P. berghei ANKA Experimental Malaria Infection Alters Drug Transporter and Drug Metabolizing Enzyme Expression in Pregnancy

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science

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A P. berghei ANKA mouse model of malaria infection was employed to investigate disease-induced changes in an array of genes that impact drug disposition in pregnancy. We demonstrate the reduced expression of a number of transporters in the placenta of infected dams relative to uninfected dams, in addition to altered expression of transporters and Cyp3a11 in maternal and fetal tissues. The impact of Complement 5a receptor (C5aR) activation during malaria infection on transporter expression in liver and placenta was also examined in C5aR−/− infected dams. Increased expression of Abcb1a and Abcg2 in the placenta of C5aR−/− infected dams was observed when compared to wildtype-infected dams. Furthermore, whereas expression of placental Abcb1a and Abc1b were decreased in infected wildtype dams, their expression was unchanged in C5aR−/− infected dams. Collectively, if these findings translate to malaria infection in human pregnancy, the systemic clearance and maternofetal disposition of therapeutics and other xenobiotics may be altered.
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<tr>
<td>AAG</td>
<td>alpha 1-acid glycoprotein</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ACT</td>
<td>artemisinin Combination Therapy</td>
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<tr>
<td>ALB</td>
<td>human serum albumin</td>
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<tr>
<td>ANKA</td>
<td>ANKA-strain <em>Plasmodium berghei</em></td>
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<tr>
<td>ANOVA</td>
<td>one-way analysis of variance</td>
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<td>ATP</td>
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<tr>
<td>AUC</td>
<td>area-under-concentration versus time curve</td>
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<td>BBB</td>
<td>blood-brain barrier</td>
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<td>Bsep/BSEP</td>
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<td>complement factor 5</td>
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<td>chondroitin Sulfate A</td>
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<td>GD</td>
<td>gestational day</td>
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<td>IFN-γ</td>
<td>interferon-gamma</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>IUGR</td>
<td>intrauterine growth restriction</td>
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<td>Keap1</td>
<td>kelch-like ECH-associated protein 1</td>
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<td>LBW</td>
<td>low birth weight</td>
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<td>rodent/human multidrug resistance protein 1 (ABCB1)</td>
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<td>rodent/human multidrug resistance-associated protein (ABCC)</td>
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<tr>
<td>Acronym</td>
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<tr>
<td>NBD</td>
<td>nucleotide binding domain</td>
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<td>Nrf2/NRF2</td>
<td>rodent/human nuclear factor erythroid 2-related factor 2</td>
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<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>Ntcp/NTCP</td>
<td>rodent/human sodium-taurocholate cotransporting polypeptide (SLCO10A1)</td>
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<td>rodent/human organic anion transporters</td>
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<td>Oct/OCT</td>
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<td>Oatp/OATP</td>
<td>rodent/human organic anion transporting polypeptide</td>
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<td>PE</td>
<td>parasitized erythrocytes</td>
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<td>pfEMP1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein 1</td>
</tr>
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<td>pfGPI</td>
<td><em>Plasmodium falciparum</em> glycosylphosphatidylinositol</td>
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<tr>
<td>Pxr/PXR</td>
<td>rodent/human pregnane X receptor</td>
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<td>qRT-PCR</td>
<td>quantitative reverse transcriptase-polymerase chain reaction</td>
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<td>RBC</td>
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<td>radical oxygen species</td>
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<td>Rxr/RXR</td>
<td>rodent/human retinoic acid receptor</td>
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<td>SLC</td>
<td>solute-carrier transporter</td>
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<tr>
<td>Sult/SULT</td>
<td>rodent/human sulfotransferase enzyme</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>transmembrane domain</td>
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<td>tumor necrosis factor-alpha</td>
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<tr>
<td>Var2CSA</td>
<td>Variant 2 Chondroitin-Sulfate A protein</td>
</tr>
<tr>
<td>VSA</td>
<td>Variant Surface Antigen</td>
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</table>
List of Symbols

$CL_{Fetal}$: overall clearance from the fetal compartment

$CL_{Fetal \rightarrow Maternal}$: clearance from the fetal compartment into the maternal compartment

$CL_{Intrinsic}$: intrinsic clearance of liver

$CL_{Hepatic}$: hepatic clearance

$CL_{Maternal \rightarrow Fetal}$: drug clearance from the maternal compartment into the fetal compartment

$f_u$: fraction of drug unbound in plasma/serum (free drug fraction)

$Q_{Hepatic}$: hepatic blood flow
Chapter 1. Introduction
1.1 Malaria Infection

In spite of substantial global health efforts directed at malaria control and the active and prophylactic treatment of malaria infection, malaria remains one of the most serious infectious diseases of humans. Recent reports have approximated that nearly one-quarter of the world’s population is at risk of infection and over 250 million cases of malaria infection occur annually [1]. Recent estimates suggest that malaria infection is implicated in the deaths of over 1.238 million people each year, >90% of which occur in Africa [2].

Malaria is a hematological disease caused by the infection of mature erythrocytes by the protozoan parasite, *Plasmodium*. As a vector-borne disease, malaria is transmitted from person to person via the bloodmeal of a female Anopheles mosquito. Within the genus *Plasmodium*, there are five species that infect humans – *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* [3,4]. *Plasmodium falciparum* is the predominant species in Sub-Saharan Africa and South-East Asia, where over 90% of all malaria deaths occur worldwide [5].

*P. falciparum* results in a number of different pathologies associated with specific organ systems [reviewed in 6]. Within the context of this thesis, the manifestations of malaria in pregnancy, namely placental malaria, will be discussed in detail.

1.1.1 Life Cycle of *P. Falciparum*

The life cycle of *P. falciparum* and the malaria infection process, as depicted in figure 1, begins with the bloodmeal of a female Anopheles mosquito infected with the *Plasmodium* parasite (fig 1A). During this initial bloodmeal, the Anopheles mosquito injects sporozoites into the host. Sporozoites then migrate rapidly through the systemic circulation to the liver, where they ultimately invade and infect hepatocytes without eliciting an immunological response (fig 1B) [7]. The sporozoite then begins a process of asexual and clinically asymptomatic reproduction known as exo-erythrocytic schizogony (fig 1C). Following the growth and maturation phase within hepatocytes, which generally lasts 7-14 days, thousands of merozoites are released into the circulation (fig 1D) upon hepatocyte rupture, beginning the symptomatic erythrocytic stage of malaria infection. During the blood-stage of infection (fig 1E-H),
*P. falciparum* parasites undergo a process of erythrocyte invasion and differentiation into merozoites, intracellular growth to become trophozoites, and rapid multiplication within erythroctes to become schizonts (fig 1E-G). Following the rupture of parasitized erythrocytes (PEs) and release of schizonts into the systemic circulation, reinvasion of host erythrocytes then occurs over a 48-hour period (fig 1H). While most intraerythrocytic parasites follow this process of intraerythrocytic schizogony, some intraerythrocytic parasites differentially develop to produce male and female *P. falciparum* gametocytes that can re-establish the sexual stage of the malaria life cycle within the mosquito midgut (fig 1I) [8].

As depicted in figure 1, pathogenesis in *P. falciparum* malaria infection is related to the latter half of the erythrocytic stages (fig 1G and 1H). Intraerythrocytic schizonts can express parasite-derived proteins at the surface of the red blood cell, also known as *Plasmodium falciparum* erythrocyte membrane protein 1 (pfEMP1), which contains a high degree of complementarity with chondroitin sulfate A (CSA), a major component of the vascular endothelium. These variant surface antigens (VSA), in particular the Variant 2 Chondroitin-Sulfate A protein (Var2CSA), are responsible for the cytoadherence of PEs to the microvasculature of essential organs such as the brain and the placenta in pregnancy. Cytoadherence and sequestration in the microvasculature of the brain and placenta allows parasitized erythrocytes to evade elimination processes by the reticuloendothelial system (i.e., the spleen and liver). Parasitized erythrocytes that have sequestered in the vasculature may then be recognized by the immune system leading to immune cell infiltration (e.g. monocytes and neutrophils) and a unique and localized inflammatory microenvironment [9]. In addition, intraerythrocytic schizonts can rupture PEs releasing infective merozoites and other parasite-derived bioactive products such as *Plasmodium falciparum* glycosylphosphatidylinositol (pfGPI) (a parasite surface receptor) and hemozoin (a parasite derived porphyrin-polymer), amongst others, which can stimulate the innate immune response through activation of toll-like receptor 2 (TLR2), TLR4 and TLR9. This can lead to systemic inflammation and pro-inflammatory cytokine release from monocytes and macrophages.
Figure 1: The life cycle of malaria infection begins with the blood meal of a female Anopheles mosquito infected with malaria. (A) During this initial bloodmeal, the Anopheles mosquito injects sporozoites into the host. (B) Sporozoites then travel rapidly through the systemic circulation to the liver where they can migrate through several cell types (e.g., Kupffer cells) before eventually infecting a hepatocyte. (C) When the sporozoite has infected the hepatocyte, it then begins an asexual and asymptomatic reproduction process known as exo-erythrocytic schizogony. Following the growth and maturation phase within hepatocytes, which generally lasts 7-14 days, thousands of merozoites are (D) released into the circulation, beginning the disease-causing erythrocytic stage of malaria infection. During the blood-stage of infection, (E-H) P. falciparum parasites undergo a process of invasion and differentiation (merozoites), intracellular growth (trophozoites) and multiplication within erythrocytes (schizonts), and reinvasion of host erythrocytes over a 48-hour period upon rupture of parasitized erythrocytes (PEs). (I) Some intraerythrocytic parasites differentially develop to produce male and female gametocytes that can re-establish the sexual stage of the malaria life cycle within the mosquito midgut.
1.1.2 The Host-Response to Malaria

As discussed above, the studies that have investigated the mechanisms underlying disease pathogenesis in malaria suggest that disease arises from site-specific localization of PEs in target organs, the local and systemic actions of bioactive parasite products, the local and systemic production of pro-inflammatory cytokines, and the activation, recruitment and infiltration of inflammatory cells such as neutrophils and monocytes to sites of PE sequestration. Generally, regulation of these processes can be maintained such that malaria infection does not progress to severe disease; however when these processes become dysregulated, fatal consequences of severe malaria can ensue.

As such, the host innate immune response to malaria infection is a key determinant of malaria severity and outcome. The innate immune response in malaria infection involves activation of a number of TLRs, a family of important receptors that are expressed on a variety of immune cells, such as monocytes and macrophages, responsible for recognition of microbial pathogen-associated molecular patterns. TLR2, TLR4, and TLR9 have been shown to be activated during malaria infection by a number of parasite derived bioactive products including hemozoin, pfGPI, etc [6]. Activation of these receptors stimulates the release of numerous pro-inflammatory cytokines and chemokines that help to eliminate the parasite and prevent disease. However, if these processes become dysregulated as in severe malaria or in localized environments where PE sequester, they can mediate disease pathogenesis leading to clinical consequences such as placental or cerebral malaria.

In addition to the role of TLR activation in malaria immunopathogenesis, the complement system has also been shown to play a role. The complement system consists of a network of proteins, which activate the innate immune system. Complement contributes to the elimination of invading *P. falciparum* parasites; however, excessive activation of this system can mediate disease pathogenesis. For example, increased complement activation is consistently observed in patients with malaria infections, in addition to numerous mouse models of malaria [10,11].

Multiple pathways of complement activation have been described in malaria infection. Three major pathways, the classical, alternative and lectin pathways have been well characterized, and all converge at complement factor 3, resulting in the
generation of activation products such as Complement factor 3a, Complement factor 5a, Complement factor 3b and the terminal membrane attack complex (MAC). Recently, two additional novel activation pathways have been identified that operate independently of C3. One pathway is mediated through actions of thrombin functioning as a C5 convertase, cleaving C5 into its active form C5a in the absence of C3 [12]. The other pathway is attributed to activity of neutrophils and monocytes, which secrete serine proteases capable of cleaving C5 to its active C5a form [13]. Due to convergence of complement activation pathways on C3a, C3b, C5a, and MAC formation, it is difficult to implicate the activation of any one specific pathway with the pathogenesis of malaria infection. Given the complexity of malaria infection, it is likely that all pathways contribute to some extent; however, recent studies support a role for specific complement activation pathways (e.g. C5a and C5aR activation) in malaria infection.

Excessive complement activation can contribute to localized and systemic inflammation, endothelial activation, and mechanical blockade of microvasculature in the brain and placenta, all of which are associated with the target organ dysfunction characteristic of cerebral malaria and poor outcomes of malaria in pregnancy [14,15]. C3a and C5a exert a wide array of biological functions through interaction with their respective G-protein coupled receptors (C3aR and C5aR), located on cells such as monocytes, macrophages, mast cells, microglia, astrocytes, and vascular endothelial cells [16]. Parasite products such as pfGPI have been shown to upregulate monocyte expression of C5aR [17] and upregulation of placental C5aR mRNA expression has been observed in P. falciparum malaria infection [17]. The contribution of C5a signaling to the pathogenesis of experimental cerebral malaria and malaria in primigravid women has been attributed to enhanced induction of inflammatory mediators downstream of C5aR [17,18]. Synergistic interaction between the complement system and TLRs has been shown to enhance C5aR-mediated pro-inflammatory responses [19] and C5a co-stimulation can potentiate human monocyte proinflammatory response to pfGPI [17,18]. This synergistic mechanism of pro-inflammatory cytokine release could contribute to the pathology observed in severe P. falciparum malaria. Additionally, C5a, and to a lesser extent C3a, regulate chemokine expression and act as chemoattractants for monocytes, neutrophils and activated
lymphocytes, which may contribute to the recruitment of immune cells to sites of inflammation and injury such as the placenta and brain [16]. This, in turn, could propagate the immune response and lead to further pro-inflammatory cytokine release and disease. Monocyte and macrophage infiltration to target organs increases the risk of poor pregnancy outcomes in placental malaria [20] and disease severity in cerebral malaria [15]. Furthermore, sequestered parasites are able to induce high-localized plasma levels of C5a and upregulate C5aR on monocytes/macrophages to further potentiate the inflammatory process [17]. These results suggest that complement pathways contribute to pro-inflammatory cytokine and chemokine secretion that is characteristic of cerebral malaria and placental malaria pathogenesis.

1.1.3 Malaria in Pregnancy

To borrow the words of Dr. Patrick E. Duffy and Dr. Robert S. Desowitz… “So long as woman has walked the earth, malaria may have stalked her” [21]. Every year, an estimated 85 million pregnancies occur in areas of P. falciparum transmission, with almost 55 million pregnancies occurring in regions with stable year-round malaria transmission [22]. Pregnant women are at an increased risk for adverse outcomes from malaria infection due to immunological changes that occur in pregnancy [23], in addition to the ability of PEs to bind to placental chondroitin sulfate-A (CSA) and sequester in placental intervillous space [24].

Maternal and fetal well-being in malaria endemic regions is associated with the rate of malaria transmission in that region. While pre-term birth often occurs and fetal health is more compromised in high-transmission regions, low pre-existing maternal immunity to malaria infection in low-transmission regions results in more severe maternal outcomes including coma, hypoglycemia, respiratory distress, and death [23]. Interestingly, there is an inverse correlation between parity and risk for adverse pregnancy outcomes, with primigravidae representing the highest risk group [25,26]. This risk declines over subsequent pregnancies and is thought to occur because women develop protective antibodies that block the adherence of PEs to the intervillous space of the placenta and promote the phagocytic clearance of PEs [27,28].
1.1.4 Impact of Malaria on Offspring

Maternal malaria infection imparts a number of adverse outcomes on newborn health. Reports suggest that malaria infection in pregnancy is implicated in up to 200,000 fetal deaths per year (stillbirths or spontaneous abortions) [29], and children born to malaria-infected mothers display a number of negative predictors for lifelong health. These include: low-birth weight (<2500g) and premature delivery (<37 weeks), which are strong predictors of risk for death in the first year of life [29,30,31,32]. An understanding of the mechanism by which malaria in pregnancy impacts these fetal outcomes is limited, however current evidence suggests that PE sequestration can lead to reduced placental vascular flow, altered placental angiogenesis, reduced nutrient and waste transfer, and a chronic localized pro-inflammatory microenvironment [33,34,35,36].

Children born to malaria infected mothers are also at an increased susceptibility for *P. falciparum* malaria infection later in life [37,38,39], an association that is particularly prominent in children born to multigravid mothers who were exposed to *P. falciparum* in previous pregnancies [40].

1.1.5 Treatment of Malaria in Pregnancy

The current World Health Organization (WHO) recommendations for treatment of malaria in pregnancy are trimester- and region-dependent [41]. The trimester dependence is due in part to the paucity of data that is currently available investigating the safety of antimalarial medications during each trimester of pregnancy [reviewed in 42,43]. As such, the WHO recommends use of therapeutics with the most substantial evidence for safety from pharmacovigilance data collected in pregnancy.

According to the WHO guidelines, for malaria infection during the first trimester, quinine plus clindamycin may be administered for 7 days. If no clinical improvement is observed, artesunate plus clindamycin is indicated. Artemisinin-combination therapy (ACT) (e.g. Artesunate + Mefloquine, Artesunate + Lumefantrine, etc.) may also be indicated only if the treatment is immediately available in that country/region, if treatment with 7-day quinine plus clindamycin fails, or if there is uncertainty about patient adherence with a 7-day treatment protocol [41]. ACT is often indicated in regions of the world where chloroquine or other drug-resistant strains predominate.
(e.g., Western Africa, Western Thailand, and Western Cambodia) [44,45,46]. Quinine plus clindamycin for a 7-day treatment regimen remains the first-line therapy for treatment of malaria in the second or third trimester. ACT may be given if it is known to be effective in the country/region [41]. A positive safety profile of ACT in the second and third trimesters of pregnancy has been established, and as such, many clinicians prescribe them as first-line therapy due to the significant detrimental outcomes of untreated malaria infection in pregnancy. It is important to note that in severe malaria at any time in gestation, intravenous artesunate is the drug of choice [47].

