The Effect of Dietary L-arginine on Malaria-Induced Dysregulation of Transporter Expression in a Murine Model of Placental Malaria.

by

Najwa Najjar

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Pharmaceutical Sciences
University of Toronto

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Najwa Najjar
Master of Science

Department of Pharmaceutical Sciences
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Abstract

Placental Malaria (PM) alters the expression of clinically important transporters in maternal and fetal tissue. Pregnancy and malaria are known to result in a state of arginine deficiency and quenching of nitric oxide (NO), respectively. We hypothesized that reducing oxidative stress during PM by dietary L-arginine supplementation, a NO precursor, would attenuate changes in transporters and improve pregnancy outcomes. Pregnant BALB/c mice receiving 1.2% L-arginine in water or plain water were infected with Plasmodium berghei on day 13 and sacrificed on day 19. The expression of numerous ABC and SLC transporters were dysregulated in PM. Supplementation with L-arginine did not improve the mRNA expression of oxidative stress markers, transporters, nor indices of fetal and maternal health. L-arginine may not have been a potent enough compound to reduce oxidative stress in PM, or the dosage received was too low. Further studies are needed in order to reduce oxidative stress in PM.
Acknowledgements

First, I would like to express my deepest gratitude to my supervisor, Dr. Micheline Piquette-Miller. Thank you for your guidance and encouragement, and for accepting me into your laboratory. Your confidence and success as a scientist have inspired me since the first day I joined your laboratory. You have provided me with many opportunities that have helped me grow in the world of Pharmaceutical Sciences.

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Table of Contents

Acknowledgements ........................................................................................................ iii
Table of Contents ........................................................................................................ iv
List of Tables ................................................................................................................ vi
List of Figures .............................................................................................................. vii
List of Abbreviations ..................................................................................................... ix

1 Introduction ................................................................................................................ 1
  1.1 Malaria .................................................................................................................. 1
  1.2 Drug-Disposition ..................................................................................................... 5
    1.2.1 Transporters ..................................................................................................... 5
    1.2.2 Drug Metabolizing Enzymes .......................................................................... 16
  1.3 Inflammation ......................................................................................................... 16
  1.4 Effect of Malaria on Gene Expression .................................................................. 17
    1.4.1 Maternal and Fetal Parameters ...................................................................... 18
    1.4.2 Placenta .......................................................................................................... 18
    1.4.3 Maternal Liver ................................................................................................ 18
    1.4.4 Fetal Liver ....................................................................................................... 18
    1.4.5 Total Plasma Bile Acid Levels ....................................................................... 19
    1.4.6 Maternal Brain (whole brain tissue) .............................................................. 19
    1.4.7 Maternal Kidney ............................................................................................. 19
  1.5 Nitric Oxide ........................................................................................................... 19
  1.6 L-arginine .............................................................................................................. 21

2 Hypothesis .................................................................................................................. 24
  2.1 Rationale ................................................................................................................ 24
  2.2 Hypothesis ............................................................................................................. 24
  2.3 Objective ................................................................................................................ 25

3 Methods ...................................................................................................................... 26
  3.1 Animal Studies ..................................................................................................... 26
    3.1.1 RNA Extractions, cDNA Synthesis and Quantitative-Polymerase Chain Reaction (RT-qPCR) ................................................................. 27
    3.1.2 RNA Extraction ............................................................................................. 27
    3.1.3 DNase I Treatment ......................................................................................... 27
    3.1.4 cDNA Synthesis ............................................................................................ 28
    3.1.5 Quantitative-Polymerase Chain Reaction (RT-qPCR) ...................................... 28
  3.2 Total Bile Acid ....................................................................................................... 28
    3.2.1 Assay Procedure ............................................................................................ 29
    3.2.2 Determining total bile acids concentration ................................................. 29
  3.3 Statistical Analysis ................................................................................................. 29

4 Results ......................................................................................................................... 30
  4.1 Plasmodium berghei ANKA Malaria Infection Impacts Maternal and Fetal Parameters 30
    4.1.1 Placental, Maternal and Fetal Weight ............................................................ 30
    4.1.2 Spleen Weight ................................................................................................. 31
  4.2 Plasmodium berghei ANKA Malaria Infection Alters Placental Transporter Expression 32
List of Tables

Chapter 1
Table 1: Clinically Important ABC Efflux Transporters.................................6
Table 2: Clinically Important SLC Uptake Transporters.................................8
Table 3: Malaria-Induced Changes on Hepatic and Placental Transporters..........9
Table 4: Endogenous substrates and expression of the SLC uptake transporters.....15

Chapter 2
No table(s).

Chapter 3
No table(s).

Chapter 4
No table(s).

Chapter 5
No table(s).

Chapter 6
No table(s).
List of Figures

Chapter 1
Figure 1: The life cycle and pathogenesis of malaria………………………………...........2
Figure 2: The Physiological Roles of NO Relevant to Malaria Pathogenesis.............20

Chapter 2
No figure(s).

Chapter 3
No figure(s)

Chapter 4
Figure 3: Impact of Plasmodium berghei ANKA Malaria Infection on (A) placental weight, (B) maternal weight, and (C) fetal weight on GD19.................................30
Figure 4: Impact of Plasmodium berghei ANKA Malaria Infection on spleen weight on GD19.................................................................................................................31
Figure 5: Impact of Plasmodium berghei ANKA Malaria Infection on ABC efflux transporters in the placenta on GD19.................................................................32
Figure 6: Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in the placenta on GD19.................................................................33
Figure 7: Impact of Plasmodium berghei ANKA Malaria Infection on ABC efflux transporters in maternal liver on GD19.................................................................34
Figure 8: Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in maternal liver on GD19.................................................................35
Figure 9: Impact of Plasmodium berghei ANKA Malaria Infection on Hepatic Expression of iNoS and HO-1 in maternal liver on GD19.............................................36
Figure 10: Impact of Plasmodium berghei ANKA Malaria Infection on ABC efflux transporters in maternal brain on GD19.................................................................37
Figure 11: Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in maternal brain on GD19.................................................................38
Figure 12: Impact of Plasmodium berghei ANKA Malaria Infection on ABC efflux transporters in maternal kidney on GD19.................................................................39
Figure 13: Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in maternal kidney on GD19.................................................................40
Figure 14: Impact of Plasmodium berghei ANKA Malaria Infection on ABC efflux transporters in fetal liver on GD19.................................................................41

Figure 15: Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in fetal liver on GD19.................................................................42

Figure 16: Impact of Plasmodium berghei ANKA Malaria Infection on Hepatic Expression of HO-1 in the Fetal Liver GD19.................................................................43

Figure 17: Impact of Plasmodium berghei ANKA Malaria Infection on Maternal Plasma Bile Acid levels on GD19.................................................................44

Figure 18: Impact of Plasmodium berghei ANKA Malaria Infection on Whole Fetus Bile Acid levels on GD19.................................................................45

Chapter 5
No figure(s)

Chapter 6
No figure(s)
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AGM</td>
<td>Agmatine</td>
</tr>
<tr>
<td>AK</td>
<td>Adenosine kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein (ABCG2)</td>
</tr>
<tr>
<td>BSEP</td>
<td>Bile salt export pump (ABCB11)</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin sulfate A</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ENT1</td>
<td>Equilibrative Nucleoside Transporter 1 (SLC29A1)</td>
</tr>
<tr>
<td>ENT2</td>
<td>Equilibrative Nucleoside Transporter 2 (SLC29A2)</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational day</td>
</tr>
<tr>
<td>GPIs</td>
<td>Glycosylphosphatidylinositols</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase 1</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>iRBC</td>
<td>Infected red blood cell</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>LBW</td>
<td>Low birth weight</td>
</tr>
<tr>
<td>MATE1</td>
<td>Multidrug and Toxin Extrusion Transporter 1 (SLC47A1)</td>
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<tr>
<td>MDR1</td>
<td>Multidrug resistance protein 1 (PGP; ABCB1A/B)</td>
</tr>
<tr>
<td>MPP</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MRP1</td>
<td>Multidrug Resistance Associated Proteins (ABCC1)</td>
</tr>
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<td>MRP4</td>
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</tr>
<tr>
<td>NBF</td>
<td>Nucleotide binding folds</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NTCP</td>
<td>Sodium-Taurocholate Co-Transporting Polypeptide (SLC10A1)</td>
</tr>
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<td>OAT1</td>
<td>Organic Anion Transporter 1 (SLC22A6)</td>
</tr>
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<td>Organic Anion Transporter 3 (SLC22A8)</td>
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<tr>
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<td>OCT1</td>
<td>Organic Cation Transporter 1 (SLC22A1)</td>
</tr>
<tr>
<td>OCT2</td>
<td>Organic Cation Transporter 2 (SLC22A2)</td>
</tr>
<tr>
<td>PAH</td>
<td>p-aminohippuric acid</td>
</tr>
<tr>
<td>PbA</td>
<td><em>Plasmodium</em> berghei ANKA</td>
</tr>
<tr>
<td>PCV2</td>
<td>Porcine circovirus type 2</td>
</tr>
<tr>
<td>PEPT2</td>
<td>Peptide Transporter 2 (SLC15A2)</td>
</tr>
</tbody>
</table>
PfEMP1  Plasmodium falciparum Erythrocyte Membrane Protein 1
PGP  P-glycoprotein (ABCB1A/B; MDR1)
PHT  Phagosome nutrient transporter (Slc15a)
PM  Placental malaria
PRBCs  Parasitized red blood cells
qRT-PCR  Quantitative reverse transcriptase-polymerase chain reaction
RBCs  Red blood cells
RNA  Ribonucleic acid
SAA  Serum amyloid A
SLC  Solute-carrier transporter
SNP  Single nucleotide polymorphism
TEA  Tetraethylammonium
TM  Transmembrane
Chapter 1. Introduction

1

1.1 Malaria

Malaria is one of the leading causes of morbidity and mortality globally, with an estimated 90% of all deaths occurring in sub-Saharan Africa [1]. There are five human malaria parasite species, *Plasmodium* (*P.*) falciparum, *P.* vivax, *P.* ovale, *P.* malaria and *P.* knowlesi [2]. *P.* falciparum is the most dangerous and deadly plasmodia among non-pregnant and pregnant individuals, because of its ability to adhere to endothelium and sequester in deep vascular beds during its intraerythrocytic developmental stage [2]–[4]. Malaria is transmitted to the host exclusively through the bite of the blood feeding Anopheles sp. Mosquito. Anopheles sp. Mosquitoes inject infectious parasites/sporozoites into the bloodstream. Within a few minutes, the sporozoites are carried to the liver, where they invade and replicate in liver cells. 10–12 days later, thousands of daughter merozoites are released back into the bloodstream, and enter red blood cells (RBCs) [5]. As the *P.* falciparum parasites mature within the red blood cells (RBCs) to trophozoite and schizont stages, they disappear from the peripheral circulation and bind to receptors expressed by endothelial cells that line the blood vessels of deep vascular beds of organs such as the brain, lungs and placenta, a process named sequestration [4], [6]–[9]. Sequestration aids in parasite survival by avoiding clearance by the spleen and appears to be an important factor in malaria pathogenesis. After 48 hours, the parasitized RBCs (PRBCs) rupture and release more daughter merozoites, perpetuating and promoting the blood-stage cycle [5]. The life cycle and pathogenesis of malaria is outlined in figure 1.
Figure 1: Lifecycle and pathogenesis of malaria. The life cycle of malaria infection begins with the bite of a female Anopheles mosquito carrying the malaria-causing parasite *P. falciparum*. The mosquito injects sporozoites into the host, which are released into the bloodstream and rapidly travel through the systemic circulation to the liver. In the liver the sporozites migrate through several cell types, and eventually infect a hepatocyte. When the sporozoite has infected the hepatocyte, it then begins an asexual and asymptomatic reproduction process. 10-12 days later, thousands of merozoites are released into the circulation and invade more red blood cells (RBCs). As they grow, they express adherent ligands that enable the maturing parasite to bind receptors expressed by endothelial cells that line the blood vessels in the deep vascular beds of organs such as the brain, lungs and placenta. After 48 hours, the parasitized RBCs (PRBCs) rupture and release more daughter merozoites, thereby perpetuating and promoting the blood-stage cycle. Figure obtained from Schofield & Grau, 2005 [5].
The severity of malaria is related to the capacity of *P. falciparum* infected red blood cells (iRBC) to sequester in microvasculature of vital organs. *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), a malarial variant antigen on infected erythrocytes, is involved in the adherence to host cell receptors. Infected erythrocytes can bind to endothelial receptors such as CD36 and intercellular adhesion molecule 1 (ICAM-1) [5]. However, studies on placental malaria have suggested that glycosaminoglycans (GAG) like chondroitin sulfate A (CSA) and hyaluronic acid (HA), play important roles as receptors for iRBC adhesion [10]–[12].

In pregnancy, there is a transient depression of cell-mediated immunity and cellular immune responses to *P. falciparum* antigens, increasing susceptibility to malaria infection [13], [14]. About 25% of pregnancies in sub-Saharan Africa are complicated by placental malaria (PM), increasing the risk of adverse events for both the mother and fetus, including maternal anemia, maternal mortality, congenital malaria, perinatal mortality, low birth weight (LBW), preterm delivery, intrauterine growth restriction (IUGR) and fetal anemia [15], [16]. LBW (low birth weight), which is defined as birth weight under 2500g, is known to be the most important risk factor for infant mortality. Children born to malaria-infected mothers are also at an increased susceptibility for *P. falciparum* malaria infection later in life [17]–[19].

The adverse events associated with placental malaria are also dependent on different epidemiologic settings and the time of infection during the pregnancy. For example, in areas with high rates of malaria transmission, infections in early pregnancy are associated with intrauterine growth restriction (IUGR) and abortions, while in areas of low disease intensity, pregnant women experience higher rates of abortion and stillbirth, which is linked to an elevated risk of maternal mortality [10], [20].

