the sole metabolite that rescues the attenuated phenotype induced by methionine depletion;
- v. Malaria parasites have the capacity to uptake exogenously supplied SAM, raising the question of whether *Plasmodium* possesses a SAM transporter.

SAM plays a pivotal role as a commitment switcher for sexual differentiation (Figure 5.3):

- i. PMT activity is closely linked to SAM metabolism and influences the rate of sexual commitment;
- ii. Parasites lacking the PMT enzyme exhibit increased SAM levels, which in turn compromises sexual commitment;
- iii. Fluctuations in SAM (and SAH) levels, resulting from variations in the availability of Met or P-cho precursors, modulate parasite sexual commitment;
- iv. Depletion of methionine and SAM or accumulation of SAH have an impact on parasite gene expression by reducing the methylation level of specific histone modifications, including H3K9me3, a marker associated with gene silencing;
- v. Changes in the balance between SAM and SAH disrupt the efficiency of H3K9me3 maintenance across the entire genome, including at the *ap2-g* locus; Activation of *ap2g* drives parasite commitment to sexual development.

Thriving in Change: Robust Sensing and Active Adaptation as Alternatives to Dormancy

Throughout evolution, virtually all living microorganisms have encountered recurring stresses that impacted their growth. To endure such periods, they have evolved the ability to enter a robust, non-replicating state, often referred to as dormancy or hibernation. A well-known model organism used to study cellular dormancy in response to nutrient starvation is *S. cerevisiae*. When faced with resource depletion, yeast enters a stage of low metabolic activity, known as G0, in which cells can survive for long periods [611]. This reversible cell cycle arrest occurs before DNA replication begins. Babbitt *et al.* documented many similar adaptive features in the *P. falciparum* response to isoleucine depletion, although a transcriptional shift that would indicate a conventional stress response was absent [612]. These features included an arrest prior to DNA synthesis, a state of low metabolic activity, a decelerated cell cycle rate, and the maintenance of viability beyond prolonged starvation.

The inability to initiate an adaptive transcription program in *P. falciparum*, a well-established signature of starvation responses, has been attributed to a lack of regulatory transcription factors encoded by the parasite genome [446]. The lack of a conventional eukaryotic nutrient-stress response pathway in the parasite, coupled with the challenge of identifying specific molecular pathways involved in this response, has led to a model proposing that the malaria parasite manages an unstable supply of amino acids in the bloodstream through a state of hibernation until additional nutrients become available. Babbitt *et al.* demonstrated that AA deficiency induces hibernation, suggesting that the control of cell cycle progression in the parasite relies on a passive mechanism, primarily by restricting reaction rates and metabolite levels (specifically isoleucine levels). In a noteworthy parallel study, McLean and Jacobs-Lorena re-examined this dormancy-like state, following the response of *P. falciparum* to isoleucine starvation for extended periods [260]. Their findings revealed that isoleucine-starved parasites do not exit the cell cycle but instead cycle at a reduced pace, successfully completing schizogony and reinventing new erythrocytes.

In Chapter II of this thesis, we present compelling evidence that supports a model combining the two contrasting viewpoints. Through extensive microscopic analysis of parasite development inside erythrocytes, we revealed that most of the parasites growing under AA-limiting conditions exhibit growth arrest (70%), thereby resembling the

hibernation phenotype first proposed by Babbitt *et al.* However, a minority of the parasite population (30%) still progresses through the cell cycle at a normal rate and successfully develops mature schizonts. Nonetheless, a noticeable decrease in merozoite numbers suggests that these schizonts replicate less, leading to fewer offspring. Interestingly, while both Ile and Met deprivation impair schizont development, we observed that Ile depletion has a stronger effect earlier in development at the trophozoite stage. Our findings reveal, for the first time, that AA depletion extensively impacts parasite development but also replication, resulting in fewer merozoites per schizont in each cycle (Figure 5.1). Moreover, we have shown that the regulation of parasite development and replication operate independently, with each process controlled by a different polyamine.

While autophagy or proteasomal degradation of preexisting proteins, including AA-containing erythrocyte proteins imported and degraded alongside Hb [457], act as cellular survival strategies that might provide some level of support for parasite growth in the absence of essential AAs, this appears rather insufficient to ensure the completion of the cell cycle for the majority of the parasites. The eukaryotic nutrient starvation response is a well-characterized mechanism for translational control in response to AA deficiency which involves crucial regulatory roles played by the TOR signaling pathway and the GCN2/eIF2 α signaling pair [342]. Nevertheless, *Plasmodium* parasites are a rare exception to the remarkable conservation of these signaling pathways observed across evolution from yeast to humans. With the exception of the *P. falciparum* orthologue *Maf1*, which has been demonstrated to maintain viability under extended starvation [400,401,405], most recognizable components of the TOR complex have been lost in the *Plasmodium* genus. Furthermore, despite having a GCN2 orthologue (*P. falciparum* eIK1), malaria parasites lack many downstream components of this pathway. Noteworthy, eIK1-deficient ring-stage parasites fail to phosphorylate eIF2 α in response to AA depletion, but the genetic disruption of this kinase had no effect on the establishment or maintenance of starvation-induced hibernation [457]. These observations raise the question of whether parasites evolved mechanisms to efficiently detect and actively respond to AA fluctuations.

An active parasite response to AA depletion becomes evident when examining our kinome screening data, which identified a trio of parasite kinases, Nek4, eIK1 and eIK2 - the latter two belonging to the eIF2 family - as key players in a stress response signaling pathway to AA deprivation. Interestingly, under AA-limiting circumstances, eIK1 and eIK2 coordinate either replication or development, while Nek4 governs both replication

and development. These findings shed new light on the intricate mechanisms that malaria parasites employ to cope with AA fluctuations, indicating that the parasite does not solely rely on passive hibernation but rather employs active mechanisms to fine-tune growth and development accordingly. Interestingly, immunoblotting analysis of eIF2 α phosphorylation status revealed that *Plasmodium* parasites exhibit stage-specific responses to AA depletion. While Δ eIK1 ring-stage parasites are unable to respond to AA depletion (but not the schizont-stages), Δ eIK2 parasites only become unresponsive later in development during the schizont stage (Figure 5.1). This stage-specificity suggests a dynamic and temporally regulated response to AA fluctuations, which may be critical for the suitable adaptation to the different environmental conditions the parasite encounters during its intricate life cycle. Moreover, analysis of eIF2 α -P levels in Δ nek4 parasites demonstrates that this kinase acts as a major regulator of growth under AA deficiency, governing both replication and development, as demonstrated at the phenotypic level. It is tempting to hypothesize that Nek4 functions upstream of eIK1 and eIK2, coordinating either development (via eIK1) or replication (via eIK2) in the absence of AAs. One of the most intriguing evidence supporting the dynamic and active regulation of parasite growth rates in response to AA fluctuations is the observation by McLean and Marcelo Jacobs-Lorena that parasites become resistant to Ile depletion once they enter S phase and DNA synthesis starts [446]. This sheds light on the ability of parasites to sense and respond to environmental cues during the G1 phase, but it also implies that such sensing mechanisms become inactive once DNA synthesis begins.

To the best of our knowledge, this is the first study to challenge the established hypothesis of passive growth regulation in AA deficiency. The results presented in Chapter II shed light on the coexistence of active and passive responses in *Plasmodium* parasites and reveal a remarkable evolutionary adaptation that allows parasites to cope with the diverse and unpredictable environmental conditions they face throughout their life cycle. These finely tuned strategies allow the parasite to effectively cope with fluctuations in amino acid availability, thus optimizing its survival and reproductive success in different host environments. Although only a small subset of parasites show an active response to AA depletion, this is remarkable for their outstanding capacity to maintain the cell cycle even in the absence of some essential AAs. The decreased rate of replication allows the parasite to move through the cell cycle and ensure the generation of viable offspring, guaranteeing its continuous transmission and persistence in the host population.

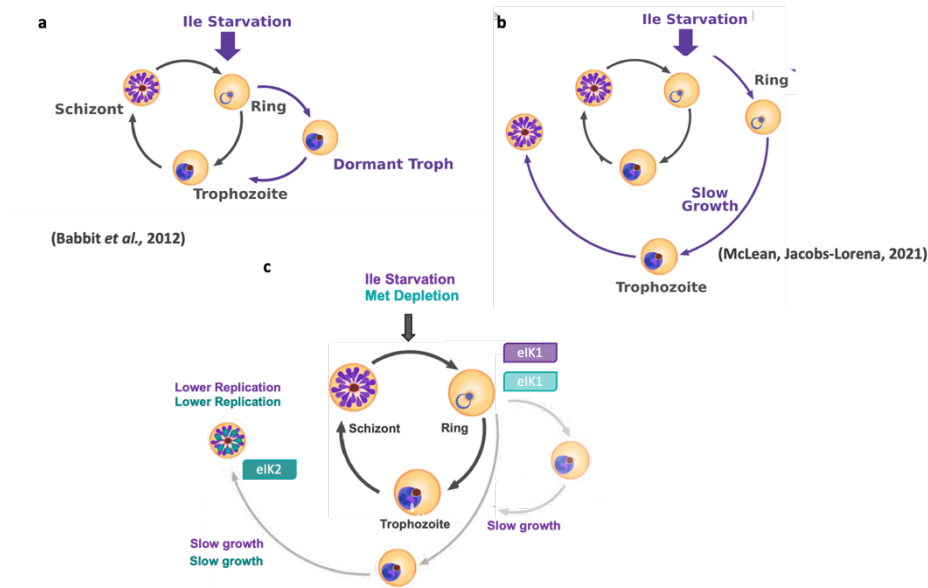


Figure 5.1 - Three separate hypotheses are illustrated to describe the response of *P. falciparum* to AA deficiency. **a.** Babbitt *et al.* suggested programmed hibernation rather than an active parasite response to Ile depletion. The data from **b.** McLean and Jacobs-Lorena showed that instead of hibernating, parasites move at a slower pace through the cell cycle, but never leave it. **c.** Our data provides a model that combines both opposing perspectives. The slow development fueled by AA deficiency is reminiscent of hibernation as most parasites never reach full maturity (mature segmented schizonts). However, a small proportion of parasites successfully complete maturation, but replication is reduced. Furthermore, while both Met and Ile affect development and replication, they exhibit some peculiarities, with isoleucine primarily affecting the trophozoite stage and methionine mostly impacting the schizont stage. Data from a kinome screen are also represented, showing the kinases that operate during AA depletion.

Is Liver Tropism in Malaria a Result of Simple Causation or an Evolutionary Adaptation Driven by Host SAM?

The *Plasmodium* genus comprises over 200 distinct parasite species, each with exceptionally complex life cycles involving a mosquito vector and a wide range of hosts, including reptiles, birds, and mammals, particularly humans. Malaria parasites have evolved to preferentially reside within mammalian erythrocytes, wherein cause disease. However, an obligatory pre-erythrocytic stage precedes blood stage infection, and for all *Plasmodium* species infecting mammals, this stage takes place in the liver. The infection of mammalian hepatocytes involves a striking phenomenon of massive parasite multiplication, with each parasite replicating into tens of thousands of erythrocytic-infectious merozoites. In sharp contrast, *Plasmodium* species infecting birds and reptiles preferentially reside in macrophages at the mosquito bite site, giving rise to only dozens of exoerythrocytic parasites. Liver stage schizogony in the mammalian host represents

one of the fastest eukaryotic replication events. For instance, rodent malaria sporozoites can transform into up to 29 000 exoerythrocytic merozoites in just 2 days [429]. Conversely, the liver stage development in *P. falciparum* takes up to 6.5 days and results in up to 90 000 erythrocytic-infectious merozoites packaged inside a fully mature parasite [224].

As obligate intracellular pathogens, *Plasmodium* parasites are auxotrophic for most nutrients within mammalian hosts, heavily relying on host resources to sustain their highly multiplying lifestyle. The parasite's remarkable growth at both cellular and genomic levels is supported by the exceptionally metabolite- and nutrient- rich milieu within liver cells. Nutrient uptake by the parasite from the extracellular environment involves passive diffusion through pores in the parasitophorous vacuole membrane (PVM) and active utilization of transporter proteins [225]. Among these nutrients, methionine (Met), an essential amino acid critical for protein synthesis and the precursor of S-adenosylmethionine (SAM), plays a crucial role. The mammalian MAT enzyme is responsible for Met metabolism and its activity is the product of two genes: the extra-hepatic *mat2a*, which is strongly inhibited by SAM, and the liver-specific *mat1a*, whose activity is never inhibited by its product [613]. This renders the host hepatocyte a unique cell, as regards to Met metabolism. Interestingly, the parasite genome encodes an orthologue of the MAT enzyme, known as S-adenosylmethionine synthetase (SAMS), along with all the necessary enzymes for completing Met metabolism [512,513].

In Chapter III of this thesis, we present data that establishes a link between hepatic methionine metabolism and parasite's high replication rates. Our findings revealed that methionine deficiency (MD) results in smaller parasite exoerythrocytic forms (EEFs) with fewer nuclei, yet no differences in liver-stage parasite numbers were observed (Figure 5.2). Moreover, the attenuated replication under MD is an outcome of diminished SAM levels within the parasite likely resulting in deregulated SAM-dependent transmethylation reactions, which predominantly occur in the liver. Of note, neither glutathione, homocysteine, nor polyamines could restore impaired replication, further underscoring the unique significance of SAM in liver stage development. These data also highlight major differences in the mechanisms regulating the development of blood- and liver-stage parasites in AA deficiency, as different metabolites regulate progression through each developmental phase. While polyamines are those coordinating parasite intra-erythrocytic replication and growth under AA deficiency (see Chapter II), SAM emerges as the chief metabolite regulating replication during liver stage development.

Remarkably, our analysis of SAMS-deficient parasites, unable to efficiently convert Met into SAM, revealed a significant reduction in intracellular SAM levels, but this had no impact on liver load neither on parasite development. Such observations are in sharp contrast to our previous findings with intra-erythrocytic parasites (see Chapter II), where the *sams* gene has proven essential and thus refractory to deletion. However, despite not relying on their own Met metabolizing enzyme while developing inside hepatic cells, liver-stage parasites are strictly dependent on the host's methionine metabolism (Figure 5.2).

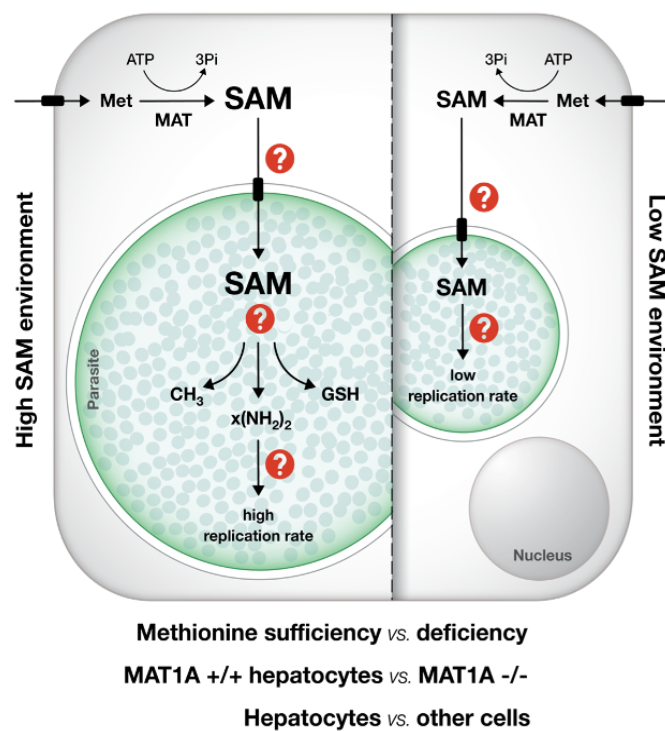


Figure 5.2 - Host liver-specific methionine metabolism sustains *Plasmodium* high replication rates by providing required SAM levels. Under physiological conditions the hepatocyte is a privileged cell as it regards to methionine metabolism, with about 50% of all dietary methionine being metabolized in the liver and up to 85% of all SAM-dependent transmethylation reactions taking place inside hepatocytes (high SAM environment). The SAM-rich environment ensures that GSH, polyamines and methylation levels are commensurate to the cell needs, allowing parasites to replicate massively. However, once Met levels or the MAT1A enzyme are disrupted, intra-hepatic SAM levels are diminished, resulting in strikingly lower replication rates (low SAM environment).

The liver cell is a much privileged and nutrient-rich environment for parasite development when compared to mature yet, non-metabolically active erythrocytes, possibly explaining the divergent data obtained for blood- and liver-stage parasites. The hypothesis that *Plasmodium* imports SAM from the host cell aligns with the knowledge that certain organisms, such as *Rickettsia* and *Pneumocystis carinii*, meet their SAM requirements by importing it through a plasma membrane transporter - since they lack or have inactivated MAT genes [550,552,614]. Other organisms, including some fungi and protozoa such as *Leishmania infantum*, *Trypanosoma brucei*, and *Toxoplasma gondii*, can both synthesize and transport SAM [474,552,614]. Despite this knowledge, the exact transporter or mechanism that the malaria parasite uses to mediate SAM uptake remains utterly unexplored. Analysis of the *Plasmodium* genome revealed the presence of six

putative SAM plasma membrane transporters with homology to *L. infantum* and *T. gondii* transporters [474,554,615]. Notably, four of these transporters have higher levels of expression in the liver stage of infection in comparison to the blood stage. To gain insight into their role, these genes have been phenotypically characterized throughout the parasite's life-cycle using reverse genetics. Three of these proved to be critical for liver stage development, while not essential for blood stage infection (PBANKA_0208300, PBANKA_0815700 and PBANKA_0918300) [616]. However, our attempts to functionally evaluate the three *Plasmodium* SAM transporter candidates using a mammalian heterologous expression system proved equivocal, leaving the possibility that one of these candidates may indeed be the *Plasmodium* SAM transporter.

The limited success of these experiments could be attributed to the use of mammalian cell lines as a tool to express recombinant proteins from the parasite. While mammalian cells are highly efficient at producing mammalian eukaryotic proteins, they present certain challenges when dealing with putative proteins from *Plasmodium*. The *Plasmodium* genome contains long stretches of AT repeats that can hinder the expression of foreign proteins in mammalian cells. In addition, the signals responsible for the proper folding and assembly of proteins in *Plasmodium* might differ from those in mammalian cells, potentially leading to misprocessing or improper transport of the proteins. These discrepancies in the cell machinery could prevent the *Plasmodium* SAM transporter from being properly expressed and characterized using the mammalian heterologous expression system.

To overcome these challenges and gain a deeper understanding of the *Plasmodium* SAM transporter, forthcoming research should explore alternative expression systems more suited to handling the unique properties of *Plasmodium* proteins. Investigating other expression systems that closely mimic the cellular environment of the parasite could present a viable alternative approach for studying the SAM transporter in *Plasmodium*. One such strategy involves employing heterologous expression in other protozoan or yeast species that share metabolic similarities with the malaria parasite. These systems may provide a more suitable environment for the correct folding, assembly, and transport of *Plasmodium* proteins, including the putative SAM transporter. Incorporating cutting-edge methodologies, such as CRISPR-Cas9 gene editing, super resolution imaging, proteomics, and single-cell analysis, could enable a more targeted and in-depth study of the putative SAM transporter. These advanced techniques have

the potential to provide valuable insights into the transporter's localization, activity, and interactions within the parasite.

It is also possible that the SAM transporter in *Plasmodium* functions via a different mechanism than other known transporters in different species. Additionally, the transporter may only be produced under specific conditions, at low levels, or during specific stages of liver development, which can hinder its identification using conventional expression systems. Alternatively, SAM uptake and metabolism in *Plasmodium* could involve intricate interactions with other host or parasite proteins. Another possibility is that the parasite evolved unique adaptations to scavenge or synthesize SAM within the hepatocyte without relying on a conventional plasma membrane transporter. The malaria parasite could have evolved to exploit the MAT1 enzyme from the hepatocyte to ensure its SAM requirements are met. Alternatively, the parasite could rely on a combination of active transport, passive diffusion, and alternate metabolic pathways to ensure adequate levels of SAM for its replication and growth, which adds further complexity to the identification of the SAM transporter.

Is SAM a Commitment Switcher? Unraveling the Metabolic Hub Governing Histone Methylation in *Plasmodium*

The lifecycle of human malaria parasites strictly depends on differentiation through multiple distinct stages within both the mammalian and the mosquito hosts, such that failure to advance to the next lifecycle stage is deleterious to the parasite. While persistence of the most widespread and lethal human malaria parasite, *P. falciparum*, relies on the continuous asexual replication inside erythrocytes, transmission requires differentiation into non-replicative sexual forms, known as gametocytes. In contrast to many apicomplexan parasites, including the closely-related *Haemoproteus* and *Leucocytozoidae spp.*, where asexual replication rounds are limited in number by the commencement of sexual differentiation [454,455,617], *P. falciparum* intra-erythrocytic stages can replicate indefinitely, with only a small fraction of the parasite population undergoing commitment to non-replicative male and female gametocytes [618]. Hence, balancing asexual replication and sexual commitment rates is of utmost importance to perpetuate the infection. Gametocyte production rates vary according to the environmental conditions, a developmental decision that is controlled via stochastic derepression of a heterochromatin-silenced locus encoding the transcription factor

PfAP2-G, which functions as a master switch for sexual commitment [235,559]. Recent work has provided evidence that derepression of the *Pfap2-g* locus is impacted by the availability of extracellular metabolites, namely phospholipid precursors. However, the molecular pathways linking such metabolites with epigenetic regulatory mechanisms, such as SAM-dependent methylations, remain hitherto unknown.