Despite the availability of antimalarial drugs to treat malaria infection during pregnancy, dramatic increases in the clearance and reductions in systemic plasma concentrations of antimalarial drugs (e.g. artemisinins) have been observed in pregnant women relative to non-pregnant women, which may impact drug effectiveness [reviewed in 41,42,43,48]. As such, a greater understanding of the mechanisms underpinning drug-disposition in pregnancy may be required to better optimize treatment outcomes. This is particularly true for the treatment of infection in regions in Southeast Asia such as Western Cambodia and Western Thailand, where resistance to ACT has been identified and is likely rapidly emerging [44,45,46]. Given that ACT medications remain the last line of defense against multidrug resistant *P. falciparum* malaria infection, it is critical that these drugs be prescribed and dosed appropriately such that treatment may be optimized to prevent further emergence and transmission of resistant strains.

### 1.2 Drug-Disposition Mechanisms

Drug-disposition defines the processes of drug absorption, distribution, metabolism and excretion/elimination (ADME) within the body [49]. Unless directly administered to the site(s) of action, a drug must gain access to the systemic circulation before distributing throughout the body. For most orally administered drugs, including many antimalarial drugs, this process primarily involves absorption from the small intestinal lumen [49]. The amount of drug reaching the splachnic circulation may be limited by: (1) the physicochemical properties of the drug (e.g. solubility, stability in stomach and intestine, size, and ionization), and/or (2) biological processes acting on the drug (e.g. efflux from the intestinal epithelia into the intestinal lumen, metabolism
in the intestinal epithelium, etc.).

Drug that is absorbed across the gastrointestinal tract enters the hepatic portal vein, which carries the drug to the liver. Upon arrival at the liver, blood from the hepatic portal vein and hepatic artery combine and this mixture advances through the hepatic sinusoidal network. Here, endogenous and exogenous chemicals suspended in the hepatic blood flow are taken up by hepatocytes at the space of Disse and subjected to metabolism and/or biliary clearance before reaching the systemic circulation, commonly called first-pass clearance. First-pass clearance can dramatically reduce a drug’s systemic availability after oral administration, a pharmacokinetic parameter known as bioavailability. Given that many antimalarials have low bioavailability and patients with acute *P. falciparum* presenting at the clinic are often severely ill, these drugs are commonly given intravenously.

Once a drug reaches the systemic circulation, the drug distributes into tissue sites based on physicochemical properties. A fraction of the drug may become bound to plasma proteins. This fraction remains in the systemic circulation because drug-plasma protein complexes are generally too large to pass through cell membranes or fenestrations in the renal glomerulus. The extent of binding in the plasma or tissues substantially impacts the apparent volume of distribution of a drug ($V_D$) [49]. Because drugs can distribute out of the blood and into tissue sites, the apparent volume of distribution can be much larger than the actual total volume of the body. This is especially evident for the antimalarial drug chloroquine, which is associated with an apparent $V_D > 100$L/kg due to its high sequestration into fat [43].

As drugs travel throughout the systemic circulation they are also distributed to elimination organs such as the liver and kidney and subject to clearance. In clearance organs, drugs may be metabolized and/or transported into the urine or bile by specialized drug metabolizing enzymes or drug transporters, respectively.

As previously mentioned, the pharmacokinetics of several antimalarial agents have been shown to be significantly altered during pregnancy [reviewed in 42,43]. For example, the drug-disposition of the antimalarial artesunate and its metabolite dihydroartemisinin has been investigated in pregnant women with acute *P. falciparum*. This study reported a 4-fold decrease in maximal systemic concentration ($C_{max}$) and a 1.8-fold decrease in area under the concentration time curve from zero to 24 hours of
artesunate in pregnant women given 4 mg/kg when compared to malaria infected non-pregnant women given 1.79 mg/kg. The metabolite (dihydroartemisinin) oral clearance in non-pregnant Thai adults was 2.7-fold lower than that in pregnant patients, with a corresponding 9-fold decrease in area under the concentration time curve from zero to 24 hours [50]. Although the underlying mechanisms were not investigated, it is clear that altered drug-disposition could lead to varied or atypical treatment outcomes as compared to non-pregnant female patients. The drug-disposition changes that can impact the pharmacokinetics of drugs in pregnancy include, but are not limited to: altered oral drug absorption through reduction of gastric emptying and gastrointestinal motility, decreased plasma binding due to reduced concentration of plasma proteins via hemodilution, increased apparent V\text{D} due to increased total body water and body fat, altered expression of drug-metabolizing enzymes, and increased renal clearance of drugs via increased glomerular filtration rate [51]. The effects of pregnancy on ADME processes have been extensively reviewed elsewhere [reviewed in 52,53], and are discussed in greater detail below.

1.2.1 Plasma Protein-Binding

Once a drug reaches the systemic circulation, it may interact with plasma proteins present in the blood. These drug-protein interactions consist of reversible-binding processes dictated by structural complementarity between the plasma protein and its substrate. For some drugs, plasma protein interactions are highly-favourable and, as such, the fraction of unbound drug in the plasma is relatively-low. These drugs are said to be “highly protein-bound”. For other drugs, plasma protein interactions occupy a lower percentage of the total drug concentration. These drugs are said to exhibit low-to-moderate protein binding. Implicit to these processes is an understanding that the ratio between unbound and bound drug in plasma is constant and maintained by a dynamic equilibrium [54]. That is, as unbound drug is eliminated from the body by the kidney and liver or distributes into tissue sites, more drug will be displaced from plasma-protein binding sites to maintain equilibrium. As a consequence of this, in theory, the fraction of unbound drug in the plasma should remain relatively constant over time.

Of particular importance to xenobiotic disposition are the interactions between
xenobiotics and the two primary plasma proteins: human serum albumin and alpha 1-acid glycoprotein. Drugs preferentially bind to either albumin or alpha 1-acid glycoprotein depending on the physicochemical properties of the drug, with weak acids primarily binding to albumin and weak bases primarily binding to alpha 1-acid glycoprotein [55].

Plasma protein binding of drugs can be altered in unique ways. First, the concentration of plasma proteins in the blood can change in response to physiological stimuli. For example, infection and inflammation are associated with decreased hepatic synthesis of albumin [56,57,58,59,60,61,62,63] and increased hepatic synthesis of alpha 1-acid glycoprotein [64,65,66,67]. Second, the concentration of substances that compete with drugs for binding sites on either albumin or alpha 1-acid glycoprotein can change. For example, increases in unconjugated bilirubin in the plasma can alter drug binding to albumin, and the consumption of other xenobiotics may displace drugs from their plasma protein binding sites. Altogether these changes in plasma protein expression and binding can lead to perturbations in the unbound fraction of drug and consequentially alter to drug-disposition.

1.2.2 Drug-Metabolizing Enzymes

Xenobiotics are often converted to polar or more polar metabolites via biotransformation before being removed from the body. The biotransformation of drugs within the body’s tissues is collectively referred to as drug metabolism and is executed by distinct families of metabolic enzymes. These enzymes can be classified as either “phase I” or “phase II” enzymes.

Phase I metabolic enzymes typically metabolize a drug (the parent) by introduction or unmasking of a functional group by way of oxidation, reduction or hydrolysis. The products of phase I metabolism can be more active, equally active, or less active than the parent compound. In some instances, the products of phase I metabolism are sufficiently polar to be removed from the body. If they are not, they may be subjected to subsequent conjugative biotransformation via further enzymatic processes. This conjugative biotransformation, called phase II metabolism, combines drugs or their metabolites with endogenous substrates to produce highly polar xenobiotic-conjugates [68]. While drug-metabolizing capabilities have been detected
in nearly every organ in the body, most drug metabolism occurs in the liver and, to a lesser extent, in the intestine [68]. For some drugs, metabolism in the kidney is also important and can be linked to renal toxicity [68].

1.2.2.1 “Phase I” Enzymes - The cytochrome P450s

The cytochrome P450 (CYP) superfamily is arguably the most clinically-important family of phase I drug metabolizing enzymes. Many of these enzymes are widely and highly expressed, with highest expression in the liver and intestine, while certain subfamilies and their gene products are expressed exclusively in extrahepatic organs [68].

CYPs are moderately sized (approximately 50-55 kDa) membrane-bound proteins capable of catalyzing a diverse array of metabolic reactions. To this end, they elicit substrate biotransformation and subsequent clearance from the body. In general, the catalytic sequence of CYPs involves the activation of molecular oxygen (O₂), in the presence of co-factors like NADPH-cytochrome P450 oxidoreductase and NADPH, to add oxygen to substrates, remove hydrogen atoms or simply remove electrons from substrates.

Currently, 57 putatively functional CYP enzymes and 58 pseudogenes have been identified in the human genome with considerably more identified in rodent species [69]. CYPs are responsible for the metabolism of approximately 75% of the 200 most commonly prescribed drugs in the United States [70]. Within the CYP superfamily, a small group of structurally dissimilar CYPs are responsible for 95% of the metabolism of these prescription drugs: CYP3A4 (approximately 50%), CYP2C9 (approximately 20%), CYP2C19 (approximately 10%), CYP2D6 (approximately 10%), and CYP1A2 (approximately 5%). CYP3A4 accounts for roughly 30% of total hepatic CYP content and 40% of total intestinal CYP content [71], and is considered the most important CYP on account of the large number of drugs it metabolizes in addition to its high expression in the liver. The substrates of CYP3A4 include, but are not limited to, antineoplastics, antibiotics, antihypertensives, antimalarials, antiretrovirals, immunosuppressants, statins, steroids, calcium channel blockers as well as many exogenous toxins [72]. A number of antimalarials are substrates for CYP3A4 including quinine [73,74], chloroquine [75], mefloquine [76], and the artemesinin and artemisinin
derivatives [75,77].

In mice, CYP3A11, not CYP3A4, is the dominant constitutive CYP3A isoform in the liver [78], and has been shown to metabolize prototypical human CYP3A4 substrates such as triazolam [79]. CYP3A11 exhibits 73% sequence homology to human CYP3A4 and displays considerable activity-correlation with human CYP3A4 (r = 0.88) for typical CYP3A4 probe-substrates [80].

1.2.2.2 Phase II Enzymes – Conjugative Biotransformation

Phase II enzymes generally add hydrophilic groups to their substrates in the presence of a co-factor, which provides the hydrophilic group and generally defines the enzyme family. Four families of phase II enzymes render their substrates more polar via conjugative biotransformation. The uridine 5’-diphospho (UDP)-glucuronosyltransferases (UGTs) metabolize many aliphatic alcohols and phenols via addition of UDP-glucuronic acid. UGT-mediated metabolism is involved in the metabolism and clearance for 10% of the top 200 prescribed drugs in the United States [70]. Members of the family of UGTs tends to have overlapping substrate specificity, differing only in relative affinities. Interestingly, UGTs also play a role in the conjugative biotransformation of endogenous substrates, including the UGT1A1-mediated conjugation of hepatic bilirubin. Second, the glutathione S-transferases (GSTs) catalyze the reaction between the tripeptide glutathione (GSH) and reactive electrophilic sites on substrates [68]. These reactions can occur spontaneously (i.e. non-enzymatically), but GSTs significantly improve the rate of reaction and increase the clearance of highly-reactive electrophiles. Third, the sulfotransferases (SULTs) catalyze the transfer of sulfonate from 3’-phosphoadenosine-5’-phosphosulfate to susceptible substrates [68]. Fourth, the acetyl CoA:arylamine N-acetyltransferases (NATs) catalyze the conjugation of aromatic amines and aromatic hydrazines with an acetyl group [68].
1.2.3 Drug Transporters – ABC and SLC Superfamilies

Xenobiotics and endobiotics must cross epithelial and endothelial membrane barriers as they make their way to their site(s) of action and elimination. For example, a drug absorbed in the small intestine must first cross the apical membrane of the polarized intestinal epithelial cell and then cross its basolateral membrane to gain access to the splanchic circulation. Drugs cross plasma membranes via passive diffusion, facilitated/carrier-mediated diffusion, endocytosis/exocytosis, and active transport. These processes occur in both inward (uptake) and outward (efflux) directions. Permeation of a drug is dictated largely by its physicochemical characteristics, including molecular weight, pKa, and octanol/water partition coefficient (K\text{ow}). The processes of passive diffusion, facilitated diffusion, and endo/exocytosis are well described in numerous textbooks \cite{103}. In this thesis, we will focus our discussion on active transporters.

Active transport is a process that utilizes energy to move drugs against a concentration gradient. Active transport is executed by large families of transport proteins that play important roles in the uptake and efflux of drugs within the body. Active transport is particularly important in the polarized cells of the intestinal epithelia, the kidney proximal tubules, the blood-brain barrier, the blood-testes barrier, hepatocytes, and the syncytiotrophoblast of the placenta.

The ATP-binding cassette (ABC) superfamily of transporters are primary active transport proteins that directly couple the hydrolysis of adenosine triphosphate (ATP) to vectorial substrate transport. In humans, there are 48 ABC transporters, in seven distinct subfamilies (ABCA-ABCG), that share a common sequence and organization of their ATP-binding domains \cite{81}. These transporters consist of one to three transmembrane domains (TMDs) and one or two nucleotide-binding domains (NBDs). ABC transporter TMDs are diverse and typically consist of \( \alpha \)-helices linked by intracellular and extracellular domains of varying length. The TMDs contain three-dimensional substrate-binding sites capable of conferring substrate-specificity to the transporter. The NBDs are highly conserved and are the sites of ATP-binding and hydrolysis. Transporters with one TMD and one NBD are referred to as half transporters and must homodimerize to function \cite{Reviewed in 82}.

ABC transporters allow the unidirectional efflux of substrates across the cellular
membrane. As efflux transporters, they serve excretory roles in the major clearance organs (e.g. kidney proximal tubules and hepatocytes) as well as barrier roles in other tissues and organs (e.g the intestinal epithelia, blood-brain barrier, blood-testes barrier, and placental syncytiotrophoblast). ABC transporter substrates are numerous and structurally diverse, ranging from phospholipids, steroid hormones and bile acids to large polypeptides and many clinically important drugs and phase II drug-conjugates. In fact, many substrates rendered more hydrophilic following phase I and phase II metabolism require ABC transporters to be cleared from the cells in which they were metabolized, in a process commonly referred to as phase III transport [83]. Key members of this family involved in xenobiotic-disposition include multidrug resistance protein 1 (ABCB1/MDR1), the multidrug resistance-associated proteins (ABCCs/MRP) and the breast cancer resistance protein (ABCG2). Other ABC transporters can play a critical role in drug-disposition, but by an indirect means through their influence on bile flow and hepatic biliary clearance (e.g., the bile salt export pump (ABCB11/BSEP) and multidrug resistance protein 3 (ABCB4/MDR3)) [84,85].

Secondary active transport proteins transport one solute against its concentration gradient by utilizing the electrochemical potential energy of a second solute being transported down its concentration gradient. This type of active transport is exemplified by many members of the solute carrier (SLC) superfamily of transporters. According to the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC) Database, there are 378 SLC superfamily members in 51 families, making the SLC superfamily one of the largest families of transmembrane proteins [69]. SLC transporters are capable of bidirectionally transporting substrates across the cell membrane but, with respect to drug-disposition, they are most frequently associated with drug-uptake processes. As uptake transporters, they serve important roles in the uptake of drugs for elimination by clearance organs (e.g., kidney proximal tubules and hepatocytes) but oppose many of the ABC efflux transporters barrier roles in other tissues and organs (e.g., intestinal epithelia, blood-brain barrier, blood-testes barrier, and placental syncytiotrophoblast). Key members of this family that transport drug substrates via secondary active transport include the organic anion transporting polypeptides (OATPs), organic anion
transporters (OATs), and organic cation transporters (OCTs). Expression and localization of the major drug transporters found in liver and placenta are illustrated in figure 3. Within this thesis, we elected to study an array of transporters that have been shown to be clinically important in determining the pharmacokinetics of drugs and other xenobiotics by the International Transporter Consortium [85].

1.2.3.1 Multidrug resistance protein 1 (MDR1/PGP; ABCB1)

MDR1 (ABCB1), commonly referred to as P-glycoprotein (P-gp), is the most extensively studied ABC transporter and was initially discovered for its role in mediating multidrug resistance to chemotherapeutics in an immortalized Chinese hamster ovary cell line [86]. A human homolog was discovered shortly after in chemoresistant tumor cells [87]. Structurally, MDR1 is a full ABC transporter with two TMDs, consisting of six α-helices each, and two NBDs (figure 2).

Figure 2: Characteristic structure of a prototypical ATP-Binding Cassette Transporter with six α-helices in each transmembrane domain and two cytosolic nucleotide binding domains that bind and hydrolyze ATP to provide chemical potential energy for thermodynamically unfavorable substrate transport across the lipid bilayer. TMD; Transmembrane domain, NBD; Nucleotide binding domain, ATP: Adenosine triphosphate.

In rodents, MDR1 is encoded by the Abcb1a and Abcb1b genes. The substrates of MDR1 are extraordinarily diverse, however they are typically neutral or cationic compounds with bulky structures, and include steroid hormones, antineoplastics, antibiotics, antiretrovirals, immunosuppresants, calcium channel
blockers, and many others [88,89,90,91,92,93,94,95,Reviewed in 96]. Many antimalarials have been shown to interact with MDR1 including mefloquine [97,98,99], quinine [100,101,102], and chloroquine [103]. The artemisinins have not been shown to interact with MDR1 in drug-resistant tumor cell lines, nor have their pharmacokinetics been shown to be influenced by varying levels of intestinal MDR1 [104,105]. However, recent studies report that the artemisinins have an inductive effect on the expression of MDR1, which could impact the pharmacokinetics of concomitant medications [106,107,108,109]. Of note, many MDR1 substrates, including many antimalarials, are also substrates for the drug-metabolizing enzyme CYP3A4 and these two proteins contribute to determining drug-disposition of a number of substrates.