Treatment of malaria in pregnancy is unique. The third edition of Guidelines for treatment of malaria by the World Health Organization states that treatment is dependent on the trimester the mother is in, whether its uncomplicated or severe malaria, and if there is regional antimalarial drug resistance. In uncomplicated malaria, quinine and clindamycin are recommended during the first trimester of pregnancy, while artemisinin derivatives, including artesunate, dihydroartemisinin, are recommended in the second and third trimester of pregnancy. In severe malaria, full doses of parenteral
artesunate is recommended during all trimesters [21]. As artemisinin derivatives have short half-lives, they are combined with longer acting compounds including lumefantrine, piperaquine, amodiaquine and mefloquine. While the short-acting compounds act to reduce the number of parasites during the first three days of treatment, the longer-acting components eliminate the remaining parasites, preventing the reoccurrence of malaria [22]. However, there is limited knowledge on the optimal safe and effective doses of anti-malarial drugs in pregnancy.

A well-established experimental model that uses Plasmodium (P.) berghi ANKA (PbA) to infect BALB/c mice during pregnancy has been used to replicate the key pathogenic features of human PM in mice, though it is important to note that rodents are susceptible to different strains of protozoa than humans are [15], [23]. PbA is a mouse plasmodium that presents pathology comparable to human PM that is evoked by P. falciparum. PbA iRBC are able to bind to receptors present in mouse placental tissues, which triggers mouse placenta pathology that is comparable to the mechanism proposed for human PM [23]. This model provides opportunities to investigate the pathogenic mechanisms of malaria during pregnancy and has been extensively used in the past.

Although the murine and human model present many similarities in terms of pathology, species differences do exist. First, the inflammatory response evoked by P. berghi entails CD11b mononuclear infiltration, up-regulation of macrophage inflammatory process 1 (MIP-1) alpha chemokine and is associated with marked reduction of placental vascular spaces [23]. Second, woman of childbearing age are nutritionally vulnerable in Sub-Saharan Africa. 5-20% of African woman have a low body mass index (BMI) as a result of inadequate food intake, poor nutritional quality of diets and frequent infections, which leads to chronic hunger. Another challenge is that HIV infection further compromises maternal nutritional status, and individuals living in areas with high Plasmodium falciparum parasite rate have increased odds of being HIV positive [24]. Thus, woman infected with placental malaria are in a state of L-arginine deficiency, while our animal model presents normal L-arginine levels. Lastly, genetics play a key role in malaria resistance in humans. In regions of high endemic malaria, such as sub-Saharan Africa, individuals can develop resistance to malaria infection, while all the mice in our study that were injected with the infected erythrocytes had an equal and
high chance of developing *P. berghei* infection [25].

### 1.2 Drug-Disposition

Xenobiotics are compounds that are foreign to the normal energy-yielding metabolism of the body, and their outcome depends on disposition (absorption, distribution, metabolism and excretion, or ADME) and pharmacokinetics. It is crucial to understand the mechanisms involved in ADME during infection in order to identify potential drug-disease interactions and improve therapeutic interventions. Humans and animals are exposed to xenobiotics on a daily basis. The process of absorption is what leads to the entry of xenobiotics into the systemic circulation of the body. Xenobiotics must overcome chemical, physical, mechanical and biological barriers in order to be absorbed. The most important site of absorption is the gastrointestinal tract, and xenobiotics must cross cell membranes to enter. The distribution of xenobiotics into the various tissues of the body following entry into the systemic circulation is then influenced by membrane permeability, active transport mechanisms, tissue hemodynamics, and binding to blood and tissue proteins [26]. Small, lipophilic compounds can cross the cell membrane by passive diffusion, while large, polar or charged compounds are dependent on the presence of an active carrier mediated transport mechanism. Plasma protein binding of compounds is dependent on the amount of available binding proteins, the affinity constant of the compound for the protein, the number of available binding sites, and the presence of endogenous compounds that may alter the drug–protein binding interaction [27]. Once in the systemic circulation, xenobiotics are also subject to clearance as they are distributed to elimination organs, such as the liver and kidney [26]. Xenobiotic metabolism and elimination will be further discussed in the *transporters (1.2.1)* and *drug metabolizing enzymes (1.2.2)* sections of this report.

#### 1.2.1 Transporters

Xenobiotics cross plasma membranes via passive diffusion, facilitated diffusion, endocytosis/exocytosis, and active transport. Many drugs are actively transported, and therefore will be the focus of this report.

The ATP-binding cassette (ABC) superfamily is the largest active transport protein gene family [28]. It uses the hydrolysis of adenosine triphosphate (ATP) to
mediate the primary active export of drugs from the intracellular to the extracellular membrane, against a concentration gradient [29]. The human genome carries 49 ABC genes, arranged in seven subfamilies, designated A to G, that share a common sequence and organization of their ATP-binding domains [30], [31]. ABC transporters contain a pair of ATP-binding domains, also known as nucleotide binding folds (NBF), and two sets of transmembrane (TM) domains, typically containing six membrane-spanning α-helices. These proteins translocate a wide variety of substrates including sugars, amino acids, metal ions, peptides, and proteins, and a large number of hydrophobic compounds and metabolites across extra and intracellular membranes. ABC transporters are essential for many processes as they serve excretory and barrier roles across blood-tissue barriers including the placenta, and mutations in these genes cause or contribute to several human genetic disorders including anemia, altered drug response, cholesterol and bile transport defects, retinal degeneration, neurological disease and cystic fibrosis [28]. Key members of this family that are involved in drug disposition of anti-malarials include multidrug resistance protein 1 (ABCB1/MDR1) which is also termed P-glycoprotein (Pgp), the multidrug resistance-associated proteins (ABCCs/MRPs) and the breast cancer resistance protein (ABCG2/BCRP)[32]. Clinically important ABC transporters are listed in Table 1 below. The main active transporters found within the placental barrier include Pgp, BCRP and MRP2 [33].

**Table 1. Clinically Important ABC Efflux Transporters. Table modified from** [34] **and** [35].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Endogenous Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1A/B</td>
<td>MDR1/PgP</td>
<td>Steroids, lipids, bilirubin, bile acids</td>
</tr>
<tr>
<td>ABCC1</td>
<td>MRP1</td>
<td>Leukotrienes, Prostaglandins, Folic acid, Glucuronide conjugates of: 17β-Estradiol, Bilirubin, hyodeoxycholate, dehydroepiandrosterone sulfatolithocholate, sulfatolithocholyl taurine</td>
</tr>
<tr>
<td>ABCC2</td>
<td>MRP2</td>
<td>Bilirubin, Leukotriene C, S-Glutathionyl, - estradiol, Cholecystokinin peptide, Ethinylestradiol-3-O-glucuronide, Estrone 3-sulfate</td>
</tr>
<tr>
<td>ABCC3</td>
<td>MRP3</td>
<td>Bile salts, Estradiol-17β-glucuronide, leukotriene C4</td>
</tr>
<tr>
<td>ABCC4</td>
<td>MRP4</td>
<td>Taurocholic acid, cAMP, cGMP, urate, DHEAS, E2-17βG, p-aminohippurate, PGE1 and PGE2</td>
</tr>
<tr>
<td>ABCG2</td>
<td>BCRP</td>
<td>Dietary flavonoids, porphyrins, estrone 3-sulfate</td>
</tr>
</tbody>
</table>
The solute carrier (SLC) superfamily is a secondary active transport protein gene family, for which transport is driven by various energy coupling mechanisms [36]–[41]. SLC transporters are membrane proteins that transport solutes, such as ions, metabolites, peptides, and drugs, across biological membranes [42]. There are 386 members grouped into 52 families based on their sequences, number of transmembrane α-helices (TMHs), and biological functions [36], [38]. Several SLC members directly mediate drug transport in the liver, kidney, and blood brain barrier (BBB), which can affect clinical outcome [32], [43]. Key members of this family that are involved in drug disposition include the organic anion transporting polypeptides (OATPs), organic anion transporters (OATs), and organic cation transporters (OCTs) [32]. Clinically important SLC transporters are listed in Table 2 below. SLC transporters in the placenta mostly facilitate energy-independent uptake of hydrophilic or charged molecules by the trophoblast cells. Once in the trophoblast, these substrates are either utilized for placentas own need or pumped out of the cell by another SLC or ABC transporter [33].
Table 2. Clinically Important SLC Uptake Transporters. Table modified from [34] and [35].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Endogenous Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC47A1</td>
<td>MATE1</td>
<td>Peptides and nucleosides, creatinine, guanidine, thiamine, E3S</td>
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<tr>
<td>SLC47A2</td>
<td>MATE2</td>
<td>Estrone sulphate, creatinine</td>
</tr>
<tr>
<td>SLC22A12</td>
<td>URAT1</td>
<td>Uric acid</td>
</tr>
<tr>
<td>SLC22A6</td>
<td>OAT1</td>
<td>Cyclic nucleotides, prostaglandin E2 and F2α, uric acids folate</td>
</tr>
<tr>
<td>SLC22A7</td>
<td>OAT2</td>
<td>cGMP, prostaglandin E2, salicylate</td>
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<tr>
<td>SLC22A8</td>
<td>OAT3</td>
<td>Prostaglandin, uric acids, bile acids; conjugated hormones</td>
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<tr>
<td>SLC22A11</td>
<td>OAT4</td>
<td>Estrone 3-sulfate, uric acid, prostaglandin E2</td>
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<td>SLC22A4</td>
<td>OCTN1</td>
<td>Ergothioneine, carnitine, acetylcholine</td>
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<td>SLC22A5</td>
<td>OCTN2</td>
<td>carnitine</td>
</tr>
<tr>
<td>SLC22A1</td>
<td>OCT1</td>
<td>Choline, acetylcholine, agmatine, monoamine neurotransmitters</td>
</tr>
<tr>
<td>SLC22A2</td>
<td>OCT2</td>
<td>Creatinine, bile acids, choline, acetylcholine and monoamine neuro-transmitters</td>
</tr>
<tr>
<td>SLC22A4</td>
<td>OCT3</td>
<td>Metabolites: Creatinine, Choline, Guanidine; Neurotransmitters: Acetylcholine, Dopamine, Norepinephrine; Hormones: Progesterone, Testosterone, Agmatine</td>
</tr>
<tr>
<td>OATP4C1</td>
<td>SLCO4C1</td>
<td>Guanidinosuccinate</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>SLCO2B1</td>
<td>Bile acids, steroid hormones</td>
</tr>
<tr>
<td>OATP1C1</td>
<td>SLCO1C1</td>
<td>T4, T4 sulfate, rT3</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>SLCO1B1</td>
<td>Bilirubin, estrone 3-sulfate, cholate</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>SLCO1B3</td>
<td>Bilirubin, estrone 3-sulfate, taurocholate</td>
</tr>
<tr>
<td>OATP1A2</td>
<td>SLCO1A2</td>
<td>Estrone 3-sulfate, taurocholate, saquinavir</td>
</tr>
</tbody>
</table>

Expression of the ABC drug transporters in a PbA-infected murine model was previously investigated in our lab, and this study demonstrated malaria-induced alterations in the expression of many transporters in maternal and fetal tissues, and the placenta (Table 3) [15]. To our knowledge, there are no studies that have investigated the effect of dietary L-arginine on the malaria-induced alterations in transporters, or extensively studied SLC transporters in a PM murine model.
Table 3. Malaria-Induced Changes on Hepatic and Placental Transporters [15].

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Expression</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcb1a</td>
<td>Liver</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>Fetal Liver</td>
<td>Significantly decreased (P &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td>Significantly decreased (P &lt; 0.001)</td>
</tr>
<tr>
<td>Abcb1b</td>
<td>Liver</td>
<td>Significantly increased (P &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>Fetal Liver</td>
<td>Significantly increased (P &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td>Significantly decreased (P &lt; 0.01)</td>
</tr>
<tr>
<td>Abcc1</td>
<td>Liver</td>
<td>Significantly increased (P &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>Fetal Liver</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td>Significantly decreased (P &lt; 0.05)</td>
</tr>
<tr>
<td>Abcc2</td>
<td>Liver</td>
<td>Significantly decreased (P &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td>Significantly decreased (P &lt; 0.05)</td>
</tr>
<tr>
<td>Abcc3</td>
<td>Liver</td>
<td>Significantly increased (P &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td>Significantly decreased (P &lt; 0.01)</td>
</tr>
<tr>
<td>Abcg2</td>
<td>Liver</td>
<td>Significantly decreased (P &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>Fetal Liver</td>
<td>Significantly decreased (P &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td>Significantly decreased (P &lt; 0.05)</td>
</tr>
<tr>
<td>Abcb11</td>
<td>Liver</td>
<td>Significantly decreased (P &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>Fetal Liver</td>
<td>Significantly decreased (P &lt; 0.01)</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>Liver</td>
<td>Significantly decreased (P &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>Fetal Liver</td>
<td>Significantly decreased (P &lt; 0.01)</td>
</tr>
<tr>
<td>iNOS</td>
<td>Liver</td>
<td>Significantly increased (P &lt; 0.001)</td>
</tr>
<tr>
<td>HO-1</td>
<td>Liver</td>
<td>Significantly increased (P &lt; 0.001)</td>
</tr>
</tbody>
</table>
1.2.1.1 ABC Drug Transporters

1.2.1.1.1 Multidrug resistance protein 1 (MDR1/PGP; ABCB1A/B)

The multidrug resistance protein 1 (MDR1) is commonly referred to as P-glycoprotein (Pgp) and plays an important protective role by secreting toxic endogenous and exogenous xenobiotics [44]. It is highly expressed in the apical membrane of epithelial cells in the liver, kidney, intestine, colon, brain, testis, and placenta [45]. It transports a broad range of chemically diverse substrates, however they are typically large, lipophilic, and uncharged or cationic structures. In rodents, MDR1 is encoded by the Abcb1a and Abcb1b genes [46].