The relationship between phospholipids and methionine metabolism is evident in *Plasmodium* parasites. Phosphatidylcholine (PtdCho) is an important phospholipid required for membrane synthesis and stability, and its de novo synthesis depends on the enzyme phosphoethanolamine methyltransferase (PMT). Importantly, PMT is a SAM-dependent methyltransferase and its activity depends on the availability of SAM as a methyl donor. Competition for SAM methyl groups is a critical aspect of cellular metabolism and because SAM is finite and serves as a methyl donor for multiple pathways, its availability can affect the regulation of various cellular processes. In the context of *Plasmodium* parasites, such competition is observed between histone methylation and phospholipid synthesis. For instance, when the parasite encounters conditions of limited phospholipid precursors such as choline and ethanolamine, PtdCho synthesis is impaired and PMT competes with histone methyltransferases for SAM methyl groups. As a result, histone methylation may be compromised, leading to changes in gene expression and particularly derepression of genes involved in sexual commitment, such the transcription factor PfAP2-G, which is responsible for controlling the switch from asexual replication to sexual commitment. The balance between asexual replication and sexual commitment rates is crucial for the parasite's survival and transmission, and the availability of SAM is intricately linked to this process.

In Chapter IV of this thesis, we successfully uncovered SAM as the missing link that closely connects phospholipid metabolism and methionine metabolism in *Plasmodium*, thus acting as a major metabolic hub. Our results demonstrate a critical association between these key pathways, such that depletion of P-cho precursors leads to a reduction in SAM availability within the parasite. This holds significant implications for the regulation of gene expression and sexual commitment in the parasite. However, it is important to acknowledge the divergent outcomes observed in the two studies examining the effects of methionine depletion on *P. falciparum* asexual replication. While the findings presented in Chapter II suggest a notable impact of methionine depletion on *P. falciparum* parasitemia from 48 hours after withdrawal, the results detailed in Chapter IV indicate no discernible impact on asexual replication, but rather on sexual commitment.

These conflicting results may stem from multiple factors, including experimental differences, variations in media formulations, potential adaptive responses of the parasite, or genetic heterogeneity between the parasite strains employed. Despite the usage of *P. falciparum* NF54 strains in both investigations, these strains were obtained from distinct repositories, potentially harboring distinct genetic backgrounds. In addition, it is important to consider that *P. falciparum* is capable of adapting to changing environments. The presence or absence of methionine might trigger compensatory mechanisms or adjustments in the parasite's metabolic pathways, resulting in differential replication responses. Moreover, the continuous passage of parasites in culture can lead to mutations that confer advantages, such as the ability to thrive in nutrient-deficient conditions. Furthermore, the composition of the growth media varied between the studies. The studies in Chapter II used homemade AlbuMAX without methionine, while Chapter IV used AlbuMAX II Lipid-Rich BSA, which included methionine as part of its composition. Although AlbuMAX makes up a small proportion (10–15%) of the medium, its presence might help support parasite growth and replication. Methionine, as an essential amino acid, plays a pivotal role in diverse cellular processes, including protein synthesis and methylation reactions. Even a low concentration of methionine in the medium could supply the parasite with vital building blocks for these processes, potentially masking the consequences of methionine depletion. The absence of methionine in homemade AlbuMAX could have resulted in a more severe methionine-deficient milieu leading to the observed effects on asexual replication. This underscores the importance of media formulation and the plausible influence of methionine availability on the dynamics of *P. falciparum* replication.

Additionally, our genome-wide mapping of protein-DNA interactions revealed that SAM depletion results in reduced histone methylation marks that are responsible for the silencing of the *Pfap2-g* locus. Therefore, *Pfap2-g* transcription is up-regulated, resulting in an increased frequency of sexual commitment (Figure 5.3). Noteworthy, we have observed that, unlike higher eukaryotes, malaria parasites' methylation of H3K9 is more sensitive to changes in SAM and SAH than H3K4 methylation. This differential sensitivity of H3K9 and H3K4 methylation to changes in SAM and SAH underscores the unique regulatory mechanisms in *Plasmodium*'s epigenetic landscape. In contrast to higher eukaryotes, where H3K4 methylation is considered to be more dynamic and responsive to environmental changes, our observations suggest that in *Plasmodium* the histone mark H3K9me3, which is associated with gene silencing and heterochromatin formation, is more sensitive to fluctuations of SAM and SAH levels. This data strongly suggests that

the histone methyltransferase *Pf*SET3, which catalyzes the methylation of lysine 9 on histone H3 (H3K9), has evolved to be finely tuned to the intracellular concentrations of SAM and SAH. In addition, we found that the K_m and K_i values of *Pf*SET3 for SAM and SAH are within the range of the physiological levels of these metabolites in the parasite, allowing the enzyme to readily respond to fluctuations in their availability. As a result, the SAM/SAH ratio in the parasite can directly regulate the accumulation of the H3K9me3 silencing mark. By modulating H3K9me3 levels, the parasite can activate or repress specific genes as needed, allowing it to adapt to changing environmental conditions and ensure the success of its life cycle.

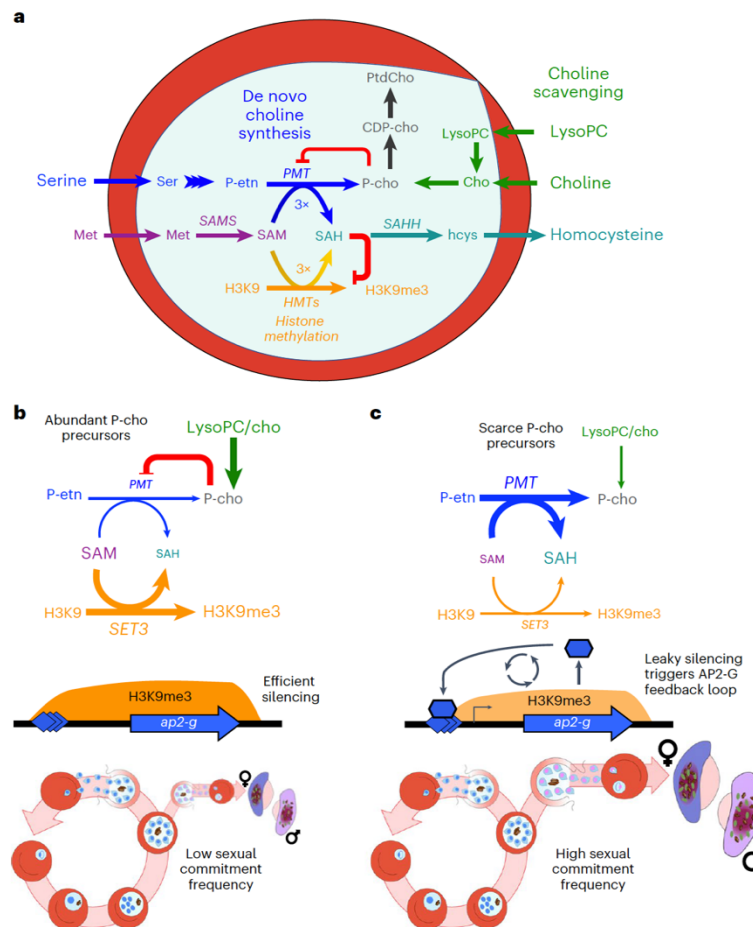


Figure 5.3 - Metabolic competition between PMT and H3K9 methylation controls the rate of sexual commitment. (a) *De novo* P-cho synthesis by PMT competes with the H3K9me3 histone methyltransferase for methyl groups provided by SAM. (b) Availability of abundant P-cho precursors reduce PMT consumption of SAM, allowing efficient maintenance of H3K9me3 heterochromatin and *ap2-g* silencing during schizogony, resulting in low sexual commitment. (c) When P-cho precursors are scarce, increased *de novo* P-cho synthesis by PMT reduces SAM and increases SAH, both of which impair deposition of H3K9me3 genome. Leaky silencing at the *ap2-g* locus increases the probability of activating the positive transcriptional feedback loop, thereby increasing the frequency of commitment to sexual differentiation.

Remarkably, the comparative analysis of rodent- and human-malaria parasites has provided valuable insight into previously reported observations. Our results demonstrate that sexual commitment in rodent malaria parasites remains unaffected by P-cho precursors. This is due to the loss of PMT in rodent malaria species, which results in the dissociation of PtdCho synthesis from SAM, SAH and histone methylation. This loss may have been compensated evolutionarily by the expansion of the lineage-specific *fam-a* gene family. Notably, the members of this gene family have been demonstrated to play a role in the transport of this phospholipid, thereby overcoming the challenges posed by the absence of PMT and ensuring the proper synthesis of PtdCho in rodent malaria species.

Understanding the precise molecular pathways and epigenetic regulatory mechanisms linking phospholipid metabolism, methionine metabolism, histone methylation, and gene expression can shed light on how the parasite responds to environmental cues and adjusts its development, virulence, and transmission accordingly. Additionally, exploring these molecular and epigenetic processes that link multiple metabolic pathways could provide valuable insight into the evolutionary history of the parasite and the factors that contributed to its ability to survive and thrive in its complex life cycle. Further investigation into the precise mechanisms by which phospholipid precursors regulate *PfAP2-G* expression and sexual commitment could unveil the intricate processes through which the parasite orchestrates its developmental decisions, but also provide insight into new potential approaches to curbing disease transmission.

Future research exploring the dynamics of SAM availability in the parasite at different stages of its life cycle and under different stressors could provide valuable insights into the central role of SAM in coordinating developmental decisions. Furthermore, examining the role of other SAM-dependent methyltransferases and the global impact of SAM availability on epigenetic regulation in *Plasmodium* could provide a deeper understanding of how SAM acts as a metabolic hub governing histone methylation and gene expression. It is important to note that SAM-dependent activities go beyond histone methylation and phospholipid production. Other biological processes such as DNA methylation, protein methylation, and metabolite production rely on SAM as a methyl donor. Furthermore, SAM availability is closely tied to the parasite's cellular metabolic status, with fluctuations in SAM levels impacting physiology and gene regulation. Thus, comprehending how the parasite employs SAM in various pathways and processes could yield valuable insights into how it regulates its genetic activities, overall functions,

and capacity to adapt to different environments. Understanding SAM methyl group competition is crucial to deciphering the complex regulatory networks that control gene expression and cellular processes in *Plasmodium* parasites. Finding the critical elements that control SAM availability and the activity of certain methyltransferases could shed light on how different metabolic pathways and epigenetic events are coordinated. Additionally, studying how host traits, environmental factors and nutrient availability affect SAM levels could reveal how the parasite adapts to different *conditions* during its life cycle. Thus, the identification of SAM as a metabolic hub in *Plasmodium* opens the possibility for the development of new therapeutic targets. Finally, the discovery of small molecules or other chemicals that modulate the activity or expression of PMT or histone methyltransferases could open the door to new avenues in antimalarial drug development.

Deciphering Adaptation: Integrating Amino Acid Metabolism in *Plasmodium* parasites

Taken together, these three independent but intertwined stories reveal the distinct roles of methionine metabolism throughout the *Plasmodium* life cycle, highlighting the extraordinary adaptability of *Plasmodium* parasites and revealing a dynamic interplay between parasite's metabolic pathways and developmental decisions. By examining both the host and parasite methionine pathway, we embark on a journey that explores the parasite's ability to sense and respond to variations in host nutrient levels, the essential role of liver-specific methionine metabolism in supporting rapid replication within hepatocytes and the central role of S-adenosylmethionine (SAM) as a key factor in sexual commitment. Each chapter uncovers an intriguing facet of *Plasmodium*'s adaptive strategies, underscoring the complex web of interactions that define the parasite-host relationship and shedding light on the strategies the malaria parasite employs to navigate its complex life cycle within the host. Despite lacking conventional signaling pathways such as TOR and GCN2 and being auxotrophic for most amino acids, *Plasmodium* has evolved an effective amino acid-sensing pathway that allows precise adjustments in replication and development based on nutritional cues. *Plasmodium*'s dual approach of active adaptation and passive dormancy simultaneously ensures survival and reproductive success and allows the parasite to cope with the complexities of differential amino acid availability. The discovery of a unique amino acid sensing pathway controlled by a trio of parasite kinases reveals the sophistication of

Plasmodium's strategies to thrive amidst changing nutritional environments. Furthermore, the close interaction between the parasite and the host's methionine/SAM metabolism sheds light on the evolutionary adaptations that facilitate the specialization of *Plasmodium* to liver tropism. Finally, the connection between phospholipid and methionine metabolism emerges as a critical determinant in epigenetic regulation, governing both gene expression and sexual commitment. The identification of SAM as a metabolic hub provides a new perspective on how *Plasmodium* orchestrates its developmental decisions in response to environmental cues. Overall, this thesis presents a comprehensive portrait of *Plasmodium's* adaptive and survival tactics, offering valuable insights into the host-parasite relationship and the dynamic interplay between metabolism and development.

By elucidating the underlying mechanisms that enable *Plasmodium* to adapt to diverse nutritional conditions and regulate its developmental decisions, this dissertation not only advances our comprehension of malaria pathogenesis but also lays the foundation for innovative approaches to combat this persistent global health threat. Delving further into the intricacies of *Plasmodium's* metabolic and developmental adaptations could unveil new avenues for therapeutic interventions and disease control, and offer hope in the ongoing fight against malaria.



"Climb the mountain not to plant your flag, but to embrace the challenge, enjoy the air and behold the view.

Climb it so you can see the world, not so the world can see you"

David McCullough Jr.

CHAPTER VI

CHAPTER VI

REFERENCES

- [1]. Laveran A. Un nouveau parasite trouvé dans le sang des malades atteints de fièvre palustre: origine parasitaire des accidents de l'impaludisme. Bull Mem Soc Med Hop. 1881;(17):158–64.
- [2]. Ross R. On some Peculiar Pigmented Cells Found in Two Mosquitos Fed on Malarial Blood. BMJ. 1897 Dec 18;2(1929):1786–8.
- [3]. Ross R. The role of the mosquito in the evolution of the malarial parasite: the recent researches of Surgeon-Major Ronald Ross, I.M.S. 1898. Yale J Biol Med. 2002;75(2):103–5.
- [4]. WHO. World Malaria Report 2021. 2021.
- [5]. Calderaro A, Piccolo G, Gorrini C, Rossi S, Montecchini S, Dell'Anna ML, et al. Accurate identification of the six human *Plasmodium* spp. causing imported malaria, including *Plasmodium ovale wallikeri* and *Plasmodium knowlesi*. Malar J. 2013 Dec 13;12(1):321.
- [6]. Loy DE, Liu W, Li Y, Learn GH, Plenderleith LJ, Sundararaman SA, et al. Out of Africa: origins and evolution of the human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. Vol. 47, International Journal for Parasitology. 2017. p. 87–97.
- [7]. Mohapatra MK. The natural history of complicated *falciparum* malaria - A prospective study. J Assoc Physicians India. 2006;54(NOV.):848–53.
- [8]. Kevin Baird J. Malaria caused by *Plasmodium vivax*: recurrent, difficult to treat, disabling, and threatening to life--the infectious bite preempts these hazards. Pathog Glob Health. 2013 Dec;107(8):475–9.
- [9]. Clark IA, Cowden BW. Why is the pathology of *falciparum* worse than that of *vivax* malaria? Vol. 15, Parasitology Today. 1999. p. 458–61.
- [10]. Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of *Plasmodium vivax* malaria. Am J Trop Med Hyg. 2001;64(1–2):97–106.
- [11]. WHO. From malaria control to elimination in the WHO European region 2006–2015. World Health Organization. 2006.
- [12]. Bousema T, Drakeley C. Epidemiology and Infectivity of *Plasmodium falciparum* and *Plasmodium vivax* Gametocytes in Relation to Malaria Control and Elimination. Clin Microbiol Rev. 2011 Apr;24(2):377–410.
- [13]. Prudêncio M, Rodriguez A, Mota MM. The silent path to thousands of merozoites: the *Plasmodium* liver stage. Nat Rev Microbiol. 2006 Nov 1;4(11):849–56.
- [14]. Bartoloni A, Zammarchi L. Clinical aspects of uncomplicated and severe malaria. Vol. 4, Mediterranean Journal of Hematology and Infectious Diseases. 2012. p. e2012026.
- [15]. Kleinschmidt I, Schwabe C, Benavente L, Torrez M, Ridl FC, Segura JL, et al. Marked increase in child survival after four years of intensive malaria control. Am J Trop Med Hyg. 2009 Jun;80(6):882–8.
- [16]. López Del Prado GR, Hernán García C, Moreno Cea L, Fernández Espinilla V, Muñoz Moreno MF, Delgado Márquez A, et al. Malaria in developing countries. J Infect Dev Ctries. 2014 Jan 15;8(1):1–4.

- [17]. Neghina R, Iacobiciu I, Neghina AM, Marincu I. Malaria, a Journey in Time: In Search of the Lost Myths and Forgotten Stories. *Am J Med Sci*. 2010 Dec;340(6):492–8.
- [18]. Buonsenso D, Cataldi L. Watch out for malaria: still a leading cause of child death worldwide. *Ital J Pediatr*. 2010;36(1):58.
- [19]. Hume J. Malaria in antiquity: a genetics perspective. *World Archaeol*. 2003 Sep;35(2):180–92.
- [20]. Bratitsis T, Demetriadis S. Research Approaches in Computer-Supported Collaborative Learning. *Int J e-Collaboration*. 2013 Jan 1;9(1):1–8.
- [21]. Bynum B. A history of malaria. *Lancet*. 2008 Apr;371(9622):1407–8.
- [22]. Humphreys M. Malaria and Rome: A History of Malaria in Ancient Italy. By Robert Sallares. New York, N.Y.: Oxford University Press, 2002. xv + 341 pp. Illustrations, maps, notes, bibliography, index. Cloth \$75.00. *Environ Hist Durh N C*. 2003 Oct 1;8(4):701–2.
- [23]. Cunha CB, Cunha BA. Brief history of the clinical diagnosis of malaria: from Hippocrates to Osler. *J Vector Borne Dis*. 2008 Sep;45(3):194–9.
- [24]. Celli LA. A History of Malaria in the Roman Campagna From Ancient Times. *Ind Med Gaz*. 1933;69(3):173–4.
- [25]. Stephens JWW. Blackwater Fever: A Historical Survey and Summary of Observations Made Over a Century. *J Am Med Assoc [Internet]*. 1937 Nov 20;109(21):1751.
- [26]. Klebs, Edwin, Tommasi-Crudeli C. Studi Sulla Natura della Malaria. Salviucci, editor. Rome: Selected Monographs of the New Sydenham Society; 1879. 1–56 p.
- [27]. Bruce-Chwatt LJ. Alphonse Laveran's discovery 100 years ago and today's global fight against malaria. *J R Soc Med*. 1981 Jul;74(7):531–6.
- [28]. Tangpukdee N, Duangdee C, Wilairatana P, Krudsood S. Malaria Diagnosis: A Brief Review. *Korean J Parasitol*. 2009;47(2):93.
- [29]. Hempelmann E, Krafts K. Bad air, amulets and mosquitoes: 2,000 years of changing perspectives on malaria. *Malar J*. 2013 Dec 9;12(1):232.
- [30]. Capanna E. Grassi versus Ross: who solved the riddle of malaria? *Int Microbiol*. 2006 Mar;9(1):69–74.
- [31]. Krafts K, Hempelmann E, Skórska-Stania A. From methylene blue to chloroquine: a brief review of the development of an antimalarial therapy. *Parasitol Res*. 2012 Jul;111(1):1–6.
- [32]. Marchiafava, Ettore, Bignami A. On summer-autumn malarial fevers. London: New Sydenham Society; 1894.
- [33]. Sinden RE. The biology of malaria transmission. In: *Advances in Malaria Research*. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2016. p. 87–124.
- [34]. King A. Insects and disease, mosquitoes and malaria. *Pop Sci*. 1883;(23):644–58.
- [35]. Patrick M. Ross's recent investigations on the mosquito-malaria theory. *Br Med J*. 1898;
- [36]. Fantini B. The concept of specificity and the Italian contribution to the discovery of the malaria transmission cycle. *Parassitologia*. 1999 Sep;41(1–3):39–47.
- [37]. Hollingdale MR. Malaria and the liver. *Hepatology*. 1985;5(2):327–35.