In normal human tissues, MDR1 is expressed in liver, kidney, intestine, the maternal and fetal blood-brain barrier, the blood-testes barrier and the placental syncytiotrophoblast [Reviewed in 96]. In the liver, MDR1 is located in the apical/canalicular membranes of hepatocytes where it effluxes substrates into the bile canaliculi for elimination via the intestinal tract. This is a major route of elimination for many lipophilic drugs. The cumulative biliary excretion of several lipophilic cationic compounds and clinically important therapeutics is reduced in Abcb1a gene knockout mice [110,111]. In the placenta, MDR1 is located in the maternal-facing apical membrane of the syncytiotrophoblast layer and its important barrier function in this tissue has been highlighted by a number of studies employing Abcb1a/b gene knockout mice and Mdr1 protein inhibitors. In a landmark paper by Lankas and colleagues, mice that carried a mutation causing a lack of placental Mdr1 were mated and the teratogenic effects of avermectin treatment on their litters were examined [112]. Pups were 100% susceptible to cleft palate if they were homozygous for the mutation, heterozygous mice were somewhat less sensitive (30%), and wildtype mice were unaffected (0%) [112]. The importance of placental Mdr1 was exemplified in another study using targeted Abcb1a/b gene knockout mice and the potent Mdr1 inhibitors, namely PSC833 and GG918 [113,114]. Here the accumulation of radiolabeled digoxin, saquinavir, and paclitaxel, all high-affinity MDR1 substrates, was dramatically increased by 2.4-, 7-, and 16-fold in fetuses from Abcb1a/b gene knockout mice, respectively, and these drugs were increased to comparable amounts
in wildtype mice treated with Mdr1 inhibitors.

1.2.3.2 The multidrug resistance-associated proteins (MRPs; ABCCs)

Nine human MRPs have been identified throughout the body, referred to as MRP1-9. Of particular importance to the studies described within this thesis are the first four MRPs. Structurally, MRP1-3 are full ABC transporters with three TMDs, totaling 17 α-helices combined, and two NBDs. MRP4, however, is considered a “short MRP” because it only contains 2 TMDs. MRP1-4 substrates are characteristically amphiphilic with at least one negative net charge. The prototypical MRP substrate is the glutathione-containing leukotriene C₄ (LTC₄). In addition to glutathione, other negatively charged moieties, such as glucuronate, sulfate, and phosphate, have been recognized as MRP substrates. Given that phase II processes often conjugatively biotransform metabolites with glucuronate (i.e. via UDPGT) and/or sulfate (i.e. via ST), the transport of these metabolites by MRPs plays a critical role in drug-disposition and clearance. Interestingly, the presence of reduced glutathione (GSH) can broaden the substrate specificity of the MRP transporters. This has been exemplified by the co-transport of vincristine with GSH for MRP1 [115,116] and co-transport of bile acids with GSH for MRP4 [117,118].

MRP1 was initially discovered and cloned by Cole and colleagues from a multidrug resistant human lung cancer cell line that did not overexpress MDR1 [119]. The expression of MRP1 in normal human tissues was subsequently determined to be ubiquitous, but at fairly low levels [Reviewed in 120]. MRP1 plays a role in the efflux of substrates from the basolateral/sinusoidal domain of hepatocytes, the blood-brain barrier, the proximal kidney tubules into the urine, and in the basolateral domain of the placental syncytiotrophoblast and within the fetal endothelium. The expression of MRP1 has also been identified in the fetal liver [121]. MRP1 substrates are known to include oxidized glutathione (GSSG), leukotriene C₄, glucuronide- and sulfate-conjugated moieties, antineoplastics, and antiretrovirals, amongst others [122]. The antimalarial mefloquine has been shown to interact with MRP1 [123].
MRP2, formerly known as the canalicular multispecific organic anion transporter (cMOAT), shares common substrate-specificity with MRP1. Substrates are known to include glutathione-, glucuronide- and sulfate-conjugated moieties, antineoplastics, antiretrovirals and statins. Of note, MRP2 shows a greater affinity for mono- and bisglucuronosyl bilirubin than MRP1. This in vitro finding in addition to many studies in Mrp2-deficient rodents, elucidated that lack of a functional MRP2 transporter in the canalicular hepatocyte domain results in hyperbilirubinemia and significantly reduced biliary clearance of organic anions and anionic conjugates. In normal human tissues, MRP2 is exclusively localized to apical membranes and is highly expressed in the hepatocytes of the liver, proximal tubule epithelia of the kidney, intestinal epithelia and placental syncytiotrophoblast [Reviewed in 120,122,124].

MRP3 substrates are known to include glutathione-, glucuronide- and sulfate-conjugated moieties (with glucuronide-conjugates being the better MRP3 substrates), antineoplastics and bile salts. In normal human tissues, MRP3 is constitutively expressed in the basolateral domain of hepatocytes, kidney, intestine, placental syncytiotrophoblast, and in the endothelia within the fetal mesenchyme [Reviewed in 120,122,124].

MRP4 substrates are characteristically nucleotide analogues and are known to include glucuronide conjugates, cyclic nucleotides (cGMP and cAMP), monoanionic bile acids, and antiretrovirals. The antimalarial mefloquine has been shown to interact with MRP4 [123]. In normal human tissues, MRP4 is ubiquitously expressed [Reviewed in 120]. In the liver MRP4 plays a role in the sinusoidal efflux of substrates into the maternal circulation [117].

In the liver, MRP1, MRP3 and MRP4 are localized in the basolateral membranes of hepatocytes where they efflux their substrates into the maternal sinusoidal blood. In contrast, MRP2 is located in the apical membranes of hepatocytes where it effluxes its substrates into the bile canaliculi for elimination via the intestinal tract. Studies have consistently shown a compensatory upregulation of MRP1, MRP3 and MRP4 in the liver, as well as the kidney, when MRP2-mediated hepatobiliary excretion is compromised [125,126,127,128,129,130]. For example, Mrp2 gene knockout mice have been shown to exhibit significantly elevated hepatic and renal
Mrp3 and Mrp4 expression [128,129,130]. For some substrates, compensation of this nature is believed to reduce intrahepatic accumulation, to reduce potential hepatotoxicity, and lead to increased renal elimination [127,129,131]. Additionally, many studies have illustrated increases in the expression of hepatic MRP1, MRP3, and MRP4 as a result of intrahepatic cholestasis or bile-duct ligation [reviewed in 132,133].

In the placenta, MRP1 and MRP3 are located in the fetal-facing basolateral membrane of the syncytiotrophoblast layer, where they promote the transfer of their substrates into the fetal compartment. MRP1 and MRP3 are also located in the apical membranes of the fetal capillary endothelial cells where they again promote the flux of their substrates into the fetal compartment. MRP2, in contrast, is localized to the maternal-facing apical membrane of the placental syncytiotrophoblast layer where it promotes the efflux of substrates into maternal blood.

1.2.3.3 The Breast cancer resistance protein (BCRP; ABCG2)

The activity of BCRP was first observed in human breast carcinoma cell lines that had grown resistant to mitoxantrone, doxorubicin and daunorubicin, but not via MDR1- or MRP-mediated upregulation [134,135,136]. Within the same year, the transporter was isolated from a MCF-7 breast cancer cell line by Doyle and colleagues [137], in the placenta by Allikmets and colleagues and called ABCP (i.e. ABC-Placenta) [138], and finally in colon cancer cells by Miyake and colleagues and denoted as MXR (Mitoxantrone Resistance Protein) [139]. Structurally, BCRP differs from MDR1 and the aforementioned MRPs in that it is a half transporter that must homodimerize to function as an ABC transporter [140]. BCRP consists of one single TMD containing six α-helices and 1 NBD [141]. The substrates of BCRP include steroid hormones, cholesterol, glucuronidated- and sulfated-conjugates, antineoplastics, antibiotics, antiretrovirals, statins, natural health products, and dietary carcinogens [142,143,144,145,146,147]. Further, it is thought that the antimalarial mefloquine is likely a BCRP substrate [98].

In normal human tissues, BCRP is primarily expressed in the liver, kidney, intestine, blood-brain barrier, lung and placental syncytiotrophoblast [148]. In the liver, BCRP is localized to the apical membrane of hepatocytes where it effluxes its
substrates into the bile canaliculi for elimination via the intestinal tract. In the placenta, BCRP is localized to the maternal-facing apical membrane of the syncytiotrophoblast layer and its important barrier function in this tissue is highlighted in numerous studies employing BCRP inhibitors or Bcrp<sup>−/−</sup> gene knockout mice. For example, relative to untreated pregnant Abcb1a/b gene knockout mice, pregnant Abcb1a/b gene knockout mice treated with the Mdr1/Bcrp inhibitor GF120918 exhibited twice the fetal penetration of the Mdr1/Bcrp substrate topotecan [149]. Placental BCRP is also partially responsible for the limited fetal exposure that occurs when women use the oral hypoglycemic agent glyburide during pregnancy [150,151].

1.2.3.4 Other ABC Efflux transporters

1.2.3.4.1 Multidrug Resistance Protein 3 (MDR3; ABCB4)

Multidrug resistance protein 3 (MDR3; ABCB4), discovered and cloned by Van der Bliek and colleagues [152], displays a very narrow substrate preference and tissue-expression compared to the ABC transporters discussed thus far. MDR3 is related to MDR1 in both structure and regulation but is believed to transport only the phospholipid phosphatidylcholine. In contrast to MDR1's expression throughout the body's major clearance organ systems, functional MDR3 protein is found primarily in the canalicular membranes of hepatocytes with some expression in the placental syncytiotrophoblast [153,154]. MDR3 is thought to play a major role in biliary phosphatidylcholine transport which in turn mediates bile flow from the biliary tree [155]. The expression of MDR3 has also been identified in the human placenta, but an understanding of its function in the placental syncytiotrophoblast remains elusive [153,154,156].

1.2.3.4.2 The Bile Salt Export Pump (BSEP; ABCB11)

The bile salt export pump (BSEP; ABCB11) was discovered in patients with progressive-familial intrahepatic cholestasis-2 (PFIC-2) [157], and was first characterized in the rodent [158]. Patients with PFIC-2 display elevated serum bile acid levels with low concentrations of biliary bile acids, low or normal serum cholesterol, and normal serum gamma glutamyltransferase [159]. Expression of the
bile salt export pump (BSEP), much like ABCB4, is also largely restricted to canalicular membranes of hepatocytes, apical membranes of cholangiocytes, and low quantities have been observed in the placental syncytiotrophoblast layer [160]. BSEP is believed to transport only conjugated monoanionic bile salts and bile salt metabolites, playing an integral role in the intestinal elimination of drugs and other xenobiotics.

While neither MDR3 nor BSEP directly contribute to drug-disposition, their expression and function may be altered by disease or through drug-mediated inhibition. This, in turn, can affect the expression of other transporters (e.g., the basolateral MRPs) and the hepatobiliary clearance of drugs and other xenobiotics by inducing intrahepatic cholestasis [161]. Additional ABC transporters believed to play important roles in the cellular efflux of drugs appear in figure 3 and are critically reviewed elsewhere [162, Reviewed in 163, Reviewed in 164].

1.2.3.5 SLC Uptake transporters

1.2.3.5.1 The Organic Anion Transporting Polypeptides (OATPs)

The organic anion transporting polypeptides (OATPs) transport amphiphilic organic compounds via anion-exchange. OATPs consist of 12 transmembrane domains/helices and a large extracellular domain [165]. They are encoded by SLCO genes but not every human OATP has a murine ortholog. There are also some species differences in OATP localization. For example, Oatp4a1 is a placental syncytiotrophoblast-specific transporter in the mouse but OATP4A1 can be found in numerous human tissues [166]. Known OATP transporter substrates include bile acids, glucuronide- and sulfate-conjugated moieties, antineoplastics, and statins.

1.2.3.5.2 The Organic Anion Transporting Polypeptide 2 (SLCO1B1; OATP2)

The organic anion transporting polypeptide 2 (SLCO1B1) is expressed exclusively in the basolateral domain of hepatocytes where it mediates the sodium-independent uptake of a number of endogenous compounds including bilirubin, bilirubin-glucuronide, bile acids, 17-ß-glucuronosyl estradiol and leukotriene C4. SLCO1B1 also transports a number of xenobiotics and clinically important
therapeutics such as bromosulfophthalein, statins, antidiabetic agents, antineoplastics, antibiotics, and antihypertensives [reviewed in 167]. The role of SLCO1B1 in the uptake of statins (and other drugs) into the liver has been widely investigated and many pharmacogenetic variants in SLCO1B1 have been identified as predictors of both reduced treatment efficacy and statin-mediated toxicity (e.g. statin-mediated myopathy) [168,169,170,171,172]. The murine gene that encodes OATP2 is Slco2b1.

1.2.3.5.3 The Sodium-Taurocholate Co-transporting Polypeptide (SLCO10A1; NTCP)

The sodium-taurocholate co-transporting polypeptide (NTCP; SLCO10A1), cloned and characterized by Hagenbuch and Meier [173], is expressed exclusively in the basolateral membrane of hepatocytes and is a major regulator of hepatocellular bile salt and cholesterol uptake [174]. NTCP transports one bile salt molecule together with two sodium ions and is therefore an electrogenic transporter [175]. As such, this allows for bile salt uptake from the blood against a substantial concentration gradient into the liver. NTCP plays a critical role in maintaining cholesterol homeostasis and displays substrate-specificity towards conjugated bile salts and sulfated compounds [176]. Of note, NTCP and BSEP act in tandem to regulate hepatic and intestinal bile salt levels within a fairly narrow range and together largely determine bile salt and cholesterol homeostasis within the body [176].

1.2.3.6 The Organic Anion and Organic Cation Transporters (The SLC22 Family)

OAT and OCT transporters also consist of 12 transmembrane domains [177]. The OATs transport amphiphilic organic anions using sodium- and dicarboxylate-gradients that are established by the sodium-dicarboxylate co-transporter and the Na⁺-K⁺ ATPase. Unlike OATPs and OATs, the OCTs simply mediate facilitated-diffusion. OCT mediated transport, therefore, occurs down the concentration gradient of substrates [Reviewed in 178]. OCT substrates are usually less than 500 Da in size and hydrophilic organic cations. Antihistamines (e.g., cimetidine and ranitidine) and the oral hypoglycemic agent metformin are classic examples of OCT substrates [179]. It has been shown that quinine is a rodent OCT1 and OCT2 substrate [180], and may
be a substrate for human OCT1 and OCT2. OATs and OCTs are expressed in a variety of normal human tissues, although the OATs and OCTs are strongly associated with the uptake and clearance of drugs by kidney proximal tubules through the urine. Additional SLC transporters that are believed to play important roles in the cellular uptake of drugs appear in figure 3 and are reviewed elsewhere [162, Reviewed in 163, Reviewed in 164, 181].
Figure 3. Localization of human transport proteins in the liver and placenta. Transporters examined within this thesis are shown in yellow. A, The basolateral (sinusoidal) membranes of hepatocytes contain Organic Anion Transporter 1 (SLC22A), Organic Anion Transporting Polypeptide 1B1 (OATP1B1) (SLCO1B1), Organic Anion Transporting Polypeptide 1B3 (OATP1B3; SLCO1B3), Organic Anion Transporting Polypeptide 2B1 (OATP2B1; SLCO2B1), the sodium/taurocholate cotransporting polypeptide (NTCP; SLCT1A1), Multidrug resistance associated protein 1 (MRP1; ABCC1), Multidrug resistance associated protein 3 (MRP3; ABCC3) and multidrug resistance associated protein 4 (MRP4; ABCC4). The apical (canalicular) membranes of these cells contain efflux transporters that transport substrates into the bile canaliculi, including multidrug resistance protein 1 (MDR1; ABCB1), multidrug resistance protein 3 (MDR3; ABCB4), the breast cancer resistance protein (BCRP; ABCG2), multidrug resistance associated protein 2 (ABCC2), and the bile salt export pump (BSEP; ABCB11). B, The syncytiotrophoblast layer of the placenta is comprised of multinucleated (N) cells that contain a number of transport proteins that contribute to the function of the placental barrier, including Organic Anion Transporting Polypeptide 4A1 (OATP4A1; SLCO4A1), MDR1, BCRP and MRP2. The basolateral membranes of these cells contain Organic cation transporter 1, OATP2B1, MRP1, MRP3, and MDR3. The apical (luminal) membranes of the fetal capillary endothelial cells, contain MRP1 and MRP3. Panels were adapted from [85] and [182].
1.2.4 Regulation of Drug Transporters and Drug Metabolizing Enzymes

Numerous networks of transcription factors regulate drug transporters and drug metabolizing enzymes. Current literature has implicated nuclear receptors as key regulators of transporter and drug metabolizing enzyme expression. The human, rat and mouse genomes contain genes for 48, 47 and 49 nuclear receptors, respectively [183]. Most ligand-activated nuclear receptors heterodimerize with the 9-cis retinoic acid receptor (RXR) upon activation and directly interact with their DNA response elements (DRE) to control the transcription of a wide array of target genes. Nuclear receptors contribute to an array of essential host processes involved in reproduction, development, energy homeostasis, responses to inflammation and xenobiotic detoxification/drug-disposition. Many nuclear receptors are dysregulated in inflammatory disease as several are suppressed by inflammation and exposure to pro-inflammatory cytokines [184,185,186,187,188,189,190]. With respect to their roles in drug-disposition, nuclear receptors are important transcription factors for all of the aforementioned drug transporters and metabolic enzymes and share many ligands with them [Reviewed in 191,Reviewed in 192]. As such, they are thought to link xenobiotic exposure to the transcription of genes encoding drug metabolizing enzymes and drug transporters that mediate xenobiotic elimination. This is particularly true of the pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which exhibit substantial ligand promiscuity. The farnesoid X receptor (FXR) is also linked to the regulation of drug transporters and metabolic enzymes and plays a role in maintaining bile acid homeostasis. More recently, the role of the nuclear erythroid 2-related factor 2 (Nrf2), a redox-sensitive transcription factor, has been implicated in drug transporter and drug-metabolizing enzyme regulation in response to oxidative stress and xenobiotic exposure [193,194].