1.2.1.2 The Multidrug Resistance Associated Proteins (MRP; ABCC Family)

The Multidrug Resistance Associated Proteins are composed of thirteen members, nine of which are primarily involved in multidrug resistance (MRP 1-9). These nine are ATP-dependent efflux transporters for endogenous substances and xenobiotics. They have a broad specificity and are primary active transporters of organic anion conjugates. MRPs are located at either the apical or basal membranes of tissues [47]. In the placenta, MRP1 is located at the apical syncytiotrophoblast membrane [48]. MRP2 is mainly expressed in the apical hepatocyte plasma membrane, small intestine, and renal proximal tubules [49]–[51]. MRP1 and MRP2 have very similar substrate specificity, with different affinities [52]. MRP3 is localized in the basolateral membrane domain of polarized cells, and is expressed in adrenal glands, kidney, small intestine, colon, pancreas, and gallbladder [53]–[56]. MRP3 shares a high degree of structural similarity with MRP1, 58% amino acid identity, however the affinity of MRP3 for conjugates is considerably lower than that of MRP1 [57], [58]. MRP4 is localized to the apical membrane of renal proximal tubule cells and brain capillary endothelium, and basolateral membranes or prostate tubuloacinar cells, hepatocytes, and choroid plexus epithelium [59]. MRP4 has a wide range of substrate specificity, including antivirals, antibiotics, cardiovascular, and cytotoxic drugs [60]–[62].

1.2.1.3 The Breast Cancer Resistance Protein (BCRP; ABCG2)

The breast cancer resistance protein (BCRP) plays an important role in the absorption, elimination, and tissue distribution of xenobiotics. It is highly expressed in normal human tissues including the small intestine, liver, brain endothelium, and
placenta [63]. BCRP substrates include chemotherapeutic agents such as mitoxantrone and methotrexate, non-chemotherapy drugs such as prazosin, glyburide and statins, and non-therapeutic compounds such as the dietary flavonoids and estrone 3-sulfate (E1S), and the carcinogen PhIP [64].

1.2.1.4 The Bile Salt Export Pump (BSEP; ABCB11)

Bile acids play an important role in several physiological processes and bile production is one of the key functions of the liver. It is involved in the absorption of lipids and fat-soluble vitamins, which provide important energy sources and are vital for many processes, respectively[65]. Therefore, disturbed bile flow can have negative consequences on the human body. Two key transporters involved in hepatic bile acid uptake and excretion are the bile salt export pump (BSEP) and the sodium-taurocholate co-transporting polypeptide (NTCP/Slc10a1) transporter. Bile acid uptake from blood into the liver is mediated by NTCP, while the efflux from the liver into bile is mediated by BSEP [66]. BSEP is predominantly expressed in the apical/canalicular membrane of hepatocytes [66].

1.2.1.5 SLC Drug Transporters

1.2.1.6 The Equilibrative Nucleoside Transporters (ENT; SLC29 Family)

The equilibrative nucleoside transporters (ENTs) are found in almost all tissues, while their abundance varies between locations [67]–[69]. ENTs are found in the basolateral and apical membranes of the placenta and brain, expressing both secretory and re-absorptive functions [70]. ENTs mediate both the influx and efflux of nucleosides across the membrane. Substrates for ENT1 and ENT2 include purine nucleosides and adenosine [71].

1.2.1.7 The Organic Cation Transporter (OCT; SLC22 Family)

The organic cation transporters (OCT) mediate the passive facilitated diffusion of a broad range of organic cations down their electrochemical gradients, thus transport may occur in either direction and is independent of either sodium or pH [72]. Substrates of OCTs include a wide variety of structurally unrelated small organic cations, both endogenous and exogenous, including toxins and many clinically used drugs. Among these substrates are catecholamines, monoamine neurotransmitters and several antiviral drugs [72]. Thus, absence of these transporters result in physiologic consequences [73]–
OCTs mediate the entry of these compounds from the blood into the excretory epithelial cells. In rodents, Oct1 (Slc22a1) is highly expressed in the sinusoidal (basolateral) membrane of hepatocytes in the liver, basolateral membrane of epithelial cells lining the proximal tubules, basolateral membrane in the placenta, and the small intestine, whereas in humans it is expressed primarily in the liver. However, mRNA expression of OCT1 has been detected in other tissues, such as heart, skeletal muscle, kidney, brain and placenta. OCT2 (Slc22a2) is generally considered to be a kidney transporter, although low levels of mRNA are found in other tissues such as spleen, placenta, small intestine and brain. In kidney, Oct2 is on the basolateral membrane of epithelial cells lining the proximal tubules.

1.2.1.8 The Peptide Transporter 2 (PEPT2; SLC15A2)

Proton-coupled oligopeptide transporters deliver peptide-bound amino nitrogen to cells and have a significant influence on the pharmacokinetics and pharmacodynamics of peptide-like drugs. There are four proton-coupled oligopeptide transporters: PEPT1 (Slc15a1), PEPT2 (Slc15a2), PHT1 (Slc15a4) and PHT2 (Slc15a3). Among these transporters, it has been suggested that the peptide transporter PEPT2 plays the primary role in renal reabsorption of drugs, due to its high expression in the kidney and high affinity. PEPT2, which is primarily expressed in the kidney and PEPT1, which is primarily expressed in intestine and kidney, mediate the cellular uptake of dipeptides and tripeptides, and selected drugs across epithelial membranes. Substrates of PEPT1 and PETP2 include β-lactam antibiotics, angiotensin-converting enzyme inhibitors and antiviral nucleoside prodrugs. In the kidney, PEPT2 is localized at the apical membrane of the renal proximal tubular cells where it is involved in the reabsorption of small peptides and drugs with peptide-like structures. PEPT2 is also expressed in the nervous system, lungs and mammary gland. Uptake into intestinal enterocytes by PEPT1 is understood to be beneficial to the bioavailability of these drugs.

1.2.1.9 The Organic Anion Transporter (OAT; SLC22 Family)

The organic anion transporter (OAT) family comprises a group of over 10 transmembrane proteins falling into the SLC22 subfamily of the major facilitator superfamily. The SLC22 subfamily also includes the organic cation transporters (OCTs)
and organic carnitine (zwitterion) transporters (OCTNs), thus OATs are highly similar within the subclass of SLC22 transporters, and many substrates are transported by more the one SLC22 transporter family [94]. OATs are localized to almost all barrier epithelia of the body, as well as endothelium and other cells, and are involved in the transcellular movement of numerous small organic anionic molecules across epithelial barriers and between body fluid compartments (i.e., blood-central nervous system, blood-urine, intestine-blood, blood-bile, blood-placenta, and others). Members of this transporter family are capable of bidirectional movement of substrates, however most of the Oats are generally viewed as facilitating the movement of organic anions into the epithelial cells, thus being referred to as influx transporters. OAT1 (Slc22a6) substrates include prostaglandins, α-ketoglutarate, NSAIDs, antivirals and anticancer drugs [72].

Oat1 and Oat3 (Slc22a8) are key transporters involved in drug excretion and drug-drug interactions [51], [52]. Oat3 works in parallel with Oat1 in mediating the entry of organic anions into renal proximal tubules from the blood [53].

Oat1 is almost exclusively expressed in the kidney, although can be found in other rodent tissue to a lesser extent [95]. Drug, toxin, and metabolite substrates of OAT1 include p-aminohippuric acid (PAH), antivirals, nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, diuretics, folate, α-ketoglutarate, cyclic nucleotides, prostaglandins, gut microbial metabolites, uremic toxins, vitamins, dietary compounds, uric acid, mercury conjugates, and other toxins [96]–[98]. Oat1 knockout mice have been found defective in the handling of many important endogenous metabolites, including creatinine, benzoate, thymidine, uracial and urate [97], [99]. Additionally, mass spectrophotometric profiling of the plasma and urine from Oat1-deficient and wild-type mice found several physiologically important metabolites, including vitamins and uremic toxins, as well as gut microbiome metabolites [97].

Oat3 is highly expressed in the renal proximal tubule and distal tubule [100], [101]. Oat3 is more broadly expressed in other tissues than Oat1. There is an overlap of substrate specificity between Oat3 and Oat1, with some differences [96], [102]. Oat3 mediates the uptake of a wide array of small molecule anions including a large number of small molecule xenobiotics, endogenous metabolites such as conjugates of signaling sex steroids, as well as vitamins and other plant-derived metabolites (e.g., flavonoids).
Oat3 deficient mice display altered uric acid handling, poor handling of antivirals, penicillin, and methotrexate, and an attenuated response to diuretics [99], [100], [102]–[106]. Additionally, data from the blood and urine of these knockout mice revealed a role of Oat3 in several metabolic pathways, including the tricarboxylic acid cycle, nucleotide and amino acid metabolism, phase I and phase II xenobiotic metabolism (i.e., hydroxylation and glucuronidation), prostaglandin and steroid metabolism, as well as the metabolism of dietary flavonoids [102]. It has also been found that the deletion of Oat3 results in impaired renal basolateral uptake of organic anions [107].

### 1.2.1.10 The Organic Anion Transporting Polypeptide 2B1 (OATP2B1; SLC20A1)

The organic anion transporting polypeptides (OATPs) are widely expressed in the body, with the highest levels found in the liver [108], [109]. They regulate the uptake of a number of organic endogenous and exogenous compounds, such as bile acids, and steroid and thyroid hormones, as well as several clinically important drugs. OATP2B1 is located at the basolateral membrane of hepatocytes, basolateral membrane of syncytiotrophoblasts in the placenta, and at the luminal membrane of the endothelial cells of the blood–brain barrier [109]–[111]. OATP2B1 is also expressed in several fetal tissues [112]. Murine Oatp2b1 has broader substrate specificity than human OATP2B1 [113].

### 1.2.1.11 The Multidrug and Toxin Extrusion Transporter 1 (MATE1; SLC47A1)

The multidrug and toxin extrusion (MATE) transporters are involved in the elimination of cationic drugs and xenobiotics, and work in combination with OCTs. MATE transporters are primarily expressed in the kidney and liver, both of which play a major role in the elimination of exogenous and endogenous compounds [114]. Mate1 is expressed in the brush-border membranes of the kidney, and in the canalicular membrane in the liver [78], [115]–[117]. Substrates for MATE1 and MATE2 include organic cations, TEA, 1-methyl-4-phenylpyridinium (MPP), metformin, cimetidine and procainamide. These substrates are also transported by OCT2 [118]. L-arginine metabolites, paraquat and agmatine, are reported to be transported by MATE1, while the anti-malaria agent pyrimethamine is a potent inhibitor of MATE1 and MATE2 in the kidney [119]–[121].
1.2.1.12 The Sodium-Taurocholate Co-Transporting Polypeptide (NTCP; SLC10A1)

The uptake of bile salts into hepatocytes occurs predominately via NTCP. Bile acids are thought to decrease Ntcp expression through the farnesoid X receptor (FXR)-small heterodimer partner (Shp) pathway, and increase Bsep expression through direct FXR activation [122]–[124]. NTCP is located on the basolateral membrane, and substrates include amidated (taurine, glycine), sulfated and unconjugated bile salts, sulfated steroids (estrone-3-sulfate, DHEAS) and thyroid hormones [125], [126].

Table 4. Endogenous substrates and expression of the SLC uptake transporters. Data has been collected from the UCSF-FDA TransPortal Database and Solvo Biotechnology [35], [127].

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrates (Endogenous)</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slco2b1 (Oatp2b1)</td>
<td>Bile acids, steroid hormones</td>
<td>Liver; placenta; heart; brain; kidney; lung; small intestine</td>
</tr>
<tr>
<td>Slc22a1 (Oct1)</td>
<td>Choline, acetylcholine, agmatine, monoamine neurotransmitters</td>
<td>Liver: hepatocytes sinusoidal membrane; Intestine: enterocytes apical membrane, neurons</td>
</tr>
<tr>
<td>Slc29a1 (Ent1)</td>
<td>Purine and pyrimidine nucleosides</td>
<td>Placenta; brain</td>
</tr>
<tr>
<td>Slc29a2 (Ent2)</td>
<td>Purine and pyrimidine nucleosides</td>
<td>Placenta; brain</td>
</tr>
<tr>
<td>Slc10a1 (Ntcp)</td>
<td>Taurocholate, bile salts, sulfated steroids, sulfated thyroid hormones</td>
<td>Sinusoidal membrane of hepatocytes</td>
</tr>
<tr>
<td>Slc47a1 (Mate1)</td>
<td>Peptides and nucleosides, creatinine, guanidine, thiamine, E3S</td>
<td>Kidney: proximal tubular cells, apical membrane; liver: hepatocytes, apical membrane; skeletal muscle</td>
</tr>
<tr>
<td>Slc15a2 (Pept2)</td>
<td>Dipeptides and tripeptides</td>
<td>Kidney; choroid plexus</td>
</tr>
<tr>
<td>Slc22a2 (Oct2)</td>
<td>Creatinine, bile acids, choline, acetylcholine and monoamine neuro-transmitters</td>
<td>Epithelial cells in renal proximal tubules; neurons</td>
</tr>
<tr>
<td>Slc22a6 (Oat1)</td>
<td>cyclic nucleotides, prostaglandin E2 and F2α, uric acids folate</td>
<td>Kidney: proximal tubule, basolateral membrane; Placenta</td>
</tr>
<tr>
<td>Slc22a8 (Oat2)</td>
<td>cGMP, prostaglandin E2, salicylate</td>
<td>Kidney; liver</td>
</tr>
</tbody>
</table>
1.2.2 Drug Metabolizing Enzymes

Numerous xenobiotics undergo enzymatic metabolism. The main site of enzymatic metabolism, also referred to as biotransformation, is the liver. Xenobiotics are often converted to polar or more polar metabolites via enzymatic metabolism before being removed from the body. Distinct families of metabolic enzymes execute the metabolism. These enzymes can be classified as either “phase I” or “phase II” enzymes [26].