- [38]. James SP, Tate P. Exo-erythrocytic schizogony in *Plasmodium gallinaceum* Brumpt, 1935. *Parasitology*. 1938 Jan 6;30(1):128–39.
- [39]. Shortt HE, Garnham PCC. The pre-erythrocytic development of *Plasmodium cynomolgi* and *Plasmodium vivax*. *Trans R Soc Trop Med Hyg*. 1948 May;41(6):785–95.
- [40]. Shortt HE, Garnham PCC, Covell G, Shute PG. The Pre-Erythrocytic Stage Of Human Malaria, *Plasmodium Vivax*. *Br Med J*. 1948;1(4550):547.
- [41]. Shortt HE, Fairley NH. The pre-erythrocytic stage of *Plasmodium falciparum*; a preliminary note. *Br Med J*. 1949 Nov 5;2(4635):1006–8, illust.
- [42]. Garnham PCC, Bray RS, Cooper W, Lainson R, Awad FI, Williamson J. Pre-erythrocytic stages of human malaria: *Plasmodium ovale*. *Br Med J*. 1954;1(4856):257.
- [43]. Krotoski WA, Krotoski DM, Garnham PCC, Bray RS, Killick-Kendrick R, Draper CC, et al. Relapses in primate malaria: Discovery of two populations of exoerythrocytic stages. Preliminary note. *Br Med J*. 1980;280(6208):153–4.
- [44]. Krotoski WA, Collins WE, Bray RS, Garnham PC, Cogswell FB, Gwadz RW, et al. Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium vivax* infection. *Am J Trop Med Hyg*. 1982 Nov;31(6):1291–3.
- [45]. Crutcher, James M; Hoffman SL. *Medical Microbiology*. 4th ed. Baron S, editor. 1996. Chapter 83.
- [46]. Huff CG. The Primate Malarias. *Am J Trop Med Hyg*. 1972 Sep 1;21(5):602–3.
- [47]. Sadanand S. Malaria: an evaluation of the current state of research on pathogenesis and antimalarial drugs. *Yale J Biol Med*. 2010 Dec;83(4):185–91.
- [48]. Campo B, Vandal O, Wesche DL, Burrows JN. Killing the hypnozoite – drug discovery approaches to prevent relapse in *Plasmodium vivax*. *Pathog Glob Health*. 2015 May 18;109(3):107–22.
- [49]. Basu S, Sahi PK. Malaria: An Update. Vol. 84, *Indian Journal of Pediatrics*. The Indian Journal of Pediatrics; 2017. p. 521–8.
- [50]. White NJ. Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. Vol. 41, *Antimicrobial Agents and Chemotherapy*. 1997. p. 1413–22.
- [51]. Brejt JA, Golightly LM. Severe malaria: update on pathophysiology and treatment. *Curr Opin Infect Dis*. 2019 Oct;32(5):413–8.
- [52]. Plewes K, Turner GDH, Dondorp AM. Pathophysiology, clinical presentation, and treatment of coma and acute kidney injury complicating *falciparum* malaria. *Curr Opin Infect Dis*. 2018;31(1):69–77.
- [53]. Wassmer SC, Taylor TE, Rathod PK, Mishra SK, Mohanty S, Arevalo-Herrera M, et al. Investigating the pathogenesis of severe malaria: A multidisciplinary and cross-geographical approach. *Am J Trop Med Hyg*. 2015 Sep 2;93(3_Suppl):42–56.
- [54]. Mawson AR. The pathogenesis of malaria: a new perspective. *Pathog Glob Health*. 2013 Apr 12;107(3):122–9.

- [55]. Cowman AF, Healer J, Marapana D, Marsh K. Malaria: Biology and Disease. *Cell*. 2016 Oct 20;167(3):610–24.
- [56]. Akenji TN, Deas J. Definition of populations at risk for *Plasmodium falciparum* infection in three endemic areas of Cameroon. *J Parasitol*. 1994 Dec;80(6):895–9.
- [57]. Van Vugt M, Van Beest A, Sicuri E, Van Tulder M, Grobusch MP. Malaria treatment and prophylaxis in endemic and nonendemic countries: Evidence on strategies and their cost-effectiveness. *Future Microbiol*. 2011;6(12):1485–500.
- [58]. Aron JL. Mathematical modelling of immunity to malaria. *Math Biosci*. 1988;90(1–2):385–96.
- [59]. Chiyaka C, Garira W, Dube S. Transmission model of endemic human malaria in a partially immune population. *Math Comput Model*. 2007;46(5–6):806–22.
- [60]. Aron JL. Acquired immunity dependent upon exposure in an SIRS epidemic model. *Math Biosci*. 1988 Mar;88(1):37–47.
- [61]. Giha HA, Rosthoj S, Dodoo D, Hviid L, Satti GMH, Scheike T, et al. The epidemiology of febrile malaria episodes in an area of unstable and seasonal transmission. *Trans R Soc Trop Med Hyg*. 2000;94(6):645–51.
- [62]. van Schalkwyk DA. History of Antimalarial Agents. In: eLS. Wiley; 2015. p. 1–5.
- [63]. Renslo AR. Antimalarial Drug Discovery: From Quinine to the Dream of Eradication. *ACS Med Chem Lett*. 2013 Dec 12;4(12):1126–8.
- [64]. de Villiers KA, Gildenhuis J, le Roex T. Iron(III) protoporphyrin IX complexes of the antimalarial Cinchona alkaloids quinine and quinidine. *ACS Chem Biol*. 2012 Apr 20;7(4):666–71.
- [65]. Egan TJ, Combrinck JM, Egan J, Hearne GR, Marques HM, Ntenti S, et al. Fate of haem iron in the malaria parasite *Plasmodium falciparum*. *Biochem J*. 2002 Jul 15;365(Pt 2):343–7.
- [66]. Jones RA, Panda SS, Hall CD. Quinine conjugates and quinine analogues as potential antimalarial agents. *Eur J Med Chem*. 2015;97(1):335–55.
- [67]. Sullivan DJ, Gluzman IY, Russell DG, Goldberg DE. On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci U S A*. 1996 Oct 15;93(21):11865–70.
- [68]. Schlitzer M. Malaria chemotherapeutics part I: History of antimalarial drug development, currently used therapeutics, and drugs in clinical development. *ChemMedChem*. 2007 Jul 9;2(7):944–86.
- [69]. C. S. Pinheiro L, M. Feitosa L, O. Gandi M, F. Silveira F, Boechat N. The Development of Novel Compounds Against Malaria: Quinolines, Triazolpyridines, Pyrazolopyridines and Pyrazolopyrimidines. *Molecules*. 2019 Nov 13;24(22):4095.
- [70]. Coatney GR. Pitfalls in a discovery: the chronicle of chloroquine. *Am J Trop Med Hyg*. 1963 Mar;12(November):121–8.

- [71]. Chou AC, Fitch CD. Control of heme polymerase by chloroquine and other quinoline derivatives. *Biochem Biophys Res Commun*. 1993;195(1):422–7.
- [72]. Singh Sidhu AB, Verdier-Pinard D, Fidock DA. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. *Science* (80-). 2002 Oct 4;298(5591):210–3.
- [73]. Jiang X. An overview of the *Plasmodium falciparum* hexose transporter and its therapeutic interventions. *Proteins*. 2022 Oct;90(10):1766–78.
- [74]. Nájera JA, González-Silva M, Alonso PL. Some lessons for the future from the global malaria eradication programme (1955-1969). Vol. 8, *PLoS Medicine*. 2011. p. e1000412.
- [75]. Greenwood BM, Fidock DA, Kyle DE, Kappe SHI, Alonso PL, Collins FH, et al. Malaria: progress, perils, and prospects for eradication. *J Clin Invest*. 2008 Apr 1;118(4):1266–76.
- [76]. Foley M, Tilley L. Quinoline antimalarials: Mechanisms of action and resistance and prospects for new agents. *Pharmacol Ther*. 1998;79(1):55–87.
- [77]. Ursos LMB, Roepe PD. Chloroquine resistance in the malarial parasite, *Plasmodium falciparum*. *Med Res Rev*. 2002 Sep;22(5):465–91.
- [78]. Payne D. Spread of chloroquine resistance in *Plasmodium falciparum*. *Parasitol today*. 1987 Aug;3(8):241–6.
- [79]. Ridley RG. Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature*. 2002 Feb 7;415(6872):686–93.
- [80]. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell*. 2000 Oct;6(4):861–71.
- [81]. Ashley EA, Recht J, White NJ. Primaquine: The risks and the benefits. *Malar J*. 2014;13(1):1–7.
- [82]. Graves PM, Gelband H, Garner P. Primaquine or other 8-aminoquinoline for reducing *P. falciparum* transmission. Vol. 2014, *Cochrane Database of Systematic Reviews*. 2014.
- [83]. White NJ. Primaquine to prevent transmission of *falciparum* malaria. *Lancet Infect Dis*. 2013 Feb;13(2):175–81.
- [84]. Alving AS, Johnson CF, Tarlov AR, Brewer GJ, Kellermeyer RW, Carson PE. Mitigation of the haemolytic effect of primaquine and enhancement of its action against exoerythrocytic forms of the Chesson strain of *Plasmodium vivax* by intermittent regimens of drug administration: a preliminary report. *Bull World Health Organ*. 1960;22(6):621–31.
- [85]. Baird JK. Reinventing primaquine for endemic malaria. Vol. 17, *Expert Opinion on Emerging Drugs*. 2012. p. 439–44.
- [86]. Greenwood B. Anti-malarial drugs and the prevention of malaria in the population of malaria endemic areas. *Malar J*. 2010 Dec 13;9(S3):S2.
- [87]. Camarda G, Jirawatcharadech P, Priestley RS, Saif A, March S, Wong MHL, et al. Antimalarial activity of primaquine operates via a two-step biochemical relay. *Nat Commun*. 2019 Jul 19;10(1):3226.

- [88]. WHO. World Malaria Report 2017. 2017.
- [89]. Galappaththy GN, Tharyan P, Kirubakaran R. Primaquine for preventing relapse in people with *Plasmodium vivax* malaria treated with chloroquine. Vol. 2013, Cochrane Database of Systematic Reviews. 2013.
- [90]. Mihaly GW, Ward SA, Edwards G, Orme ML, Breckenridge AM. Pharmacokinetics of primaquine in man: identification of the carboxylic acid derivative as a major plasma metabolite. *Br J Clin Pharmacol*. 1984 Apr;17(4):441–6.
- [91]. Watson J, Taylor WRJ, Menard D, Kheng S, White NJ. Modelling primaquine-induced haemolysis in G6PD deficiency. *Elife*. 2017 Feb 4;6:1–19.
- [92]. Howes RE, Piel FB, Patil AP, Nyangiri OA, Gething PW, Dewi M, et al. G6PD deficiency prevalence and estimates of affected populations in malaria endemic countries: a geostatistical model-based map. *PLoS Med*. 2012;9(11):e1001339.
- [93]. Jason W. Bennett, M.D. Brandon S. Pybus, Ph.D. Anjali Yadava, Ph.D. Donna Tosh, R.N. Jason C. Sousa PD. Primaquine Failure and Cytochrome P-450 2D6 in *Plasmodium vivax* Malaria. *N Engl J Med*. 2013;369(14):1380–1.
- [94]. O'Neill PM, Barton VE, Ward SA. The molecular mechanism of action of artemisinin--the debate continues. *Molecules*. 2010 Mar 12;15(3):1705–21.
- [95]. White NJ, Hien TT, Nosten FH. A Brief History of Qinghaosu. *Trends Parasitol*. 2015 Dec;31(12):607–10.
- [96]. Su XZ, Miller LH. The discovery of artemisinin and the Nobel Prize in Physiology or Medicine. *Sci China Life Sci*. 2015 Nov 19;58(11):1175–9.
- [97]. Kumar N, Zheng H. Stage-specific gametocytocidal effect in vitro of the antimalaria drug qinghaosu on *Plasmodium falciparum*. *Parasitol Res*. 1990;76(3):214–8.
- [98]. Lin AJ, Klayman DL, Milhous WK. Antimalarial activity of new water-soluble dihydroartemisinin derivatives. *J Med Chem*. 1987 Nov;30(11):2147–50.
- [99]. Brossi A, Venugopalan B, Dominguez Gerpe L, Yeh HJC, Flippen-Anderson JL, Buchs P, et al. Arteether, a new antimalarial drug: synthesis and antimalarial properties. *J Med Chem*. 1988 Mar;31(3):645–50.
- [100]. Davis TME, Karunajeewa HA, Ilett KF. Artemisinin-based combination therapies for uncomplicated malaria. *Med J Aust*. 2005 Feb 21;182(4):181–5.
- [101]. Terkuile F, White NJ, Holloway P, Pasvol G, Krishna S. *Plasmodium falciparum*: In Vitro Studies of the Pharmacodynamic Properties of Drugs Used for the Treatment of Severe Malaria. *Exp Parasitol*. 1993 Feb;76(1):85–95.
- [102]. Gordi T, Dinh XH, Trinh NH, Nguyen TN, Ashton M. Artemisinin pharmacokinetics and efficacy in uncomplicated-malaria patients treated with two different dosage regimens. *Antimicrob Agents Chemother*. 2002 Apr;46(4):1026–31.
- [103]. de Vries PJ, Dien TK. Clinical pharmacology and therapeutic potential of artemisinin and its derivatives in the treatment of malaria. *Drugs*. 1996 Dec;52(6):818–36.
- [104]. WHO. WHO Guidelines for Malaria. 2021;

- [105]. Pousibet-Puerto J, Salas-Coronas J, Sánchez-Crespo A, Molina-Arrebola MA, Soriano-Pérez MJ, Giménez-López MJ, et al. Impact of using artemisinin-based combination therapy (ACT) in the treatment of uncomplicated malaria from *Plasmodium falciparum* in a non-endemic zone. *Malar J*. 2016 Jul 2;15(1):339.
- [106]. Tse EG, Korsik M, Todd MH. The past, present and future of anti-malarial medicines. *Malar J*. 2019 Dec 22;18(1):93.
- [107]. Sugiarto SR, Davis TME, Salman S. Pharmacokinetic considerations for use of artemisinin-based combination therapies against *falciparum* malaria in different ethnic populations. Vol. 13, *Expert Opinion on Drug Metabolism and Toxicology*. Taylor & Francis; 2017. p. 1115–33.
- [108]. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, et al. Artemisinin Resistance in *Plasmodium falciparum* Malaria. *N Engl J Med*. 2009 Jul 30;361(5):455–67.
- [109]. Straimer J, Gnädig NF, Witkowski B, Amaratunga C, Duru V, Ramadani AP, et al. K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. *Science*. 2015 Jan 23;347(6220):428–31.
- [110]. Mita T, Tanabe K. Evolution of *Plasmodium falciparum* drug resistance: Implications for the development and containment of artemisinin resistance. Vol. 65, *Japanese Journal of Infectious Diseases*. 2012. p. 465–75.
- [111]. Beshir K, Sutherland CJ, Merinopoulos I, Durrani N, Leslie T, Rowland M, et al. Amodiaquine resistance in *Plasmodium falciparum* malaria in Afghanistan is associated with the *pfprt* SVMNT allele at codons 72 to 76. *Antimicrob Agents Chemother*. 2010 Sep;54(9):3714–6.
- [112]. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet*. 2004;364(9432):438–47.
- [113]. Witkowski B, Duru V, Khim N, Ross LS, Saintpierre B, Beghain J, et al. A surrogate marker of piperazine-resistant *Plasmodium falciparum* malaria: a phenotype–genotype association study. *Lancet Infect Dis*. 2017 Feb;17(2):174–83.
- [114]. Arie F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, et al. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. 2016;1(1):23–30.
- [115]. Leang R, Taylor WRJ, Bouth DM, Song L, Tarning J, Char MC, et al. Evidence of *Plasmodium falciparum* Malaria Multidrug Resistance to Artemisinin and Piperazine in Western Cambodia: Dihydroartemisinin-Piperazine Open-Label Multicenter Clinical Assessment. *Antimicrob Agents Chemother*. 2015 Aug;59(8):4719–26.
- [116]. Imwong M, Suwannasin K, Kunasol C, Sutawong K, Mayxay M, Rekol H, et al. The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong subregion: a molecular epidemiology observational study. *Lancet Infect Dis*. 2017 May;17(5):491–7.
- [117]. WHO. World Malaria Report 2022. 2022.

- [118]. Uwimana A, Umulisa N, Venkatesan M, Savigel SS, Zhou Z, Munyaneza T, et al. Association of *Plasmodium falciparum* kelch13 R561H genotypes with delayed parasite clearance in Rwanda: an open-label, single-arm, multicentre, therapeutic efficacy study. *Lancet Infect Dis*. 2021 Aug;21(8):1120–8.
- [119]. Uwimana A, Legrand E, Stokes BH, Ndikumana JLM, Warsame M, Umulisa N, et al. Emergence and clonal expansion of in vitro artemisinin-resistant *Plasmodium falciparum* kelch13 R561H mutant parasites in Rwanda. *Nat Med*. 2020 Oct;26(10):1602–8.
- [120]. van der Pluijm RW, Tripura R, Hoglund RM, Pyae Phy A, Lek D, ul Islam A, et al. Triple artemisinin-based combination therapies versus artemisinin-based combination therapies for uncomplicated *Plasmodium falciparum* malaria: a multicentre, open-label, randomised clinical trial. *Lancet*. 2020 Apr 25;395(10233):1345–60.
- [121]. WHO. Consolidated Guidelines on Tuberculosis Treatment. World Health Organization. 2019. 99 p.
- [122]. WHO. The use of antiretroviral drugs for treating and preventing HIV infection. World Health Organization. 2016.
- [123]. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, et al. Spread of Artemisinin Resistance in *Plasmodium falciparum* Malaria. *N Engl J Med*. 2014 Jul 31;371(5):411–23.
- [124]. Asenso-Okyere K, Asante FA, Tarekegn J, Andam KS. A review of the economic impact of malaria in agricultural development. *Agric Econ*. 2011 May;42(3):293–304.
- [125]. Hay SI, Guerra CA, Tatem AJ, Noor AM, Snow RW. The global distribution and population at risk of malaria : past , present , and future. Vol. 4. 2004.
- [126]. Autino B, Noris A, Russo R, Castelli F. Epidemiology of malaria in endemic areas. Vol. 4, *Mediterranean Journal of Hematology and Infectious Diseases*. 2012. p. e2012060.
- [127]. WHO. World Malaria Report 2010. 2010.
- [128]. WHO. World Malaria Report 2019. 2019.
- [129]. WHO. World Malaria Report 2020. 2020.
- [130]. Sherrard-Smith E, Hogan AB, Hamlet A, Watson OJ, Whittaker C, Winskill P, et al. The potential public health consequences of COVID-19 on malaria in Africa. *Nat Med*. 2020 Sep;26(9):1411–6.
- [131]. WHO Global Malaria Programme. The potential impact of health service disruptions on the burden of malaria: a modelling analysis for countries in sub-Saharan Africa. 2020.
- [132]. Chuma JM, Thiede M, Molyneux CS. Rethinking the economic costs of malaria at the household level: Evidence from applying a new analytical framework in rural Kenya. *Malar J*. 2006;5:1–14.
- [133]. Attanayake N, Fox-Rushby J, Mills A. Household costs of “malaria” morbidity: a study in Matale district, Sri Lanka. *Trop Med Int Health*. 2000 Sep;5(9):595–606.
- [134]. Ettling M, McFarland DA, Schultz LJ, Chitsulo L. Economic impact of malaria in Malawian households. *Trop Med Parasitol*. 1994 Mar;45(1):74–9.

- [135]. Sachs J, Malaney P. The economic and social burden of malaria. *Nature*. 2002 Feb 7;415(6872):680–5.
- [136]. Mia MS, Begum RA, Er AC, Datuk R, Raja Z, Abidin Z, et al. Malaria and Climate Change: Discussion on Economic Impacts. *Am J Environ Sci*. 2011 Jan 1;7(1):65–74.
- [137]. Russell S. The economic burden of illness for households in developing countries: a review of studies focusing on malaria, tuberculosis, and human immunodeficiency virus/acquired immunodeficiency syndrome. *Am J Trop Med Hyg*. 2004 Aug 1;71(2 Suppl):147–55.
- [138]. Brooker S, Guyatt H, Omumbo J, Shretta R, Drake L, Ouma J. Situation Analysis of Malaria in School-aged Children in Kenya – What Can Be Done? *Parasitol Today*. 2000 May;16(5):183–6.
- [139]. Halliday KE, Witek-McManus SS, Opondo C, Mtali A, Allen E, Bauleni A, et al. Impact of school-based malaria case management on school attendance, health and education outcomes: a cluster randomised trial in southern Malawi. *BMJ Glob Heal*. 2020 Jan;5(1):e001666.
- [140]. Greenwood BM, Bojang K, Whitty CJ, Targett GA. Malaria. *Lancet*. 2005 Apr;365(9469):1487–98.
- [141]. WHO. Twenty-Second World Health Assembly, Part I: Resolutions and Decisions. Official Records of the World Health Organization. 1969.
- [142]. WHO. Re-examination of the global strategy of malaria eradication. In 1969.
- [143]. WHO. Eliminating malaria [Internet]. 2016. Available from: http://www.who.int/about/licensing/copyright_form/en/index.html
- [144]. Mendis K, Rietveld A, Warsame M, Bosman A, Greenwood B, Wernsdorfer WH. From malaria control to eradication: The WHO perspective. *Trop Med Int Heal*. 2009 Jul;14(7):802–9.
- [145]. Narasimhan V, Attaran A. Roll Back Malaria? The scarcity of international aid for malaria control. *Malar J*. 2003;2(8):1–8.
- [146]. Breman JG, Kilama WL, Greenwood B, Druilhe P, Nabarro D, Mendis K. Rolling back malaria: Action or rhetoric? *Bull World Health Organ*. 2000;78(12):1450–5.
- [147]. WHO Technical Report Series. Implementation of the Global Malaria Control Strategy. 1993.
- [148]. Malaria No More. How Can We End Malaria? Lessons from Around the World. 2021.
- [149]. WHO. World Malaria Report 2015. 2015.
- [150]. WHO. World Malaria Report 2016. Geneva; 2016.
- [151]. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature*. 2015 Oct 8;526(7572):207–11.
- [152]. WHO. Global technical strategy for malaria 2016-2030 [Internet]. World Health Organization. Geneva; 2015. Available from: http://apps.who.int/iris/bitstream/10665/176712/1/9789241564991_eng.pdf?ua=1

- [153]. WHO. WHO Guidelines for malaria. 2021.
- [154]. Toé KH, Jones CM, N’Fale S, Ismail HM, Dabiré RK, Ranson H. Increased pyrethroid resistance in malaria vectors and decreased bed net effectiveness, Burkina Faso. *Emerg Infect Dis.* 2014 Oct;20(10):1691–6.
- [155]. Lengeler C. Insecticide-treated bednets and curtains for preventing malaria. *Cochrane database Syst Rev.* 2000;(2):CD000363.
- [156]. WHO. Guidelines for Malaria Vector Control. Guidelines for Malaria Vector Control. World Health Organization; 2019. 161 p.
- [157]. Vatandoost H, Hanafi-Bojd AA. Indication of pyrethroid resistance in the main malaria vector, *Anopheles stephensi* from Iran. *Asian Pac J Trop Med.* 2012 Sep;5(9):722–6.
- [158]. Pryce J, Medley N, Choi L. Indoor residual spraying for preventing malaria in communities using insecticide-treated nets. *Cochrane database Syst Rev.* 2022 Jan 17;1(1):CD012688.
- [159]. Gleave K, Lissenden N, Richardson M, Choi L, Ranson H. Piperonyl butoxide (PBO) combined with pyrethroids in insecticide- treated nets to prevent malaria in Africa (Review). *Cochrane Database Syst Rev.* 2018;
- [160]. Ngufor C, Fongnikin A, Rowland M, N’Guessan R. Indoor residual spraying with a mixture of clothianidin (a neonicotinoid insecticide) and deltamethrin provides improved control and long residual activity against pyrethroid resistant *Anopheles gambiae* sl in Southern Benin. *PLoS One.* 2017;12(12):e0189575.
- [161]. WHO. Recommended insecticides for indoor residual spraying against malaria vectors. Geneva; 2018.
- [162]. York A. Seasonal malaria chemoprevention in the Sahel. *Lancet Infect Dis.* 2017 Jun;17(6):588.
- [163]. Coldiron ME, Von Seidlein L, Grais RF. Seasonal malaria chemoprevention: Successes and missed opportunities. *Malar J.* 2017;16(1):18–21.
- [164]. Fried M, Duffy PE. Malaria during Pregnancy. *Cold Spring Harb Perspect Med.* 2017 Jun 1;7(6):a025551.
- [165]. Briand V, Cottrell G, Massougbodji A, Cot M. Intermittent preventive treatment for the prevention of malaria during pregnancy in high transmission areas. *Malar J.* 2007 Dec 4;6:160.
- [166]. Schlagenhauf P, Petersen E. Malaria chemoprophylaxis: strategies for risk groups. *Clin Microbiol Rev.* 2008 Jul;21(3):466–72.
- [167]. van Vugt M, van Beest A, Sicuri E, van Tulder M, Grobusch MP. Malaria treatment and prophylaxis in endemic and nonendemic countries: evidence on strategies and their cost-effectiveness. *Future Microbiol.* 2011 Dec;6(12):1485–500.
- [168]. Hill DR, Baird JK, Parise ME, Lewis LS, Ryan ET, Magill AJ. Primaquine: Report from CDC expert meeting on malaria chemoprophylaxis I. Vol. 75, *American Journal of Tropical Medicine and Hygiene.* 2006. p. 402–15.