1.2.4.1 The Pregnane X Receptor (PXR; NR1I2)

PXR is primarily expressed in the liver, kidney, intestine, placenta, and blood-brain barrier [195,196,197,198]. PXR is activated by a diverse array of drug compounds [199]. The most robust PXR drug ligands are those that are also established CYP3A inducers, such as dexamethasone, phenobarbital and
spironolactone \([200,201]\). Other notable ligands include: antiretrovirals, hyperforin (St. John’s Wort), nicotine, omeprazole, paclitaxel, phenytoin and many others \([191]\). Bile acids, bilirubin and steroid hormones are endogenous PXR ligands \([202]\). PXR activation has been implicated in the induction of many drug transporters, such as MDR1, MRP2, MRP3, BCRP, OATP1A1 and OATP1B1 \([203,204,205,206,207,208,209]\). PXR has also been implicated in the transcription of many drug metabolizing enzymes, such as CYP2As, CYP2Bs, CYP2Cs, CYP3As, UGT1As and SULTs \([210,211,212,213,214,215]\). The role of PXR in drug-metabolizing enzyme and drug transporter expression has been extensively studied in the context of inflammatory disorders, infection, and pro-inflammatory cytokine regulation. As noted above, many studies have identified inflammation-mediated decreases in the expression of PXR \([164,167,168]\).

### 1.2.4.2 The Constitutive Androstane Receptor (CAR; NR1I3)

CAR is primarily expressed in the liver, kidney, intestine and blood-brain barrier \([216,217,218]\). As its name suggests, CAR is constitutively active in the absence of ligand but is regulated by agonists and inverse agonists. The CAR/RXR heterodimer complex binds to the phenobarbital response element – so named because the prototypical CAR activator is phenobarbital. CAR and PXR have many xenobiotic and endobiotic ligands in common. For example, the artemisinins, which are first-line therapeutics for the treatment of severe malaria, are CAR/PXR ligands and have been shown to induce the expression of prototypical CAR/PXR responsive genes such as CYP3A and MDR1 \([106,107,108]\). Furthermore, through use of selective CAR-agonists, CAR activation has been shown to regulate many of the enzymes and transporters involved in the hepatobiliary clearance of bilirubin \([219]\). There is a great deal of overlap in the drug transporters and metabolic enzymes that CAR and PXR modulate. For example, CAR has been implicated in the transcriptional induction of MDR1, MRP2, MRP3 CYP3A, OATP2B1, and BCRP \([203,204,207,220]\).

### 1.2.4.3 The Farnesoid X Receptor (FXR; NR1H4)

FXR is predominantly expressed in the liver, kidney and intestine \([221]\) where it plays a role in regulating bile acid homeostasis by mediating the expression of a
battery of genes involved in bile acid synthesis and the enterohepatic recirculation of bile acids between the liver and intestine upon ligand-activation. FXR is regulated by bile acids, and as a result, plays an important role in triggering adaptive responses to high intracellular bile acid levels [222,223,224]. For example, FXR-activation indirectly suppresses the transcription of CYP7A1, the rate-limiting biosynthetic enzyme involved in initiation of bile acid synthesis [225], by increasing the expression a protein (SHP) that interferes with CYP7A1 transcription [226]. Studies examining the drug transporter and metabolic enzyme target genes of FXR suggest that FXR regulates MRP2, BSEP, NTCP, OATP1B1 and OATP1B3 [227,228], as well as CYP3A4, SULT2A1 and various UGTs [204,229,230,231,232].

1.2.4.4 The Nuclear Erythroid 2-Related Factor 2 (NRF2; NFE2L2)

NRF2 plays a pivotal role in maintaining redox homeostasis during oxidative stress. Although normally sequestered in the cytosol via binding to Keap1 (kelch-like ECH-associated protein 1), Keap1 dissociates and NRF2 is activated in response to electrophilic and oxidative stress and enters the nucleus where it binds to antioxidant response elements (ARE) and consequentially upregulates a number of genes involved in antioxidant response and detoxification. These gene products include proteins that catalyze reduction reactions (e.g. NADPH:quinone oxidoreductase 1), conjugative biotransformation reactions (e.g. GST and UGT), heme metabolism (e.g. heme-oxygenase 1), as well as the efflux of potentially toxic xenobiotics and conjugated metabolites (e.g. MRP1-MRP4) [233]. The significance of Nrf2 in the liver has been well established, as livers of Nrf2 gene knockout mice are more susceptible to various oxidative and electrophilic stress-induced pathologies than wildtype Nrf2+/+ mice. Furthermore, activation of Nrf2 has been shown to play a pivotal role in regulating the innate immune response in sepsis and malaria infection [234,235].

1.2.5 Pharmacokinetic Concepts Relevant to this Work

The alterations to drug transporter and drug metabolizing enzyme expression discussed within this thesis may impact several pharmacokinetic parameters leading to alterations in drug-disposition.
1.2.5.1 Hepatic clearance

Hepatic clearance (CL$_{\text{Hepatic}}$) is a parameter used to characterize efficiency of drug elimination through the activity of a consort of hepatic drug transporters and drug metabolizing enzymes [236,237]. Hepatic clearance is calculated as a function of hepatic blood flow (Q$_{\text{Hepatic}}$), plasma protein binding (f$_u$) and the intrinsic clearance capacity of the liver (CL$_{\text{intrinsic}}$).

Q$_{\text{Hepatic}}$ determines the rate at which a xenobiotic is delivered to the liver via the hepatic portal vein and hepatic artery. The f$_u$ value determines the free-fraction of drug presented to the liver and available for hepatic clearance processes. CL$_{\text{intrinsic}}$ represents the inherent ability of the liver to metabolize/excrete the drug in the absence of flow-limitations. As such, CL$_{\text{intrinsic}}$ plays a major role in determining the total clearance capacity of the liver and can change in response to normal physiological processes, disease, and drug-drug interactions.

The extraction ratio for a given drug is defined as the fraction of drug entering the liver that is irreversibly extracted in one pass of blood and is determined by Q$_{\text{Hepatic}}$, f$_u$ and CL$_{\text{intrinsic}}$ [236]. An extraction ratio of 0 indicates no extraction while a ratio of 1 indicates total extraction in a single pass. Drugs are often dichotomized into those with low extraction ratios (~0.3 or lower) and high extraction ratios (~0.7 or higher). Although this distinction exists, it is important to note that a number of drugs fall within intermediate extraction ratios (~0.3-0.7).

Variation in Q$_{\text{Hepatic}}$ has a greater effect on the hepatic clearance of high extraction ratio drugs than it does for low extraction ratio drugs. High extraction ratio drugs can theoretically be removed at a rate much higher than hepatic blood flow because CL$_{\text{intrinsic}}$ is high and efficient. Since drugs cannot be cleared at a rate faster than they are presented to an eliminating organ, clearance of high extraction ratio drugs is said to be flow-limited. Low extraction ratio drugs, on the other hand, are removed at a rate far below the rate of hepatic blood flow. As such, hepatic clearance of low extraction ratio drugs is said to exhibit capacity-limited clearance since they are insensitive to changes in blood flow and CL$_{\text{intrinsic}}$ and f$_u$ are the limiting factors.

For low extraction ratio drugs, slight changes in CL$_{\text{intrinsic}}$ will produce corresponding changes in CL$_{\text{Hepatic}}$. For CL$_{\text{Hepatic}}$ of high extraction ratio drugs, it is accepted that changes to Q$_{\text{Hepatic}}$ and f$_u$ are more significant contributors to CL$_{\text{Hepatic}}$ than
However, to contradict this point, significant reductions in CL\textsubscript{intrinsic} have been observed in a rodent model of arthritis where dramatic inflammation-mediated changes in hepatic metabolism resulted in a high extraction ratio drug (e.g. propranolol), being converted to an intermediate extraction ratio drug [238].

It is important to note that in disease, it is common for more than one of Q\textsubscript{Liver}, f\textsubscript{u} and CL\textsubscript{intrinsic} to change simultaneously. For instance, in most scenarios where changes in protein-binding (i.e., changes to f\textsubscript{u}) are believed to result in clinically-relevant alterations in CL\textsubscript{Hepatic}, a change in drug transporter and/or metabolic enzyme activity/expression may also be probable.

### 1.2.5.2 The Fetal:Maternal Free Drug Concentration Ratio

An understanding of the fetal:maternal (F:M) free drug concentration ratio, a parameter used to characterize the extent of fetal drug accumulation, is useful to discuss the potential impact of changes in placental transporter expression.

This concept, extensively reviewed by Hutson and colleagues in the context of the human placental perfusion model, may be used to help interpret perturbations to fetal drug concentration ratios as a result of disease-induced changes or transporter gene knockout [239]. Critical parameters that influence maternofetal drug-disposition include: (1) maternal and fetal protein binding, (2) maternal and fetal compartmental pH, and (3) maternofetal/fetomaternal placental uptake and efflux transport processes [240,241].

The percentage of free drug in the maternal circulation, pH of the fetal compartment, and the clearance of drug from maternal to fetal circulation (CL\textsubscript{Maternal $\rightarrow$ Fetal}) influence the transfer of drugs in the maternofetal direction. As discussed, alterations in plasma protein binding can lead to aberrations in maternal plasma free drug concentration, and in theory, would increase the amount of drug available for passive or active transport across the placenta. Drug transporters in the placental syncytiotrophoblast layer also play a major role in determining CL\textsubscript{Maternal $\rightarrow$ Fetal}. For example, many of the SLC uptake transporters e.g. OATP4A1 in the apical membrane of the syncytiotrophoblast and the ABC efflux transporters MRP1 and MRP3 in the basolateral membrane of the syncytiotrophoblast contribute to the CL\textsubscript{Maternal $\rightarrow$ Fetal} of
substrates (Figure 3B). Furthermore, drug transporters in fetal capillary endothelium, located in the fetal mesenchyme layer may also contribute to $CL_{\text{Maternal} \rightarrow \text{Fetal}}$ (e.g., MRP1 and MRP3; Figure 3B).

Once a drug has crossed the placenta, the marginal difference in fetal compartmental pH (pH ~ 7.35) from maternal compartmental pH (pH ~ 7.40) can alter the relative proportion of ionized drug to unionized drug [241]. This is particularly important for weakly basic drugs (i.e., pKa within the range of 7-8) that have permeated the placenta as they may become trapped once they enter the fetal circulation [242].

The percentage of unbound drug in the fetal circulation, $CL_{\text{Fetal} \rightarrow \text{Maternal}}$, and $CL_{\text{Fetal}}$ impact transfer in the fetomaternal direction. Drug transporters in the placental syncytiotrophoblast layer play a critical role in determining the $CL_{\text{Fetal} \rightarrow \text{Maternal}}$. For example, the efflux transporters MDR1, MRP2 and BCRP in the apical membrane and the uptake transporters OCT1 and OATP2B1 in the basolateral membrane of the placental syncytiotrophoblast layer contribute to the $CL_{\text{Fetal} \rightarrow \text{Maternal}}$ of substrates (Figure 3B). The importance of these placental transporters in determining fetal xenobiotic exposure has been well-established in placental primary cultures, mutant/knockout mice, and in ex vivo placental perfusion models [151, 243, 244, 245, 246, 247, 248, 249].

Fetal hepatic clearance ($CL_{\text{Fetal}}$) is determined by fetal drug metabolism and drug elimination processes which are believed to develop in utero, but do not reach adult activity and expression levels until weeks to months after parturition [as reviewed in 250]. Many of the ABC transporters and drug metabolizing enzymes discussed within this thesis are expressed in the developing human and murine fetal liver and brain and their ontogenic expression has been extensively reviewed elsewhere [251, 252, 253, 254]. An understanding of the contribution of fetal hepatic clearance mechanisms to in utero drug-disposition remains limited and there is a paucity of data on the impact of disease or inflammation on these processes.
1.3 Impact of inflammation on drug-disposition mechanisms

The association between inflammation and altered drug-disposition has been well characterized through research conducted over the last 35 years. Inflammation is the body’s defensive response to harmful stimuli, such as pathogen exposure or tissue damage, and includes local and systemic effects executed by the immune system. The local effects of inflammation are initiated by the movement of neutrophils from the blood compartment into the affected site(s). As the inflammatory response propagates, macrophages infiltrate the affected site(s). The systemic effects of inflammation, collectively referred to as the acute phase response, are triggered by the release of pro-inflammatory cytokines from monocyte, macrophages and other immune cells into the systemic circulation. Systemic pro-inflammatory cytokines, primarily interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), stimulate the synthesis of acute phase proteins in the liver [255]. These proteins include C-reactive protein (CRP), alpha 1-acid glycoprotein, serum amyloid A, glucocorticoids and other proteins that are associated with the fever, increased blood pressure, somnolence, lethargy, and decreased appetite that accompany systemic inflammation [256].

While the increased release of acute phase proteins is generally beneficial, the preceding processes can also affect drug-disposition. For example, increased synthesis of acute phase proteins in the liver can impact the plasma protein binding of drugs. Increased alpha 1-acid glycoprotein is observed in patients with diseases that are associated with inflammation, such as inflammatory bowel disease [64,65], rheumatoid arthritis [66], or infections such as malaria [257], which can translate into increased plasma protein binding of basic drugs and altered \( f_u \). Conversely, the acute phase response is also characterized by alterations involving decreased synthesis of proteins. Decreased synthesis of albumin has been observed in inflammation and many other inflammatory disorders this can translate into decreased plasma protein binding of acidic drugs and altered \( f_u \) [56,57].

One of the first clinical examples of inflammation-induced changes in human drug response, as described by Chang and colleagues, reported decreased
theophylline clearance in patients with upper-respiratory tract infections [258]. This observation was attributed not to alterations in plasma protein binding and \( f_u \), but to a down-regulation of a number of CYPs responsible for theophylline metabolism [259,260]. In the decades that followed, numerous studies utilized human and rodent cell lines, and in vivo animal models of inflammation and infection to demonstrate CYP suppression in response to pro-inflammatory cytokines such as IL-1\( \beta \), IL-6 and TNF-\( \alpha \) [Reviewed in 260, Reviewed in 261,262]. Consequently, it is now widely accepted that inflammation imposes a down-regulation of hepatic and extrahepatic CYPs, and that this effect is mediated by pro-inflammatory cytokines.

Data over the past 10 years also suggest a similar relationship for inflammation and drug transporter expression. The injection of rats with bacterial endotoxins has been shown to decrease Mdr1 expression and activity in isolated rat hepatocytes [263]. Furthermore, it was found that incubating cultured hepatocytes with the pro-inflammatory cytokines IL-1\( \beta \) (for 3 days) or IL-6 (for 24 hours) also decreased Mdr1 expression and substrate transport activity [264,265]. In addition to replicating these findings, a number of subsequent studies have demonstrated inflammation-mediated changes in hepatic Mrp2, Mrp3, Bcrp, Ntcp, Bsep, several Oatps, Oats, and Octs [266,267,268,269,270,271,272].

While inflammation-mediated changes in expression have also been observed in the intestine, kidney, and CNS [reviewed in 182,273,274,275,276], very little is known about inflammation-mediated changes to drug transporter expression in the placenta or maternal and fetal tissues during pregnancy. One in vitro study demonstrated decreased MDR1 and BCRP mRNA and protein expression in primary cultures of human placental syncytiotrophoblasts following incubation with the pro-inflammatory cytokines IL-1\( \beta \) and TNF-\( \alpha \) [277]. In addition, Wang and colleagues observed a significant downregulation of placental Abcb1a/b mRNA in pregnant rats injected with bacterial endotoxin versus saline [278]. In these pregnant rats, significant increases in IL-1\( \beta \), IL-6, and TNF-\( \alpha \) were observed and hypothesized to mediate the observed changes in placental Abcb1a/b. Fetal tissues from endotoxin-injected rats also accumulated 3.5 times more \([^{99mTc}]\)-sestamibi, a radiopharmaceutical agent and high-affinity Mdr1 substrate [278]. Further studies found that Abcb1a, Abcb1b, Abcc1-3, Abcg2 and several Oatps were significantly downregulated in placental samples.
from endotoxin-injected pregnant rats [279]. The functional consequences of BCRP downregulation were investigated using a biodistribution study with the Bcrp substrate and antidiabetic agent, glyburide, and it was confirmed that fetuses of endotoxin-treated pregnant rats accumulated 1.6-fold higher levels of glyburide, with comparable systemic plasma concentrations and protein binding in the maternal circulation. The impact of polyinosinic:polycitydylic acid (poly I:C), a double-stranded RNA and viral mimetic, on the expression of transporters in the maternal liver and placenta has also been investigated. In this study, significant decreases in the expression of placental Abcb1a, Abcb1b, Abcc1-3, Abcg2, and several Oatps were observed. Also, as expected, several ABC transporters, Oatps, and Cyp3a were reduced in maternal liver [272]. Recently, this line of work has been extended to human pregnancy where Mason and colleagues investigated the impact of intra-amnionic inflammation and preterm labor on placental ABC transporter expression [154]. In this study, Mason et al reported increased expression of MDR1 and BCRP in the placenta, however it is difficult to decipher if this may have been a consequence of variability in inflammatory stimuli and/or a differential regulation of transporters in the human placenta. Furthermore, the impact of localized placental inflammation on transporter expression is poorly understood, and these divergent results may be due to local inflammation rather than systemic inflammation that is associated with endotoxin-administration.

To date, the impact of malaria infection on drug-disposition mechanisms is poorly understood. The effect of malaria infection on hepatic clearance of drugs and other xenobiotics has been assessed in rat liver microsomes, isolated perfused rat hepatocytes, or through the use of probe drugs in humans [74,280,281,282,283,284,285,286,287,288]. These studies unanimously report decreased hepatic clearance of drugs (e.g., quinine, dihydroartemisinin, caffeine, pyrimethamine, primaquine) and other xenobiotics (e.g. phenol) and this is believed to occur as a result of decreased CYP and phase II enzymatic activity. These mechanistic claims have been confirmed in the literature where the altered expression of many drug-metabolizing enzymes (Cyp3a11, Cyp2e1, Cyp1a2, Cyp2a5, Cyp2e1) in male rodents infected with malaria have been identified [289,290,291].

Given the important role of drug transporters in mediating hepatobiliary clearance, it is plausible that alterations in drug transporter expression may also
contribute to the observed changes in xenobiotic clearance during malaria infection. To the best of our knowledge, no studies have examined the impact of malaria infection on drug transporter expression in any tissue, be it maternal, fetal, or in the placenta. Also, no studies currently exist where the impact of malaria infection on drug metabolizing enzyme expression in pregnancy has been investigated.