The most important enzyme system involved in phase I metabolism is the cytochrome P450 (CYP) superfamily of isoenzymes. The P450 enzymes are grouped together into families that share sequence identity. There are 10 mammalian gene families compromised of 18 subfamilies [128]. Individual enzymes metabolize specific substrates, all via the same catalytic mechanism [129]. Within the CYP superfamily, a small group of structurally dissimilar CYPs are responsible for the metabolism of about 80-90% of clinically used drugs [130]–[133]. CYP3A4 is considered the most important CYP because it is involved in the metabolism of approximately 50% of clinically used drugs, and is the most highly expressed enzyme in the human liver [130], [134]. CYP3A4 is involved in the metabolism of a number of antimalarial agents including chloroquine, quinine, mefloquine, and artemisinin and artemisinin derivatives [135]–[139]. Cyp3a11 is the dominant constitutive CYP3A4 isoform in the liver in mice, exhibiting 73% sequence homology to human CYP3A4 [128], [140].

Since CPY3A4 is involved in the metabolism of many antimalarials, it is important to investigate the impact of treatment on Cyp3a11 in a PM murine model.

1.3 Inflammation

Inflammation is the body’s defensive response to harmful stimuli and is a key component in many acute and chronic diseases states. Inflammation plays a significant role in disease resolution or progression, depending on the type, duration and magnitude of response [141]. Inflammatory processes are essential for the early control of parasitemia following malaria infection, however, dysregulated production of inflammatory mediators can lead to severe immunopathology [5]. Glycosylphosphatidylinositol (GPIs) of intraerythrocytic *P. falciparum* contributes to malaria pathogenesis by inducing pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, all
of which promote systemic inflammation [142]. Significant hepatic oxidative stress is also associated with this inflammatory response.

Many key transcription factors are activated during inflammation, including NF-κB and nuclear factor IL-6, and it is thought that these transcription factors play a key role in the regulation of transporters and metabolizing enzymes [143]–[147]. Thus ADME determinants of drug disposition may be modified during this inflammatory response.

Another key biomarker of inflammation in severity of malaria is C-reactive protein (CRP). CRP is an acute phase reactant that has been identified to play a key pathogenic role in malaria [148]. It is thought to bind to infected erythrocytes and help in their clearance [149]. Several studies have shown that serum CRP levels correlate with parasite burden and complications in malaria, especially in *P. falciparum*, thus measurement of CRP can be useful in understanding the pathogenesis of placental malaria.

Lastly, serum amyloid A (SAA), is a multifunctional protein that is involved in cholesterol transport and metabolism, as well as inflammatory response [150]. It is an acute phase reactant that has been identified as a marker of assessing severity of *P. falciparum* malaria and response to antimalarial treatment [151].

### 1.4 Effect of Malaria on Gene Expression

In the study conducted in our lab by Cressman et al. [141], the same study design outlined in section 3.1 was used, however all the mice were administered regular drinking water, and no L-arginine supplemented water. The focus of the study was on the effect of malaria infection on the expression of key ABC drug transporters and drug-metabolizing enzyme Cyp3a11 in a *Plasmodium berghei* infected pregnant Balb/c mouse model. The mice were infected on gestation day (GD) 13, and euthanized on gestational day (GD) 19. Results from the study are summarized in Table 3. Results from the study conclude that malaria-induced alterations in the expression of transporters and drug-metabolizing enzymes in maternal and fetal tissues may alter the disposition of endogenous and therapeutic substrates, potentially impacting maternal and fetal outcomes.
1.4.1 Maternal and Fetal Parameters

Results showed a decrease in maternal body weight in PbA-infected dams relative to uninfected dams (P < 0.05). Spleen weight was significantly higher in PbA-infected dams relative to uninfected control dams (P < 0.001). Fetal weight was also significantly lower in fetuses obtained from PbA-infected dams relative to those from uninfected control dams (P < 0.001).

1.4.2 Placenta

Compared with controls, there were significant decreases in placental mRNA expression of Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, and Abcg2 in malaria-infected dams (P < 0.05). A significant decrease in the protein expression of MDR1 (multidrug resistance protein 1) was seen in placenta isolated from infected dams compared with uninfected controls (P < 0.05).

1.4.3 Maternal Liver

There was a pronounced increase in the hepatic expression of both iNOS and HO-1 in the maternal liver of PbA-infected dams, relative to uninfected control dams (P < 0.001). Expression of Cyp3a11 was significantly decreased (P < 0.001) in the livers of infected dams relative to uninfected control dams. Significant decreases in the expression of the canalicular transporters Abcc2, Abcg2, Abcb11 were seen in the livers of infected dams. A dramatic increase in mRNA expression of Abcb1b (P < 0.001) was seen in the infected group, whereas Abcb1a was unaffected. Dramatic increases in the expression of the basolateral transporters Abcc1 and Abcc3 were observed in the livers of infected dams (P < 0.001). They also found a significant decrease in the expression of the organic anion transporter Slco2b1 (P < 0.01), whereas the expression of Slco10a1 was not significantly changed. Protein expression of MDR1 was not significantly different between infected and control dams.

1.4.4 Fetal Liver

Significant 40–70% decreases in expression of fetal hepatic Cyp3a11, Abcb1a, Abcg2, and Abcb11 was seen in pups isolated from infected dams compared with uninfected controls, whereas the expression of Abcb1b was significantly increased (P < 0.01). Contrary to changes observed in the maternal liver, the expression of fetal liver Abcc1 (P < 0.097) was not significantly altered.
1.4.5 **Total Plasma Bile Acid Levels**

A significant and dramatic 80-fold increase in total serum bile acid concentrations in PbA-infected dams relative to uninfected control dams was observed. Relative total bile acid levels were also significantly increased in the livers of PbA infected dams (P < 0.05).

1.4.6 **Maternal Brain (whole brain tissue)**

A significant increase in Abcb1b was observed in infected dams relative to control dams (P < 0.01). No significant differences were observed in the expression of Abcb1a or Abcg2. A significant increase in the protein expression of MDR1 in brain isolated from PbA-infected dams compared with controls was observed (P < 0.05).

1.4.7 **Maternal Kidney**

Significant 2- to 3.5-fold increases in the expression of Abcb1a (P < 0.05) and Abcb1b (P < 0.05) in infected compared with control dams were observed. Malaria infection was associated with a significantly higher renal protein expression of MDR1 (P < 0.05).

1.5 **Nitric Oxide**

Nitric oxide is a molecule that has been proposed to have a crucial role in malaria pathogenesis [152]. NO is an uncharged free radical that is produced during the enzymatic conversion of L-arginine to L-citrulline by members of the nitric oxide synthase (NOS) family; endothelial NOS (eNOS or NOS3), neuronal NOS (nNOS or NOS1) and iNOS [153]. NO plays an important role in many physiological events during parasitic infection. NO modulates the production of cytokines, generally decreasing the amount of pro-inflammatory cytokines, and increasing the amount of anti-inflammatory cytokines [1]. NO also has several anti-inflammatory effects including inhibition of T-cell and B-cell proliferation, and leukocyte rolling and adhesion on microvascular endothelial cells [26], [154], [155]. The figure below outlines the physiological roles of NO relevant to malaria pathogenesis.
Figure 2. The Physiological Roles of NO Relevant to Malaria Pathogenesis. Homeostatic and anti-inflammatory pathways that can be disrupted by NO quenching during malaria are depicted by the red lines, while green lines indicate enhancement, and black lines indicate modulation. Figure obtained from Sobolewski et al., 2005 [1].

Of particular importance are the homeostatic and anti-inflammatory pathways, depicted by the red arrows, which can be disrupted by NO quenching during malaria. Endothelial dysfunction is a measure of endothelial activation, and may play a role in the pathogenesis of severe malaria by increasing the adhesion of parasitized erythrocytes to the endothelium and thereby worsening microcirculatory obstruction and oxygen delivery [156].

Studies have reported that low NO bioavailability contributes to pathologic activation of the immune system, and the endothelium and the coagulation system, factors observed during severe P. falciparum malaria [26], [157], [158]. Additionally, pregnancy and malaria infection have been reported to be a state of relative arginine deficiency and NO bioavailability, respectively [26], [159], [160]. A study conducted by Ren et al. in 2012 showed that dietary arginine supplementation in porcine circovirus type 2 (PCV2) infected mice resulted in significantly improved serum NO levels, and
fetal outcomes, including survival and abortion rate [161]. Direct supplementation of NO is not feasible, as it is a short-lived free radical and results in severe side effects. Thus, L-arginine is a safe amino acid that has been used in humans for decades to restore NO levels, as it is the sole substrate for NO [162].

Research from our lab showed significant increases in the hepatic expression of heme-oxygenase 1 (HO-1) and inducible nitric oxide synthase 1 (iNOS) following PM infection. HO-1 and iNOS are both enzymes that play a protective role in host response to malaria infection [15], [163]–[165].

There are many factors that can impact that bioavailability of nitric oxide. First, free hemoglobin released during the asexual cycle of blood-stage Plasmodium might quench NO, thereby having an important role in limiting NO bioavailability during malaria [1]. Second, NO bioavailability is limited by superoxide. The rupture of infected erythrocytes cause the release of malaria glycosylphosphatidylinositol (GPI)-anchored proteins and Hz, which elicit a strong pro-inflammatory response in monocytes and neutrophils, causing oxidative stress. Studies of several fields have implicated oxidative stress in reducing the bioavailability of NO [166], [167]. Lastly, asymmetric dimethylarginine (ADMA) is a major endogenous inhibitor of the nitric oxide synthase (NOS) family. Increased plasma ADMA concentrations cause impaired NO synthesis leading to endothelial dysfunction and atherosclerotic vascular disease [168].

1.6 L-arginine

L-arginine is a semi-essential amino acid as endogenous synthesis is not sufficient to fulfill requirements under increased demands (i.e. infection). It is involved in many metabolic processes, and plays a crucial role in the treatment of heart diseases and high blood pressure. It improves circulation and strengthens the immune system [169]. It is a substrate for four enzymes, some of which exist as multiple isoforms: arginase, nitric oxide synthase (NOS), arginine: glycine amidinotransferase, and arginine decarboxylase [170]. Arginase enzymes are the endogenous antagonists to iNOS as they compete for the same L-arginine substrate. Arginase metabolizes L-arginine to L-ornithine and urea, whereas iNOS metabolizes L-arginine to NO and L-citrulline. L-ornithine, one of three amino acids involved in urea cycle, is then metabolized by the enzyme ornithine decarboxylase (ODC) to produce polyamines. In some studies, the
antagonism between iNOS and arginase has lead to decreased L-arginine availability [171].

NO is an important mediator of vasodilation and inhibition of platelet aggregation via increased formation of cyclic guanosine monophosphate (cGMP). NO is synthesized in endothelial cells from the terminal guanidino nitrogen of L-arginine by the activity of the endothelial, calcium-dependent isoform of NO synthase. NO is then rapidly oxidized to nitrite (NO–2) and nitrate (NO–3) in vivo, and NO–3 and cGMP are subsequently eliminated via the kidneys [172]–[176].

The mean baseline plasma concentration of L-arginine in healthy individuals is 15.1±2.6 µg ml−1. Plasma concentration reached 1390±596 µg ml−1, after intravenous administration of 30 grams of L-arginine over 30 minutes. Substantial urinary clearance of L-arginine occurred in the first 90 minutes due to concentration-dependent renal clearance followed by a slower fall in plasma concentrations due to non-renal elimination. Plasma concentration reached 50.0±13.4µg ml−1 after one hour of oral administration of 10 grams of L-arginine. Renal elimination was not observed after oral administration of this dose. The absolute bioavailability of a single oral 10 gram dose of L-arginine is approximately 20% [177].

Activation of macrophages has been shown to result in a substantial enhancement of L-arginine transport [178]. Levels of L-arginine are altered in animal models of inflammation, including ischemic colitis and inflammatory bowel disease [179]. Additionally, significant decreases in serum L-arginine concentrations are seen in mice with infectious colitis as compared to control mice [180]. On the other hand, dietary L-arginine supplementation has been found to enhance immune response in various pre-clinical models of immunological challenges [181]. In a rat model, L-arginine supplementation attenuated the degree of tissue damage in intestinal ischemia and promoted healing of intestinal mucosa. L-arginine was administered orally by gavage in three equal doses, 22, 8 hours and 1 hour before surgery. The dose of L-arginine was 20 mg/kg of body weight [182].

Several clinical trials have investigated the effects of dietary L-arginine on different disease states. Most importantly, clinical trials studying the effects of L-arginine on pre-eclampsia saw a significant reduction of the occurrence of pre-eclampsia in
patients supplemented with 6.6 g/day L-arginine along with anti-oxidant vitamins [183]. Pre-eclampsia is a state in which there is defective synthesis of nitric oxide, similar to that of malaria infection, and thus L-arginine is used at greater amounts to help restore the quenching of nitric oxide [184]. Clinical trials have reported that L-arginine administration improves endothelial NO production in patients with moderately severe falciparum malaria. L-arginine supplementation did not improve NO production in patients with severe falciparum malaria. Differences in efficacy could be due to the lower dosage rate of L-arginine that was administered to the severe group as compared to that administered to the moderately severe patients[185]. Patients with severe falciparum received L-arginine as one 12g dose in 10% in normal saline and given by an infusion pump over 8 hours (1.5g/hour). Patients with moderately severe malaria received one 12g dose of L-arginine over 30 minutes. Though significantly higher, the dosage received by the moderately severe malaria group was deemed safe with no significant adverse events, and significantly improved endothelial function [186].
Chapter 2. Hypothesis & Rationale

2

2.1 Rationale
Research in our lab has shown that PM infection alters the expression of ABC efflux transporters and the drug-metabolizing enzyme Cyp3a11 in maternal and fetal tissue in a PM murine model. In a previous study, placental mRNA expression of key ABC drug transporters (Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3 and Abcg2), and expression of Cyp3a11 in the liver were found to be significantly down regulated in the malaria-infected group. Placental MDR1 protein was also significantly down regulated. There was significant down regulation of Abcb1a, Abcg2 and Cyp3a11 in the fetal liver of infected dams as well, indicating that fetal tissue is subject to malaria-mediated changes in gene expression. These changes are thought to be related to oxidative stress and inflammation. Additionally, there is evidence that there is a quenching of nitric oxide during malaria, which can disrupt homeostatic and anti-inflammatory pathways, thus increasing oxidative stress. Research shows that dietary L-arginine supplementation generally improves the reproductive performance of mice, rats and pigs, and that NO is an important effector molecule of the immune system in eliminating numerous pathogens. Therefore our principle goal was to study whether supplementation of PbA-infected animals with dietary L-arginine would reverse or attenuate the effects of malaria on genes involved in drug disposition mechanisms. Moreover as numerous SLC transporters are also involved in drug disposition, another goal was to examine the impact of malaria on these transporters. Thus the expression of several drug transporters and drug metabolizing enzymes were examined in maternal and fetal tissues in pregnant PbA-infected mice that are receiving or not receiving supplementation with L-arginine.