- [169]. Baird JK. 8-Aminoquinoline Therapy for Latent Malaria. *Clin Microbiol Rev.* 2019 Sep 18;32(4):1–68.
- [170]. Peters W, Robinson BL, Milhous WK. The chemotherapy of rodent malaria. LI. Studies on a new 8-aminoquinoline, WR 238,605. *Ann Trop Med Parasitol.* 1993 Jan 15;87(6):547–52.
- [171]. RTS SCTP. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet.* 2015 Jul 4;386(9988):31–45.
- [172]. Cohen J, Nussenzweig V, Nussenzweig R, Vekemans J, Leach A. From the circumsporozoite protein to the RTS, S/AS candidate vaccine. *Hum Vaccin.* 2010 Jan;6(1):90–6.
- [173]. RTSS Clinical Trials Partnership. A Phase 3 Trial of RTS,S/AS01 Malaria Vaccine in African Infants. *N Engl J Med.* 2012;365(20):2284–95.
- [174]. Gordon DM, Mc Govern TW, Krzych U, Cohen JC, Schneider I, La Chance R, et al. Safety, immunogenicity, and efficacy of a recombinantly produced plasmodium falciparum circumsporozoite protein-hepatitis b surface antigen subunit vaccine. *J Infect Dis.* 1995;171(6):1576–85.
- [175]. Kaslow DC, Biernaux S. RTS , S: Toward a first landmark on the Malaria Vaccine Technology Roadmap &. *Vaccine.* 2015;33(52):7425–32.
- [176]. Crompton PD, Pierce SK, Miller LH. Advances and challenges in malaria vaccine development. Vol. 120, *Journal of Clinical Investigation.* 2010. p. 4168–78.
- [177]. Hill AVS. Vaccines against malaria. Vol. 366, *Philosophical Transactions of the Royal Society B: Biological Sciences.* 2011. p. 2806–14.
- [178]. Yeboah D, Owusu-Marfo J, Agyeman YN. Predictors of malaria vaccine uptake among children 6–24 months in the Kassena Nankana Municipality in the Upper East Region of Ghana. *Malar J.* 2022 Nov 16;21(1):339.
- [179]. Sinnis P, Fidock DA. The RTS,S vaccine—a chance to regain the upper hand against malaria? *Cell.* 2022 Mar;185(5):750–4.
- [180]. Olotu A, Fegan G, Wambua J, Nyangweso G, Leach A, Lievens M, et al. Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children. *N Engl J Med.* 2016 Jun 30;374(26):2519–29.
- [181]. Datto MS, Natama MH, Somé A, Traoré O, Rouamba T, Bellamy D, et al. Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial. *Lancet.* 2021 May 15;397(10287):1809–18.
- [182]. Moorthy V, Binka F. R21/Matrix-M: a second malaria vaccine? *Lancet.* 2021;397(10287):1782–3.

- [183]. Gosling J, Case P, Tulloch J, Chandramohan D, Wegbreit J, Newby G, et al. Effective program management: a cornerstone of malaria elimination. *Am J Trop Med Hyg.* 2015 Jul;93(1):135–8.
- [184]. Hemingway J, Shretta R, Wells TNC, Bell D, Djimdé AA, Achee N, et al. Tools and Strategies for Malaria Control and Elimination: What Do We Need to Achieve a Grand Convergence in Malaria? *PLOS Biol.* 2016 Mar 2;14(3):e1002380.
- [185]. Alonso S, Chaccour CJ, Elobolobo E, Nacima A, Candrinho B, Saifodine A, et al. The economic burden of malaria on households and the health system in a high transmission district of Mozambique. *Malar J.* 2019;18(1):1–10.
- [186]. Payne D. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. *Bull World Health Organ.* 1988;66(5):621–6.
- [187]. Endeshaw T, Gebre T, Ngondi J, Graves PM, Shargie EB, Ejigsemahu Y, et al. Evaluation of light microscopy and rapid diagnostic test for the detection of malaria under operational field conditions: a household survey in Ethiopia. *Malar J.* 2008 Jul 3;7:118.
- [188]. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH. A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). *Am J Trop Med Hyg.* 2007 Dec;77(6 Suppl):119–27.
- [189]. Kattenberg JH, Ochodo EA, Boer KR, Schallig HD, Mens PF, Leeftang MM. Systematic review and meta-analysis: Rapid diagnostic tests versus placental histology, microscopy and PCR for malaria in pregnant women. *Malar J.* 2011;10(1):321.
- [190]. Chiodini PL, Bowers K, Jorgensen P, Barnwell JW, Grady KK, Luchavez J, et al. The heat stability of Plasmodium lactate dehydrogenase-based and histidine-rich protein 2-based malaria rapid diagnostic tests. *Trans R Soc Trop Med Hyg.* 2007 Apr 1;101(4):331–7.
- [191]. Selvarajah D, Naing C, Htet NH, Mak JW. Loop-mediated isothermal amplification (LAMP) test for diagnosis of uncomplicated malaria in endemic areas: a meta-analysis of diagnostic test accuracy. *Malar J.* 2020 Jun 19;19(1):211.
- [192]. Mohon AN, Getie S, Jahan N, Alam MS, Pillai DR. Ultrasensitive loop mediated isothermal amplification (US-LAMP) to detect malaria for elimination. *Malar J.* 2019 Dec 16;18(1):350.
- [193]. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 2000 Jun 15;28(12):E63.
- [194]. Thekisoe OMM, Bazie RSB, Coronel-Servian AM, Sugimoto C, Kawazu SI, Inoue N. Stability of Loop-Mediated Isothermal Amplification (LAMP) reagents and its amplification efficiency on crude trypanosome DNA templates. *J Vet Med Sci.* 2009 Apr;71(4):471–5.
- [195]. Sirichaisinthop J, Buates S, Watanabe R, Han ET, Suktawonjaroenpon W, Krassaesub S, et al. Evaluation of loop-mediated isothermal amplification (LAMP) for malaria diagnosis in a field setting. *Am J Trop Med Hyg.* 2011 Oct;85(4):594–6.
- [196]. Alonso PL, Brown G, Arevalo-herrera M, Binka F, Chitnis C, Doumbo OK, et al. A Research Agenda to Underpin Malaria Eradication. *PLoS Med.* 2011;8(1).

- [197]. Douglas NM, Lampah DA, Kenangalem E, Simpson JA, Poespoprodjo JR, Sugiarto P, et al. Major burden of severe anemia from non-falciparum malaria species in Southern Papua: a hospital-based surveillance study. *PLoS Med.* 2013 Dec;10(12):e1001575; discussion e1001575.
- [198]. Poespoprodjo JR, Fobia W, Kenangalem E, Lampah DA, Hasanuddin A, Warikar N, et al. Vivax malaria: A major cause of morbidity in early infancy. *Clin Infect Dis.* 2009;48(12):1704–12.
- [199]. Tekwani BL, Walker LA. 8-Aminoquinolines: future role as antiprotozoal drugs. *Curr Opin Infect Dis.* 2006 Dec;19(6):623–31.
- [200]. Kwiatkowski DP. How malaria has affected the human genome and what human genetics can teach us about malaria. *Am J Hum Genet.* 2005 Aug;77(2):171–92.
- [201]. Yang T, Otilie S, Istvan ES, Godinez-Macias KP, Lukens AK, Baragaña B, et al. MalDA, Accelerating Malaria Drug Discovery. *Trends Parasitol.* 2021 Jun;37(6):493–507.
- [202]. Fernández-Álvaro E, Hong WD, Nixon GL, O'Neill PM, Calderón F. Antimalarial Chemotherapy: Natural Product Inspired Development of Preclinical and Clinical Candidates with Diverse Mechanisms of Action. *J Med Chem.* 2016;59(12):5587–603.
- [203]. Baggish AL, Hill DR. Antiparasitic Agent Atovaquone. *Antimicrob Agents Chemother.* 2002;46(5):1163–73.
- [204]. Mossallam SF, Amer EI, El-Faham MH. Efficacy of Synriam™, a new antimalarial combination of OZ277 and piperaquine, against different developmental stages of *Schistosoma mansoni*. *Acta Trop.* 2015 Mar;143:36–46.
- [205]. Phyto AP, Jittamala P, Nosten FH, Pukrittayakamee S, Imwong M, White NJ, et al. Antimalarial activity of artefenomel (OZ439), a novel synthetic antimalarial endoperoxide, in patients with *Plasmodium falciparum* and *Plasmodium vivax* malaria: an open-label phase 2 trial. *Lancet Infect Dis.* 2016;16(1):61–9.
- [206]. MMV. Medicines for Malaria Venture [Internet]. 2021. Available from: <https://www.mmv.org/research-development/mmv-supported-projects>
- [207]. Favuzza P, de Lera Ruiz M, Thompson JK, Triglia T, Ngo A, Steel RWJ, et al. Dual Plasmepsin-Targeting Antimalarial Agents Disrupt Multiple Stages of the Malaria Parasite Life Cycle. *Cell Host Microbe.* 2020 Apr 8;27(4):642-658.e12.
- [208]. Yang T, Otilie S, Istvan ES, Godinez-Macias KP, Lukens AK, Baragaña B, et al. MalDA, Accelerating Malaria Drug Discovery. *Trends Parasitol.* 2021;37(6):493–507.
- [209]. Chang HH, Hartl DL. Recurrent bottlenecks in the malaria life cycle obscure signals of positive selection. *Parasitology.* 2015;142:S98–107.
- [210]. Smith RC, Vega-Rodríguez J, Jacobs-Lorena M. The *Plasmodium* bottleneck: malaria parasite losses in the mosquito vector. *Mem Inst Oswaldo Cruz.* 2014 Aug;109(5):644–61.
- [211]. Sinden RE. A biologist's perspective on malaria vaccine development. *Hum Vaccin.* 2010 Jan 27;6(1):3–11.

- [212]. Porter RJ, Laird RL, Dusseau EM. Studies on malarial sporozoites. II. Effect of age and dosage of sporozoites on their infectiousness. *Exp Parasitol*. 1954 May;3(3):267–74.
- [213]. Hopp CS, Chiou K, Ragheb DRT, Salman AM, Khan SM, Liu AJ, et al. Longitudinal analysis of *Plasmodium* sporozoite motility in the dermis reveals component of blood vessel recognition. *Elife*. 2015 Aug 13;4(AUGUST2015):1–21.
- [214]. Ménard R, Tavares J, Cockburn I, Markus M, Zavala F, Amino R. Looking under the skin: the first steps in malarial infection and immunity. *Nat Rev Microbiol*. 2013 Oct;11(10):701–12.
- [215]. Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, et al. Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nat Med*. 2006;12(2):220–4.
- [216]. Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevert U, Nussenzweig RS, et al. Migration of *Plasmodium* sporozoites through cells before infection. *Science* (80-). 2001 Jan 5;291(5501):141–4.
- [217]. Baer K, Roosevelt M, Clarkson AB, van Rooijen N, Schnieder T, Frevert U. Kupffer cells are obligatory for *Plasmodium yoelii* sporozoite infection of the liver. *Cell Microbiol*. 2007 Feb;9(2):397–412.
- [218]. Tavares J, Formaglio P, Thiberge S, Mordelet E, Van Rooijen N, Medvinsky A, et al. Role of host cell traversal by the malaria sporozoite during liver infection. *J Exp Med*. 2013 May 6;210(5):905–15.
- [219]. Risco-Castillo V, Topçu S, Marinach C, Manzoni G, Bigorgne AE, Briquet S, et al. Malaria Sporozoites Traverse Host Cells within Transient Vacuoles. *Cell Host Microbe*. 2015 Nov 11;18(5):593–603.
- [220]. Vaughan AM, Kappe SHI. Malaria Parasite Liver Infection and Exoerythrocytic Biology. *Cold Spring Harb Perspect Med*. 2017 Jun 1;7(6):1–22.
- [221]. Kaushansky A, Kappe SH. Selection and refinement: the malaria parasite's infection and exploitation of host hepatocytes. *Curr Opin Microbiol*. 2015 Aug;26(1):71–8.
- [222]. Zuzarte-Luis V, Mota MM, Vigário AM. Malaria infections: what and how can mice teach us. *J Immunol Methods*. 2014 Aug;410:113–22.
- [223]. Prudêncio M, Mota MM, Mendes AM. A toolbox to study liver stage malaria. *Trends Parasitol*. 2011 Dec;27(12):565–74.
- [224]. Baer K, Klotz C, Kappe SHI, Schnieder T, Frevert U. Release of hepatic *Plasmodium yoelii* merozoites into the pulmonary microvasculature. *PLoS Pathog*. 2007;3(11):1651–68.
- [225]. Vaughan AM, Mikolajczak SA, Wilson EM, Grompe M, Kaushansky A, Camargo N, et al. Complete *Plasmodium falciparum* liver-stage development in liver-chimeric mice. *J Clin Invest*. 2012 Oct;122(10):3618–28.
- [226]. Frevert U, Späth GF, Yee H. Exoerythrocytic development of *Plasmodium gallinaceum* in the White Leghorn chicken. *Int J Parasitol*. 2008 May;38(6):655–72.
- [227]. Wells TNC, Burrows JN, Baird JK. Targeting the hypnozoite reservoir of *Plasmodium vivax*: the hidden obstacle to malaria elimination. *Trends Parasitol*. 2010 Mar;26(3):145–51.

- [228]. Hulden L, Hulden L. Activation of the hypnozoite: a part of *Plasmodium vivax* life cycle and survival. *Malar J.* 2011 Dec 16;10(1):90.
- [229]. Cowman AF, Tonkin CJ, Tham WH, Duraisingh MT. The Molecular Basis of Erythrocyte Invasion by Malaria Parasites. *Cell Host Microbe.* 2017;22(2):232–45.
- [230]. Graewe S, Stanway RR, Rennenberg A, Heussler VT. Chronicle of a death foretold: *Plasmodium* liver stage parasites decide on the fate of the host cell. *FEMS Microbiol Rev.* 2012 Jan;36(1):111–30.
- [231]. Heussler V, Sturm A, Langsley G. Regulation of host cell survival by intracellular *Plasmodium* and *Theileria* parasites. *Parasitology.* 2006;132(SUPPL. 1).
- [232]. Rankin KE, Graewe S, Heussler VT, Stanway RR. Imaging liver-stage malaria parasites. *Cell Microbiol.* 2010 May;12(5):569–79.
- [233]. Gaur D, Mayer DCG, Miller LH. Parasite ligand-host receptor interactions during invasion of erythrocytes by *Plasmodium* merozoites. *Int J Parasitol.* 2004;34(13–14):1413–29.
- [234]. Riglar DT, Richard D, Wilson DW, Boyle MJ, Dekiwadia C, Turnbull L, et al. Super-Resolution Dissection of Coordinated Events during Malaria Parasite Invasion of the Human Erythrocyte. *Cell Host Microbe.* 2011 Jan;9(1):9–20.
- [235]. Josling G a, Llinás M. Sexual development in *Plasmodium* parasites: knowing when it's time to commit. *Nat Rev Microbiol.* 2015 Sep;13(9):573–87.
- [236]. Arnot DE, Gull K. The *Plasmodium* cell-cycle: facts and questions. *Ann Trop Med Parasitol.* 1998 Jun;92(4):361–5.
- [237]. Bannister L., Hopkins J., Fowler R., Krishna S, Mitchell G. A Brief Illustrated Guide to the Ultrastructure of *Plasmodium falciparum* Asexual Blood Stages. *Parasitol Today.* 2000 Oct;16(10):427–33.
- [238]. Read M, Sherwin T, Holloway SP, Gull K, Hyde JE. Microtubular organization visualized by immunofluorescence microscopy during erythrocytic schizogony in *Plasmodium falciparum* and investigation of post-translational modifications of parasite tubulin. *Parasitology.* 1993 Apr;106 (Pt 3(3):223–32.
- [239]. Oakley MS, Gerald N, McCutchan TF, Aravind L, Kumar S. Clinical and molecular aspects of malaria fever. *Trends Parasitol.* 2011 Oct;27(10):442–9.
- [240]. Reece SE, Drew DR, Gardner A. Sex ratio adjustment and kin discrimination in malaria parasites. *Nature.* 2008 May 29;453(7195):609–14.
- [241]. Sinden RE, Carter R, Drakeley C, Leroy D. The biology of sexual development of *Plasmodium*: the design and implementation of transmission-blocking strategies. *Malar J.* 2012 Mar 16;11:70.
- [242]. Billker O, Lindo V, Panico M, Etienne AE, Paxton T, Dell A, et al. Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. *Nature.* 1998 Mar 19;392(6673):289–92.
- [243]. Sinden RE. *Plasmodium* differentiation in the mosquito. *Parassitologia.* 1999 Sep;41(1–3):139–48.

- [244]. Whitten MMA, Shiao SH, Levashina EA. Mosquito midguts and malaria: cell biology, compartmentalization and immunology. *Parasite Immunol.* 2006 Apr;28(4):121–30.
- [245]. Gouagna LC, Gouagna LC, Mulder B, Mulder B, Noubissi E, Noubissi E, et al. The early sporogonic cycle of *Plasmodium falciparum* in laboratory-infected *Anopheles gambiae* : an estimation of parasite efficacy. *Trop Med Int Heal.* 1998 Jan 21;3(1):21–8.
- [246]. Zuccala ES, Baum J. Cytoskeletal and membrane remodelling during malaria parasite invasion of the human erythrocyte. *Br J Haematol.* 2011 Sep;154(6):680–9.
- [247]. Mbengue A, Yam XY, Braun-Breton C. Human erythrocyte remodelling during *Plasmodium falciparum* malaria parasite growth and egress. *Br J Haematol.* 2012 Apr;157(2):171–9.
- [248]. Ginsburg H, Krugliak M, Eidelman O, Cabantchik ZI. New permeability pathways induced in membranes of *Plasmodium falciparum* infected erythrocytes. *Mol Biochem Parasitol.* 1983 Jun;8(2):177–90.
- [249]. Francia ME, Striepen B. Cell division in apicomplexan parasites. Vol. 12, *Nature Reviews Microbiology.* Nature Publishing Group; 2014. p. 125–36.
- [250]. Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. *Cell.* 2006 Feb 24;124(4):755–66.
- [251]. Naughton JA, Bell A. Studies on cell-cycle synchronization in the asexual erythrocytic stages of *Plasmodium falciparum*. *Parasitology.* 2007 Mar;134(Pt 3):331–7.
- [252]. Lew, Virgilio L., Tiffert T. Volume Control. In: *Encyclopedia of Malaria.* Springer, New York, NY; 2014. p. 1–9.
- [253]. Prudêncio M, Rodrigues CD, Mota MM. The relevance of host genes in malaria. *SEB Exp Biol Ser.* 2007;58:47–91.
- [254]. Nyalwidhe J, Maier UG, Lingelbach K. Intracellular parasitism: cell biological adaptations of parasitic protozoa to a life inside cells. *Zoology (Jena).* 2003 Jan;106(4):341–8.
- [255]. Zomorodipour A, Andersson SGE. Obligate intracellular parasites: *Rickettsia prowazekii* and *Chlamydia trachomatis*. *FEBS Lett.* 1999 Jun 4;452(1–2):11–5.
- [256]. Sakharkar KR, Dhar PK, Chow VTK. Genome reduction in prokaryotic obligatory intracellular parasites of humans: a comparative analysis. *Int J Syst Evol Microbiol.* 2004 Nov;54(Pt 6):1937–41.
- [257]. Gil R, Latorre A, Moya A. Bacterial endosymbionts of insects: insights from comparative genomics. *Environ Microbiol.* 2004 Nov;6(11):1109–22.
- [258]. Fraser-Liggett CM. Insights on biology and evolution from microbial genome sequencing. *Genome Res.* 2005 Dec;15(12):1603–10.
- [259]. Nerima B, Nilsson D, Måser P. Comparative genomics of metabolic networks of free-living and parasitic eukaryotes. *BMC Genomics.* 2010 Dec 31;11(1):217.
- [260]. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature.* 2002 Oct 3;419(6906):498–511.