As a number of commonly used antimalarial drugs discussed within this thesis have been shown to interact with drug transporters and drug metabolizing enzymes, an understanding of the factors that modulate expression of these genes is important in considering antimalarial drug disposition.

1.3.1 Malaria-associated inflammation during pregnancy

Inflammatory processes are essential for the early control of parasitemia following malaria infection. However, dysregulated production of inflammatory mediators can lead to severe immunopathology [6]. As malaria infection progresses, parasitized erythrocytes bind, sequester and mature in the placental and cerebral microvasculature, where they produce and release a variety of bioactive molecules that mediate pathogenic processes [6]. These are thought to occur through their effects on the innate immune system as reviewed in 1.1.2 [9]. It is currently believed that *Plasmodium falciparum* glycosylphosphatidylinositol, a plasma membrane glycolipid, stimulates the immune system resulting in the induction of the expression of many genes implicated in malaria pathogenesis including the pro-inflammatory cytokines IL-1β, IFN- and TNF-α [292,293,294]. Furthermore, it has been shown that Complement 5a, a signaling molecule involved in the complement cascade, potentiates the release of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α by pfGPI in peripheral blood mononuclear cells [17,18]. Notably, blockade of C5a with selective antibodies has been shown to attenuate the extent of release of these pro-inflammatory mediators [17,18].

In the liver, the accumulation of parasitized erythrocytes and the parasite hemoglobin biopolymer, hemozoin, in resident macrophages (e.g. Kupffer cells) results in the release of numerous pro-inflammatory cytokines that have been implicated in inflammation-mediated changes in transporter and drug-metabolizing enzyme expression. An in vitro study has illustrated profound increases in the
expression of IL-1β and TNF-α in response to Kupffer cell exposure to hemozoin [295]. Given that Kupffer cells can be considered a tissue-resident monocyte, numerous lines of evidence converge on the activation of monocytes and consequential increases in secretion of TNF-α, IL-1β, and a variety of other cytokines and chemokines in the liver during malaria infection [296,297,298]. The localized uptake of hemozoin and parasitized erythrocytes can lead to a unique pro-inflammatory cytokine milieu in the liver that may play a role in regulating drug transporter and drug-metabolizing enzyme expression. As a consequence of inflammation and other factors associated with malaria infection, it has been shown that significant hepatic oxidative stress can occur. This is believed to ensue, in part, due to the accumulation of free heme in the liver following intravascular hemolysis [299]. Free heme both induces, and is metabolized by, heme-oxygenase 1 (HO-1), an inducible enzyme that releases free iron, biliverdin, and carbon monoxide from the NADPH-dependent oxidative metabolism of heme [reviewed in 300]. As a consequence of increased levels of free iron, a pro-oxidant environment is produced in the liver and in the systemic circulation, which can reduce antioxidant capacity via depletion of glutathione and other antioxidants leading to hepatic or other organ injury [301,302,303]. An additional enzyme that has been shown to be induced by both inflammation and oxidative stress is inducible nitric oxide synthase 1 (iNOS). iNOS catalyzes the formation of nitric oxide (NO), a messenger molecule with diverse biological functions throughout the body including maintenance of vascular integrity and homeostasis. Furthermore, iNOS is involved in host defense against a number of invading pathogens including protozoa, bacteria, fungi and viruses. iNOS is transcriptionally induced in response to bacterial endotoxins (LPS) and proinflammatory cytokines in macrophages and various other cell types [304].

In the placenta, parasitized erythrocytes expressing chondroitin sulfate A binding proteins are known to bind chondroitin sulfate A expressed on the placental syncytiotrophoblast. This mediates their sequestration in the intervillous space and prevents their elimination via the reticuloendothelial system (i.e. the spleen and liver) [24,305]. Products from ruptured parasitized erythrocytes within the intervillous space can stimulate resident macrophages in the syncytiotrophoblast or circulating monocytes and promote the generation of C5a, TNF-α and other pro-inflammatory
cytokines and chemokines [9,17,306,307,308]. This in turn can contribute to further macrophage infiltration, low birth weight and detrimental outcomes of malaria in pregnancy [309]. The function of both HO-1 and iNOS in the placenta have been widely studied and these enzymes have been shown to be implicated in pregnancy outcomes and their expression to be altered by infection and inflammation [310,311,312,313,314,315,316]. However, an understanding of how malaria infection impacts their placental expression remains limited.

TNF-α is one of the few factors associated with malaria in pregnancy that has been shown to mediate placental pathology. An increase of TNF-α and other pro-inflammatory cytokines in cord blood of women with placental malaria has been shown in several studies [317,318,319] and the role of TNF-α as a mediator of fetal loss associated with placental malaria has been directly demonstrated by TNF-α-blocking experiments in Plasmodium-infected pregnant mice and non-human primates [320,321,322].

While activation of the complement cascade represents a crucial step in the immune response to malaria infection, complement signaling in placenta is thought to contribute to adverse fetal outcomes during pregnancy, via the potentiation of pro-inflammatory cytokine release and dysregulation of angiogenesis [10,11,17]. Unpublished data from the laboratory of Dr. Kevin C. Kain suggests that global knockout of the C5a receptor in pregnant rodents improves fetal outcomes (e.g. birth weight and viability) during P. berghei ANKA malaria infection [35]. Since several transcription factors (e.g. NF-κB) known to regulate the expression of drug-metabolizing enzymes and drug transporters are located downstream of C5aR activation, it remains to be determined whether C5aR activation mediates changes in maternofetal drug transport and/or drug metabolism during pregnancy [190,323,324,325,326,327].
1.4 Rationale

Every year, an estimated 85 million pregnancies occur in areas of *P. falciparum* transmission, with almost 55 million pregnancies occurring in regions with stable malaria transmission [22]. Pregnant women are at an increased risk for adverse outcomes from malaria infection due to immunological changes that occur in pregnancy [23], in addition to the parasitized erythrocytes ability to bind to placental chondroitin sulfate-A (CSA) and to sequester in placental intervillous space [24].

Given the necessity to safely and effectively treat pregnant women infected with malaria, research investigating the pharmacokinetic processes that impact the maternofetal disposition of antimalarial drugs and other xenobiotics is warranted. The current understanding of the impact of maternal malaria infection on drug-disposition mechanisms in pregnancy is limited. As such, many pregnant women infected with malaria may be incorrectly dosed. This may result in maternal and/or fetal toxic effects stemming from supra-therapeutic drug concentrations or sub-therapeutic drug dosing resulting in drug failure [41,48]. This in turn may lead to poor pregnancy outcomes, including maternal and/or fetal death. Moreover, the development of drug-resistant malaria infections is a concern for women receiving sub-therapeutic drug concentrations. This is particularly of concern given the recent emergence of ACT-resistant parasites [44].

As previously described, pregnancy is associated with significant physiological changes that lead to alterations in key pharmacokinetic parameters that influence systemic drug concentrations and drug clearance. These include, but are not limited to, increased apparent V_D of drugs, reduced gut motility, increased renal blood flow, alterations in protein binding, and alterations in drug-metabolism processes. Reports in the literature have confirmed that many of these physiological changes alter the pharmacokinetic parameters (i.e. AUC, apparent oral clearance, and V_D) of antimalarial drugs such as artemisinin derivatives and quinine leading to markedly reduced systemic plasma concentrations of drug in pregnant women in comparison to term matched non-pregnant controls [reviewed in 42,43]. To further complicate the understanding of drug-disposition mechanisms in malaria-infected pregnancies, *P. falciparum* infection itself can alter drug-disposition leading to changes in the pharmacokinetics of antimalarials and other drugs (reviewed in 1.3).
To the best of our knowledge, no study currently exists where the impact of malaria infection on mechanisms of maternofetal xenobiotic-disposition in pregnancy has been investigated. Given the high prevalence of malaria infection in pregnancy and the array of drugs and other xenobiotics that malaria-infected women may be exposed to, a thorough investigation into this field is warranted. Furthermore, evidence for the distinct molecular contributions of the complement cascade to malaria pathogenesis has recently emerged, and given the role of C5a in mediating inflammatory mediator production in malaria, we examined if C5aR activation played a role malaria-induced changes in drug transporter and drug metabolizing enzyme expression.

To this end, we sought an experimental malaria model in pregnancy to answer our important study questions. *P. falciparum* is the main parasite involved in the human pathogenesis of malaria, however it does not infect any rodent strains. *P. berghei* is one of the four known strains of *Plasmodium* known to infect mice. Although a number of animal models for malaria in pregnancy (primarily in rodents) are currently available [reviewed in 328], the *P. berghei* ANKA animal model best reflects the clinical features of *P. falciparum* malaria. It is the only strain that has been used to study placental malaria. The features of this infection include, but are not limited to, placental zonal necrosis, mononuclear cell infiltration, sinusoid constriction, accumulation of PEs and hemozoin in placental intravascular spaces, marked placental inflammation, intrauterine growth restriction, low-birth weight, premature birth, and reduced fetal viability [14,329,330,331,332,333,334,335,336,337,338]. Furthermore, this model has been widely used in the literature and represents the current “gold standard” for studying malaria in pregnancy [328].
1.5 Hypotheses and Objectives

We hypothesized that malaria infection during pregnancy causes alterations in xenobiotic-disposition mechanisms, particularly in the expression of ABC efflux transporters, SLC uptake transporters, and drug-metabolizing enzymes in maternal tissues, fetal tissues, and the placenta. To test this hypothesis, we examined the impact of malaria on expression of the ABC efflux transporters MDR1, MRP1-3, BCRP, and BSEP, SLC transporters NTCP and SLCO2B1, and the drug-metabolizing enzyme CYP3A11 in maternal and fetal brain and liver, as well as in maternal kidney and the placenta using the *P. berghei* ANKA animal model.

We further hypothesized that activation of the complement factor 5a (C5a) innate immune pathway during malaria infection modulates the expression of transporters (MDR1, MRP1, BCRP, BSEP) and drug-metabolizing enzymes (CYP3A11) in pregnancy. To test this hypothesis, we examined whether global knockout of the C5a receptor (Gene: C5AR1) attenuates malaria-induced alterations in transporter and Cyp3a11 expression in the maternal liver and placenta using C5aR−/− mice on a Balb/c background in the *P. berghei* ANKA animal model.
Chapter 2. Materials and Methods
2.1 *Plasmodium berghei* ANKA Balb/c Experimental Malaria Pregnancy Model

To facilitate the investigation of our research objectives, collaboration was sought and formed with the laboratory of Dr. Kevin C Kain, a malaria research laboratory at the McLaughlin-Rotman Centre for Global Health (affiliated with the University of Toronto). All animal work described within this thesis up to gestational day (GD) 18 was completed by Dr. Karlee L. Silver, Chloe McDonald, and Kathleen Zhong for concurrent studies investigating placental malaria pathophysiology. At GD 18 and beyond, our group was involved with the weighing of dams, animal sacrifices, harvesting of all the maternal and fetal tissues, fetal viability checks, as well as the weighing of pups and maternal spleens. We completed all analytical work, data analyses, and interpretation of results ourselves, with some minor insights provided by the Kain group.

2.1.1 Animal Studies

In collaboration with the laboratory of Dr. Kevin C. Kain (Dr. Karlee L. Silver, Chloe MacDonald, and Kathleen Zhong) at the Sandra-Rotman Centre for Global Health, an experimental placental malaria model was employed to investigate the impact of malaria infection on drug transporter and drug-metabolizing enzyme expression in maternal, fetal, and placental tissue in pregnancy. All animal model protocols were approved by the Toronto University Health Network Animal Care Committee and performed in accordance with current institutional and national regulations, including the Canadian Council on Animal Care’s Guide to the Care and Use of Experimental Animals and the Ontario Society for the Prevention of Cruelty to Animals Act. Furthermore, all research adhered to the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985).

Eight to ten week old Balb/c female mice were obtained from Jackson Laboratories Inc. (Bar Harbor, Maine, USA) and upon arrival at the Toronto General Hospital Animal Research Facilities, mice were maintained on a 12-hour dark and 12-hour light cycle with access to standard rodent chow and water *ad libitum*. Following acclimatization, female eight to ten week-old Balb/c female mice were mated with
males, and checked for the presence of a vaginal plug (marked as GD1). Meanwhile, cryopreserved *Plasmodium berghei* ANKA strain malaria (MR4; Manassas, VA, USA) was thawed and passaged through a male Balb/c mouse via intravenous tail-vein injection. Peripheral parasitemia levels in the passage mouse were determined from thin blood smears using the modified Giemsa stain (Protocol Hema3 Stain Set, Sigma, Oakville, ON, Canada) and these values were utilized in calculation for preparation of the experimental malaria inoculum. Once peripheral parasitemia in the passage mouse rose to >10%, the passage mouse was sacrificed, exact peripheral parasitemia determined, and the experimental inoculum was then prepared.

Upon confirmation of pregnancy by observation of a 3-4 gram increase in maternal body weight; pregnant females were inoculated on GD13 with $10^6$ *Plasmodium berghei* ANKA infected erythrocytes in RPMI media (Sigma-Aldrich, Oakville, ON, Canada) via lateral tail-vein injection. Control pregnant Balb/c females were injected with an equivalent volume of RPMI media alone. Maternal peripheral parasitemia was monitored daily over the course of infection (GD13-GD19) by thin blood smear with modified Giemsa stain (Protocol Hema3 Stain Set, Sigma, Oakville, ON, Canada). Pregnant female mice were euthanized by CO$_2$ on either GD16 or GD19 (i.e. days 3 and 6 post-inoculation). Maternal blood was collected by cardiac puncture, centrifuged at 13,000 rpm for 5 minutes, and plasma was stored at -80°C until further use. Maternal tissues (liver, brain, and kidney) were removed immediately after sacrifice and snap frozen in liquid nitrogen. Uteri were removed and examined for evidence of resorptions. Yolk sacs were dissected from uteri and fetuses were removed and weighed using a Mettler-Toledo® analytical balance (Mettler Toledo, Mississauga, ON, Canada). Fetal viability was determined by assessing the pedal withdrawal reflex and placentae and fetal tissues (fetal liver and brain) from viable fetuses were removed immediately and snap frozen in N$_2$. Samples were subsequently stored at -80°C until further use. Non-viable fetuses were considered aborted and experimental studies on these fetuses were not conducted.

**Complement 5a Receptor Knockout Mouse Model:**

Based upon studies completed in the laboratory of our collaborators that have shown improved outcome in the context of cerebral malaria [18] and evidence that
C5aR knockout may result in improved outcomes in placental-malaria [10,11,17], we investigated this model in the context of C5aR acting as a possible regulator of placental and hepatic transporter expression. To do this, the same animal husbandry and experimental parameters were utilized as described for the wildtype Balb/c mice above, but experiments were completed using the C5ar1<sup>tm1Cge</sup>/J (Jackson Laboratories Inc., Bar Harbour, MA, USA) strain bred on a Balb/c background [developed in 339].
Figure 4. Pictorial representation of *P. berghei* ANKA experimental malaria pregnancy animal model as described within this thesis [developed in 329].
2.2 QPCR Methodology

The subsequent reporting of qPCR methodology follows the recommendations of the MIQE (Minimum information for publication of Quantitative Real-Time PCR Experiments) guidelines wherever applicable [340].

2.2.1 Total RNA Extraction

Total cellular RNA was extracted from ~75 mg of snap-frozen maternal, placental, or fetal tissues using the TriZol® RNA isolation method (Invitrogen, Carlsbad, CA, USA) following manufacturer’s instructions. Briefly, tissue was homogenized using a motorized pestle in 1 mL of TriZol® reagent (Invitrogen, Carlsbad, CA, USA) for 3 minutes. Tissue homogenate was then allowed to sit for 5 minutes at room temperature to allow complete dissociation of the nucleoprotein complex. Following this, 200 µL of chloroform was added to each sample and the suspension was vigorously shook for 15 seconds and allowed to sit for 3 minutes at room temperature. The suspension was then centrifuged at 12,000 x g for 15 minutes at 4°C. 500 µL of 100% isopropanol was then added to 500 µL of whole cell supernatant in a fresh microfuge tube and was allowed to sit for 10 minutes at room temperature. The sample was then spun at 12,000 x g for 10 minutes at 4°C. Supernatant was discarded and the RNA pellet was then washed with 1mL of 75% ethanol/25% ddH2O and the sample was briefly vortexed and then centrifuged at 7,500 x g for 5 minutes at 4°C. The RNA pellet was then air-dried for 10 minutes. Total extracted RNA was reconstituted in nuclease-free diethylpropylcarbonate (DEPC) H2O (Life Technologies Inc., CA, USA) and heated for 10 minutes at 55-60°C to promote RNA pellet dissolution. After heating, RNA was immediately centrifuged on bench top centrifuge and placed on ice for 10 minutes to cool. RNA was then subjected to qualitative and quantitative measurements i.e. purity such that A260/280 ≥ 1.90 and A260/320 ≥ 1.80 and yield of > 800ng RNA/µL DEPC-H2O using a NanoDrop®-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). As a means of quality control for subsequent downstream applications (e.g., qPCR), if RNA purity was not above these a priori cutoffs, total RNA was re-extracted from these samples.
2.2.2 DNAse I Treatment

2 µg of total extracted RNA was DNase treated using 25 mM MgCl₂ (Sigma Aldrich, Oakville, ON) and 4 U/µL DNAse I enzyme (Invitrogen, Burlington, ON) adjusted with nuclease-free DEPC H₂O (Life Technologies Inc., CA) to a final volume of 20 µL. 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.

2.2.3 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.