2.2 Hypothesis
Restoring systemic levels of NO during PM by dietary L-arginine supplementation will attenuate changes in transporters and pro-inflammatory cytokines that are observed in maternal and fetal tissues during PbA infection.
2.3 Objective
Previous work in our laboratory focused on the impact of PbA infection on the expression of the ABC efflux transporters, therefore our first objective is to examine the impact of PbA infection on the expression of SLC uptake transporters in maternal and fetal tissues (placenta, maternal liver, maternal kidney, maternal brain, and fetal liver). The impact on dietary L-arginine on inflammation and gene dysregulation in PM has never been examined. Thus, our second objective is to examine the impact of dietary L-arginine on the expression of clinically important ABC and SLC transporters, in addition to the drug-metabolizing enzyme Cyp3a11 and inflammatory responses in maternal and fetal tissues in the PbA murine model of placental malaria. Lastly, previous work in our laboratory correlated the dysregulation of transporters with dramatic increases in maternal bile acid levels, therefore we wanted to examine whether this translated to fetal bile acid levels.
Chapter 3. Methods

3

3.1 Animal Studies

Eight to ten week old Balb/c female mice were obtained and maintained on a 12-hour dark and 12-hour light cycle with access to rodent chow and water. The female mice were mated with males, and checked for presence of a vaginal plug, marked as gestational day 1 (GD1). Pregnant mice received (1) 1.2% L-arginine in drinking water, or (2) regular drinking water daily, starting on GD1. Meanwhile, PbA was passaged through BALB/c mice. These mice were monitored twice daily and daily thin blood smears with 2 uL of tail vein blood were used to monitor parasitemia. Once sufficient parasitemia was obtained (day 5 post infection), the passage mice were euthanized with carbon dioxide and blood was collected via cardiac puncture. Infected erythrocytes from these mice were used to infect the experimental, pregnant mice. Pregnant mice were infected on GD13 with 1x10⁶ PbA infected erythrocytes in RPMI media, or 2) RPMI alone, via lateral tail-vein injection. Maternal peripheral parasitemia was monitored daily over the course of infection (GD13-GD19) by thin blood smear with modified Giemsa stain. There was a total of four treatment groups, eight animals per group: (1) Uninfected dams, (2) PbA infected dams, (3) Uninfected dams receiving L-arginine (1.2% in drinking water) and (4) PbA infected dams receiving L-arginine (1.2% in drinking water). The dams were euthanized on GD 19, and fetuses were checked for viability by assessing the pedal withdrawal reflex. Maternal blood was collected by cardiac puncture and maternal, and fetal and placental tissue from viable fetuses were isolated, snap frozen in liquid nitrogen and stored at -80°C until further use. The placenta, maternal brain, maternal liver, maternal kidney, fetal liver and fetal brain were also isolated, snap frozen in liquid nitrogen and stored at -80°C until analysis. The animal study outlined above was repeated twice over two separate occasions (3 months apart). The first animal study included 14 dams (4 groups of 4 dams, while the PbA + L-arginine group had 2 dams). The second animal study included 25 dams (Control group had 7 dams, Control + L-arginine group had 5 dams, PbA group had 8 dams, and PbA + L-arginine group had 5 dams).
3.1.1 RNA Extractions, cDNA Synthesis and Quantitative-Polymerase Chain Reaction (RT-qPCR)

RNA was isolated using TRIzol reagent, assessed for purity, and reverse transcribed to cDNA. The mRNA expression of transporters, proinflammatory cytokines and drug metabolizing enzymes were examined by reverse transcription quantitative polymerase chain reaction (qRT-PCR).

3.1.2 RNA Extraction

1 mL of TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) was added to 50–100 mg of tissue sample and homogenized using a homogenizer. The samples were then incubated for 5 minutes to permit complete dissociation of the nucleoprotein complex. 0.2 mL of chloroform was added to each sample for lysis, and samples were incubated for 2–3 minutes. The samples were centrifuged for 15 minutes at 12,000 × g at 4°C to allow the mixture to separate into a lower red phenol-chloroform, an interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred to a new tube.

0.5 mL of isopropanol was added to the aqueous phase, incubated for 10 minutes and centrifuged for 10 minutes at 12,000 × g at 4°C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube. The supernatant was discarded with a micropipette.

The pellet was then re-suspended in 1 mL of 75% ethanol. The samples were vortexed briefly, then centrifuged for 5 minutes at 7500 × g at 4°C. The supernatant was discarded and the RNA pellet was allowed to air dry for 15-20 minutes. The pellet was re-suspended in 50 µL of RNase-free water by pipetting up and down until the RNA pellet was completely dissolved.

The RNA yield and quality was determined by UV absorbance. Basically, samples were diluted in RNase-free water (Life Technologies Inc., CA, USA) and measured using a NanoDrop®-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Concentrations ranged from 500-1000ng/ul and only samples with desired purity were used (A260/A280 ratio = 2; A260/A230 ratio = 1.8).

3.1.3 DNAse I Treatment

2 µg of total extracted RNA was treated with 4 U / µL DNAse I enzyme (Invitrogen, Burlington, ON) in 25 mM MgCl2 (Sigma Aldrich, Oakville, ON) and
adjusted to a final volume of 20 µL with nuclease-free DEPC H2O (Life Technologies Inc., CA). DNAse I enzyme treatment was completed in an Eppendorf® Mastercycler EPgradient thermocycler (Eppendorf AG, Hamburg, Germany) with the following thermocycling parameters: 37°C for 30 minutes, 75°C for 10 minutes, and held at 4°C until removal from thermocycler.

### 3.1.4 cDNA Synthesis

Single-stranded cDNA was synthesized from 2 µg of DNase I treated RNA using the High-Capacity cDNA Reverse Transcription Kit for qPCR (Applied Biosystems, Burlington, ON, Canada). Briefly, a master mix of 10X reverse transcriptase buffer, 25X dNTP mix (100mM), 10X reverse transcriptase random hexamer primers, MultiScribe™ reverse transcriptase enzyme, and nuclease-free DEPC H2O was prepared and 20 µL of this master mix was added to each RNA sample. cDNA preparation was completed in an Eppendorf® Mastercycler EPgradient thermocycler with the following thermocycling parameters: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C, and samples were held at 4°C until removal from the thermocycler.

### 3.1.5 Quantitative-Polymerase Chain Reaction (RT-qPCR)

mRNA expression of drug transporters, drug-metabolizing enzymes, and disease-relevant genes in maternal tissues, fetal tissues, and the placenta were determined by real-time reverse-transcriptase quantitative polymerase chain reaction (qPCR). Each RNA sample was plated in triplicate and assayed for genomic cDNA specific for each primer set using Power SYBR Green detection system (ABI 7900HT; Applied Biosystems, Burlington, ON, Canada). Relative mRNA levels were calculated using the comparative threshold cycle method (ΔΔCt), where each gene of interest was normalized to GAPDH or 36B4, depending on the tissue [187]. The primers used are listed in Table 2 in the appendix.

### 3.2 Total Bile Acid

Total bile acids in plasma was analyzed in infected and control dams using the Crystal Chem, Inc. Mouse Total Bile Acids Kit according to manufacturer directions. The whole fetus was homogenized in 1mL of 75% ethanol, and then incubated for 2 hours at 50°C. The homogenate was then centrifuged for 10 minutes at 6000 x g at 4°C, and the supernatant fraction which contains the bile acids was retained.
3.2.1 Assay Procedure

150µL of reconstituted Reagent CC3 and 20µl of sample, calibrator, or control were added into each well of a microplate and mixed well by repeat pipetting. The microplate was then placed in the incubator and allowed to equilibrate to 37°C over 5 minutes, and the absorbance was measured using a plate reader at 540nm. 30µl of Reagent CC2 was added to each well and mixed by repeated pipetting. The increase in absorbance was measured using a plate reader at 540nm, after the wells were allowed to equilibrate to 37°C over 5 minutes.

3.2.2 Determining total bile acids concentration

The mouse total bile acids concentration was calculated using the following equation:

\[
\text{BA concentration} = \frac{(\text{sample A540} - \text{blank A540})}{(\text{calibrator A540} - \text{blank A540})} \times \text{calibrator concentration}
\]

The calibrator concentration value was obtained from the calibrator label.

3.3 Statistical Analysis

All data were analyzed with GraphPad Prism 6.0 for Macintosh (Graphpad Software, Inc., San Diego, CA, USA). Data from the two separate experiments were combined, as the results from each study were not significantly different. To assess for differences in maternal and fetal parameters i.e. maternal weight, fetal weight, bile acid levels, and epigenetic changes the Student’s unpaired two-tailed t-test was used. To assess for differences in gene expression levels between groups, the non-parametric Mann-Whitney U test was conducted. All results are presented as mean % change from control ± standard error of the mean (SEM). Levels of significance for all statistical analyses were set at or below \( \alpha = 0.05 \), with the following symbols denoting statistical significance: *, \( p < 0.05 \), **, \( p < 0.01 \) and ***, \( p < 0.001 \); #, \( p < 0.05 \), ##, \( p < 0.01 \) and ###, \( p < 0.001 \). Asterisks indicate values that are significantly different from the water versus l-arginine groups, whereas pound signs indicate values that are significantly different from respective control dam group.
Chapter 4. Results

4

4.1 *Plasmodium berghei* ANKA Malaria Infection Impacts Maternal and Fetal Parameters

4.1.1 Placental, Maternal and Fetal Weight

While placental weight was not significantly altered in PbA infected dams (Fig 3A), we saw a significant decreases in maternal weight (Fig 3B.) and fetal weight (Fig. 3C) in PbA infected dams receiving regular and L-arginine supplemented water, as compared to the control dams.

![Figure 3](image)

Figure 3. Impact of *Plasmodium berghei* ANKA Malaria Infection on (A) placental weight, (B) maternal weight, and (C) fetal weight on GD19. Results presented as mean weight as % control ± SEM. Mann-Whitney U test was used for statistical analysis. (A) N= 19-42 placentae/group, (B) N= 7-12 dams/group, and (C) N= 19-42 pups/group. #, p<0.01; and ###, p <0.001. # denotes statistical significance from the respective control dam group.
4.1.2 Spleen Weight

As compared to controls, there was a two-fold increase in the weight of spleens isolated from the PbA infected dams (Fig. 4). L-arginine supplemented water showed no improvements in maternal and fetal parameters.

Figure 4. Impact of *Plasmodium berghei* ANKA Malaria Infection on spleen weight on GD19. Results presented as mean spleen weight as % control ± SEM. Mann-Whitney U test was used for statistical analysis. N= 7-12 dams/group. ###, p <0.001. # denotes statistical significance from the respective control dam group.
4.2 *Plasmodium berghei* ANKA Malaria Infection Alters Placental Transporter Expression

As compared to controls, the placental mRNA expression of Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3 and Abcg2, were significantly down regulated in both PbA infected dams receiving regular or L-arginine supplemented water (Fig. 5). As compared to the PbA infected dams receiving regular water, Abcb1a and Abcb1b levels were significantly lower in the PbA infected dams receiving L-arginine supplemented water. L-arginine supplemented water showed no improvements in the mRNA expression of placental ABC transporters.

![Figure 5. Impact of *Plasmodium berghei* ANKA Malaria Infection on ABC efflux transporters in the placenta on GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to Gapdh. Mann-Whitney U test was used for statistical analysis. N= 14-20 placentae/group. *, p < 0.05; #, p < 0.05; ##, p < 0.01; and ###, p <0.001. * denotes statistical difference from the water versus l-arginine groups; and # denotes statistical significance from the respective control dam group.](image)
As compared to controls, there was a significant decrease in placental mRNA levels of Slc22a1, Slc29a1 and Slc29a2 in the PbA infected dams receiving either regular or L-arginine supplemented water (Fig 6). On the other hand, the expression of Slco2b1 was only significantly affected in PbA infected dams receiving L-arginine. Slc29a2 was significantly lower in control animals receiving L-arginine supplemented water as compared to controls receiving regular drinking water. L-arginine supplemented water showed no improvements in the mRNA expression of placental SLC transporters.

**Figure 6. Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in the placenta on GD19.** Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to Gapdh. Mann-Whitney U test was used for statistical analysis. N= 14-20 placentae/group. *, p < 0.05; #, p < 0.05; ##, p < 0.01; and ### p < 0.001. * denotes statistical difference from the water versus l-arginine groups; and # denotes statistical significance from the respective control dam group.
4.3 Impact of *Plasmodium berghei* ANKA Malaria Infection on Hepatic Transporters and Cyp3a11 Expression

As compared to controls, the hepatic mRNA expression of Abcc2, Abcb11, Abcg2, and drug metabolizing enzyme Cyp3a11 were significantly down regulated in both PbA infected dams receiving regular or L-arginine supplemented water (Fig. 7). As compared to the control dams, Abcb1a levels were significantly lower in the PbA infected dams receiving regular water but not those receiving L-arginine. As compared to the control dams receiving L-arginine supplemented water, Abcb1b levels were significantly higher in the PbA infected dams receiving L-arginine supplemented water. As compared to the control dams receiving regular water, Cyp3a11 was significantly lower in the control dams receiving L-arginine supplemented water. L-arginine supplemented water showed no improvements in the mRNA expression of hepatic ABC transporters.