- [261]. Olszewski KL, Morrissey JM, Wilinski D, Burns JM, Vaidya AB, Rabinowitz JD, et al. Host-Parasite Interactions Revealed by *Plasmodium falciparum* Metabolomics. *Cell Host Microbe*. 2009 Feb 19;5(2):191–9.
- [262]. Roth E. *Plasmodium falciparum* carbohydrate metabolism: a connection between host cell and parasite. *Blood Cells*. 1990;16(2–3):453–60; discussion 461–6.
- [263]. Asahi H, Kanazawa T, Kajihara Y, Takahashi K, Takahashi T. Hypoxanthine: a low molecular weight factor essential for growth of erythrocytic *Plasmodium falciparum* in a serum-free medium. *Parasitology*. 1996 Jul;113 (Pt 1(1):19–23.
- [264]. Spry C, van Schalkwyk DA, Strauss E, Saliba KJ. Pantothenate utilization by *Plasmodium* as a target for antimalarial chemotherapy. *Infect Disord Drug Targets*. 2010 Jun 1;10(3):200–16.
- [265]. Payne SH, Loomis WF. Retention and Loss of Amino Acid Biosynthetic Pathways Based on Analysis of Whole-Genome Sequences. *Eukaryot Cell*. 2006 Feb;5(2):272–6.
- [266]. Palm W, Thompson CB. Nutrient acquisition strategies of mammalian cells. *Nature*. 2017 Jun 7;546(7657):234–42.
- [267]. Saliba KJ, Kirk K. Nutrient acquisition by intracellular apicomplexan parasites: staying in for dinner. *Int J Parasitol*. 2001 Oct;31(12):1321–30.
- [268]. Sinai AP, Joiner KA. Safe haven: the cell biology of nonfusogenic pathogen vacuoles. *Annu Rev Microbiol*. 1997;51:415–62.
- [269]. Beck JR, Ho CM. Transport mechanisms at the malaria parasite-host cell interface. *PLoS Pathog*. 2021;17(4):1–27.
- [270]. Counihan NA, Modak JK, de Koning-Ward TF. How Malaria Parasites Acquire Nutrients From Their Host. *Front cell Dev Biol*. 2021 Mar 25;9(March):649184.
- [271]. Spillman NJ, Beck JR, Goldberg DE. Protein export into malaria parasite-infected erythrocytes: mechanisms and functional consequences. *Annu Rev Biochem*. 2015;84(January):813–41.
- [272]. Boddey JA, Cowman AF. *Plasmodium* nesting: Remaking the erythrocyte from the inside out. *Annu Rev Microbiol*. 2013;67:243–69.
- [273]. Maier AG, Cooke BM, Cowman AF, Tilley L. Malaria parasite proteins that remodel the host erythrocyte. *Nat Rev Microbiol*. 2009 May;7(5):341–54.
- [274]. Slavic K, Krishna S, Derbyshire ET, Staines HM. Plasmodial sugar transporters as anti-malarial drug targets and comparisons with other protozoa. *Malar J*. 2011 Jun 15;10(1):165.
- [275]. Ginsburg H. Transport pathways in the malaria-infected erythrocyte: characterization and their use as potential targets for chemotherapy. *Mem Inst Oswaldo Cruz*. 1994;89 Suppl 2(September):99–109.
- [276]. Lang-Unnasch N, Murphy AD. Metabolic changes of the malaria parasite during the transition from the human to the mosquito host. *Annu Rev Microbiol*. 1998;52:561–90.

- [277]. van Dooren GG, Stimmler LM, McFadden GI. Metabolic maps and functions of the Plasmodium mitochondrion. *FEMS Microbiol Rev.* 2006 Jul;30(4):596–630.
- [278]. Joët T, Eckstein-Ludwig U, Morin C, Krishna S. Validation of the hexose transporter of Plasmodium falciparum as a novel drug target. *Proc Natl Acad Sci U S A.* 2003;100(13):7476–9.
- [279]. Meireles P, Sales-Dias J, Andrade CM, Mello-Vieira J, Mancio-Silva L, Simas JP, et al. GLUT1-mediated glucose uptake plays a crucial role during Plasmodium hepatic infection. *Cell Microbiol.* 2017 Feb;19(2).
- [280]. Kirk K, Saliba KJ. Targeting nutrient uptake mechanisms in Plasmodium. *Curr Drug Targets.* 2007 Jan 1;8(1):75–88.
- [281]. Brahm J. Kinetics of glucose transport in human erythrocytes. *J Physiol.* 1983 Jun;339(1):339–54.
- [282]. Manolescu AR, Witkowska K, Kinnaird A, Cessford T, Cheeseman C. Facilitated hexose transporters: new perspectives on form and function. *Physiology.* 2007 Aug;22(4):234–40.
- [283]. Desai SA, Rosenberg RL. Pore size of the malaria parasite's nutrient channel. *Proc Natl Acad Sci U S A.* 1997;94(5):2045–9.
- [284]. Qureshi AA, Suades A, Matsuoka R, Brock J, McComas SE, Nji E, et al. The molecular basis for sugar import in malaria parasites. *Nature.* 2020 Feb;578(7794):321–5.
- [285]. Low LM, Azasi Y, Sherling ES, Garten M, Zimmerberg J, Tsuboi T, et al. Deletion of Plasmodium falciparum Protein RON3 Affects the Functional Translocation of Exported Proteins and Glucose Uptake. *MBio.* 2019 Jul 9;10(4):1–11.
- [286]. Woodrow CJ, Penny JI, Krishna S. Intraerythrocytic Plasmodium falciparum expresses a high affinity facilitative hexose transporter. *J Biol Chem.* 1999 Mar 12;274(11):7272–7.
- [287]. Belen Cassera M, Zhang Y, Z. Hazleton K, L. Schramm V. Purine and Pyrimidine Pathways as Targets in Plasmodium falciparum. *Curr Top Med Chem.* 2011;11(16):2103–15.
- [288]. Chaudhary K, Darling JA, Fohl LM, Sullivan WJ, Donald RGK, Pfefferkorn ER, et al. Purine salvage pathways in the apicomplexan parasite Toxoplasma gondii. *J Biol Chem.* 2004;279(30):31221–7.
- [289]. De Koning HP, Bridges DJ, Burchmore RJS. Purine and pyrimidine transport in pathogenic protozoa: From biology to therapy. *FEMS Microbiol Rev.* 2005;29(5):987–1020.
- [290]. Deitsch KW, Driskill CL, Wellems TE. Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. *Nucleic Acids Res.* 2001;29(3):850–3.
- [291]. Hyde J. Targeting Purine and Pyrimidine Metabolism in Human Apicomplexan Parasites. *Curr Drug Targets.* 2006;8(1):31–47.
- [292]. Parker MD, Hyde RJ, Yao SYM, McRobert L, Cass CE, Young JD, et al. Identification of a nucleoside/nucleobase transporter from Plasmodium falciparum, a novel target for anti-malarial chemotherapy. *Biochem J.* 2000 Jul 1;349(1):67.

- [293]. Quashie NB, Dorin-Semblat D, Bray PG, Biagini GA, Doerig C, Ranford-Cartwright LC, et al. A comprehensive model of purine uptake by the malaria parasite *Plasmodium falciparum*: identification of four purine transport activities in intraerythrocytic parasites. *Biochem J*. 2008 Apr 15;411(2):287–95.
- [294]. Carter NS, Ben Mamoun C, Liu W, Silva EO, Landfear SM, Goldberg DE, et al. Isolation and functional characterization of the PfNT1 nucleoside transporter gene from *Plasmodium falciparum*. *J Biol Chem*. 2000 Apr 7;275(14):10683–91.
- [295]. Saliba KJ, Kirk K. H⁺-coupled pantothenate transport in the intracellular malaria parasite. *J Biol Chem*. 2001 May 25;276(21):18115–21.
- [296]. Michael AJ. Biosynthesis of polyamines and polyamine-containing molecules. *Biochem J*. 2016 Aug 1;473(15):2315–29.
- [297]. Phillips MA. Polyamines in protozoan pathogens. *J Biol Chem*. 2018 Nov;293(48):18746–56.
- [298]. Minois, N, Carmona-Gutierrez, D, Madeo, F. Polyamines in aging and disease. *Aging (Albany NY)*. 2011;3(8).
- [299]. Gerner EW, Meyskens FL. Polyamines and cancer: Old molecules, new understanding. *Nat Rev Cancer*. 2004;4(10):781–92.
- [300]. Birkholtz LM, Williams M, Niemand J, Louw AI, Persson L, Heby O. Polyamine homeostasis as a drug target in pathogenic protozoa: Peculiarities and possibilities. *Biochem J*. 2011;438(2):229–44.
- [301]. Casero RA, Marton LJ. Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. *Nat Rev Drug Discov*. 2007;6(5):373–90.
- [302]. Clark K, Niemand J, Reeksting S, Smit S, van Brummelen AC, Williams M, et al. Functional consequences of perturbing polyamine metabolism in the malaria parasite, *Plasmodium falciparum*. *Amino Acids*. 2010 Feb;38(2):633–44.
- [303]. Pegg AE. Regulation of ornithine decarboxylase. *J Biol Chem*. 2006 May 26;281(21):14529–32.
- [304]. Xiao Y, McCloskey DE, Phillips MA. RNA interference-mediated silencing of ornithine decarboxylase and spermidine synthase genes in *Trypanosoma brucei* provides insight into regulation of polyamine biosynthesis. *Eukaryot Cell*. 2009 May;8(5):747–55.
- [305]. Mangold U, Leberer E. Regulation of all members of the antizyme family by antizyme inhibitor. *Biochem J*. 2005;385(1):21–8.
- [306]. Bale, Shridhar, E. Ealick S. Structural Biology of S-Adenosylmethionine Decarboxylase. *Amino Acids*. 2010;38(2):451–60.
- [307]. Ikeguchi Y, Bewley MC, Pegg AE. Aminopropyltransferases: Function, Structure and Genetics. *J Biochem*. 2006 Jan 1;139(1):1–9.

- [308]. Haider N, Eschbach ML, Dias S de S, Gilberger TW, Walter RD, Lüersen K. The spermidine synthase of the malaria parasite *Plasmodium falciparum*: Molecular and biochemical characterisation of the polyamine synthesis enzyme. *Mol Biochem Parasitol*. 2005 Aug;142(2):224–36.
- [309]. Birkholtz LM, Wrenger C, Joubert F, Wells GA, Walter RD, Louw AI. Parasite-specific inserts in the bifunctional S-adenosylmethionine decarboxylase/ornithine decarboxylase of *Plasmodium falciparum* modulate catalytic activities and domain interactions. *Biochem J*. 2004;377(2):439–48.
- [310]. Wrenger C, Luersen K, Krause T, Muller S, Walter RD. The *Plasmodium falciparum* bifunctional ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase, enables a well balanced polyamine synthesis without domain-domain interaction. *J Biol Chem*. 2001 Aug 10;276(32):29651–6.
- [311]. Krause T, Lüersen K, Wrenger C, Gilberger TW, Müller S, Walter RD. The ornithine decarboxylase domain of the bifunctional ornithine decarboxylase/S-adenosylmethionine decarboxylase of *Plasmodium falciparum*: Recombinant expression and catalytic properties of two different constructs. *Biochem J*. 2000;352(2):287–92.
- [312]. Müller S, Da'dara A, Lüersen K, Wrenger C, Das Gupta R, Madhubala R, et al. In the human malaria parasite *Plasmodium falciparum*, polyamines are synthesized by a bifunctional ornithine decarboxylase, S-adenosylmethionine decarboxylase. *J Biol Chem*. 2000 Mar 17;275(11):8097–102.
- [313]. Wells GA, Birkholtz LM, Joubert F, Walter RD, Louw AI. Novel properties of malarial S-adenosylmethionine decarboxylase as revealed by structural modelling. *J Mol Graph Model*. 2006 Jan;24(4):307–18.
- [314]. Gugliucci A. Polyamines as clinical laboratory tools. *Clin Chim Acta*. 2004;344(1–2):23–35.
- [315]. Assaraf YG, Golenser J, Spira DT, Bachrach U. Polyamine levels and the activity of their biosynthetic enzymes in human erythrocytes infected with the malarial parasite, *Plasmodium falciparum*. *Biochem J*. 1984;222(3):815–9.
- [316]. Das Gupta R, Krause-Ihle T, Bergmann B, Müller IB, Khomutov AR, Müller S, et al. 3-Aminooxy-1-Aminopropane and Derivatives Have an Antiproliferative Effect on Cultured *Plasmodium falciparum* by Decreasing Intracellular Polyamine Concentrations. *Antimicrob Agents Chemother*. 2005 Jul;49(7):2857–64.
- [317]. Ramya TNC, Surolia N, Surolia A. Polyamine synthesis and salvage pathways in the malaria parasite *Plasmodium falciparum*. *Biochem Biophys Res Commun*. 2006 Sep 22;348(2):579–84.
- [318]. Teng R, Junankar PR, Bubb WA, Rae C, Mercier P, Kirk K. Metabolite profiling of the intraerythrocytic malaria parasite *Plasmodium falciparum* by (1)H NMR spectroscopy. *NMR Biomed*. 2009 Apr;22(3):292–302.
- [319]. Pegg AE, Shuttleworth K, Hibasami H. Specificity of mammalian spermidine synthase and spermine synthase. *Biochem J*. 1981 Aug 1;197(2):315–20.

- [320]. Müller S, Coombs GH, Walter RD. Targeting polyamines of parasitic protozoa in chemotherapy. *Trends Parasitol.* 2001 May;17(5):242–9.
- [321]. Assaraf YG, Golenser J, Spira DT, Messer G, Bachrach U. Cytostatic effect of DL-alpha-difluoromethylornithine against *Plasmodium falciparum* and its reversal by diamines and spermidine. *Parasitol Res.* 1987;73(4):313–8.
- [322]. Bitonti AJ, McCann PP, Sjoerdsma A. *Plasmodium falciparum* and *Plasmodium berghei*: Effects of ornithine decarboxylase inhibitors on erythrocytic schizogony. *Exp Parasitol.* 1987;64(2):237–43.
- [323]. Wright PS, Byers TL, Cross-Doersen DE, McCann PP, Bitonti AJ. Irreversible inhibition of S-adenosylmethionine decarboxylase in *Plasmodium falciparum*-infected erythrocytes: growth inhibition in vitro. *Biochem Pharmacol.* 1991 Jun 1;41(11):1713–8.
- [324]. Kay JE, Lindsay VJ. Control of ornithine decarboxylase activity in stimulated human lymphocytes by putrescine and spermidine. *Biochem J.* 1973 Apr;132(4):791–6.
- [325]. McCann PP, Tardif C, Pegg AE, Diekema K. The dual action of the non-physiological diamines 1,3 diaminopropane and cadaverine on ornithine decarboxylase of HTC cells. *Life Sci.* 1980 Jun;26(23):2003–10.
- [326]. Porter CW, Berger FG, Pegg AE, Ganis B, Bergeron RJ. Regulation of ornithine decarboxylase activity by spermidine and the spermidine analogue N 1 N 8-bis(ethyl)spermidine. *Biochem J.* 1987 Mar 1;242(2):433–40.
- [327]. Bitonti AJ, Dumont JA, Bush TL, Edwards ML, Stemerick DM, McCann PP, et al. Bis(benzyl)polyamine analogs inhibit the growth of chloroquine-resistant human malaria parasites (*Plasmodium falciparum*) in vitro and in combination with alpha-difluoromethylornithine cure murine malaria. *Proc Natl Acad Sci U S A.* 1989 Jan;86(2):651–5.
- [328]. Sherman IW. Amino acid metabolism and protein synthesis in malarial parasites. *Bull World Health Organ.* 1977;55(2–3):265–76.
- [329]. Sherman IW. Biochemistry of *Plasmodium* (malarial parasites). *Microbiol Rev.* 1979 Dec;43(4):453–95.
- [330]. Krugliak M, Zhang J, Ginsburg H. Intraerythrocytic *Plasmodium falciparum* utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. *Mol Biochem Parasitol.* 2002 Feb;119(2):249–56.
- [331]. Liu J, Istvan ES, Gluzman IY, Gross J, Goldberg DE. *Plasmodium falciparum* ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. *Proc Natl Acad Sci U S A.* 2006;103(23):8840–5.
- [332]. Kirk K. Membrane Transport in the Malaria-Infected Erythrocyte. *Physiol Rev.* 2001 Apr 1;81(2):495–537.
- [333]. Smith CC, McCormick GJ, Canfield CJ. *Plasmodium knowlesi*: in vitro biosynthesis of methionine and thymidylic acid. *Exp Parasitol.* 1977 Oct;43(1):248–54.

- [334]. Olszewski KL, Llinás M. Central carbon metabolism of *Plasmodium* parasites. *Mol Biochem Parasitol.* 2011 Feb;175(2):95–103.
- [335]. Groman NB. Dynamic aspects of the nitrogen metabolism of *Plasmodium gallinaceum* in vivo and in vitro. *J Infect Dis.* 1951;88(2):126–50.
- [336]. Cenedella RJ, Rosen H, Angel CR, Saxe LH. Free amino-acid production in vitro by *Plasmodium berghei*. *Am J Trop Med Hyg.* 1968 Nov;17(6):800–3.
- [337]. Loria P, Miller S, Foley M, Tilley L. Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials. *Biochem J.* 1999 Apr 15;339 (Pt 2(Pt 2):363–70.
- [338]. Sullivan, David, Krishna S. *Malaria: Drugs, Disease and Post-genomic Biology.* 1st ed. R. W. Compans, M. D. Cooper, T. Honjo, H. Koprowski, F. Melchers, M. B. A. Oldstone, S. Olsnes, M. Potter, P. K. Vogt, H. Wagner, David J. Sullivan SK, editor. Berlin: Springer; 2006. 275–291 p.
- [339]. Francis SE, Gluzman IY, Oksman A, Knickerbocker A, Mueller R, Bryant ML, et al. Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. *EMBO J.* 1994 Jan 15;13(2):306–17.
- [340]. Rudzinska MA, Trager W, Bray RS. Pinocytotic uptake and the digestion of hemoglobin in malaria parasites. *J Protozool.* 1965 Nov;12(4):563–76.
- [341]. Lew VL, Tiffert T, Ginsburg H. Excess hemoglobin digestion and the osmotic stability of *Plasmodium falciparum*-infected red blood cells. *Blood.* 2003 May 15;101(10):4189–94.
- [342]. Sherman IW. Transport of amino acids and nucleic acid precursors in malarial parasites. *Bull World Health Organ.* 1977;55(2–3):211–25.
- [343]. Martin RE, Kirk K. Transport of the essential nutrient isoleucine in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. *Blood.* 2007;109(5):2217–24.
- [344]. Cobbold SA, Martin RE, Kirk K. Methionine transport in the malaria parasite *Plasmodium falciparum*. *Int J Parasitol.* 2011 Jan;41(1):125–35.
- [345]. Liu J, Gluzman IY, Drew ME, Goldberg DE. The role of *Plasmodium falciparum* food vacuole plasmepsins. *J Biol Chem.* 2005 Jan 14;280(2):1432–7.
- [346]. Divo AA, Geary TG, Davis NL, Jensen JB. Nutritional requirements of *Plasmodium falciparum* in culture. I. Exogenously supplied dialyzable components necessary for continuous growth. *J Protozool.* 1985 Feb;32(1):59–64.
- [347]. Siddiqui, W.A., Schnell JV. In-vitro and in-vivo studies with *Plasmodium falciparum* and *Plasmodium knowlesi*. *Proc Helm Soc Wash.* 1972;39:204–210.
- [348]. Ginsburg H, Kutner S, Krugliak M, Cabantchik ZI. Characterization of permeation pathways appearing in the host membrane of *Plasmodium falciparum* infected red blood cells. *Mol Biochem Parasitol.* 1985 Mar;14(3):313–22.
- [349]. Elford BC, Haynes JD, Chulay JD, Wilson RJM. Selective stage-specific changes in the permeability to small hydrophilic solutes of human erythrocytes infected with *Plasmodium falciparum*. *Mol Biochem Parasitol.* 1985 Jun;16(1):43–60.