2.2.4 Quantitative-Polymerase Chain Reaction (RT-qPCR):

mRNA expression of drug transporters (Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, Abcb11, Abcg2, Slco2b1, and Slco10a1), drug-metabolizing enzymes (Cyp3a11), and disease-relevant genes (HO-1 and iNOS) in maternal tissues, fetal tissues, and the placenta were determined by real-time reverse-transcriptase quantitative polymerase chain reaction (qPCR). qPCR was performed on cDNA using LightCycler® technology with the Roche LightCycler® 2.0 FastStart DNA Master SYBR Green I fluorescence detection kit (Roche Diagnostics, Montreal, QC, Canada). qPCR oligonucleotides were synthesized at The Hospital for Sick Children (DNA Synthesis Centre, Toronto, ON, Canada) and reconstituted in nuclease-free DEPC H₂O prior to
use. qPCR primers were designed to recognize one distinct target sequence and to span adjacent exon-exon junctions to eliminate the potential for amplification of residual genomic DNA or off-target products. Also, primers were designed to be approximately 20-25 base pairs in length, with guanine/cytosine content between 45-60%, melting temperatures between 57°C and 62°C, and to produce amplicons of less than 150 base pairs in length. The full array of qPCR primer sequences and National Library of Medicine (NLM) target mRNA sequences are available in table 1.
Table 1: qPCR primer sequences for quantitative determination of drug transporter (Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, Abcg2, Abcb11, Slco10a1, Slco2b1), drug-metabolizing enzyme (Cyp3a11), and disease-relevant gene (HO-1 and iNOS) expression. National Library of Medicine target mRNA sequences from *Mus musculus* are included in addition to gene nomenclature as per the Human Gene Nomenclature Committee (HGNC).

<table>
<thead>
<tr>
<th>Gene Nomenclature</th>
<th>NLM Target mRNA Sequence</th>
<th>Forward Primer Sequence (5' → 3')</th>
<th>Reverse Primer Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normalizing Gene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CycA</td>
<td>NM_008907.1</td>
<td>GGAGATGCCACAGGAGGAA</td>
<td>GCCCGTAGTGCTTCAGCTT</td>
</tr>
<tr>
<td>ATP-Binding Cassette Transporters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abcb1a</td>
<td>NM_011076.2</td>
<td>GGAGCTGCTGTCCCATCTTTCA</td>
<td>GACCTCACGTGTCTCTACCTCCCG</td>
</tr>
<tr>
<td>Abcb1b</td>
<td>NM_011075.2</td>
<td>AGGCCGCTGTCTCCCATCTTTGA</td>
<td>CATCACACCTCAGTCGCCACCT</td>
</tr>
<tr>
<td>Abcc1</td>
<td>NM_008576.3</td>
<td>TGAGTGTGCACAGGGTGAGG</td>
<td>ACCCGCCTGTAGCTCCATTAT</td>
</tr>
<tr>
<td>Abcc2</td>
<td>NM_013806.2</td>
<td>GGTATATGAGGCCGCGTGGGT</td>
<td>CCGGCCGATACCGCATTGA</td>
</tr>
<tr>
<td>Abcc3</td>
<td>NM_029600.3</td>
<td>CCCACAGCTGCAGTCTATAC</td>
<td>GATCAATGTCAGTGGCGACCT</td>
</tr>
<tr>
<td>Abcg2</td>
<td>NM_011920.3</td>
<td>ATGGAGCAACCTAGTCCAGG</td>
<td>GTCAGGGTGCCCACATCAACGT</td>
</tr>
<tr>
<td>Abcb11</td>
<td>NM_021022.3</td>
<td>CCCCTCGGAAGGCATGGTGAGG</td>
<td>ACCAAGCGAGGATTCTTCTGCGA</td>
</tr>
<tr>
<td>Cytochrome P450 Enzymes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>NM_007818.3</td>
<td>AGGGAAGCATGAGGAGGATCATCA</td>
<td>TGCTAGGAGCACCAGGTTCTGA</td>
</tr>
<tr>
<td>Solute Carrier Transporters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slco10a1</td>
<td>NM_001177561.1</td>
<td>CCCTACGTCCCAAGGAGG</td>
<td>GCCATCAGGAGGAGGTA</td>
</tr>
<tr>
<td>Slco2b1</td>
<td>NM_001252530.1</td>
<td>CCTAACAGCAGCAAGGAGG</td>
<td>ACTACTGCAGACAGAC</td>
</tr>
<tr>
<td>Disease-Relevant Genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HO-1</td>
<td>NM_010442.2</td>
<td>CTCGAGCATAGCAGGAGG</td>
<td>AGACAAAGTCTGGGCGCAGT</td>
</tr>
<tr>
<td>iNOS</td>
<td>NM_010927.3</td>
<td>CACTTTGAGTTCTACAGG</td>
<td>ACCAAGACTACTTTGAGG</td>
</tr>
</tbody>
</table>
For each qPCR sample, 2 µL of sample cDNA was added to 18 µL of reaction mix containing 4 µL of Roche SYBR green (Roche Diagnostics, Montreal, QC), 100 pmol of forward and reverse qPCR oligonucleotide primers, and nuclease-free DEPC H₂O. No template control (NTC) samples were also used on each qPCR run to confirm that samples and primers were not amplifying residual DNA contaminants that is, any residual genomic DNA or other nucleic acid contaminants that could theoretically be present in laboratory reagents. A calibrator sample i.e. a pool of all “control” cDNA samples, was used on each qPCR run to allow for mean fold-change quantification and to account for any inter-run variability.

Amplicon sequences were amplified using the following thermocycling parameters: 1 cycle of 10 minutes at 95°C, 50 cycles (6 seconds at 95°C, 6 seconds at 60°C, and 6 seconds at 72°C), followed by a continuous 180-second melting curve cycle ramping from 65-99°C. Melt-curve analysis was used after qPCR amplification to ensure primer-specificity such that all samples exhibited a single quantifiable amplicon and that no off-target amplification or primer product amplification occurred.

Expression levels of drug transporter, drug-metabolizing enzyme, disease-relevant gene, and normalizing gene mRNA were denoted as C_q (the point at which gene-of-interest and reference gene could be reliably quantified by instrumental software) and these values were determined using the Roche LightCycler® II software (Ver. 3.5) configured with the Roche LightCycler® II Real-Time qPCR instrument (Roche Diagnostics GmbH, Hamburg, Germany). Gene-of-interest expression in each sample was normalized internally to cyclophilin A mRNA expression using the efficiency-corrected ΔC_q qPCR method [as described in 341]. The normalizing gene cyclophilin A was selected as its expression has been shown to be stable in a number of other studies and we observed equivalent expression levels between control and infected dams.

The efficiency-corrected ΔC_q qPCR method was used because there were slight differences in the efficiencies of our primer sets as determined by standard-curve analysis of serially-diluted calibrator cDNA. qPCR efficiencies were always >90% with coefficient-of-determination (R²) > 0.95 and as such, it is unlikely that subtle differences in primer efficiency had any substantial impact on our analysis and/or conclusions. For qPCR analysis, gene-to-cyclophilin ratios were converted to each
run’s calibrator sample and then presented as mean fold-change in expression compared to control ± SEM.

2.3 Western Blots

300 mg of snap-frozen tissue was homogenized using a motorized pestle in radioimmunoprecipitation assay buffer (RIPA) (Cell Signaling Technologies, Pickering, ON, Canada) containing freshly added dithiothreitol (1 mM; DTT; Sigma-Aldrich, Oakville, ON), phenylmethylsulfonyl fluoride (0.5 mM; PMSF; BioShop Canada Inc., Burlington, ON, Canada) and 1X protease-inhibitor cocktail (Sigma-Aldrich, Oakville, ON, Canada) at 4°C. Homogenates were then incubated on ice for 30 minutes with brief vortexing at 15 minutes and subsequently centrifuged at 18,000 x g for 15 minutes at 4°C. For each sample, the supernatant was isolated and subjected to a Bradford protein assay [342].

Protein samples were prepared in Laemmli sample loading buffer, heated at 36°C for 20 minutes, separated via 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred for 2 hours to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories Canada, Ltd., Mississauga, ON, Canada). PVDF membranes were then blocked in 5% non-fat dry milk in Tris-Buffere Saline + 0.1% Tween 20 (TBST) and incubated with an anti-MDR1 mouse monoclonal antibody (1:500; 1mg/mL mC219 clone, ID Labs Biotechnology, Inc., London, ON, Canada) overnight at 4°C. The mC219 antibody clone is a commercially available and widely-used antibody to detect protein expression of P-glycoprotein and produces distinct quantifiable bands in tissue samples of murine placenta, liver, kidney, and brain [343,344].

After a series of washes with TBST, membranes were incubated with an anti-mouse horseradish peroxidase-labeled secondary antibody (1:300; goat-anti mouse Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 hour in 2% non-fat milk TBST. Membranes were again subjected to a series of washes with TBST and immunoreactive proteins were detected using an ECL Plus chemiluminescence kit (Amersham Biosciences, Baie d’Urfé, QC, Canada). An Alpha Innotech (San Leandro, CA, USA) FluorChem imaging system was used to capture the image of the immunoreactive proteins present on the blots. The optical density (OD) of each Mdr1
band was determined using ImageJ (Software version 1.44o) for Macintosh.

To confirm equivalent protein loading, an amido black total protein stain was employed such that the OD of MDR1 could be normalized internally to total protein loading. Each blot was stained using submersion in Amido Black (0.03% Napthol Blue Black in 3% acetic acid) (BioShop Canada Inc., Burlington, ON, Canada) for 3 minutes and allowed to air-dry overnight on plastic wrap before scanning at 600 dpi using an HP Scanjet 7400C. The optical density (OD) of each lane was determined using ImageJ (Software version 1.44o) for Macintosh. This method was chosen due to its superiority in detection of total protein loading, stability between the respective study groups, and high-degree of linearity between protein loading and band optical density [345,346]. Recent reports suggest that the expression of many common “normalizing” genes and proteins may vary between control and treated or diseased groups [347]. In fact, while optimizing we did see variable expression in ß-actin (data not shown), so we elected to use a total protein stain to circumvent this issue.

2.4 Total Cellular Antioxidant Assay

A commercially available total cellular antioxidant assay (Cayman Chemical Company, Ann Arbor, MI, USA) was used to assess the impact of P. berghei ANKA malaria infection on the total cellular antioxidant capacity of the maternal liver. The antioxidant assay was used to measure total antioxidant capacity of cellular constituents including both aqueous and lipid-soluble antioxidants (e.g., vitamins C and E, proteins, lipids, glutathione, bilirubin, uric acid, etc.).

This assay relies on the ability of antioxidants present in the sample to inhibit the oxidation of ABTS® (2,2’-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS®“ by metmyoglobin. The ability of total antioxidants to prevent ABTS® oxidation is then compared to that of a Trolox standard-curve, a water-soluble tocopherol analogue and results are presented as antioxidant capacity of maternal liver relative to the Trolox standard curve.

The antioxidant assay was conducted all according to the manufacturer’s instructions. Briefly, ~150 mg of maternal liver was homogenized on ice in 1 mL of cold homogenization buffer (5mM potassium phosphate, pH 7.4 containing 0.9% NaCl
and 0.1% glucose) using a motorized pestle. Homogenates were then vortexed and centrifuged at 12,000 x g for 15 minutes at 4°C. Whole cell lysate supernatant was removed and stored on ice until assay, all completed on the same day. Whole cell lysate supernatant was isolated and subjected to a Bradford assay for total-protein normalization [342]. Whole cell lysate supernatant was assayed according to the commercially available total cellular antioxidant kit and absorbance of sample was determined using a UV-Vis spectrophotometer at 750nm. Results were then calculated and reported as µmol of total antioxidant levels relative to the Trolox standard, normalized to total hepatic protein content.

2.5 Serum Chemistry Analysis

Total serum bile acids were analyzed in plasma samples obtained from infected and control dams at a certified GLP laboratory (IDEXX laboratories, Inc., Markham, ON, Canada) using current standard methods of the International Federation of Clinical Chemistry.

2.6 Statistical Analysis

All data were analyzed with GraphPad Prism 5.0 for Macintosh (GraphPad Software, Inc., San Diego, CA, USA). Tests on maternal and fetal physiological parameters i.e. maternal weight, fetal weight, bile acid levels, and total hepatic cellular antioxidant levels were completed using Student’s unpaired two-tailed t-test. Spearman rank correlation coefficient analysis was used to assess for association of placental gene expression to fetal weight.

Gene and protein expression results are presented as mean % change from control ± standard error of the mean (SEM). To assess for differences in gene and protein expression between the infected and control groups, Student’s unpaired two-tailed t-tests were conducted. 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 ***, $p < 0.001$. Performing the non-parametric Mann-Whitney $U$ test yielded comparable results to Student’s unpaired two-tailed t-test. For the C5aR$^+$ study, gene expression results are presented and analyzed as above, with # indicating
significantly different gene expression as compared to the *P. berghei* ANKA infected wildtype mouse with *p* < 0.05.
Chapter 3. Results
3.1 *P. berghei* ANKA Malaria Infection Impacts Maternal and Fetal Parameters

Given the established impact of *P. falciparum* malaria infection on maternal weight, spleen weight, and fetal weight in humans, we confirmed whether these changes were also seen in our animal model of malaria. As previously reported in studies using the *P. berghei* ANKA malaria model in pregnancy [36,329,334], numerous changes to maternal and fetal physiological parameters were observed.

A significant decrease in maternal body weight was observed in *P. berghei* infected dams from gestational day (GD) 16 to GD19 (Figure 5). *P. berghei* infected dams weighed a mean of 7.53g ± 1.21g less at GD19, relative to uninfected dams (Figure 5). No significant differences were seen on GD13 or GD16. The marked weight loss in infected dams from GD16 to GD19 was consistent with increasing peripheral parasitemia over this time period, from 2.37% ± 0.38% to 49.88% ± 2.67% at GD16 and GD19, respectively (Figure 5).
Figure 5: Impact of increasing maternal *P. berghei* ANKA parasitemia on pregnancy weight gain in Balb/c dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. Maternal weight of infected and control dams was measured on GD 13, 16, and 19 using an analytical balance and results are presented as mean maternal weight (g) ± SEM; peripheral parasitemia was measured using a thin blood smear with modified Giemsa stain and maternal parasitemia (as % infected erythrocytes in blood smear) was counted using light microscopy. N = 6 dams/group, ***, p < 0.001.
As splenic- and hepatic-macrophages sequester parasitized erythrocytes and hemozoin as part of the reticuloendothelial system, we next looked to see if maternal spleen weight was significantly altered. Not surprisingly, spleen weight was significantly higher in *P. berghei* ANKA infected dams relative to uninfected control dams (208.1mg ± 13.0mg vs. 102.2mg ± 8.9mg; p < 0.001), as depicted in Figure 6.

![Figure 6](image)

**Figure 6.** Impact of *P. berghei* ANKA infection on maternal spleen weight at GD19 in Balb/c dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. Spleen weight was measured using an analytical balance and results are presented as mean spleen weight (mg) ± SEM. N = 6 dams/group, ***, p <0.001.
In terms of fetal changes, we observed a significant decrease in the mean total body weight of fetuses from *P. berghei* ANKA infected dams, relative to fetuses from uninfected control dams (748.50mg ± 40.38mg vs. 1082.00mg ± 44.71mg, respectively; p < 0.001), as depicted in Figure 7. Moreover, *P. berghei* ANKA infection was associated with significantly decreased fetal viability (59% in *P. berghei* ANKA infected dams vs. 97.6% in uninfected dams; p < 0.001).

**Figure 7.** *P. berghei* ANKA malaria infection reduces fetal weight at GD19 in pregnant Balb/c mice inoculated with 10⁶ *P. berghei* ANKA infected erythrocytes on GD 13. Fetal weight was measured using an analytical balance and data presented are mean fetal weights (in mg) ± SEM. N = 10 pups/group, ***, p <0.001.
3.2 *P. berghei* ANKA Malaria Infection Alters Hepatic Transporter and Cyp3a11 Expression

Infection and inflammatory disorders significantly impact drug transporter and drug metabolizing enzyme expression. As such, we investigated the expression of Cyp3a11 and key hepatic transporters as these mechanisms contribute to the hepatobiliary clearance of drugs and other xenobiotics. We asked whether *P. berghei* ANKA malaria infection is associated with changes to Cyp3a11 and drug transporter expression in the maternal liver during pregnancy.

The expression of maternal Cyp3a11 was significantly decreased to 35.45% of control (± 18.93%; \( p < 0.001 \)) in *P. berghei* ANKA infected dams relative to uninfected control dams (Figure 8). In this study to gain a holistic view of transporter expression in malaria infection, we examined both canalicular and basolateral domain transporters. We first analyzed transporters expressed in the canalicular domain and noted an increase in Abcb1b mRNA expression by 359.6% (± 40.50%; \( p < 0.001 \)) in *P. berghei* ANKA infected dams relative to uninfected control dams (Figure 8). In contrast, there were significant decreases in the expression of Abcc2 to 54.45% of the level of control (± 11.40%; \( p < 0.01 \)), Abcg2 to 49.21% of the level of control (± 9.15%; \( p < 0.01 \)), and Abcb11 to 11.19% of the level of control (± 3.89%; \( p < 0.001 \)) in *P. berghei* infected dams. Finally, the expression of maternal hepatic Abcb1a was not altered by *P. berghei* ANKA malaria infection (Figure 8).

Given the changes observed in the mRNA expression of canalicular transporters, we elected to investigate the protein expression of hepatic Mdr1. We did not observe alterations in the expression of hepatic Mdr1 protein in *P. berghei* ANKA infected dams when compared to uninfected control Balb/c dams (124.1% ± 11.11% the level of control; \( p = 0.1229 \)) (Figure 9).
Figure 8. Impact of *P. berghei* ANKA malaria infection on maternal hepatic mRNA expression of Cyp3a11 and canalicular transporters at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. mRNA expression of genes in *P. berghei* infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Results are presented as mean transporter mRNA expression as % control ± SEM. N = 6 dams/group. Student's unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: **, p < 0.01 and ***, p <0.001.
Figure 9: Impact of *P. berghei* ANKA malaria infection on the maternal hepatic protein expression of Mdr1 at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. Mdr1 protein expression was quantified using Western Blotting and normalized to total-protein levels via Amido Black staining. Results presented as mean Mdr1 protein expression % control ± SEM. N = 4 dams/group. Student’s unpaired t-test was used for statistical analysis.