*Figure 7. Impact of Plasmodium berghei ANKA Malaria Infection on ABC efflux transporters in maternal liver on GD19.* Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to 36B4. Mann-Whitney U test was used for statistical analysis. N= 7-8 dams/group. **, p < 0.01; #, p < 0.05; ##, p <0.01; and ###, p <0.001. * denotes statistical difference from the water versus l-arginine groups; and # denotes statistical significance from the respective control dam group.
As compared to controls, there was a significant decrease in hepatic SLC levels of Slc02b1, Slc10a1, Slc22a1 and Slc47a1 in the PbA infected dams receiving either regular or L-arginine supplemented water (Fig 8). L-arginine supplemented water showed no improvements in the mRNA expression of hepatic SLC transporters.

Figure 8. Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in maternal liver on GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to 36B4. Mann-Whitney U test was used for statistical analysis. N= 7-8 dams/group. #, p < 0.05; ##, p <0.01; and ###, p <0.001. # denotes statistical significance from the respective control dam group.
4.4 Impact of *Plasmodium berghei* ANKA Malaria Infection on Hepatic Expression Inducible Nitric Oxide Synthase and Heme-Oxygenase 1

As compared to controls, there was a significant increase in hepatic levels of iNOS and HO-1 in the PbA infected dams receiving either regular or L-arginine supplemented water (Fig 9). L-arginine supplemented water showed no improvements in the mRNA expression of these hepatic transporters.

![Graph showing mRNA expression of iNOS and HO-1](image)

**Figure 9.** Impact of *Plasmodium berghei* ANKA Malaria Infection on Hepatic Expression of iNoS and HO-1 in maternal liver on GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in *P. berghei* infected and control dams at GD19 were normalized to 36B4. Mann-Whitney U test was used for statistical analysis. N= 7-8 dams/group. #, p < 0.05; ##, p <0.01; and ###, p <0.001. # denotes statistical significance from the respective control dam group.
4.5 Impact of *Plasmodium berghei* ANKA Malaria Infection on Transport Expression in the Maternal Brain

As compared to controls, the maternal brain mRNA expression of Abcc2, Abcb1a, Abcb1b and Abcc4 were not significantly altered in both PbA infected dams receiving regular or L-arginine supplemented water (Fig. 10). L-arginine supplemented water showed no improvements in the mRNA expression of maternal brain ABC transporters.

![Figure 10](image.png)

**Figure 10.** Impact of *Plasmodium berghei* ANKA Malaria Infection on ABC efflux transporters in maternal brain on GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to Gapdh. Mann-Whitney U test was used for statistical analysis. N= 4-6 dams/group.
As compared to controls, there was a significant decrease in maternal brain levels of Slc29a1 and Slc29a2 in the PbA infected dams receiving regular water (Fig 11). As compared to the PbA infected receiving dams receiving regular water, there was a significant increase in the PbA infected dams receiving L-arginine supplemented water. L-arginine supplemented water showed no improvements in the mRNA expression of maternal brain SLC transporters.

**Figure 11. Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in maternal brain on GD19.** Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to Gapdh. Mann-Whitney U test was used for statistical analysis. N= 4-6 dams/group. **, p < 0.01; #, p < 0.05; and ###, p <0.01. * denotes statistical difference from the water versus l-arginine groups; and # denotes statistical significance from the respective control dam group.
4.6 Impact of *Plasmodium berghei* ANKA Malaria Infection on Transporter Expression in the Maternal Kidney

As compared to controls receiving L-arginine supplemented water, the renal mRNA expression of Abcc2, Abcb1a and Abcb1b were significantly up regulated in PbA infected dams L-arginine supplemented water (Fig. 12). As compared to the control dams receiving regular water, Abcb1a levels were significantly higher in the PbA infected dams receiving regular water. As compared to the control dams receiving regular water, Abcc4 levels were significantly lower in the PbA infected dams receiving regular water.

![Figure 12. Impact of *Plasmodium berghei* ANKA Malaria Infection on ABC efflux transporters in maternal kidney on GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in *P. berghei* infected and control dams at GD19 were normalized to Gapdh. Mann-Whitney U test was used for statistical analysis. N= 6-7 dams/group. #, p < 0.05; and ##, p < 0.01. # denotes statistical significance from the respective control dam group.](image-url)
As compared to controls receiving regular water, there was a significant decrease in renal SLC levels of Slc15a2 and Slc22a1 in the PbA infected dams receiving regular water (Fig 13). As compared to controls receiving L-arginine supplemented water, there was a significant decrease in Slc22a2 in the PbA infected dams receiving L-arginine supplemented water. L-arginine supplemented water showed no improvements in the mRNA expression of renal SLC transporters.

Figure 13. Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in maternal kidney on GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to Gapdh. Mann-Whitney U test was used for statistical analysis. N= 6-7 dams/group. #, p < 0.05; and ##, p <0.01. # denotes statistical significance from the respective control dam group.
4.7 Impact of *Plasmodium berghei* ANKA Malaria Infection on Hepatic Transporters in the Fetal Liver

As compared to controls, fetal hepatic mRNA expression of Abcb11 were significantly down regulated in PbA infected dams receiving bother regular and L-arginine supplemented water (Fig. 14). As compared to the control dams receiving L-arginine supplemented water, Abcc1 levels were significantly lower in the PbA infected dams receiving L-arginine supplemented water. L-arginine supplemented water showed no improvements in the mRNA expression of fetal hepatic ABC transporters.

![Graph showing mRNA expression of ABC transporters in PbA infected dams](image)

**Figure 14. Impact of Plasmodium berghei ANKA Malaria Infection on ABC efflux transporters in fetal liver on GD19.** Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to 36B4. Mann-Whitney U test was used for statistical analysis. N= 5-7 dams/group. *, p < 0.05; #, p < 0.05; and ##, p <0.01. * denotes statistical difference from the water versus l-arginine groups; and # denotes statistical significance from the respective control dam group.
As compared to controls receiving regular water, there was a significant decrease in fetal hepatic SLC levels of Slc10a1, Slc22a1 and Slc47a1 in the PbA infected dams receiving regular water (Fig 15). As compared to controls receiving L-arginine supplemented water, there was a significant decrease in Slc22a1 and Slc47a1 in the PbA infected dams receiving L-arginine supplemented water. As compared to controls receiving regular water, there was a significant decrease in Slc47a1 in the control dams receiving L-arginine supplemented water. L-arginine supplemented water showed no improvements in the mRNA expression of fetal hepatic SLC transporters. L-arginine supplemented water showed no improvements in the mRNA expression of fetal hepatic SLC transporters.

Figure 15. Impact of Plasmodium berghei ANKA Malaria Infection on SLC uptake transporters in fetal liver on GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to 36B4. Mann-Whitney U test was used for statistical analysis. N= 5-7 dams/group. *, p < 0.05; #, p < 0.05; and ##, p <0.01. * denotes statistical difference from the water versus l-arginine groups; and # denotes statistical significance from the respective control dam group.
4.8 Impact of *Plasmodium berghei* ANKA Malaria Infection on Hepatic Expression of Heme Oxygenase-1 in the Fetal Liver

As compared to controls, there was no significant change in fetal hepatic levels of HO-1 in the PbA infected dams receiving either regular or L-arginine supplemented water (Fig 16). L-arginine supplemented water showed no improvements in the mRNA expression of fetal HO-1 levels.

![Graph showing HO-1 mRNA expression in different groups](image)

Figure 16. Impact of *Plasmodium berghei* ANKA Malaria Infection on Hepatic Expression of HO-1 in the Fetal Liver GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in P. berghei infected and control dams at GD19 were normalized to 36B4. Mann-Whitney U test was used for statistical analysis. N= 4-6 dams/group.
4.9 Impact of *Plasmodium berghei* ANKA Malaria Infection on Total Plasma Bile Acid Levels

4.9.1 Maternal Plasma Bile Acid Levels

As compared to controls, there was no significant change in maternal plasma bile acid levels in the PbA infected dams receiving either regular or L-arginine supplemented water (Fig 17). L-arginine supplemented water showed no improvements.

![Graph showing bile acid levels](image)

**Figure 17. Impact of *Plasmodium berghei* ANKA Malaria Infection on Maternal Plasma Bile Acid levels on GD19.** Results presented as mean plasma bile acid levels as concentration (µmol/L) ± SEM. Mann-Whitney U test was used for statistical analysis. N= 4 dams/group.
4.9.2 Whole Fetal Tissue Bile Acid Levels

As compared to controls, there was no significant change in fetal plasma bile acid levels in the PbA infected dams receiving either regular or L-arginine supplemented water (Fig 18). L-arginine supplemented water showed no improvements.

**Figure 18. Impact of Plasmodium berghei ANKA Malaria Infection on Whole Fetus Bile Acid levels on GD19.** Results presented as mean bile acid levels as concentration (µmol/L) ± SEM. Mann-Whitney U test was used for statistical analysis. N= 4 pups/group.
Chapter 5. Discussion

I hypothesized that reducing oxidative stress during PM by dietary L-arginine supplementation would attenuate or normalize expression of transporters during infection. The findings from the experiments I conducted did not support my hypothesis. Overall, I did not detect changes in the expression of transporters following dietary L-arginine supplementation. These findings could be a result of L-arginine not functioning as a potent enough anti-oxidant or anti-inflammatory agent. Additionally, arginase enzymes, the endogenous antagonists to iNOS, could be competing for the L-arginine substrate by metabolizing it to l-Orn and urea, hence very little iNOS is metabolizing L-arginine to NO and l-citrulline.

My findings on ABC transporter expression levels in various tissues were consistent with those observed in Cressman et al. in the PbA infected and control dams receiving regular drinking water. The expression of ABC transporters and drug metabolizing enzyme Cyp3a11 were significantly altered across all tissues.

Numerous endogenous compounds and xenobiotics are organic anions or cations. Their disposition and elimination depends on the function of drug transporters that belong to the ATP and SLC transport protein families [72]. As the ABC transporters have been previously discussed and published, my discussion will focus on the SLC uptake transporters. Many SLCs are involved in the epithelial transfer of drug substrates during the process of absorption, distribution and elimination therefore modulation of these transport systems may alter drug efficacy and toxicity. Substrates have different affinities for the transporters that direct their transfer, thus the interaction between inflammatory stimuli and transporter activities should be assessed very carefully when considering medication usage or treatment during pregnancy. Additionally, since there is an overlap of substrates across transporters, there is a mutual functioning relationship between transporters with endogenous metabolites and xenobiotics.

SLC transporters transport solutes, such as ions, metabolites, peptides, and drugs, across biological membranes. Slco2b1 (OAT2B1), Slc22a1 (OCT1), Slc29a1 (ENT1), Slc29a2 (ENT2), Slc47a1 (MATE1) and Slc10a1 (NTCP) were all significantly down
regulated in maternal and fetal tissues in the PbA infected dams in our study, with no observed improvements following dietary l-arginine supplementation.

Additionally, there were no significant differences in results obtained from the animal studies conducted on two separate occasions, three months apart. Although Dr. Kain’s laboratory observed improved fetal and maternal parameters in a study performed using the same animal model outlined above (section 3.1), a large sample size (n>100) was needed in order to reach significance (manuscript in preparation).

5.1 Impact of P. berghei ANKA Malaria Infection on Maternal and Fetal Parameters

Maternal infection with Plasmodium falciparum resulted in decreased maternal and fetal weight (Figure 3). Although very little is known about the precise mechanism by which parasite accumulation and placental inflammation result in fetal adverse events, it is believed complement C5a is partially involved [19]. C5a levels in maternal plasma and the placental messenger RNA that encode the C5a receptor are both increased during PM infection [20]. C5a is also implicated with poor fetal outcome in an antibody-independent mouse model of spontaneous miscarriage and intrauterine growth restriction IUGR [188], [189]. Additionally, a recent study published by Lybbert et al. provided first evidence that the megalin transport/signaling system and its reduced abundance during PM may also contribute to LBW [22].

Dietary arginine supplementation has been found to have beneficial effects in pregnant rats and pigs, where dietary arginine supplementation has been shown to increase the live born litter weight in pigs and rats by 24 and 30%, respectively, and the number of live-born piglets increased by two [190], [191]. A study published by Ren et al. (2012) observed a non-significant trend toward increased birth weight in porcine circovirus (PCV2) infected mice following dietary arginine supplementation [161]. While litter number, daily weight gain and litter birth weight were not significantly altered by L-arginine supplementation, they tended to be higher than that seen in the control PCV2 group. Thus, the effect of dietary arginine supplementation may change in cases of disease and infection. Therefore it is plausible that L-arginine supplementation may not have been effective in improving outcomes in PbA infected mice due to the severity of the PM infection.
Ren et al. also saw significant decreases in the mortality and abortion rates in PCV2 infected mice receiving L-arginine compared to the control group (PCV2 infected mice receiving alanine). It was believed that this drop in in abortion rate was related to progesterone, which was significantly increased following dietary arginine supplementation [161]. Progesterone is a major hormone in the maintenance and establishment of pregnancy, which in turn decreases abortion rate [192]. However, Li and Bazer (2010) observed that 0.8% dietary arginine supplementation resulted in reduced maternal plasma concentrations of progesterone in pregnant pigs. These differences could be due to species differences in metabolic pathways of progesterone or arginine. In the study by Ren et al., serum NO levels in the arginine group were significantly higher than in the control group. This was in agreement with other studies, which indicates that arginine is the sole substrate for NO synthesis and dietary arginine deficiency in young rats decreases plasma concentrations of arginine and impaired NO synthesis [193]. NO plays an important role in physiology, as it is a major endothelium-derived factor, a mediator of immune response, a neurotransmitter, a cytotoxic free radical and a widespread signaling molecule; L-arginine is the immediate precursor of NO [194]. Furthermore, NO is an antimicrobial agent effective against intracellular pathogens, extracellular parasites and bacteria [195],[196], [197]. Thus, higher NO levels are advantageous and explain the improved outcomes observed in the arginine group. Although previous studies indicate that dietary L-arginine could improve maternal and fetal outcomes, there were no beneficial effects observed in our study. This may possibly be due to the fact that PbA infection in this PM model is very severe so it may mask any beneficial effects of L-arginine, or that there was an insufficient supplementation of L-arginine.