- [350]. Tunncliffe G. Amino acid transport by human erythrocyte membranes. *Comp Biochem Physiol Comp Physiol*. 1994 Aug;108(4):471–8.
- [351]. Rosenberg R. Na-independent and Na-dependent transport of neutral amino acids in the human red blood cell. *Acta Physiol Scand*. 1982 Dec;116(4):321–30.
- [352]. Barker GA, Ellory JC. The identification of neutral amino acid transport systems. *Exp Physiol*. 1990 Jan;75(1):3–26.
- [353]. Huber SM, Duranton C, Lang F. Patch-clamp analysis of the “new permeability pathways” in malaria-infected erythrocytes. *Int Rev Cytol*. 2005;246(05):59–134.
- [354]. Merckx A, Bouyer G, Thomas SLY, Langsley G, Egée S. Anion channels in *Plasmodium falciparum*-infected erythrocytes and protein kinase A. *Trends Parasitol*. 2009 Mar;25(3):139–44.
- [355]. Upston JM, Gero AM. Parasite-induced permeation of nucleosides in *Plasmodium falciparum* malaria. *Biochim Biophys Acta*. 1995 Jun 14;1236(2):249–58.
- [356]. Rosenberg R. Amino acid transport in human red blood cells. *Acta Psychiatr Scand Suppl*. 1988;345(345 S):25–8.
- [357]. Martin RE, Henry RI, Abbey JL, Clements JD, Kirk K. The “permeome” of the malaria parasite: an overview of the membrane transport proteins of *Plasmodium falciparum*. *Genome Biol*. 2005;6(3).
- [358]. Le Roch KG, Zhou Y, Blair PL, Grainger M, Moch JK, Haynes JD, et al. Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* (80-). 2003 Sep 12;301(5639):1503–8.
- [359]. Bozdech Z, Llinás M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. Gary Ward, editor. *PLoS Biol*. 2003 Oct 18;1(1):E5.
- [360]. Cornet S, Bichet C, Larcombe S, Faivre B, Sorci G. Impact of host nutritional status on infection dynamics and parasite virulence in a bird-malaria system. *J Anim Ecol*. 2014 Jan;83(1):256–65.
- [361]. Chandra RK. Nutrition, immunity and infection: from basic knowledge of dietary manipulation of immune responses to practical application of ameliorating suffering and improving survival. *Proc Natl Acad Sci U S A*. 1996 Dec 10;93(25):14304–7.
- [362]. Carreiro AL, Dhillon J, Gordon S, Higgins KA, Jacobs AG, McArthur BM, et al. The Macronutrients, Appetite, and Energy Intake. *Annu Rev Nutr*. 2016 Jul 17;36:73–103.
- [363]. Saliba KJ, Ferru I, Kirk K. Provitamin B5 (pantothenol) inhibits growth of the intraerythrocytic malaria parasite. *Antimicrob Agents Chemother*. 2005 Feb;49(2):632–7.
- [364]. Zuzarte-Luís V, Mota MM. Parasite Sensing of Host Nutrients and Environmental Cues. *Cell Host Microbe*. 2018 Jun 13;23(6):749–58.
- [365]. Kumar M, Skillman K, Duraisingh MT. Linking nutrient sensing and gene expression in *Plasmodium falciparum* blood-stage parasites. *Mol Microbiol*. 2021 May;115(5):891–900.

- [366]. Rosenberg E, Ben-Shmuel A, Shalev O, Sinay R, Cowman A, Pollack Y. Differential, positional-dependent transcriptional response of antigenic variation (var) genes to biological stress in *Plasmodium falciparum*. Hansen IA, editor. PLoS One. 2009 Sep 10;4(9):e6991.
- [367]. Becker K, Tilley L, Vennerstrom JL, Roberts D, Rogerson S, Ginsburg H. Oxidative stress in malaria parasite-infected erythrocytes: host–parasite interactions. Int J Parasitol. 2004 Feb;34(2):163–89.
- [368]. Engelbrecht D, Coetzer TL. Turning up the heat: Heat stress induces markers of programmed cell death in *Plasmodium falciparum* in vitro. Cell Death Dis. 2013;4(12):e971-11.
- [369]. Tebben K, Bradwell K, Serre D. Variation in selective constraints along the *Plasmodium* life cycle. Infect Genet Evol. 2021 Aug;92:104908.
- [370]. Howick VM, Russell AJC, Andrews T, Heaton H, Reid AJ, Natarajan K, et al. The malaria cell atlas: Single parasite transcriptomes across the complete *Plasmodium* life cycle. Science (80-). 2019;365(6455).
- [371]. Sà JM, Cannon M V., Caeon RL, Wellems TE, Serre D. Single-cell transcription analysis of *Plasmodium vivax* blood-stage parasites identifies stage- and species-specific profiles of expression. PLoS Biol. 2020 May;18(5):e3000711.
- [372]. Srivastava A, Philip N, Hughes KR, Georgiou K, MacRae JI, Barrett MP, et al. Stage-Specific Changes in *Plasmodium* Metabolism Required for Differentiation and Adaptation to Different Host and Vector Environments. PLoS Pathog. 2016 Dec;12(12):e1006094.
- [373]. Mancio-Silva L, Slavic K, Grilo Ruivo MT, Grosso AR, Modrzynska KK, Vera IM, et al. Nutrient sensing modulates malaria parasite virulence. Nature. 2017 Jul 5;547(7662):213–6.
- [374]. Srivastava A, Creek DJ, Evans KJ, De Souza D, Schofield L, Müller S, et al. Host reticulocytes provide metabolic reservoirs that can be exploited by malaria parasites. PLoS Pathog. 2015 Jun;11(6):e1004882.
- [375]. Tewari SG, Swift RP, Reifman J, Prigge ST, Wallqvist A. Metabolic alterations in the erythrocyte during blood-stage development of the malaria parasite. Malar J. 2020 Feb 27;19(1):94.
- [376]. Narula AS, Gopinathan VP, Narula HS. Hypoglycaemia in falciparum malaria-an unusual manifestation. J Assoc Physicians India. 1985 Nov;33(11):740–1.
- [377]. Rawat M, Srivastava A, Johri S, Gupta I, Karmodiya K. Single-Cell RNA Sequencing Reveals Cellular Heterogeneity and Stage Transition under Temperature Stress in Synchronized *Plasmodium falciparum* Cells. Cuomo CA, editor. Microbiol Spectr. 2021 Sep 3;9(1):e0000821.

- [378]. Gunnar R. Mair, Joanna A. M. Braks, Lindsey S. Garver, George Dimopoulos, Neil Hall, Joop C.A.G. Wiegant, Roeland W. Dirks, Shahid M. Khan, Chris J. Janse APW. Translational Repression is essential for Plasmodium sexual development and mediated by a DDX6-type RNA helicase. *Science* (80-). 2006;313(5787):667–9.
- [379]. Müller K, Matuschewski K, Silvie O. The Puf-family RNA-binding protein Puf2 controls sporozoite conversion to liver stages in the malaria parasite. *PLoS One*. 2011;6(5):e19860.
- [380]. Ke H, Lewis IA, Morrissey JM, McLean KJ, Ganesan SM, Painter HJ, et al. Genetic investigation of tricarboxylic acid metabolism during the Plasmodium falciparum life cycle. *Cell Rep*. 2015 Apr 7;11(1):164–74.
- [381]. Hohmann S, Mager WH. Yeast Stress Responses. In: *Topics in Current Genetics*. 1997.
- [382]. Tewari R, Straschil U, Bateman A, Böhme U, Cherevach I, Gong P, et al. The Systematic Functional Analysis of Plasmodium Protein Kinases Identifies Essential Regulators of Mosquito Transmission. *Cell Host Microbe*. 2010 Oct 21;8(4):377–87.
- [383]. Itoe MA, Sampaio JL, Cabal GG, Real E, Zuzarte-Luis V, March S, et al. Host cell phosphatidylcholine is a key mediator of malaria parasite survival during liver stage infection. *Cell Host Microbe*. 2014 Dec 10;16(6):778–86.
- [384]. Sonia Gulati, Eric H. Ekland, Kelly V. Ruggles, Robin B. Chan, Bamini Jayabalasingham¹, Bowen Zhou, Pierre-Yves Mantel, Marcus C. S. Lee, Natasha Spottiswoode, Olivia Coburn-Flynn, Daisy Hjelmqvist, Tilla S. Worgall, Matthias Marti, Gilbert Di Paolo, and. Profiling the Essential Nature of Lipid Metabolism in Asexual Blood and Gametocyte Stages of Plasmodium falciparum Sonia. *Cell Host Microbe*. 2016;17(3):139–48.
- [385]. Brancucci NMB, De Niz M, Straub TJ, Ravel D, Sollelis L, Birren BW, et al. Probing Plasmodium falciparum sexual commitment at the single-cell level. *Wellcome Open Res*. 2018;3:70.
- [386]. Brancucci NMB, Gerdt JP, Wang C, De Niz M, Philip N, Adapa SR, et al. Lysophosphatidylcholine Regulates Sexual Stage Differentiation in the Human Malaria Parasite Plasmodium falciparum. *Cell*. 2017 Dec 14;171(7):1532-1544.e15.
- [387]. Witola WH, El Bissati K, Pessi G, Xie C, Roepe PD, Mamoun C Ben. Disruption of the Plasmodium falciparum PfPMT gene results in a complete loss of phosphatidylcholine biosynthesis via the serine-decarboxylase-phosphoethanolamine-methyltransferase pathway and severe growth and survival defects. *J Biol Chem*. 2008 Oct 10;283(41):27636–43.
- [388]. Pessi G, Kociubinski G, Mamoun C Ben. A pathway for phosphatidylcholine biosynthesis in Plasmodium falciparum involving phosphoethanolamine methylation. *Proc Natl Acad Sci*. 2004 Apr 20;101(16):6206–11.
- [389]. Pessi G, Choi JY, Reynolds JM, Voelker DR, Mamoun C Ben. In Vivo Evidence for the Specificity of Plasmodium falciparum Phosphoethanolamine Methyltransferase and Its Coupling to the Kennedy Pathway. *J Biol Chem*. 2005 Apr 1;280(13):12461–6.

- [390]. Tintó-Font E, Cortés A. Malaria parasites do respond to heat. *Trends Parasitol.* 2022 Jun;38(6):435–49.
- [391]. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. *Int J Cell Biol.* 2010;2010:214074.
- [392]. Bruce MC, Alano P, Duthie S, Carter R. Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology.* 1990 Apr;100 Pt 2(2):191–200.
- [393]. Smith TG, Lourenço P, Carter R, Walliker D, Ranford-Cartwright LC. Commitment to sexual differentiation in the human malaria parasite, *Plasmodium falciparum*. *Parasitology.* 2000 Aug;121 (Pt 2(2):127–33.
- [394]. Sowunmi A, Fateye BA, Adediji AA, Fehintola FA, Happi TC. Risk factors for gametocyte carriage in uncomplicated *falciparum* malaria in children. *Parasitology.* 2004 Sep;129(Pt 3):255–62.
- [395]. Peatey CL, Spicer TP, Hodder PS, Trenholme KR, Gardiner DL. A high-throughput assay for the identification of drugs against late-stage *Plasmodium falciparum* gametocytes. *Mol Biochem Parasitol.* 2011 Dec;180(2):127–31.
- [396]. White NJ. The role of anti-malarial drugs in eliminating malaria. *Malar J.* 2008;7(SUPPL. 1):1–6.
- [397]. Vonlaufen N, Kanzok SM, Wek RC, Sullivan WJ. Stress response pathways in protozoan parasites. *Cell Microbiol.* 2008 Dec;10(12):2387–99.
- [398]. Gonzalez S, Rallis C. The TOR Signaling Pathway in Spatial and Temporal Control of Cell Size and Growth. *Front Cell Dev Biol.* 2017 Jun 7;5(JUN):1–6.
- [399]. Kim J, Guan KL. Amino acid signaling in TOR Activation. *Annu Rev Biochem.* 2011 Jul 7;80(1):1001–32.
- [400]. González A, Hall MN. Nutrient sensing and TOR signaling in yeast and mammals. *EMBO J.* 2017 Feb 15;36(4):397–408.
- [401]. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell.* 2006 Feb 10;124(3):471–84.
- [402]. Schmelzle T, Hall MN. TOR, a Central Controller of Cell Growth. *Cell.* 2000 Oct 13;103(2):253–62.
- [403]. Adams B, Musiyenko A, Kumar R, Barik S. A Novel Class of Dual-family Immunophilins. *J Biol Chem.* 2005 Jul;280(26):24308–14.
- [404]. Kumar R, Adams B, Musiyenko A, Shulyayeva O, Barik S. The FK506-binding protein of the malaria parasite, *Plasmodium falciparum*, is a FK506-sensitive chaperone with FK506-independent calcineurin-inhibitory activity. *Mol Biochem Parasitol.* 2005;141(2):163–73.
- [405]. Wek RC, Jiang HY, Anthony TG. Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans.* 2006 Feb 1;34(1):7.
- [406]. Holcik M, Sonenberg N. Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol.* 2005;6(4):318–27.

- [407]. Wek SA, Zhu S, Wek RC. The Histidyl-tRNA Synthetase-Related Sequence in the eIF-2 α Protein Kinase GCN2 Interacts with tRNA and Is Required for Activation in Response to Starvation for Different Amino Acids. *Mol Cell Biol*. 1995 Aug 1;15(8):4497–506.
- [408]. Hinnebusch AG, Natarajan K. Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot Cell*. 2002 Feb;1(1):22–32.
- [409]. Gebauer F, Hentze MW. Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol*. 2004 Oct;5(10):827–35.
- [410]. Humeau J, Leduc M, Cerrato G, Loos F, Kepp O, Kroemer G. Phosphorylation of eukaryotic initiation factor-2 α (eIF2 α) in autophagy. *Cell Death Dis*. 2020;11(6).
- [411]. Donnelly N, Gorman AM, Gupta S, Samali A. The eIF2 α kinases: Their structures and functions. *Cell Mol Life Sci*. 2013;70(19):3493–511.
- [412]. Lu PD, Jousse C, Marciniak SJ, Zhang Y, Novoa I, Scheuner D, et al. Cytoprotection by pre-emptive conditional phosphorylation of translation initiation factor 2. *EMBO J*. 2004 Jan 14;23(1):169–79.
- [413]. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep*. 2006 Sep;7(9):880–5.
- [414]. Nakamura T, Furuhashi M, Li P, Cao H, Tuncman G, Sonenberg N, et al. Double-Stranded RNA-Dependent Protein Kinase Links Pathogen Sensing with Stress and Metabolic Homeostasis. *Cell*. 2010 Feb;140(3):338–48.
- [415]. Onuki R, Bando Y, Suyama E, Katayama T, Kawasaki H, Baba T, et al. An RNA-dependent protein kinase is involved in tunicamycin-induced apoptosis and Alzheimer's disease. *EMBO J*. 2004 Feb 25;23(4):959–68.
- [416]. Chen JJ, Zhang S. Heme-regulated eIF2 α kinase in erythropoiesis and hemoglobinopathies. *Blood*. 2019 Nov 14;134(20):1697–707.
- [417]. Zhang P, McGrath BC, Reinert J, Olsen DS, Lei L, Gill S, et al. The GCN2 eIF2 α kinase is required for adaptation to amino acid deprivation in mice. *Mol Cell Biol*. 2002 Oct;22(19):6681–8.
- [418]. Nanduri S, Rahman F, Williams BR, Qin J. A dynamically tuned double-stranded RNA binding mechanism for the activation of antiviral kinase PKR. *EMBO J*. 2000 Oct 16;19(20):5567–74.
- [419]. Padyana AK, Qiu H, Roll-Mecak A, Hinnebusch AG, Burley SK. Structural Basis for Autoinhibition and Mutational Activation of Eukaryotic Initiation Factor 2 α Protein Kinase GCN2. *J Biol Chem*. 2005 Aug;280(32):29289–99.
- [420]. Qiu H, Dong J, Hu C, Francklyn CS, Hinnebusch AG. The tRNA-binding moiety in GCN2 contains a dimerization domain that interacts with the kinase domain and is required for tRNA binding and kinase activation. *EMBO J*. 2001 Mar 15;20(6):1425–38.

- [421]. Qiu H, Garcia-Barrio MT, Hinnebusch AG. Dimerization by translation initiation factor 2 kinase GCN2 is mediated by interactions in the C-terminal ribosome-binding region and the protein kinase domain. *Mol Cell Biol*. 1998 May 1;18(5):2697–711.
- [422]. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. Nutritional control of gene expression: How mammalian cells respond to amino acid limitation. *Annu Rev Nutr*. 2005;25:59–85.
- [423]. Romano PR, Garcia-Barrio MT, Zhang X, Wang Q, Taylor DR, Zhang F, et al. Autophosphorylation in the activation loop is required for full kinase activity in vivo of human and yeast eukaryotic initiation factor 2 α kinases PKR and GCN2. *Mol Cell Biol*. 1998 Apr;18(4):2282–97.
- [424]. Dever TE, Feng L, Wek RC, Cigan AM, Donahue TF, Hinnebusch AG. Phosphorylation of initiation factor 2 α by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. *Cell*. 1992;68(3):585–96.
- [425]. Ward P, Equinet L, Packer J, Doerig C. Protein kinases of the human malaria parasite *Plasmodium falciparum*: the kinome of a divergent eukaryote. *BMC Genomics*. 2004 Oct 12;5:79.
- [426]. Zhang M, Mishra S, Sakthivel R, Rojas M, Ranjan R, Sullivan WJ, et al. PK4, a eukaryotic initiation factor 2 α (eIF2 α) kinase, is essential for the development of the erythrocytic cycle of *Plasmodium*. *Proc Natl Acad Sci U S A*. 2012 Mar 6;109(10):3956–61.
- [427]. Zhang M, Fennell C, Ranford-Cartwright L, Sakthivel R, Gueirard P, Meister S, et al. The *Plasmodium* eukaryotic initiation factor-2 α kinase IK2 controls the latency of sporozoites in the mosquito salivary glands. *J Exp Med*. 2010 Jul 5;207(7):1465–74.
- [428]. Solyakov L, Halbert J, Alam MM, Semblat JP, Dorin-Semblat D, Reininger L, et al. Global kinomic and phospho-proteomic analyses of the human malaria parasite *Plasmodium falciparum*. *Nat Commun*. 2011 Nov 29;2(1):565.
- [429]. Fennell C, Babbitt S, Russo I, Wilkes J, Ranford-Cartwright L, Goldberg DE, et al. Pfk1K1, a eukaryotic initiation factor 2 kinase of the human malaria parasite *Plasmodium falciparum*, regulates stress-response to amino-acid starvation. *Malar J*. 2009;8(1):1–15.
- [430]. Witkowski B, Lelièvre J, Barragán MJL, Laurent V, Su X zhuan, Berry A, et al. Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. *Antimicrob Agents Chemother*. 2010 May;54(5):1872–7.
- [431]. Stepniewska K, Ashley E, Lee SJ, Anstey N, Barnes KI, Binh TQ, et al. In vivo parasitological measures of artemisinin susceptibility. *J Infect Dis*. 2010 Feb 15;201(4):570–9.
- [432]. Storey KB, Storey JM. Tribute to P. L. Lutz: putting life on 'pause'-molecular regulation of hypometabolism. *J Exp Biol*. 2007 May;210(Pt 10):1700–14.
- [433]. Sullivan WJ, Jeffers V. Mechanisms of *Toxoplasma gondii* persistence and latency. *FEMS Microbiol Rev*. 2012 May;36(3):717–33.
- [434]. Teklehaimanot and Paola Mejia A. Malaria and Poverty. *Ann N Y Acad Sci*. 2008 Jul 25;1136(1):32–7.

- [435]. Edirisinghe JS. Infections in the malnourished: with special reference to malaria and malnutrition in the tropics. *Ann Trop Paediatr*. 1986 Dec;6(4):233–7.
- [436]. Shankar AH, Genton B, Semba RD, Baisor M, Paino J, Tamja S, et al. Effect of vitamin A supplementation on morbidity due to *Plasmodium falciparum* in young children in Papua New Guinea: a randomised trial. *Lancet*. 1999 Jul 17;354(9174):203–9.
- [437]. Oppenheimer SJ, Gibson FD, Macfarlane SB, Moody JB, Harrison C, Spencer A, et al. Iron supplementation increases prevalence and effects of malaria: report on clinical studies in Papua New Guinea. *Trans R Soc Trop Med Hyg*. 1986;80(4):603–12.
- [438]. Ahmad SH, Moonis R, Shahab T, Khan HM, Jilani T. Effect of nutritional status on total parasite count in malaria. *Indian J Pediatr*. 1985;52(416):285–7.
- [439]. Ahmad, S.H., Moonis, R., Shahab T et al. Effect of nutritional status on total parasite count in malaria. *Indian J Pediatr*. 1985;52:285–7.
- [440]. Purtilo DT, Connor DH. Fatal infections in protein-calorie malnourished children with thymolymphatic atrophy. *Arch Dis Child*. 1975 Feb 1;50(2):149–52.
- [441]. Ray AP. Haematological studies in simian malaria. II. Blood picture in monkeys during acute and chronic stages of *P. knowlesi* infection. *Indian J Malariol*. 1957 Dec;11(4):369–88.
- [442]. Bakker NPM, Eling WMC, De Groot AMT, Sinkeldam EJ, Luyken R. Attenuation of malaria infection, paralysis and lesions in the central nervous system by low protein diets in rats. *Acta Trop*. 1992 Apr;50(4):285–93.
- [443]. Edirisinghe JS, Fern EB, Targett GAT, Edirisinghe JS. Resistance to superinfection with *Plasmodium berghei* in rats fed a protein-free diet. *Trans R Soc Trop Med Hyg*. 1982;76(3):382–6.
- [444]. Fern EB, Edirisinghe JS, Targett GAT. Increased severity of malaria infection in rats fed supplementary amino acids. *Trans R Soc Trop Med Hyg*. 1984;78(6):839–41.
- [445]. Riggs A. The Amino Acid Composition of Some Mammalian Hemoglobins: Mouse, Guinea Pig, and Elephant. *J Biol Chem*. 1963 Sep;238(9):2983–7.
- [446]. Babbitt SE, Altenhofen L, Cobbold SA, Istvan ES, Fennell C, Doerig C, et al. *Plasmodium falciparum* responds to amino acid starvation by entering into a hibernatory state. *Proc Natl Acad Sci U S A*. 2012;109(47).
- [447]. McLean KJ, Jacobs-Lorena M. The response of *Plasmodium falciparum* to isoleucine withdrawal is dependent on the stage of progression through the intraerythrocytic cell cycle. *Malar J*. 2020 Dec 8;19(1):147.
- [448]. Mclean KJ, Jacobs-lorena M. *Plasmodium falciparum* Maf1 Confers Survival upon Amino Acid Starvation. 2017;8(2):1–19.
- [449]. Baertl JM, Placko RP, Graham GG. Serum proteins and plasma free amino acids in severe malnutrition. *Am J Clin Nutr*. 1974 Jul;27(7):733–42.
- [450]. McMenamy RH, Lund CC, Oncley JL. Unbound Amino Acid Concentrations in Human Blood Plasmas. *J Clin Invest*. 1957 Dec 1;36(12):1672–9.