We next analyzed the expression of several hepatic basolateral (sinusoidal) transporters. As depicted in Figure 10, we observed significantly elevated expression of Abcc1 (733.8% ± 95.91% relative to control; $p < 0.001$) and Abcc3 (474.2% ± 71.17% relative to control; $p < 0.001$). In contrast, the expression of the uptake transporter Slco2b1 was significantly decreased (39.79% ± 32.87% relative to control; $p < 0.01$) whereas the expression of Slco10a1 was not significantly changed relative to control.
Figure 10: Impact of *P. berghei* ANKA malaria infection on the maternal hepatic mRNA expression of basolateral transporters at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. mRNA expression of genes in *P. berghei* infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Results presented as mean transporter mRNA expression as % control ± SEM. N = 6 dams/group. Student’s unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: ***, p < 0.001 and **, p < 0.01.
3.3 *P. berghei* ANKA Malaria Infection Alters Total Plasma Bile Acid Levels

Next, we investigated a functional consequence of the *P. berghei* ANKA induced changes in hepatic transporter expression by examining the levels of endogenous serum bile acids. We hypothesized that the decreased expression of canalicular Abcb11 and Abcc2, along with the increased expression of sinusoidal Abcc1 and Abcc3, would result in decreased canalicular transport of bile acids out of the liver via Abcb11 and Abcc2 and increased bile transport into the maternal circulation by Abcc1 and Abcc3 via salvage pathways at the sinusoidal domain. These processes in turn would result in significantly increased serum bile acids. As illustrated in Figure 11, we observed a significant and dramatic increase of ~75-fold in total serum bile acid concentrations in *P. berghei* ANKA infected dams relative to uninfected control dams (382.0 ± 152.3 µmol/L vs. 4.833 ± 0.1667 µmol/L, respectively).
Figure 11: Impact of *P. berghei* ANKA malaria infection total maternal serum bile acid levels at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. Results presented as mean serum bile acid levels (µmol/L) ± SEM as described in methods. N = 6 dams/group. Student’s unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: ***, p <0.001.
3.4 *P. berghei* ANKA Malaria Infection Dramatically Alters the Hepatic Expression of inducible Nitric Oxide Synthase, Heme Oxygenase-1 and Hepatic Cellular Antioxidant Capacity:

We next examined the expression of hepatic inducible Nitric Oxide Synthase (iNOS) and Heme Oxygenase-1 (HO-1) as these enzymes are induced by hepatic inflammation, oxidative stress, and have been shown to be induced in the liver as a host-response to malaria infection [300,348,349,350,351]. As depicted in Figure 12, we identified significant increases in the hepatic expression of both iNOS and HO-1 in the maternal liver of *P. berghei* ANKA infected dams by 209.6-fold and 21.16-fold, respectively (p < 0.001), relative to uninfected control dams (Figure 12A). We also observed a significant decrease in total cellular antioxidant level in the livers of *P. berghei* ANKA infected dams relative to uninfected control dams (263.3 uM vs. 208uM; p < 0.001) (Figure 12B).

![Figure 12](image)

**Figure 12:** (A) Impact of *P. berghei* ANKA malaria infection on the maternal hepatic mRNA expression of iNOS and HO-1 and (B) total hepatic cellular antioxidant capacity at GD19 in dams inoculated with 10⁶ *P. berghei* ANKA infected erythrocytes on GD 13. mRNA expression of genes in *P. berghei* infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Total cellular antioxidant capacity was determined using a commercially available kit as described in the methodology. Results presented as mean mRNA expression as % control ± SEM. N = 6 dams/group; Student’s unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: ***, p <0.001.
3.5  *P. berghei* ANKA Malaria Infection Alters Transporter Expression in the Maternal Brain:

The expression of *Abcb1a*, *Abcb1b*, and *Abcg2* were assessed in maternal brain homogenate due to their protective role in the extrusion of xenobiotics in the endothelium of the blood-brain barrier [352]. As depicted in figure 13, we observed modest alterations in the expression of transporters in the maternal brain. A significant increase in *Abcb1b* was observed in infected dams relative to control dams (162.57% ± 26.91%; p < 0.01). No significant changes were observed in the expression of *Abcb1a* or *Abcg2* relative to control.
Figure 13: Impact of *P. berghei* ANKA malaria infection on the mRNA expression of transporters in maternal brain homogenate at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. mRNA expression of genes in *P. berghei* infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Results presented as mean transporter mRNA expression as % control ± SEM. N = 4-6 dams/group. Student's unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: ***, p < 0.01.
Given the changes observed in the mRNA expression of Abcb1b in the brain, we investigated the protein expression of cerebral Mdr1. In line with what was observed with the expression of Abcb1b, we observed a significant increase in the expression of maternal brain Mdr1 in *P. berghei* ANKA infected dams when compared to uninfected control Balb/c dams (227.6% ± 46.65%) the level of control; \( p < 0.05 \) (Figure 14).

**Figure 14:** Impact of *P. berghei* ANKA malaria infection in pregnancy on maternal brain Mdr1 expression at GD19 in dams inoculated with \( 10^6 \) *P. berghei* ANKA infected erythrocytes on GD 13. Mdr1 quantified using Western Blotting and normalized to total-protein levels via Amido Black stain. Results presented as mean Mdr1 protein expression as % control ± SEM. N = 4-5 dams/group. Student’s unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: *, \( p < 0.05 \).
3.6 *P. berghei* ANKA Malaria Infection Upregulates Transporter Expression in the Maternal Kidney:

The expression of *Abcb1a* and *Abcb1b* were assessed in whole kidney homogenate due to their role in the extrusion of drugs and other xenobiotics from the kidney proximal tubule into the tubule lumen [85]. As depicted in figure 15, we observed significant increases in the expression of both *Abcb1a* (350.37% ± 101.53% the level of control; p < 0.01) and *Abcb1b* (225.93% ± 98.91% the level of control; p < 0.05) in comparison to uninfected Balb/c dams.
Figure 15: Impact of *P. berghei* ANKA malaria infection on the maternal renal mRNA expression at GD19 in dams inoculated with 10⁶ *P. berghei* ANKA infected erythrocytes on GD 13. mRNA expression of genes in *P. berghei* infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Results presented as mean transporter mRNA expression as % control ± SEM N = 6 dams/group. Student’s unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: ***, p <0.001.
Given the changes observed in the mRNA expression of renal Abcb1a and Abcb1b, we investigated the protein expression of renal Mdr1. In line with what was observed with the expression of both Abcb1a and Abcb1b, we observed a significant increase in the expression of renal Mdr1 in *P. berghei* ANKA infected dams when compared to uninfected control Balb/c dams (144.4% ± 3.76% the level of control; p < 0.05) (Figure 16).

**Figure 16:** Impact of *P. berghei* ANKA malaria infection on the maternal renal expression of Mdr1 at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. Mdr1 quantified using Western Blotting and normalized to total-protein levels via Amido Black stain. Results presented as mean Mdr1 protein expression as % change of control ± SEM. N = 4 dams/group. Student’s unpaired t-test used for statistical analysis with the following symbols denoting statistical significance: *, p < 0.05.
3.7 *P. berghei ANKA* Malaria Infection Decreases Transporter Expression in the Placenta:

Given the critical role of placental transporters in determining fetal drug accumulation and their function as a protective barrier limiting fetal exposure to toxic xenobiotics, we chose to investigate the impact of *P. berghei* ANKA infection on the placental expression of several ABC efflux transporters.

*P. berghei* ANKA malaria infection led to significant changes in placental ABC transporter expression at GD19. As depicted in figure 17, we observed a significant decrease in the placental mRNA expression of Abcb1a (65.31% ± 16.33% the level of control; \( p < 0.001 \)), Abcb1b (74.29% ± 21.00% the level of control; \( p < 0.01 \)), Abcc1 (70.76% ± 17.20% the level of control; \( p < 0.05 \)), Abcc2 (41.63% ± 26.79% the level of control; \( p < 0.05 \)), Abcc3 (53.55% ± 28.77% the level of control; \( p < 0.01 \)), and Abcg2 (59.11% ± 15.84% the level of control; \( p < 0.05 \)) at GD19 in *P. berghei* ANKA infected Balb/c dams. Significant changes were not observed at an earlier time-point in malaria infection at GD16 where peripheral parasitemia of 2.370% ± 0.377% was observed.
Figure 17: Impact of *P. berghei* ANKA malaria infection on the mRNA expression of ABC transporters in the placenta at GD19 in dams inoculated with $10^6 *P. berghei* ANKA infected erythrocytes on GD 13. mRNA expression of genes in *P. berghei* infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Results presented as mean transporter mRNA expression as % control ± SEM. N = 10 placentae/group. Student’s unpaired t-test used for statistical analysis with the following symbols denoting statistical significance: *, p < 0.05, **, p < 0.01 and ***, p <0.001.
Given the changes observed in the mRNA expression of placental Abcb1a and Abcb1b, we further examined the protein expression of placental Mdr1. We observed a significant decrease in the expression of placental Mdr1 in *P. berghei* ANKA infected dams when compared to uninfected control Balb/c dams (46.40% ± 11.09%) the level of control; *p* < 0.05) (Figure 18).

![Bar graph showing protein expression of Mdr1 in control (C) and infected (I) dams.](image)

**Figure 18:** Impact of *P. berghei* ANKA malaria infection on the expression of Mdr1 in the placenta at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. Mdr1 quantified using Western Blotting and normalized to total-protein levels via Amido Black stain. Results presented as mean Mdr1 protein expression as % control ± SEM. *N* = 4 placentae/group. Student’s unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: *, *p* < 0.05.
3.8 Placental expression of inducible Nitric Oxide Synthase and Heme-Oxygenase 1 are altered in *P. berghei* ANKA infection and negatively and positively correlate with fetal weight:

We next examined the expression of placental iNOS and HO-1 as these enzymes in the liver have been reported to be induced by infection, inflammation and pro-inflammatory cytokines, oxidative stress, and have been shown to be increased in response to malaria and other infections [349,350]. The function of both HO-1 and iNOS in the placenta have been widely studied and these enzymes have been shown to be implicated in pregnancy outcomes and their placental expression to be altered by infection and inflammation [310,311,312,313,314,315,316]. Their role in the placenta has also been extensively studied in the pathogenesis of preeclampsia [353] and the HELLP syndrome (hypertension, elevated liver enzymes, low platelet counts) [354], but limited work has been completed investigating the role of these enzymes in malaria infection or malaria outcomes.

As depicted in figure 19, we identify significant decreases in the placental expression of iNOS (77.06% ± 19.77%; p < 0.05) and a significant increase in the placental expression of HO-1 (169.37% ± 43.32%; p < 0.001) in comparison to uninfected Balb/c control dams. Interestingly, these changes in expression positively and negatively correlated with fetal weight, respectively (iNOS: r = 0.5293, p < 0.05; HO-1: r = -0.7564, p < 0.001).
Figure 19: Impact of *P. berghei* ANKA malaria infection on the placental mRNA expression of inducible nitric oxide synthase and heme-oxygenase 1 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13 and correlation with fetal weight. (A) iNOS and HO-1 mRNA expression quantified using QPCR and normalized to cyclophilin expression with results presented as mean mRNA expression as % control ± SEM, N = 10 placentae/group. Student's unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: *, p < 0.05, **, p < 0.01 and ***, p < 0.001. (B) Placental expression of heme-oxygenase 1 (HO-1) and inducible-nitric oxide synthase 1 (iNOS) differentially correlate with fetal weight in offspring of *P. berghei* ANKA malaria infected dams. Correlation analyzed via Spearman Coefficient Analysis.
3.9  *P. berghei* ANKA Malaria Infection Alters Fetal Hepatic Cyp3a11 and Transporter expression:

There are few reports of the impact of maternal infection on fetal hepatic drug-metabolizing enzyme expression [355,356]. To the best of our knowledge, no such reports exist that have investigated the impact of maternal infection on fetal hepatic transporter expression or drug metabolizing enzyme expression in the context of malaria. Given that metabolism and transport processes may influence the distribution of xenobiotics and their metabolites within the fetal compartment, we asked if *P. berghei* ANKA infection would alter their expression.

As depicted in figure 20, we observed significant changes in the expression of Cyp3a11 and numerous ABC efflux transporters in the fetal liver. Significant decreases in the expression of fetal hepatic Cyp3a11 (50.81% ± 10.38% of control; p < 0.01), Abcb1a (28.51% ± 8.27% of control; p < 0.001), Abcg2 (62.84% ± 6.8% of control; p < 0.01), and Abcb11 (48.57% ± 7.77% of control; p < 0.01) were all seen in pups from *P. berghei* dams in comparison to pups from uninfected control dams. Abcb1b was significantly increased (320.4% ± 55.09% of control; p < 0.01) above that which was observed in the control pups. Contrary to changes observed in the maternal liver, the expression of fetal liver Abcc1 (151.94% ± 62.00% of control; p = 0.097) was not altered.
Figure 20: Impact of \textit{P. berghei} ANKA malaria infection in on the mRNA expression of fetal hepatic Cyp3a11 and ABC transporters at GD19 in dams inoculated with $10^6$ \textit{P. berghei} ANKA infected erythrocytes on GD 13. mRNA expression of genes in \textit{P. berghei} infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Results presented as mean transporter mRNA expression as \% control $\pm$ SEM, N = 6 fetuses/group. Student’s unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$. 

Cyp3a11

Abcg2

Abcb1b

Abcb1a

Abcg2

Abcc1
3.10 Impact of *P. berghei* ANKA Malaria Infection on Fetal Brain Transporter Expression:

The expression of *Abcb1a*, *Abcb1b*, and *Abcg2* were assessed in fetal brain homogenate due to their known role in the extrusion of drugs in the developing fetal blood-brain barrier [reviewed in 357]. As depicted in figure 21, we observed a significant increase in *Abcb1a* expression (123.49% ± 19.76% of control; p < 0.05) whereas the expression of *Abcb1b* and *Abcg2* were not significantly changed.
Figure 21. Impact of *P. berghei* ANKA malaria infection on fetal whole brain mRNA expression of transporters at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. mRNA expression of genes in *P. berghei* infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Results presented as mean transporter mRNA expression as % control ± SEM, N = 6 fetuses/group. Student’s unpaired t-test was used for statistical analysis with the following symbols denoting statistical significance: *, p < 0.05.
3.11 Impact of *P. berghei* ANKA Malaria Infection on Fetal weight from pregnant Balb/c WT and C5aR−/− Dams:

As the complement cascade is thought to contribute to disease pathogenesis in malaria infection, we examined if C5aR and its associated signaling cascade was involved in: (1) the fetal outcomes of *P. berghei* ANKA malaria infection, namely fetal weight gain and fetal viability and (2) the regulation of drug transporter and drug metabolizing enzyme expression in maternal liver and placenta.

As depicted in figure 22, we investigated if inactivation of C5aR had any impact on fetal weight and/or fetal viability between infected wildtype and C5aR−/− Balb/c dams. *P. berghei* infection imposed significant decreases in fetal weight of both C5aR−/− and wildtype dams. However, the extent of weight change was diminished in *P. berghei* ANKA infected C5aR−/− dams, corresponding to a significantly higher fetal weight of 74.60 ± 23.64mg (p < 0.01) as compared to *P. berghei* infected wildtype dams. The observed increase in weight was independent of changes in mean weight of control wildtype Balb/c and control C5aR−/− Balb/c pups.
Figure 22: Impact of *P. berghei* ANKA infection on viable fetal weight at GD19 in Balb/c and C5aR<sup>−/−</sup> gene knockout mice inoculated with 10<sup>6</sup> *P. berghei* ANKA infected erythrocytes on GD 13. Fetal weight was measured using an analytical balance and data presented are mean fetal weights (in mg) ± SEM. One-Way ANOVA and Newman-Kuels post-test analysis were utilized to test for statistical significance with the following symbols denoting statistical significance: *, p < 0.05, **, p < 0.01 and ***, p < 0.001.
3.12 Impact of *P. berghei* ANKA Malaria Infection on Hepatic Cyp3a11 and Hepatic Transporters in C5aR<sup>-/-</sup> Dams:

As depicted in figure 23, we examined the expression of transporters and Cyp3a11 in infected and control maternal livers of C5aR<sup>-/-</sup> Balb/c dams. The expression of Cyp3a11 and transporters in the maternal liver of wildtype Balb/c dams in figure 23 is derived from figures 8 and 10 and described earlier within this thesis.

In C5aR<sup>-/-</sup> dams, we observed significant changes in the expression of Cyp3a11 and many transporters in the maternal liver when compared to uninfected C5aR<sup>-/-</sup> control dams. We observed significant decreases in the expression of maternal hepatic Cyp3a11 (46.57% ± 23.79% of control; p < 0.01) and Abcb11 (9.32% ± 1.45% of control; p < 0.001), significant increases in Abcc1 (741.36% ±155.2% of control; p < 0.001), and no change in the expression of Abcb1a or Slco2b1. We observed an increase in the expression of Abcb1b (484.84% ± 75.06% of control; p < 0.001) when compared to uninfected C5aR<sup>-/-</sup> dams.

Of note, the increased expression of Abcb1b was significantly higher than that which was observed in the wildtype *P. berghei* ANKA infected Balb/c dams (134.83% ± 81.50% of wildtype *P. berghei* ANKA infected Balb/c dams; p < 0.05). No significant differences in the expression of Abcb1a, Cyp3a11, Slco2b1, Abcb11, and Abcc1 were observed when comparing C5aR<sup>-/-</sup> and wildtype infected dams.
Figure 23: Impact of *P. berghei* ANKA malaria infection on the maternal hepatic mRNA expression of Cyp3a11 and drug transporters in C5aR−/− dams at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. mRNA expression of genes in *P. berghei* infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Results presented as mean transporter mRNA expression as % control ± SEM. N = 4-6 dams/group, statistical analysis via Student’s unpaired t-test, p-value *< 0.05, ** <0.01, ***<0.001, # denotes significantly different from wildtype infected dams with a p < 0.05.
3.13 Impact of *P. berghei* ANKA Malaria Infection on Placental Transporters in C5aR\(^{-}\) Dams:

Given the role of C5a receptor activation in the release of pro-inflammatory cytokines and inflammatory mediators during infection, it is plausible that inactivation of this pathway could alter the expression of transporters during infections such as malaria. We asked if the expression of placental transporters in infected and control wildtype dams differed when compared to their expression in infected and control C5aR\(^{-}\) dams. The expression of placental transporters in wildtype dams within figure 24 is derived from figure 17 described earlier within this thesis.