Malaria-infected RBCs contain an increasingly large and rigid parasite, therefore the spleen plays a pivotal role in Plasmodium clearance. The spleen is made up of distinct micro anatomical zones and microcirculations, and is responsible for the removal of senescent red blood cells (RBCs), infectious microorganisms and Plasmodium-parasitized RBCs [198]–[201]. The filtering capacity of the spleen is related to its trabecular structure formed by the white pulp, the red pulp, and the marginal zone lying between the white pulp and red pulp, where inert particles, bacteria and viruses such as
parasitized RBCs are eliminated [199]. During infection, the weight of the spleen increases as a sign of activation of the immune system, therefore spleen weight and size is used to determine the intensity of infection and transmission intensity. Infection by malaria is the most common cause of spleen rupture and splenomegaly [202]. A study conducted by Engwerda et al. in 2005 found the spleen weight of P. berghei infected pregnant female mice to be increased by 5-fold when compared to the uninfected controls, representing an activation of the maternal immune system [199]. We observed a 2-fold increase in spleen weight in the infected dams when compared to the uninfected controls (Figure 4). There was no difference in the infected dams receiving regular drinking water or L-arginine supplemented water. Thus, it remains consistent in our study that the spleen is the main organ involved in the elimination of pRBCs, and dietary L-arginine supplementation has no effect on parasitized RBCs.

5.2 Impact of P. berghei ANKA Malaria Infection on SLC Uptake Transporters in Maternal Tissues

5.2.1 Impact on Placenta

The placenta plays a protective role between the mother and fetus, playing a critical role in fetal growth and development, and providing nutrients. P. berghl ANKA malaria infection led to a significant decrease in mRNA expression levels of Slco2b1 (Oatp2b1), Slc22a1 (Oct1), Slc29a1 (Ent1) and Slc29a2 (Ent2) (Figure 6) in PbA infected mice compared to control mice, which will be further discussed in the sections below.

PM was associated with a down regulation in the placental expression of Ent1 (Slc29a1) and Ent2 (Slc29a2). These transporters are involved in the transport of purine and pyrimidine nucleosides, including adenosine. These transporters also play a key role in the cellular uptake and efficacy of antivirals; therefore the malaria-induced downregulation observed could result in diminished responsiveness in HIV-Malaria co-infected pregnant woman. Individuals living in areas with high Plasmodium falciparum parasite rate have increased odds of being HIV positive [203]. Additionally, adenosine poses an anti-inflammatory effect, however this protective effect is limited due to rapid cellular re-uptake of adenosine by ENT1 or break down by adenosine kinase (AK), a key enzyme in adenosine clearance pathway [71]. Mice treated with an AK inhibitor have
demonstrated a significant reduction of ENT1, TNF-α, IL-6, and iNOS protein and mRNA expression as revealed by western blot and real time PCR. The AK inhibitor has a protective role against marked traumatic optic neuropathy induced retinal inflammation and damage by increasing the therapeutic effects of site and event specific accumulation of endogenous extracellular adenosine [204]. Additionally, in vivo experiments using a murine model of inflammatory lung injury showed that the pharmacological inhibition of ENT1 and ENT2 resulted in improved pulmonary barrier function and reduced signs of acute inflammation of the lung [71]. The decrease in ENTs in the placenta could be a protective response to the malarial infection. However, this is not a conclusive statement, as the effect of adenosine on inflammatory responses in the placenta is not known. Lastly, L-arginine does not seem to normalize the expression of either of these transporters.

The clinical relevance of murine Oatp2b1 is far less understood than that of human OATP2B1. OATPs have broad substrate specificity and many are highly expressed in epithelial membranes, thus they are thought to play an important role in drug disposition. Oatp2b1 knock out mice were found to be viable and fertile, and did not show clear pathological abnormalities, however they displayed marked conjugated hyperbilirubinemia and associated jaundice [205].

Due to its broad substrate specificity and high expression in the intestine, liver and blood brain barrier, OATP2B1 is involved in the absorption and disposition of numerous endogenous compounds, xenobiotics and clinically important drugs, such as statins, fexofenadine and rifampicin [127]. Moreover, evidence suggests that the transport of OATP2B1 substrates requires co-expression of an efflux transporter in the corresponding apical membrane, such as BCRP [206]. Bcrp plays a large role in protecting placental and fetal tissue by removing potentially toxic xenobiotics and endogenous metabolites [207].

Previous studies in pregnant rats have seen down regulation of Bcrp and Oatp2b1 in placenta following endotoxin-induced inflammation. Likewise, in human placenta significant and similar downregulation of OATP2B1 and BCRP were seen in in placentas from women with chorioamnionitis infection [206]. Thus, there may be a functional interaction between OATP2B1 and BCRP in the human placenta. Such cooperation is of
functional importance because compounds from the fetal circulation enter the syncytiotrophoblast cells through OATP2B1 and are subsequently eliminated into the maternal blood by BCRP. In the placenta, Oatp2b1 and Bcrp were both significantly decreased following PbA infection regardless of L-arginine supplementation. This down regulation can affect the transplacental transport of bile acids and steroid hormones. Although the effect of steroids is poorly understood, they play an important role in regulating key physiological events essential to the maintenance of pregnancy and development of the fetus [208]. Hormones are crucial for the normal fetal development and growth, and a positive correlation has been reported between fetal birth weight, and serum placental growth hormone and placental lactogen levels in pregnant women [209]. Additionally, estrogen enhances uteroplacental blood flow and possibly placental neovascularization to provide optimal gas exchange and the nutrients required for the rapidly developing fetus and placenta [208]. Thus, fetal growth may be comprised when Oatp2b1 and Bcrp are down regulated. Additionally, L-arginine does not seem to normalize the expression of either of these transporters.

5.2.2 Impact on Liver

The liver plays an extremely critical role in drug metabolism. Enzymes located in the endoplasmic reticulum of the liver cells protect the organism against an accumulation of lipid-soluble exogenous and endogenous compounds by converting them to water-soluble metabolites, which can be then be 1) easily excreted by the kidney, or 2) subject to biliary clearance before reaching systemic circulation [210].

OCT1 is highly expressed in the liver and is involved in the uptake of xenobiotic and endogenous compounds, and other important cationic drugs [89]. The uptake of the antimalarial quinine, depends in part on the expression and function of OCT1 [211]. Studies in OCT1 knockout mice have demonstrated that Oct1 plays an important role in both the hepatic uptake and intestinal excretion of organic cations. Oct1 knockout mice had four-to-six fold lower hepatic accumulation of the OCT substrate tetraethylammonium (TEA), as compared to wild type mice. Our results demonstrated that hepatic expression of Oct1 was significantly decreased in PbA infected dams regardless of L-arginine supplementation (Figure 8). A down regulation of Oct1 has also been reported in cholestatic liver disease and advanced hepatocellular carcinoma [37].
Choline, a macronutrient involved in liver function, and agmatine (AGM), a metabolite of L-arginine, are substrates of OCT1. Choline influences the enterohepatic circulation of bile and cholesterol, and choline deficiency can result in hepatosteatosis and liver cell death [212], [213]. Thus, the decrease in the expression levels of Oct1 can result in compromised liver function.

Agmatine (AGM) is an amine that is formed by decarboxylation of L-arginine by the enzyme arginine decarboxylase, and hydrolyzed by the enzyme agmatinase to putrescine [214]. It is involved in many physiological and pharmacological effects, and the liver plays a crucial physiological role in the maintenance of AGM homeostasis [215], [216]. AGM can decrease LPS-induced acute hepatic injury in mice via suppression of NF-κB translocation, and decrease the synthesis and release of cytokines [216]. AGM protective effects have also been shown in mice with fulminant hepatic failure, and it is believed to be related to its ability to suppress NO synthesis, and TNF-α production [217]. Therefore, AGM may play a crucial role in hepatic inflammatory diseases. Thus, the decrease in the expression levels of Oct1 in our study diminishes the anti-inflammatory effects caused by L-arginine supplementation.

Similar to the results observed in the placenta, the expression of Oatp2b1 and Bcrp were significantly decreased in maternal liver following PbA infection regardless of L-arginine supplementation (Figure 7 and Figure 8). Oatp2b1 is involved in the hepatic uptake of many endogenous substrates including bile acids and steroids hormones as well as clinically important drugs, such as lopinavir and rifampicin. On the other hand Bcrp is involved in the hepatobiliary secretion of many of these substrates, including estrone-3-sulfate and taurocholate [218]–[220]. Therefore, this indicates that there is reduced hepatic uptake of many xenobiotics, a reduced biliary clearance of toxins, and exogenous substrates, and an increase systemic exposure to xenobiotics.

Results from our study showed a significant decrease in Ntcp (Slc10a1) levels in the maternal liver of PbA infected mice (Figure 8). The sodium bile acid transporter, Ntcp, is predominately responsible for the uptake of bile salts. It is well recognized that down regulation of the Bile Salt Export Pump (BSEP/ Abcb11), such as that seen in the PbA infected dams, causes increased accumulation of bile acids, which in turn triggers down regulation of NTCP (Slc10a1). Human liver disease, progressive familial
intrahepatic cholestasis, and numerous animal models of inflammation have demonstrated a pattern of down regulation of NTCP expression [221]. This minimizes the hepatic uptake of bile salts, thus serving to protect hepatocytes from the accumulation of cytotoxic bile salts and acids [65]. It is also believed that NTCP is inversely correlated with serum bilirubin and bile salt levels [222], [223].

The bile salt export transporter, ABCB11, is a critical determinant of bile acid homeostasis in the liver and facilitates hepatobiliary clearance of lipophilic substrates. As previously described by Cressman et al., PbA infection imposes a pronounced decrease in the expression of Abcb11, along with a dramatic upregulation in the basolateral expression of Abcc1 and Abcc3, while levels of Ntcp were unchanged. It was hypothesized that due to the impaired canalicular secretion, a compensatory increase transport of bile acids into the maternal circulation by Abcc1 and Abcc3, imposed the observed 80-fold increase in total serum concentrations of total bile acids in the PbA-infected dams [15]. While we observed a pronounced trend of increased plasma bile acid levels in the PbA infected dams, it did not reach significance (Figure 17). Moreover, while we detected a significant infection-induced reduction in Ntcp, the expression of Abcc3 was not significantly increased. Therefore altered hepatobiliary compensatory mechanisms could be responsible for the differences. On the other hand, the differences between these studies could be due to technical differences in measurements as the previous study outsourced quantification of total bile acids while the current study utilized an Elisa kit (Crystal Chem, Inc. Mouse Total Bile Acids Kit) for quantification. Therefore this could be due to issues of sensitivity. The Cyrstal Chem Kit we used is a highly quality enzymatic assay, while IDEXX laboratories use a potentiometric assay. Additionally, while the kit we used is mouse specific, the method used by IDEXX laboratories is non-species specific.

Since Cressman et al. saw such a pronounced increase in maternal bile acid levels; we wanted to investigate whether fetal levels of bile acids were affected. It has been reported that maternal cholestasis elevates bile acid levels in fetal circulation. We found that total bile acid levels in whole fetuses were not altered by PbA infection (Figure 18). The fetus is able to eliminate increases in bile acids by transporting excessive amounts of bile acid. This is facilitated by reduced fetal plasma albumin
concentration, allowing bile acids in free solution to be diffused more easily into the placenta, lowering fetal serum bile acid concentrations [224], [225]. Moreover, Abcg2 is able to export bile acids from the fetus and placenta and is as important in the transport of bile acids in the placenta, as BSEP is in the liver [226].

In this study we found a significant decrease in MATE1 in the maternal liver in the PbA infected dams receiving regular and L-arginine supplemented water (Figure 8). MATE1 plays an important role in the biliary secretion of many endogenous and exogenous organic cations. Decreased MATE1 expression has been observed in animal models of human diseases including acute kidney injury and chronic renal failure [227]– [230]. MATE1 dysfunction is known to cause a marked elevation in the metformin concentration in the liver which can lead to lactic acidosis in mice [231]. Decreased elimination of the endogenous substrate of MATE1, polyhexamethylene guanidinium hydrochloride (PHMG), an antimicrobial biocide of the guanidine family, causes acute liver injury [232]. Thus, the decrease in Mate1 observed in our study may contribute to hepatic inflammation and the significant increases observed in the hepatic inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1).

5.2.3 Impact on Fetal Liver

In the fetal liver, PbA induced changes in the expression of SLC uptake transporters, Slc10a1, Slc22a1 and Slc47a1, were consistent with those observed in maternal liver (Figure 15). This indicates that PbA imposes a similar trend of dysregulation of transporters in both maternal and fetal tissues [233].

On the other hand, while Oatp2b1 was significantly down regulated in maternal liver of PbA-infected dams, no significant changes were observed in fetal liver. This could be due to the fact that Slco2b1 may not be fully developed in the fetus, resulting in extremely low basal levels of expression. Alternatively this could also be due to an underdeveloped transcription regulatory pathway for Slco2b1. [233].

5.2.4 Impact on Kidney

Renal tubular secretion is performed by transporters located at the basolateral membranes that mediate the cellular uptake of substrates from blood, and transporters at the brush-border membranes that mediate the efflux of cellular substrates into the tubular lumen [234]. Renal elimination is a major route of clearance for many drugs and their
metabolites. Decreased function of the kidney, and thus kidney filtration is linked to serious kidney diseases [235].