- [451]. Jahoor F. Effects of decreased availability of sulfur amino acids in severe childhood undernutrition. *Nutr Rev.* 2012 Mar;70(3):176–87.
- [452]. Kaushansky A, Kappe SHI. Selection and refinement: the malaria parasite's infection and exploitation of host hepatocytes. *Curr Opin Microbiol.* 2015 Aug;26:71–8.
- [453]. Lindner SE, Miller JL, Kappe SHI. Malaria parasite pre-erythrocytic infection: preparation meets opportunity. *Cell Microbiol.* 2012 Mar;14(3):316–24.
- [454]. Blume M, Hliscs M, Rodriguez-Contreras D, Sanchez M, Landfear S, Lucius R, et al. A constitutive pan-hexose permease for the *Plasmodium* life cycle and transgenic models for screening of antimalarial sugar analogs . *FASEB J.* 2011;25(4):1218–29.
- [455]. Labaied M, Jayabalasingham B, Bano N, Cha SJ, Sandoval J, Guan G, et al. *Plasmodium* salvages cholesterol internalized by LDL and synthesized de novo in the liver. *Cell Microbiol.* 2011;13(4):569–86.
- [456]. Glushakova S, Busse BL, Garten M, Beck JR, Fairhurst RM, Goldberg DE, et al. Exploitation of a newly-identified entry pathway into the malaria parasite-infected erythrocyte to inhibit parasite egress. *Sci Rep.* 2017 Sep 25;7(1):12250.
- [457]. McLean KJ, Jacobs-Lorena M. *Plasmodium falciparum* Maf1 Confers Survival upon Amino Acid Starvation. *MBio.* 2017 Mar 28;8(2):1–19.
- [458]. Pombo DJ, Lawrence G, Hirunpetcharat C, Rzepczyk C, Bryden M, Cloonan N, et al. Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. *Lancet.* 2002;360(9333):610–7.
- [459]. Penha-Gonçalves C. Genetics of Malaria Inflammatory Responses: A Pathogenesis Perspective. *Front Immunol.* 2019 Jul;10:1771.
- [460]. White NJ, Turner GDH, Day NPJ, Dondorp AM. Lethal malaria: Marchiafava and Bignami were right. *J Infect Dis.* 2013 Jul;208(2):192–8.
- [461]. Guindo A, Fairhurst RM, Doumbo OK, Wellems TE, Diallo DA. X-Linked G6PD Deficiency Protects Hemizygous Males but Not Heterozygous Females against Severe Malaria. Krishna S, editor. *PLoS Med.* 2007 Mar;4(3):e66.
- [462]. Cunnington AJ, Bretscher MT, Nogaro SI, Riley EM, Walther M. Comparison of parasite sequestration in uncomplicated and severe childhood *Plasmodium falciparum* malaria. *J Infect.* 2013 Sep;67(3):220–30.
- [463]. Hill RJ, Konigsberg W, Guidotti G, Craig LC. The structure of human hemoglobin. I. The separation of the alpha and beta chains and their amino acid composition. *J Biol Chem.* 1962;237(5):1549–54.
- [464]. Braunitzer G, Gehring-Müller R, Hilschmann N, Hilse K, Hobom G, Rudloff V, et al. Die Konstitution des normalen adulten Humanhämoglobins. *Hoppe-Seyler's Zeitschrift für Physiol Chemie.* 1961 Jan;325(Jahresband):283–6.
- [465]. Walls J, Sinclair L, Finlay D. Nutrient sensing, signal transduction and immune responses. *Semin Immunol.* 2016;28(5):396–407.

- [466]. Tsalikis J, Croitoru DO, Philpott DJ, Girardin SE. Nutrient sensing and metabolic stress pathways in innate immunity. *Cell Microbiol.* 2013;15(10):1632–41.
- [467]. Kilber MS, Shan J, Su N. ATF4-Dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol Metab.* 2009;20(9):436–43.
- [468]. Soeters PB, Poll MCG Van De, Gemert WG Van, Dejong CHC. Amino Acid Adequacy in Pathophysiological States. *J Nutr.* 2004;134(6):1575S–1582S.
- [469]. Harris CT, Tong X, Campelo R, Marreiros IM, Vanheer LN, Nahiyaan N, et al. Sexual differentiation in human malaria parasites is regulated by competition between phospholipid metabolism and histone methylation. *Nat Microbiol.* 2023 Jul 1;8(7):1280–92.
- [470]. Janse CJ, Franke-Fayard B, Mair GR, Ramesar J, Thiel C, Engelmann S, et al. High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. *Mol Biochem Parasitol.* 2006;145(1):60–70.
- [471]. Iwamoto M. A general chemical method to regulate protein stability in the mammalian central nervous system. *Chem Biol.* 2011;17(9):981–8.
- [472]. Yu D, Yang SE, Miller BR, Wisinski JA, Sherman DS, Brinkman JA, et al. Short-Term methionine deprivation improves metabolic health via sexually dimorphic, mTORC1-independent mechanisms. *FASEB J.* 2018;32(6):3471–82.
- [473]. Hutson SM, Sweatt AJ, Lanoue KF. Branched-Chain Amino Acid Metabolism: Implications for Establishing Safe Intakes. *J Nutr.* 2005;1557–64.
- [474]. Dridi L, Ahmed Ouameur A, Ouellette M. High affinity S-Adenosylmethionine plasma membrane transporter of *Leishmania* is a member of the folate bipterin transporter (FBT) family. *J Biol Chem.* 2010 Jun 25;285(26):19767–75.
- [475]. le Roux D, Burger PB, Niemand J, Grobler A, Urbán P, Fernández-Busquets X, et al. Novel S-adenosyl-L-methionine decarboxylase inhibitors as potent antiproliferative agents against intraerythrocytic *Plasmodium falciparum* parasites. *Int J Parasitol Drugs Drug Resist.* 2014;4(1):28–36.
- [476]. Biljon R Van, Niemand J, Wyk R Van. Inducing controlled cell cycle arrest and re-entry during asexual proliferation of. *Sci Rep.* 2018;(August):1–27.
- [477]. van Brummelen AC, Olszewski KL, Wilinski D, Llinás M, Louw AI, Birkholtz LM. Co-inhibition of *Plasmodium falciparum* S-adenosylmethionine decarboxylase/ornithine decarboxylase reveals perturbation-specific compensatory mechanisms by transcriptome, proteome, and metabolome analyses. *J Biol Chem.* 2009 Feb 13;284(7):4635–46.
- [478]. Reininger L, Garcia M, Tomlins A, Müller S, Doerig C. The *Plasmodium falciparum*, Nima-related kinase Pfnek-4: A marker for asexual parasites committed to sexual differentiation. *Malar J.* 2012;11(250):1–11.
- [479]. Baird TD, Wek RC. Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. *Adv Nutr.* 2012;3(3):307–21.

- [480]. Hinnebusch AG. Transcriptional and Translational Regulation of Gene Expression in the General Control of Amino- Acid Biosynthesis in *Saccharomyces cerevisiae*. *Prog Nucleic Acid Res Mol Biol*. 1990;38:195–240.
- [481]. Zhang M, Gallego-Delgado J, Fernandez-Arias C, Waters NC, Rodriguez A, Tsuji M, et al. Inhibiting the Plasmodium eIF2 α Kinase PK4 Prevents Artemisinin-Induced Latency. *Cell Host Microbe*. 2017;22(6):766-776.e4.
- [482]. Reininger L, Billker O, Tewari R, Mukhopadhyay A, Fennell C, Dorin-Semblat D, et al. A NIMA-related protein kinase is essential for completion of the sexual cycle of malaria parasites. *J Biol Chem*. 2005;280(36):31957–64.
- [483]. Zhan K, Narasimhan J, Wek RC. Differential activation of eIF2 kinases in response to cellular stresses in *Schizosaccharomyces pombe*. *Genetics*. 2004;168(4):1867–75.
- [484]. Augusto L, Amin PH, Wek RC, Sullivan WJ. Regulation of arginine transport by GCN2 eIF2 kinase is important for replication of the intracellular parasite *Toxoplasma gondii*. *PLoS Pathog*. 2019;15(6):1–19.
- [485]. Reinert RB, Oberle LM, Wek SA, Bunpo P, Wang XP, Mileva I, et al. Role of Glutamine Depletion in Directing Tissue-specific Nutrient Stress Responses to L-Asparaginase. *J Biol Chem*. 2006;281(42):31222–33.
- [486]. Huang BPH, Wang Y, Wang X, Wang Z, Proud CG. Blocking eukaryotic initiation factor 4F complex formation does not inhibit the mTORC1-dependent activation of protein synthesis in cardiomyocytes. *Am J Physiol - Hear Circ Physiol*. 2009;296(2).
- [487]. Kimball SR, Horetsky RL, Jefferson LS. Implication of eIF2B rather than eIF4E in the regulation of global protein synthesis by amino acids in L6 myoblasts. *J Biol Chem*. 1998;273(47):30945–53.
- [488]. Anthony TG, McDaniel BJ, Byerley RL, McGrath BC, Cavener DB, McNurlan MA, et al. Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eIF2 kinase GCN2. *J Biol Chem*. 2004;279(35):36553–61.
- [489]. Mazor KM, Dong L, Mao Y, Swanda R V., Qian SB, Stipanuk MH. Effects of single amino acid deficiency on mRNA translation are markedly different for methionine versus leucine. *Sci Rep*. 2018;8(1):1–13.
- [490]. Klonis N, Crespo-Ortiz MP, Bottova I, Abu-Bakar N, Kenny S, Rosenthal PJ, et al. Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. *Proc Natl Acad Sci U S A*. 2011;108(28):11405–10.
- [491]. Klonis N, Xie SC, McCaw JM, Crespo-Ortiz MP, Zaloumis SG, Simpson JA, et al. Altered temporal response of malaria parasites determines differential sensitivity to artemisinin. *Proc Natl Acad Sci U S A*. 2013;110(13):5157–62.

- [492]. Saroj, Chakraborty, Sarah, Galla, Xi, Cheng, Ji-Youn Yeo, Blair Mell, Vishal Singh, BengSan Yeoh, Piu Saha, Anna V. Mathew, Matam Vijay-Kumar, Bina J. Inhibiting the Plasmodium eIF2 α kinase PK4 prevents artemisinin-induced latency. *Cell Host Microbe*. 2017;176(5):139–48.
- [493]. Janse CJ, Ramesar J, Waters AP. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat Protoc*. 2006;1(1):346–56.
- [494]. Moll K, Kaneko A, Scherf A, Wahlgren M. *Methods in Malaria Research*. 6th ed. Methods in Malaria Research. Manassas, USA; 2013.
- [495]. Trager W, Jensen JB, Journal T. Human Malaria Parasites in Continuous. *J Parasitol*. 2005;91(3):484–6.
- [496]. Lambros,Chris, Vanderberg, Jerome P. Synchronization of *Plasmodium falciparum* Erythrocytic Stages in Culture. *J Parasitol*. 1979;65(3):418–20.
- [497]. Izumiyama S, Omura M, Takasaki T, Ohmae H, Asahi H. *Plasmodium falciparum*: Development and validation of a measure of intraerythrocytic growth using SYBR Green I in a flow cytometer. *Exp Parasitol*. 2009;121(2):144–50.
- [498]. Garg A, Lukk T, Kumar V, Choi JY, Augagneur Y, Voelker DR, et al. Structure, Function and Inhibition of the Phosphoethanolamine Methyltransferases of the Human Malaria Parasites *Plasmodium vivax* and *Plasmodium knowlesi*. *Sci Rep*. 2015;5:1–13.
- [499]. Ścibior D, Skrzycki M, Podsiad M, Czeczot H. Glutathione level and glutathione-dependent enzyme activities in blood serum of patients with gastrointestinal tract tumors. *Clin Biochem*. 2008;41(10–11):852–8.
- [500]. Wendel A, Cikryt P. The level and half-life of glutathione in human plasma. *FEBS Lett*. 1980;120(2):209–11.
- [501]. Jeevanandam M, Begay CK, Shahbazian LM, Petersen SR. Altered plasma cytokines and total glutathione levels in parenterally fed critically ill trauma patients with adjuvant recombinant human growth hormone (rhGH) therapy. *Crit Care Med*. 2000;28(2):324–9.
- [502]. Chillemi R, Zappacosta B, Simporè J, Persichilli S, Musumeci M, Musumeci S. Hyperhomocysteinemia in acute *Plasmodium falciparum* malaria: an effect of host-parasite interaction. *Clin Chim Acta*. 2004 Oct;348(1–2):113–20.
- [503]. Rzepczyk CM, Saul AJ, Ferrante A. Polyamine oxidase-mediated intraerythrocyte killing of *Plasmodium falciparum*: Evidence against the role of reactive oxygen metabolites. *Infect Immun*. 1984;43(1):238–44.
- [504]. Mastrodomenico V, Esin JJ, Qazi S, Khomutov MA, Ivanov A V., Mukhopadhyay S, et al. Virion-Associated Polyamines Transmit with Bunyaviruses to Maintain Infectivity and Promote Entry. *ACS Infect Dis*. 2020;6(9):2490–501.
- [505]. Madhubala R. Thin-Layer Chromatographic Method for Assaying Polyamines. 79th ed. David M. L. Morgan, editor. *Methods in molecular biology*. Clifton, NJ: Humana Press; 1998. 131–136 p.

- [506]. Mato J, Alvarez L, Ortiz P, Pajares MA. S-adenosylmethionine synthesis: Molecular mechanisms and clinical implications. *Pharmacol Ther.* 1997 Jan;73(3):265–80.
- [507]. Mato JM, Martínez-Chantar ML, Lu SC. Methionine metabolism and liver disease. *Annu Rev Nutr.* 2008;28:273–93.
- [508]. Finkelstein JD. Methionine metabolism in mammals. *J Nutr Biochem.* 1990 May;1(5):228–37.
- [509]. Mudd SH, Poole JR. Labile methyl balances for normal humans on various dietary regimens. *Metabolism.* 1975 Jun;24(6):721–35.
- [510]. Frau M, Feo F, Pascale RM. Pleiotropic effects of methionine adenosyltransferases deregulation as determinants of liver cancer progression and prognosis. *J Hepatol.* 2013 Oct;59(4):830–41.
- [511]. Salomon B, Bluestone JA. Complexities of CD28/B7: CTLA-4 Costimulatory Pathways in Autoimmunity and Transplantation. *Annu Rev Immunol.* 2001 Apr;19(1):225–52.
- [512]. Pretzel J, Gehr M, Eisenkolb M, Wang L, Fritz-Wolf K, Rahlfs S, et al. Characterization and redox regulation of *Plasmodium falciparum* methionine adenosyltransferase. *J Biochem.* 2016 Dec;160(6):355–67.
- [513]. Chiang PK, Chamberlin ME, Nicholson D, Soubes S, Su X zhuan, Subramanian G, et al. Molecular characterization of *Plasmodium falciparum* S-adenosylmethionine synthetase. *Biochem J.* 1999 Dec 1;344 Pt 2(Pt 2):571–6.
- [514]. Aurrecochea C, Brestelli J, Brunk BP, Dommer J, Fischer S, Gajria B, et al. PlasmoDB: A functional genomic database for malaria parasites. *Nucleic Acids Res.* 2009;37(SUPPL. 1):539–43.
- [515]. Pollack Y, Kogan N, Golenser J. *Plasmodium falciparum*: Evidence for a DNA methylation pattern. *Exp Parasitol.* 1991 May;72(4):339–44.
- [516]. Ponts N, Fu L, Harris EY, Zhang J, Chung DWD, Cervantes MC, et al. Genome-wide Mapping of DNA Methylation in the Human Malaria Parasite *Plasmodium falciparum*. *Cell Host Microbe.* 2013 Dec 11;14(6):696–706.
- [517]. Lüersen K, Walter RD, Müller S. *Plasmodium falciparum*-infected red blood cells depend on a functional glutathione de novo synthesis attributable to an enhanced loss of glutathione. *Biochem J.* 2000 Mar 1;346 Pt 2(Pt 2):545–52.
- [518]. Marreiros IM, Marques S, Parreira A, Mastrodomenico V, Mounce BC, Harris CT, et al. A non-canonical sensing pathway mediates *Plasmodium* adaptation to amino acid deficiency. *Commun Biol.* 2023 Feb;6(1):205.
- [519]. Dembélé L, Franetich J François, Lorthiois A, Gego A, Zeeman A marie, Kocken CHM, et al. Persistence and activation of malaria hypnozoites in long-term primary hepatocyte cultures. *Nat Med.* 2014;20(3):307–12.
- [520]. Giuliodori P, Galli-Kienle M, Catto E, Stramentinoli G. Transmethylation, transsulfuration, and aminopropylation reactions of S-adenosyl-L-methionine in vivo. *J Biol Chem.* 1984 Apr 10;259(7):4205–11.

- [521]. Marreiros IM, Marques S, Parreira A, Mastrodomenico V, Mounce BC, Harris CT, et al. A non-canonical sensing pathway mediates Plasmodium adaptation to amino acid deficiency. *Commun Biol.* 2023 Feb 21;6(1):205.
- [522]. Lu SC, Mato JM. S-adenosylmethionine in liver health, injury, and cancer. *Physiol Rev.* 2012 Oct;92(4):1515–42.
- [523]. Sullivan DM, Hoffman JL. Fractionation and kinetic properties of rat liver and kidney methionine adenosyltransferase isozymes. *Biochemistry.* 1983 Mar 1;22(7):1636–41.
- [524]. A Research Agenda for Malaria Eradication: Basic Science and Enabling Technologies. *PLoS Med.* 2011 Jan 25;8(1):e1000399.
- [525]. Gueirard P, Tavares J, Thiberge S, Bernex F, Ishino T, Milon G, et al. Development of the malaria parasite in the skin of the mammalian host. *Proc Natl Acad Sci.* 2010 Oct 26;107(43):18640–5.
- [526]. Sinnis P, Zavala F. The skin: where malaria infection and the host immune response begin. *Semin Immunopathol.* 2012 Nov 2;34(6):787–92.
- [527]. Coppi A, Tewari R, Bishop JR, Bennett BL, Lawrence R, Esko JD, et al. Heparan Sulfate Proteoglycans Provide a Signal to Plasmodium Sporozoites to Stop Migrating and Productively Invade Host Cells. *Cell Host Microbe.* 2007 Nov;2(5):316–27.
- [528]. Rodrigues CD, Hannus M, Prudêncio M, Martin C, Gonçalves LA, Portugal S, et al. Host scavenger receptor SR-BI plays a dual role in the establishment of malaria parasite liver infection. *Cell Host Microbe.* 2008 Sep 11;4(3):271–82.
- [529]. Yalaoui S, Huby T, Franetich JF, Gego A, Rametti A, Moreau M, et al. Scavenger Receptor BI Boosts Hepatocyte Permissiveness to Plasmodium Infection. *Cell Host Microbe.* 2008 Sep;4(3):283–92.
- [530]. Kaushansky A, Douglass AN, Arang N, Vigdorovich V, Dambrauskas N, Kain HS, et al. Malaria parasites target the hepatocyte receptor EphA2 for successful host infection. *Science (80-).* 2015 Nov 27;350(6264):1089–92.
- [531]. Meireles P, Mendes AM, Aroeira RI, Mounce BC, Vignuzzi M, Staines HM, et al. Uptake and metabolism of arginine impact Plasmodium development in the liver. *Sci Rep.* 2017;7(1):1–12.
- [532]. Epiphany S, Mikolajczak SA, Gonçalves LA, Pamplona A, Portugal S, Albuquerque S, et al. Heme Oxygenase-1 Is an Anti-Inflammatory Host Factor that Promotes Murine Plasmodium Liver Infection. *Cell Host Microbe.* 2008 May;3(5):331–8.
- [533]. Epiphany S, Campos MG, Pamplona A, Carapau D, Pena AC, Ataíde R, et al. VEGF promotes malaria-associated acute lung injury in Mice. *PLoS Pathog.* 2010;6(5):1–10.
- [534]. Portugal S, Carret C, Recker M, Armitage AE, Gonçalves LA, Epiphany S, et al. Host-mediated regulation of superinfection in malaria. *Nat Med.* 2011 Jun 15;17(6):732–7.
- [535]. Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphany S, et al. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat Med.* 2007 Jun 13;13(6):703–10.

- [536]. Mancio-Silva L, Slavic K, Grilo Ruivo MT, Grosso AR, Modrzynska KK, Vera IM, et al. Nutrient sensing modulates malaria parasite virulence. *Nature*. 2017;547(7662).
- [537]. Zuzarte-Luís V, Mello-Vieira J, Marreiros IM, Liehl P, Chora ÂF, Carret CK, et al. Dietary alterations modulate susceptibility to *Plasmodium* infection. *Nat Microbiol*. 2017;2(12):1600–7.
- [538]. Meireles P, Brás D, Fontinha D, Chora ÂF, Serre K, Mendes AM, et al. Elimination of Hepatic Rodent *Plasmodium* Parasites by Amino Acid Supplementation. *iScience*. 2020 Dec;23(12):101781.
- [539]. Cobbold SA, Martin RE, Kirk K. Methionine transport in the malaria parasite *Plasmodium falciparum*. *Int J Parasitol*. 2011;41(1):125-35 OD-2010/09/21.
- [540]. Pretzel J, Gehr M, Eisenkolb M, Wang L, Fritz-Wolf K, Rahlfs S, et al. Characterization and redox regulation of *Plasmodium falciparum* methionine adenosyltransferase OD - 2016/07/29. *J Biochem*. 2016;
- [541]. Chiang PK, Chamberlin ME, Nicholson D, Soubes S, Su X, Subramanian G, et al. Molecular characterization of *Plasmodium falciparum* S-adenosylmethionine synthetase. *Biochem J*. 1999;344 Pt 2:571-6 OD-1999/11/24.
- [542]. Aurrecoechea C, Brestelli J, Brunk BP, Dommer J, Fischer S, Gajria B, et al. PlasmoDB: a functional genomic database for malaria parasites. *Nucleic Acids Res*. 2009;37(Database issue):D539-43 OD-2008/10/30.
- [543]. Luersen K, Walter RD, Muller S. *Plasmodium falciparum*-infected red blood cells depend on a functional glutathione de novo synthesis attributable to an enhanced loss of glutathione. *Biochem J*. 2000;346 Pt 2:545-52 OD-2000/03/24.
- [544]. van Brummelen AC, Olszewski KL, Wilinski D, Llinas M, Louw AI, Birkholtz LM. Co-inhibition of *Plasmodium falciparum* S-adenosylmethionine decarboxylase/ornithine decarboxylase reveals perturbation-specific compensatory mechanisms by transcriptome, proteome, and metabolome analyses. *J Biol Chem*. 2009;284(7):4635-46 OD-2008/12/17.
- [545]. Pollack Y, Kogan N, Golenser J. *Plasmodium falciparum*: evidence for a DNA methylation pattern. *Exp Parasitol*. 1991;72(4):339-44 OD-1991/05/01.
- [546]. Ponts N, Fu L, Harris EY, Zhang J, Chung DW, Cervantes MC, et al. Genome-wide mapping of DNA methylation in the human malaria parasite *Plasmodium falciparum*. *Cell Host Microbe*. 2013;14(6):696-706 OD-2013/12/18.
- [547]. Meireles P, Mendes AM, Aroeira RI, Mounce BC, Vignuzzi M, Staines HM, et al. Uptake and metabolism of arginine impact *Plasmodium* development in the liver. *Sci Rep*. 2017 Jun;7(1):4072.
- [548]. Lu SC, Mato JM. S-Adenosylmethionine in cell growth, apoptosis and liver cancer. *J Gastroenterol Hepatol*. 2008 Mar;23 Suppl 1(Suppl 1):S73-7.
- [549]. Mato JM, Corrales FJ, Lu SC, Avila M a. S-Adenosylmethionine: a control switch that regulates liver function. *FASEB J*. 2002;16(1):15–26.

- [550]. Tucker AM, Winkler HH, Driskell LO, Wood DO. S-adenosylmethionine transport in *Rickettsia prowazekii*. *J Bacteriol.* 2003 May;185(10):3031–5.
- [551]. Merali S, Clarkson AB. S-adenosylmethionine and *Pneumocystis*. *FEMS Microbiol Lett.* 2004 Aug;237(2):179–86.
- [552]. Merali S, Saric M, Chin K, Clarkson AB. Effect of a bis-benzyl polyamine analogue on *Pneumocystis carinii*. *Antimicrob Agents Chemother.* 2000 Feb;44(2):337–43.
- [553]. Dridi L, Ahmed Ouameur A, Ouellette M. High affinity S-Adenosylmethionine plasma membrane transporter of *Leishmania* is a member of the folate bipterin transporter (FBT) family. *J Biol Chem.* 2010;285(26):19767-75 OD-2010/04/22.
- [554]. Goldberg B, Rattendi D, Lloyd D, Sufrin JR, Bacchi CJ. Effects of Intermediates of Methionine Metabolism and Nucleoside Analogs on S-Adenosylmethionine Transport by *Trypanosoma brucei brucei* and a Drug-resistant *Trypanosoma brucei rhodesiense*. *Biochem Pharmacol.* 1998 Jul;56(1):95–103.
- [555]. Behnke MS, Khan A, Sibley LD. Genetic mapping reveals that sinefungin resistance in *Toxoplasma gondii* is controlled by a putative amino acid transporter locus that can be used as a negative selectable marker. *Eukaryot Cell.* 2015;14(2):140-8 OD-2014/12/07.
- [556]. Zhang W, Sargis RM, Volden PA, Carmean CM, Sun XJ, Brady MJ. PCB 126 and Other Dioxin-Like PCBs Specifically Suppress Hepatic PEPCK Expression via the Aryl Hydrocarbon Receptor. Andrabi SA, editor. *PLoS One.* 2012 May 16;7(5):e37103.
- [557]. Drakeley C, Sutherland C, Bousema JT, Sauerwein RW, Targett GAT. The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. *Trends Parasitol.* 2006;22(9):424–30.
- [558]. Sinha A, Hughes KR, Modrzynska KK, Otto TD, Pfander C, Dickens NJ, et al. A cascade of DNA-binding proteins for sexual commitment and development in *Plasmodium*. *Nature.* 2014 Mar 13;507(7491):253–7.
- [559]. Kafsack BFC, Rovira-Graells N, Clark TG, Bancells C, Crowley VM, Campino SG, et al. A transcriptional switch underlies commitment to sexual development in malaria parasites. *Nature.* 2014 Mar 23;507(7491):248–52.
- [560]. Brancucci NMB, Witmer K, Schmid C, Voss TS. A var Gene Upstream Element Controls Protein Synthesis at the Level of Translation Initiation in *Plasmodium falciparum*. Spielmann T, editor. *PLoS One.* 2014 Jun 17;9(6):e100183.
- [561]. Fräschka SA, Filarsky M, Hoo R, Niederwieser I, Yam XY, Brancucci NMB, et al. Comparative Heterochromatin Profiling Reveals Conserved and Unique Epigenome Signatures Linked to Adaptation and Development of Malaria Parasites. *Cell Host Microbe.* 2018 Mar 14;23(3):407-420.e8.
- [562]. Lopez-Rubio JJ, Mancio-Silva L, Scherf A. Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. *Cell Host Microbe.* 2009 Feb 19;5(2):179–90.

- [563]. Coleman BI, Skillman KM, Jiang RHY, Childs LM, Altenhofen LM, Ganter M, et al. A *Plasmodium falciparum* Histone Deacetylase Regulates Antigenic Variation and Gametocyte Conversion. *Cell Host Microbe*. 2014 Aug;16(2):177–86.
- [564]. Filarsky M, Fraschka SA, Niederwieser I, Brancucci NMB, Carrington E, Carrió E, et al. GDV1 induces sexual commitment of malaria parasites by antagonizing HP1-dependent gene silencing. *Science* (80-). 2018 Mar 16;359(6381):1259–63.
- [565]. Poran A, Nötzl C, Aly O, Mencia-Trinchant N, Harris CT, Guzman ML, et al. Single-cell RNA sequencing reveals a signature of sexual commitment in malaria parasites. *Nature*. 2017 Nov 2;551(7678):95–9.
- [566]. Josling GA, Russell TJ, Venezia J, Orchard L, van Biljon R, Painter HJ, et al. Dissecting the role of PfAP2-G in malaria gametocytogenesis. *Nat Commun*. 2020 Mar 20;11(1):1503.
- [567]. Llorà-Batlle O, Michel-Todó L, Witmer K, Toda H, Fernández-Becerra C, Baum J, et al. Conditional expression of PfAP2-G for controlled massive sexual conversion in *Plasmodium falciparum*. *Sci Adv*. 2020 Jun;6(24):eaaz5057.
- [568]. Kent RS, Modrzynska KK, Cameron R, Philip N, Billker O, Waters AP. Inducible developmental reprogramming redefines commitment to sexual development in the malaria parasite *Plasmodium berghei*. *Nat Microbiol*. 2018 Nov;3(11):1206–13.
- [569]. Neveu G, Beri D, Kafsack BF. Metabolic regulation of sexual commitment in *Plasmodium falciparum*. *Curr Opin Microbiol*. 2020 Dec;58:93–8.
- [570]. Pollitt LC, Mideo N, Drew DR, Schneider P, Colegrave N, Reece SE. Competition and the evolution of reproductive restraint in malaria parasites. *Am Soc Nat*. 2011 Mar;177(3):358–67.
- [571]. Joice R, Nilsson SK, Montgomery J, Dankwa S, Egan E, Morahan B, et al. *Plasmodium falciparum* transmission stages accumulate in the human bone marrow. *Sci Transl Med*. 2014 Jul 9;6(244):244re5.
- [572]. Venugopal K, Hentzschel F, Valkiūnas G, Marti M. *Plasmodium* asexual growth and sexual development in the haematopoietic niche of the host. *Nat Rev Microbiol*. 2020 Mar;18(3):177–89.
- [573]. Gulati S, Ekland EH, Ruggles K V., Chan RB, Jayabalasingham B, Zhou B, et al. Profiling the Essential Nature of Lipid Metabolism in Asexual Blood and Gametocyte Stages of *Plasmodium falciparum*. *Cell Host Microbe*. 2015 Sep;18(3):371–81.
- [574]. Wein S, Ghezal S, Buré C, Maynadier M, Périgaud C, Vial HJ, et al. Contribution of the precursors and interplay of the pathways in the phospholipid metabolism of the malaria parasite. *J Lipid Res*. 2018 Aug;59(8):1461–71.
- [575]. Kilian N, Choi JY, Voelker DR, Mamoun C Ben. Role of phospholipid synthesis in the development and differentiation of malaria parasites in the blood. *J Biol Chem*. 2018;293(45):17308–16.

- [576]. Witola WH, Ben Mamoun C. Choline induces transcriptional repression and proteasomal degradation of the malarial phosphoethanolamine methyltransferase. *Eukaryot Cell*. 2007 Sep;6(9):1618–24.
- [577]. Ye C, Sutter BM, Wang Y, Kuang Z, Tu BP. A Metabolic Function for Phospholipid and Histone Methylation. *Mol Cell*. 2017 Apr 20;66(2):180-193.e8.
- [578]. Prommana P, Uthaipibull C, Wongsombat C, Kamchonwongpaisan S, Yuthavong Y, Knuepfer E, et al. Inducible Knockdown of Plasmodium Gene Expression Using the glmS Ribozyme. Craig AG, editor. *PLoS One*. 2013 Aug 30;8(8):e73783.
- [579]. Reyes P, Rathod PK, Sanchez DJ, Mrema JE, Rieckmann KH, Heidrich HG. Enzymes of purine and pyrimidine metabolism from the human malaria parasite, *Plasmodium falciparum*. *Mol Biochem Parasitol*. 1982 May;5(5):275–90.
- [580]. Déchamps S, Maynadier M, Wein S, Gannoun-Zaki L, Maréchal E, Vial HJ. Rodent and nonrodent malaria parasites differ in their phospholipid metabolic pathways. *J Lipid Res*. 2010 Jan;51(1):81–96.
- [581]. Ye C, Sutter BM, Wang Y, Kuang Z, Zhao X, Yu Y, et al. Demethylation of the Protein Phosphatase PP2A Promotes Demethylation of Histones to Enable Their Function as a Methyl Group Sink. *Mol Cell*. 2019 Mar;73(6):1115-1126.e6.
- [582]. Mentch SJ, Mehrmohamadi M, Huang L, Liu X, Gupta D, Mattocks D, et al. Histone Methylation Dynamics and Gene Regulation Occur through the Sensing of One-Carbon Metabolism. *Cell Metab*. 2015 Nov 3;22(5):861–73.
- [583]. Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng Y, Teo RY, Ratanasirintrawoot S, et al. Influence of threonine metabolism on S-adenosylmethionine and histone methylation. *Science* (80-). 2013 Jan 11;339(6116):222–6.
- [584]. Li S, Swanson SK, Gogol M, Florens L, Washburn MP, Workman JL, et al. Serine and SAM Responsive Complex SESAME Regulates Histone Modification Crosstalk by Sensing Cellular Metabolism. *Mol Cell*. 2015;60(3):408–21.
- [585]. Sutter BM, Wu X, Laxman S, Tu BP. Methionine inhibits autophagy and promotes growth by inducing the SAM-responsive methylation of PP2A. *Cell*. 2013 Jul 18;154(2):403–15.
- [586]. Morillo RC, Harris CT, Kennedy K, Henning SR, Kafsack BF. Genome-wide profiling of histone modifications in *Plasmodium falciparum* using CUT&RUN. *Life Sci Alliance*. 2023 Jan 15;6(1):e202201778.
- [587]. Salcedo-Amaya AM, van Driel MA, Alako BT, Trelle MB, van den Elzen AMG, Cohen AM, et al. Dynamic histone H3 epigenome marking during the intraerythrocytic cycle of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A*. 2009 Jun 16;106(24):9655–60.
- [588]. Karmodiya K, Pradhan SJ, Joshi B, Jangid R, Reddy PC, Galande S. A comprehensive epigenome map of *Plasmodium falciparum* reveals unique mechanisms of transcriptional regulation and identifies H3K36me2 as a global mark of gene suppression. *Epigenetics Chromatin*. 2015 Dec 17;8(1):32.

- [589]. Reguera RM, Redondo CM, Pérez-Pertejo Y, Balaña-Fouce R. S-Adenosylmethionine in protozoan parasites: functions, synthesis and regulation. *Mol Biochem Parasitol.* 2007 Mar;152(1):1–10.
- [590]. Luo M. Chemical and Biochemical Perspectives of Protein Lysine Methylation. *Chem Rev.* 2018 Jul 25;118(14):6656–705.
- [591]. Chiang PK. Biological effects of inhibitors of S-adenosylhomocysteine hydrolase. *Pharmacol Ther.* 1998 Feb;77(2):115–34.
- [592]. Bujnicki JM, Prigge ST, Caridha D, Chiang PK. Structure, evolution, and inhibitor interaction of S-adenosyl-L-homocysteine hydrolase from *Plasmodium falciparum*. *Proteins.* 2003 Sep 1;52(4):624–32.
- [593]. Beri D, Balan B, Chaubey S, Subramaniam S, Surendra B, Tatu U. A disrupted transsulphuration pathway results in accumulation of redox metabolites and induction of gametocytogenesis in malaria. *Sci Rep.* 2017 Jan 16;7(1):40213.
- [594]. Neveu G, Richard C, Dupuy F, Behera P, Volpe F, Subramani PA, et al. *Plasmodium falciparum* sexual parasites develop in human erythroblasts and affect erythropoiesis. *Blood.* 2020 Sep 17;136(12):1381–93.
- [595]. Tibúrcio M, Niang M, Deplaine G, Perrot S, Bischoff E, Ndour PA, et al. A switch in infected erythrocyte deformability at the maturation and blood circulation of *Plasmodium falciparum* transmission stages. *Blood.* 2012 Jun 14;119(24):e172–80.
- [596]. Trager W, Gill GS, Lawrence C, Nagel RL. *Plasmodium falciparum*: Enhanced Gametocyte Formation in Vitro in Reticulocyte-Rich Blood. *Exp Parasitol.* 1999 Feb;91(2):115–8.
- [597]. Hentzschel F, Gibbins MP, Attipa C, Beraldi D, Moxon CA, Otto TD, et al. Host cell maturation modulates parasite invasion and sexual differentiation in *Plasmodium berghei*. *Sci Adv.* 2022 Apr 29;8(17).
- [598]. Trager W, Gill GS. Enhanced gametocyte formation in young erythrocytes by *Plasmodium falciparum* in vitro. *J Protozool.* 1992 May;39(3):429–32.
- [599]. Percy AK, Schmell E, Earles BJ, Lennarz WJ. Phospholipid biosynthesis in the membranes of immature and mature red blood cells. *Biochemistry.* 1973 Jun 1;12(13):2456–61.
- [600]. Huang NJ, Lin YC, Lin CY, Pishesha N, Lewis CA, Freinkman E, et al. Enhanced phosphocholine metabolism is essential for terminal erythropoiesis. *Blood.* 2018 Jun 28;131(26):2955–66.
- [601]. Otto TD, Böhme U, Jackson AP, Hunt M, Franke-Fayard B, Hoeijmakers WAM, et al. A comprehensive evaluation of rodent malaria parasite genomes and gene expression. *BMC Biol.* 2014 Dec 30;12(1):86.
- [602]. Fougère A, Jackson AP, Paraskevi Bechti D, Braks JAM, Annoura T, Fonager J, et al. Variant Exported Blood-Stage Proteins Encoded by *Plasmodium* Multigene Families Are Expressed in Liver Stages Where They Are Exported into the Parasitophorous Vacuole. *PLoS Pathog.* 2016;12(11):1–37.

- [603]. McLean KJ, Straimer J, Hopp CS, Vega-Rodriguez J, Small-Saunders JL, Kanatani S, et al. Generation of Transmission-Competent Human Malaria Parasites with Chromosomally-Integrated Fluorescent Reporters. *Sci Rep*. 2019 Sep 11;9(1):13131.
- [604]. Ballinger E, Mosior J, Hartman T, Burns-Huang K, Gold B, Morris R, et al. Opposing reactions in coenzyme A metabolism sensitize *Mycobacterium tuberculosis* to enzyme inhibition. *Science* (80-). 2019;363(6426).
- [605]. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009 Jul 15;25(14):1754–60.
- [606]. Li, H. & Handsaker, B. & Wysoker, Alec & Fennell, T. & Ruan, Jue & Homer, N. & Marth, G. & Abecasis, Goncalo & Durbin, R.. 1000 genome project data processing subgroup. The sequence alignment/map (SAM) format and SAMtools. *Bioinformatics*. 2010;25:2078–9.
- [607]. Zhang Y, Liu T, Meyer CA, Eeckhoutte J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol*. 2008;9(9):R137.
- [608]. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*. 2010 Mar 15;26(6):841–2.
- [609]. Lawrence M, Huber W, Pagès H, Aboyoun P, Carlson M, Gentleman R, et al. Software for Computing and Annotating Genomic Ranges. *Prlic A, editor. PLoS Comput Biol*. 2013 Aug 8;9(8):e1003118.
- [610]. Hahne F, Ivanek R. Visualizing Genomic Data Using Gviz and Bioconductor. In: *Methods in Molecular Biology*. 2016. p. 335–51.
- [611]. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, et al. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods*. 2015;12(2):115–21.
- [612]. Brunet S, Sardon T, Zimmerman T, Wittmann T, Pepperkok R, Karsenti E, et al. Characterization of the TPX2 Domains Involved in Microtubule Nucleation and Spindle Assembly in *Xenopus* nucleation around chromatin and functions in a network of other molecules , some of which also are regulated by. *Mol Biol Cell*. 2004;15(December):5318–28.
- [613]. García-Trevijano ER, Latasa MU, Carretero MV, Berasain C, Mato JM, Avila MA. S-Adenosylmethionine regulates MAT1A and MAT2A gene expression in cultured rat hepatocytes: a new role for S-adenosylmethionine in the maintenance of the differentiated status of the liver. *FASEB J*. 2000 Dec;14(15):2511–8.
- [614]. Merali S, Clarkson AB. S-Adenosylmethionine and *Pneumocystis*. *FEMS Microbiol Lett*. 2004 Aug;237(2):179–86.
- [615]. Behnke MS, Khan A, Sibley LD. Genetic mapping reveals that sinefungin resistance in *Toxoplasma gondii* is controlled by a putative amino acid transporter locus that can be used as a negative selectable marker. *Eukaryot Cell*. 2015 Feb;14(2):140–8.
- [616]. Kenthirapalan S, Waters AP, Matuschewski K, Kooij TWA. Functional profiles of orphan membrane transporters in the life cycle of the malaria parasite. *Nat Commun*. 2016 Jan 22;7(1):10519.

- [617]. Slavic K, Delves MJ, Prudêncio M, Talman AM, Straschil U, Derbyshire ET, et al. Use of a selective inhibitor to define the chemotherapeutic potential of the plasmodial hexose transporter in different stages of the parasite's life cycle. *Antimicrob Agents Chemother*. 2011 Jun;55(6):2824–30.
- [618]. Watson RB. Malaria Parasites and Other Haemosporidia. *Am J Trop Med Hyg*. 1967 Jul 1;16(4):561–3.