While OCT1 is primarily expressed in the liver in humans, OCT1 is also expressed in the proximal tubule epithelial cells of rodents [80]. OCT2 is generally considered to be a kidney transporter and mainly localized to the luminal membrane of the distal convoluted tubules. We observed a significant and pronounced trend of decreased expression in Slc22a1 in the PbA infected dams when compared to the control dams, and Slc22a2 in the PbA infected dams receiving dietary L-arginine supplementation (Figure 13). Studies in Oct1/2 knockout mice have demonstrated that both Oct1 and Oct2 are involved and essential in the renal clearance of tetraethylammonium (TEA), a common substrate of OCTs [236]. Substrates for Oct2 include the antimalarial drug, quinine, in addition to endogenous cations such as creatinine, bile acids and monoamine neurotransmitters [127], [237]. Thus, since Oct1/2 knockout mouse studies have observed a decrease in the renal clearance of OCT substrates, it is likely that the observed malaria-induced decrease in Oct1 and Oct2 expression could result in decreased renal clearance of circulating toxins and metabolites. The decrease in bile acid excretion, a substrate of Oct2, can also explain the increase in plasma bile acid levels. Lastly, L-arginine does not seem to normalize the expression of either of these transporters.

We observed a significant decrease in the renal expression of Pept2 (Slc15a2) in PbA infected mice (Figure 13). While a trend was seen in both the water and L-arginine groups, this only reached significance in the water group. Studies in Pept2 knockout mice have demonstrated that Pept2, in conjunction with Pept1, is the principle system responsible for tubular reabsorption of peptide-bound amino acids, and overall amino acid homeostasis [238]. Therefore changes in Pept2 can affect the reabsorption of di- and tripeptides, resulting in a loss of amino acids into urine. Thus, this could help to explain why supplementation with L-arginine did not have beneficial effects in the PbA infected mice.

### 5.2.5 Impact on Brain

In the brain, both Ent1 (Slc29a1) and Ent2 (Slc29a2) were significantly down regulated in the PbA infected dams receiving regular drinking water, while Ent1 was up
regulated in the PbA infected dams receiving L-arginine (Figure 11). These transporters are involved in the cellular uptake of purine and pyrimidine nucleosides, which have essential physiological functions. Evidence suggests a role for ENT1 in modulating glutamate levels, and that inhibition or knockdown of ENT1 diminishes glutamate transport activity of excitatory amino acid transporter 2 (EAAT2). Glutamate is involved in most aspects of normal brain function including cognition, memory and learning. Decreased expression may contribute to glutamatergic abnormalities.

As previously discussed, ENT transporters are also involved in the uptake and efficacy of antiviral nucleosides, therefore the decrease observed could result in greater adverse events in HIV-Malaria co-infected pregnant woman.

Lastly, ENT substrates, nucleosides and nucleotides, are key signaling molecules in modulating brain function, and have limited de novo synthesis of purine and pyrimidine bases [239]. Brain pathologies including epilepsy and cerebrovascular ischemia result from decreased levels of nucleosides and their metabolites, and nucleoside analogs are used clinically in the treatment of brain cancer and viral infections. The transport of nucleosides across cell membranes is a critical determinant of their metabolism [240]. Additionally, inflammatory stimuli (exposure of inflammatory cytokines IL-6, IL-1β, and TNF-α to confluent vascular endothelial HMEC-1 cells) in in-vitro experiments resulted in a repression of ENT1 and ENT2, which was associated with an attenuation of extracellular adenosine uptake [71]. Adenosine is a potent endogenous anti-inflammatory agent [241]. It interacts with specific G-protein-coupled receptors on astrocytes, microglia and infiltrating immune cells to regulate the function of the immune system in the brain and protect neuronal integrity. Additionally, the receptors it stimulates inhibit the expression of inducible nitric oxide synthase (iNOS), and thus the production of nitric oxide (NO) [242]. Thus, the changes in ENTs observed in our study may explain in part the inflammation caused by P. berghei, and contribute to the pathophysiology of many central nervous system diseases.
Chapter 6. Conclusion, Limitations & Future Direction

6.1 Conclusion

Studies have shown that oxidative stress is reduced with L-arginine supplementation, improving inflammation-mediated changes, and improving maternal and fetal outcomes. Inflammation-mediated changes did not seem to be improved with L-arginine supplementation in our study. Expression of oxidative stress markers, iNos and HO-1 were significantly increased following PbA infection, and no improvements in these oxidative markers or maternal and fetal parameters were seen following dietary L-arginine supplementation. This is in contrast to results from Dr. Kain’s laboratory, which demonstrated significant improvements in maternal and fetal parameters in PbA-infected pregnant mice receiving dietary L-arginine. However a very large sample size (n>100) was used in that study (manuscript in preparation). The same animal model outline in section 3.1 was used.

Likewise, while the expression of numerous ABC and SLC transporters, along with the drug-metabolizing enzyme Cyp3a11, were significantly altered following PbA infection, these changes were not improved in dams receiving dietary L-arginine supplementation. My hypothesis was that restoring systemic levels of NO during PM by dietary L-arginine supplementation would attenuate PbA infection-mediated changes in transporters due to its anti-oxidant properties. Use of L-arginine supplementation in placental malaria therefore does not appear to benefit. However it is possible that a stronger L-arginine dosage, in combination with antioxidant vitamins could attenuate inflammation-mediated dysregulation of transporter and drug metabolizing enzyme expression, along with improved maternal and fetal parameters. This thesis confirms that PbA infection significantly alters the expression of ABC efflux transporters. Further study is required before these findings can be translated to pregnant women infected with malaria.
6.2 Limitations

There are a few limitations in the work presented. The biggest limitation is that the study was conducted in a preclinical murine model. This may not be entirely representative or a valid predictor of human reactions to exposures and treatments; therefore this research may not be applicable to humans. Species differences exist and include, but are not limited to disease progression, anatomy, metabolizing enzymes, transporters and biochemical pathways. Since it is not ethically possible to conduct these types of studies in humans, animal work is the best available option. The mouse model we chose to work with replicates the key pathogenic features of human PM, and presents pathology comparable to human PM that is evoked by P. falciparum. While dietary L-arginine could be supplemented in humans and outcomes measured based solely on maternal and fetal characteristics, such as birth weight, maternal and fetal iron levels, and preterm deliveries. However, this would provide no insight on drug disposition mechanisms involved in PbA infection.

Another limitation in the work conducted is that we did not measure the absolute amount of dietary L-arginine consumed by the mice as it was dissolved in their drinking water and subject to spills or other losses. Therefore, we are unsure if a sufficient amount of L-arginine entered the mice’s system to achieve therapeutic benefits. However, since the mice were monitored on a daily basis and no signs of dehydration were observed, we believe this was the most appropriate method to administer this dietary supplementation.

Another limitation is that the regular chow the Balb/c mice receive contains >1% L-arginine. This could be a confounding factor as the mice are at a sufficient baseline level of L-arginine before infection, while pregnant woman in Sub-Saharan Africa are malnourished, and thus present low levels of L-arginine. Therefore, effects of dietary L-arginine could be masked due to sufficient baseline levels of L-arginine in our model. Future research is recommend to use an L-arginine deficient chow which has been confirmed to not cause any adverse impacts on the general health and well-being of the dams.

Another limitation is that proteins levels were not measured. Posttranscriptional modifications can occur, and thus may result in differences between mRNA and protein expression levels. While mRNA expression values have shown their usefulness in a
broad range of applications, including the diagnosis and classification of cancers, these results are only correlative, rather than causative. A combination of mRNA and protein expression patterns should be simultaneously considered to fully develop a conceptual understanding of effect of PbA and L-arginine supplementation on transporters, drug metabolizing enzymes and inflammatory markers. Future studies are recommended to confirm our results by obtaining protein expression levels.

Another limitation is that L-arginine levels in serum were not measured. This poses a level of uncertainty as to whether L-arginine is entering the system circulation. However, since the same animal model and percentage of L-arginine outlined in this thesis has been extensively used in the scope of research of our collaborators, the degree of uncertainty decreases. Future studies are recommended to measure L-arginine levels in serum.

Another limitation is that mice were injected with the infected erythrocytes on GD13. This may not be representative of the trimester pregnant woman are at the highest risk of infection. In humans, the second trimester carries the highest risk of infection[243]. However, previous research has found that infecting the mice earlier than GD13 does not allow the mice to carry out their pregnancies to term, therefore although GD13 may not translate to the second trimester, it is the best option that allows us to study the effect of P. falciparum on the mother and fetus.

Lastly, all samples were collected at a single time point (GD19). This provides only a snapshot of the effect of PbA on the mother and fetus. Including additional time points may provide a more holistic view on the mechanisms involved in malaria infection and allow us to monitor the effect of dietary L-arginine in the fetus after birth. However, adding additional time points has ethical and financial disadvantages. Additionally, the PbA model is extremely sensitive, and sacrificing mice earlier than GD19, may not allow parasitemia levels to reach that of severe malaria. Lastly, Cressman et al. euthanized mice on GD16, and found low parasite levels and no significant effects on oxidative markers or transporters.

6.3 Future Direction

This thesis confirmed the impact of PbA infection on ABC efflux transporter expression in maternal and fetal tissues, and oxidative stress markers previously
investigated by our lab. However, the impact on SLC uptake transporters, and dietary L-arginine supplementation is novel in placental malaria. This thesis opens up the door for future studies that would expand the scope of my work.

The dosage of dietary L-arginine could be increased, as other research groups have used a higher L-arginine dosage and observed no significant adverse events. However, this was conducted in a different animal model, therefore a pilot study is first needed to confirm the safety of different L-arginine dosages in our animal model, before conducting future research. The dosage used in our study is considered acceptable, as our collaborators at the McLaughlin-Rotman Centre for Global Health have used it extensively for their scope of research, and observed no adverse events.

The combination of dietary L-arginine and antioxidant vitamins have been previously investigated and found to significantly reduce the reoccurrence of pre-eclampsia in humans. Introducing antioxidant vitamins to the dietary L-arginine supplemented water may result in improved outcomes in the expression of ABC efflux transporters, SLC uptake transporters, and drug metabolizing enzymes, and maternal and fetal parameters in our PbA mouse model.

Future research should reconfirm bile acid levels as Cressman et al. observed a significant and dramatic increase of 80-fold in total bile acid serum concentrations in PbA-infected dams relative to uninfected control dams [15], while we only observed a non-significant two fold increase. The difference between these studies could be due to technical differences in measurements as Cressman et al. outsourced bile acid level changes, while in our study the Crystal Chem, Inc. Mouse Total Bile Acids Kit was used.

It has been shown that epigenetic changes occur in the developing fetus due to maternal disease or conditions. Moreover there are long-term developmental changes, including autism and mental disorders, in the offspring that are thought to be due to these maternal diseases. It has been proposed that epigenetic changes in the fetus may be involved. Future studies should investigate global DNA methylation in fetal brains following PbA infection.

Investigating the regulatory mechanisms that govern the expression and function of drug transporters and drug metabolizing enzymes provides a critical in-depth insight to the mechanisms altered by PbA infection. This may provide an insight to different
affordable treatments that can be used in PbA infection in developing countries. Specifically, investigating nuclear receptors is of great interest and importance, as they are involved in the regulation of many ABC efflux transporters, SLC uptake transporters, and drug metabolizing enzymes. Studies investigating the involvement of nuclear receptors in infection-mediated changes have been conducted in our laboratory.

Lastly, the final step would be to determine if the effects described in this thesis and suggested studies translate into human pregnancies. I would suggest expanding future studies to include placenta samples obtained from women receiving dietary L-arginine and/or other antioxidants. There are a number of studies that have utilized human placental samples from women with infections or other complications. Dr. Kain’s laboratory has collected human placental samples from studies conducted in developing countries looking at infectious diseases, including placental malaria.
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## 8 Appendix

### Table A1. QPCR prime sequences and National Library of Medicine (NLM) target mRNA sequences.

<table>
<thead>
<tr>
<th>Reference Genes</th>
<th>Forward Primer Sequence (5' → 3')</th>
<th>Reverse Primer Sequence (5' → 3')</th>
<th>NLM Target</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gapdh</strong></td>
<td>CGTGCCGGCTGGGAGAA</td>
<td>GATGCCTGTCTTCACCACCTT</td>
<td>NM_001289726.1</td>
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<tr>
<td><strong>β4</strong></td>
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<td>CGTGCTGGCTGACGGGATG</td>
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<tr>
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<td></td>
</tr>
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<tr>
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<td>CCGACAAAGGAAACCA</td>
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Pilot Study

A pilot study was initially conducted on previously frozen placental samples. However, no conclusions can be made as the samples were degraded. The RNA yield and quality was low and unsatisfactory.

Figure A.23. Impact of *Plasmodium berghei* ANKA Malaria Infection on ABC efflux transporters in the placenta on GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in *P. berghei* infected and control dams at GD19 were normalized to Gapdh. One-way ANOVA was used for statistical analysis. N= 5-8 placentae/group. *, p < 0.05; **, p < 0.01; and *** p < 0.001.
Figure A.24. Impact of *Plasmodium berghei* ANKA Malaria Infection on SLC uptake transporters in the placenta on GD19. Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in *P. berghei* infected and control dams at GD19 were normalized to Gapdh. One-way ANOVA was used for statistical analysis. N= 3-9 placentae/group. **, p < 0.01; and *** p < 0.001.
Figure A.25. Impact of *Plasmodium* berghei ANKA Malaria Infection on Inducible Nitric Oxide Synthase and Proinflammatory Cytokines in the placenta on GD19.

Results presented as mean transporter mRNA expression as % control ± SEM. mRNA expression of genes in *P*. berghei infected and control dams at GD19 were normalized to Gapdh. One-way ANOVA was used for statistical analysis. N= 3-8 placentae/group. *, p < 0.05; **, p < 0.01; and *** p < 0.001.