As compared to uninfected C5aR\(^{-}\) dams, we observed a significant decrease in the expression of Abcc2 (48.05% ± 26.59% of control; \(p < 0.001\)) and Abcg2 (79.77% ± 17.56% of control; \(p < 0.05\)) in *P. berghei* infected C5aR\(^{-}\) dams. No significant changes were observed in the expression of Abcb1a or Abcb1b in infected C5aR\(^{-}\) dams compared to uninfected C5aR\(^{-}\) dams.

As compared to *P. berghei* infected wildtype dams, we observed a significant increase in the expression of both Abcb1a and Abcg2 in the placenta of C5aR\(^{-}\) infected dams (\(p < 0.05\)). The expression of Abcb1a in C5aR\(^{-}\) infected dams was 81.09% ± 16.89% of control; \(p = 0.085\), whereas expression of Abcb1a in wildtype infected dams was reduced to 65.31% ± 16.33% from control; \(p < 0.001\). The expression of Abcg2 in C5aR\(^{-}\) infected dams was 79.77% ± 17.56% of control; \(p < 0.05\), whereas the expression of Abcg2 in wildtype infected dams was reduced to 59.11% ± 15.84% of control; \(p < 0.05\). Contrary to what was observed in the wildtype infected dams, it is noteworthy that the expression of both Abcb1a and Abcb1b in the placentae of C5aR\(^{-}\) infected dams was not altered when compared to control C5aR\(^{-}\) dams.
Figure 21: Impact of *P. berghei* ANKA infection in C5aR⁻/⁻ Balb/c dams on the expression of ABC efflux transporters in the placenta at GD19 in dams inoculated with $10^6$ *P. berghei* ANKA infected erythrocytes on GD 13. mRNA expression of genes in *P. berghei* infected and control groups at GD19 was quantified using QPCR and normalized to cyclophilin expression. Results presented as mean transporter mRNA expression as % control ± SEM. N = 10 placentae/group, statistical analysis using Student’s unpaired t-test with the following symbols denoting statistical significance: *, p < 0.05, **, p < 0.01 and ***, p <0.001, # denotes significantly different from wildtype infected with a p < 0.05.
Chapter 4. Discussion, Conclusions, and Recommendations
This thesis describes a set of experiments using a well-characterized *P. berghei* ANKA animal model of malaria infection in pregnancy to investigate the impact of infection on maternal, fetal, and placental xenobiotic-disposition mechanisms.

We hypothesized that malaria infection during pregnancy causes alterations in xenobiotic-disposition mechanisms, particularly in the expression of ABC efflux transporters, SLC uptake transporters, and drug-metabolizing enzymes in maternal tissues, fetal tissues, and the placenta. To test this hypothesis, we utilized the the *P. berghei* ANKA animal model to investigate the expression of the ABC efflux transporters Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, Abcg2, and Abcb11, the SLC uptake transporters Slco10a1/NTCP and Slco2b1/Oatp2, and the drug-metabolizing enzyme Cyp3a11 in maternal and fetal brain and liver, as well as in maternal kidney and the placenta.

The importance of drug transporters and drug-metabolizing enzymes in determining the disposition of drugs and other xenobiotics is becoming ever more apparent, and has been well-characterized and reviewed within the scientific literature. In the liver, these transporters and drug-metabolizing enzymes in tandem play a critical role in determining the CL\textsubscript{Hepatic} and CL\textsubscript{intrinsic} of drugs and other xenobiotics. In the kidney, uptake and efflux transport processes mediated largely by the SLC uptake and ABC efflux transporters play a role in the excretion of drugs and other xenobiotics from blood into the proximal tubule lumen and urine. As such, these processes play a role in CL\textsubscript{Renal} of drugs and other xenobiotics and competitive inhibition of transport processes can lead to drug-drug interactions. In the brain, a number of efflux transporters (e.g. MDR1 and BCRP) at the blood-brain barrier limit the permeation of drugs and other xenobiotics into the cerebral space [reviewed in 358].

The expression of transporters and drug-metabolizing enzymes in many pharmacokinetically-relevant tissues has been shown to be modulated by physiological processes (e.g. pregnancy), and diseases such as cancer, inflammatory disorders, and infectious disease [reviewed in 182]. This may lead to changes in overall systemic drug exposure, drug clearance or distribution into tissue or sensitive sites such as the fetal compartment. Given the high prevalence of malaria infection in pregnancy and the array of drugs and other xenobiotics that malaria-infected women may be exposed to, a thorough investigation of maternofetal drug-disposition mechanisms was warranted. As
such, we investigated the expression of drug-transporters and drug-metabolizing enzymes in a mouse model of malaria infection in pregnancy.

4.1 *P. berghei* ANKA Malaria Infection Alters Transporter and Cyp3a11 Expression in Maternal Tissues

Consistent with previous reports using the *P. berghei* ANKA animal model in pregnancy, as parasitic load increased from 3 to 6 days after inoculation with *P. berghei*-infected erythrocytes, we observed significant decreases in maternal weight at GD19 in infected dams compared to control dams. In these same animals, we observed significant increases in spleen weight as a consequence of the uptake of parasitized and ruptured erythrocytes by the spleen. This is in line with previous reports in the literature demonstrating reduced maternal weight gain and splenomegaly as a consequence of *P. falciparum* malaria infection during pregnancy [359, 360, 361].

4.1.1 Impact on the Maternal Liver

In the maternal liver, we observed marked changes in the expression of many hepatic ABC efflux transporters, SLC uptake transporters and the drug metabolizing enzyme Cyp3a11. We observed a substantial decrease in the hepatic expression of Cyp3a11, arguably the most clinically-important drug-metabolizing enzyme present in the maternal liver (reviewed in 1.2.2.1 of this thesis). This decreased Cyp3a11 expression is in agreement with a growing body of literature that has illustrated that infection and inflammatory disorders in pregnant and non-pregnant rodents can mediate the down-regulation of hepatic Cyp3a expression [reviewed in 260, 262, 362, 363]. Furthermore, this observation is in line with previous reports demonstrating significant decreases in the hepatic expression and activity of Cyp3a11 in *P. berghei* ANKA malaria-infected non-pregnant Swiss Webster mice [290, 291].

In the canalicular hepatocyte domain, we identified significant decreases in the expression of Abcg2, Abcc2, and Abcb11 with malaria infection. In contrast, malaria infection caused a significant increase in the expression of Abcb1b, and no change in the expression of Abcb1a or Mdr1 protein. Apart from the absence of change in Abcb1a expression, the observed perturbations in canalicular transporter expression are in line
with what has been observed in numerous studies investigating the effect of bacterial endotoxin-induced inflammation in maternal liver [268,364]. The role of Abcb1b in mediating hepatic Mdr1 protein expression remains controversial and the current consensus is that Abcb1a is the predominant gene isoform responsible for hepatic Mdr1 expression. To illustrate this point, while endotoxin induced a ten-fold increase in Abcb1b expression, a significant decrease in hepatic Mdr1 protein expression was seen which was in line with significant decreases observed in Abcb1a mRNA expression [272].

Endotoxin-induced systemic inflammation has consistently been shown to decrease Abcb1a mRNA and Mdr1 protein expression as a response to the hepatic acute phase response [263,264,265,266,267,268,269,270,271,272]. However, these studies only examined the acute effects after LPS-mediated inflammation occurring within 24 hours after endotoxin-injection. It is plausible that long term infection or chronic inflammation may differentially affect gene and protein expression of Mdr1. Furthermore, the mechanism by which bacterial LPS-mediates the downregulation of Abcb1a mRNA and Mdr1 protein may differ from that which occurs in malaria infection. LPS is a characteristic toll-like receptor 4 (TLR4) ligand and may downregulate gene expression via the activation of NF-κB downstream of TLR4 signaling. *P. falciparum* glycosylphosphatidylinositol has been shown to activate TLR2, TLR4, and TLR9 [365,366], and this broad TLR activation may result in a unique effect on Abcb1a mRNA and Mdr1 protein expression. It has been reported that poly I:C mediated activation of TLR2 and TLR3 pathways did not result in alterations in hepatic Abcb1a mRNA expression [272]. Paradoxically in this study, the expression of hepatic Mdr1 protein was significantly reduced [272]. We hypothesized that it was plausible that similar post-transcriptional changes could occur due to malaria infection. As such, we further examined whether malaria infection impacted the protein expression of Mdr1. In line with what was observed for the expression of Abcb1a, we did not observe alterations in the expression of hepatic Mdr1 in *P. berghei* ANKA infected dams when compared to uninfected control Balb/c dams.

The changes in canalicular transporter expression, particularly Abcc2 and Abcg2, provide an additional explanation for the decreased hepatobiliary clearance of several xenobiotics previously reported in malaria-infected isolated rodent hepatocytes
and perfused rodent liver [74,281,284,285,286,287,288]. For example, reduced biliary clearance of a number of drugs and glucuronide conjugates has been identified in malaria-infected rodents, and the reduction in Abcc2-mediated transport of glucuronide conjugates may contribute to this. Also, the reduction in bile salt transport by Abcb11 may decrease bile flow rate thereby indirectly impairing substrate excretion into the bile canaliculus.

Overall, the altered expression of these transporters would likely contribute to the decreased canalicular transport of an array of substrates including clinically-important therapeutics, bile salts, xenobiotic-conjugates, and bilirubin-glucuronide conjugates. Although many of the antimalarials have not been investigated in the context of their interaction with drug transporters and it remains unknown if many of the antimalarials are ABC transporter substrates, these results may highlight a potential mechanism whereby hepatobiliary clearance of drugs may be altered during malaria infection. For example, the clearance of quinine, a PGP substrate has been shown to be reduced in severe malaria infection [74,367].

In the basolateral domain, malaria infection significantly increased the expression of Abcc1 (Mrp1) and Abcc3 (Mrp3). Mrp1 is not normally expressed at appreciable levels in the adult liver, however reports have identified that expression of Mrp1, Mrp3, and Mrp4 are compensatory upregulated when Mrp2-mediated hepatobiliary excretion is compromised [125,126,127,128,129,130]. For some substrates, such as bilirubin and bilirubin glucuronides, compensation of this nature is believed to reduce intrahepatic accumulation of these substrates and lead to increased renal elimination [127,129,131]. Additionally, many studies have illustrated increases in the expression of Mrp1, Mrp3, and Mrp4 as a result of intrahepatic cholestasis or experimental bile-duct ligation [reviewed in 132,133]. Pertaining to our findings, the increased Mrp1 and Mrp3 expression likely arose as a result of decreased expression of Mrp2 and Abcb11 (Bsep), which are important in the biliary clearance of a number of xeno- and endobiotics including bilirubin and bile acids. Furthermore, the substantial induction of Mrp1 and Mrp3 expression may have arisen due to the steady influx of bile acids via Ntcp despite their compromised canalicular clearance. Mrp4 can mediate the sinusoidal efflux of bile acids in the presence of glutathione [117] and as such it is
plausible that levels of Mrp4 may have also been altered, however this was not investigated.

We also observed a significant decrease in the expression of the uptake transporter Slco2b1 in the basolateral domain of maternal liver. Decreased expression of Slco2b1 has been identified in numerous models of inflammation and would likely contribute to the decreased hepatic uptake of a number of drugs and other xeno- and endobiotics substrates including bilirubin, bilirubin-glucuronide, bile acids, 17-ß-glucronosyl estradiol, leukotriene C4, the statins, antidiabetic agents, antineoplastics, antibiotics, and antihypertensives [reviewed in 167]. This could have potential therapeutic consequences for drugs that act in the liver such as the HMG-CoA reductase inhibitors that have been identified as substrates for SLCO1B1 in humans, the human isoform of rodent Slco2b1.

The apparent lack of change in Slco10a1 is in disagreement with other models of infection and inflammatory disorders available in the literature in which a downregulation in Slco10a1 has been reported. This may be due to differential pathways involved in malaria-induced changes in transporter expression. However, if Slco10a1 expression were maintained at a normal level, it would lead to continual influx of bile acids into the liver despite a significant reduction in hepatobiliary clearance mechanisms.

As a consequence of the malaria-induced changes in transporter expression, we observed a dramatic and significant increase in plasma bile acids within our animal model of malaria infection in pregnancy. Given the decreased expression of Abcb11 and Abcc2 in the canalicular domain, the increased expression of Abcc1 and Abcc3 in the basolateral domain, and the decreased expression of Slco2b1 in the basolateral domain, the increases in serum bile acid levels are not surprising and are consistent with animal models of infection and inflammation that have illustrated intrahepatic cholestasis. These transporters play a critical role in the hepatic uptake and efflux of a number of endogenous products including bile acids and bilirubin conjugates. As Abcb11 and Slco10a1 are key mediators of bile acid homeostasis and Slco2b1 is involved in the uptake of bile acids from the basolateral domain, it is likely that the nearly 10-fold decrease in Abcb11 expression and decreases in Slco2b1 expression contributed to the increased plasma bile acid levels.
Collectively, the decreased hepatic expression of important bile and drug transporters including Abcg2, Abcc1, Abcc2, Abcc3, Abcb11, and Slco2b1 suggest the potential for the development of both intrahepatic cholestasis and reduced biliary clearance of a number of lipophilic drugs and other xenobiotics in pregnant women infected with malaria.

4.1.2 Impact on the Maternal Kidney

The expression of Abcb1a and Abcb1b were assessed in whole kidney homogenate due to their role in the extrusion of drugs and other xenobiotics from the kidney proximal tubule into the tubule lumen [85]. Malaria infection significantly increased the expression of both Abcb1a and Abcb1b in comparison to uninfected Balb/c dams. In line with what was observed with the mRNA expression of both Abcb1a and Abcb1b, we observed a significant increase in the expression of renal Mdr1 protein in *P. berghei* ANKA infected dams when compared to uninfected control dams.

Few studies have examined the effect of infection and inflammation on the expression of renal Mdr1. The few that do exist have all reported increased expression of renal Abcb1a, Abcb1b, and Mdr1 protein [276,368,369]. In one such study, endotoxin-induced inflammation imposed a significant increase in Mdr1 expression which resulted in increased renal elimination of the prototypical PGP substrate, doxorubicin [276]. In the context of pregnancy, the increased expression of renal Mdr1 may potentiate the effect of increased glomerular filtration rate [51] on increasing the CL\textsubscript{Renal} of drugs and other xenobiotics that are Mdr1 substrates. This increased urinary excretion could have consequences for drugs cleared primarily via the kidney, including many antibiotics and antimalarial drugs (e.g. quinine), resulting in subtherapeutic plasma concentrations and exacerbated disease.

4.1.3 Impact on the Maternal Brain

The expression of Abcb1a, Abcb1b, and Abcg2 were assessed in maternal brain homogenate due to their role in the extrusion of xenobiotics at the endothelium of the blood-brain barrier [352]. We observed very little change in the expression of transporters in the maternal brain with only a modest increase in Abcb1b. In line with what was observed with the expression of Abcb1b, a corresponding increase in the protein expression of Mdr1 was also seen in the maternal brain of *P. berghei* ANKA.
infected dams. Although Mdr1 expression was not examined, Farinotti and colleagues observed an approximately 2-fold decrease in mefloquine concentration (an antimalarial and PGP substrate) in the brain of Balb/c mice infected with *P. berghei* ANKA malaria compared to controls [370]. It is plausible that changes in Mdr1 expression may have likely contributed to this change.

The induction of Mdr1 expression in brain may be a consequence of pro-inflammatory cytokine-mediated NF-κB activation by parasitized erythrocytes sequestered in the brain endothelial cells [325,371,372]. Studies in the cerebral endothelia of rodents injected with bacterial endotoxin or the pro-inflammatory cytokine TNF-α have demonstrated acute and transient (<6 hours) decreases in the expression of cerebral Abcb1a and Abcb1b mRNA and Mdr1 protein [324,373]. In contrast, after 6 hours, TNF-α exposure imposes a two-fold increase in Mdr1 expression and activity in the blood-brain barrier [324]. The increased levels of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) in the cerebral vasculature following parasitized erythrocytes sequestration in our model [371,372] may explain the increased expression of Abcb1b and Mdr1 protein in the brain.

### 4.2 Impact of *P. berghei* ANKA Malaria Infection on Drug Transporter and Cyp3a11 Expression in Fetal Tissues

Maternal malaria infection imparts a number of adverse outcomes on newborn health. Reports suggest that malaria infection in pregnancy is implicated in up to 200,000 fetal deaths per year (stillbirths or spontaneous abortions) [29], and children born to mothers infected with malaria possess a number of characteristics predicting future morbidity and mortality. These include: low-birth weight (<2500g) and premature delivery (<37 weeks), which increase the risk of death in the first year of life [29,30,31,32]. An understanding of the mechanism(s) by which malaria in pregnancy impacts these fetal outcomes is limited, however current evidence suggests that PE sequestration leads to reduced placental vascular flow, altered placental angiogenesis [35,36], reduced nutrient and waste transfer, and a chronic localized pro-inflammatory microenvironment [6,9,33,34].
4.2.1 Impact on Fetal Liver

There are few reports in the literature that have investigated the impact of maternal infection on the expression of drug-metabolizing enzyme expression in the fetal liver \([355,356]\), and to the best of our knowledge, the impact of maternal infection on the expression of transporters in fetal tissues has never been reported. Given that fetal expression of drug transporters and metabolizing enzymes may contribute to fetal exposure to many potentially toxic agents, we therefore examined the impact of \(P.\) \(\textit{berghei}\) infection on transporter and drug-metabolizing enzyme gene expression in the fetus.

We observed significant changes in the expression of Cyp3a11 and several ABC transporters in the fetal liver. The expression of hepatic Cyp3a11, Abcb1a, Abcg2, and Abcb11 were significantly decreased in malaria-exposed pups compared to pups from uninfected control dams. In contrast, Abcb1b was significantly increased. Many of these changes were comparable to those seen in the maternal liver. However, some interesting differences were seen. For example, Abcb1a expression was downregulated in fetal but not maternal liver and Abcc1 expression was upregulated in maternal but not fetal liver.

It is important to note that there are many differences between maternal and fetal livers that may result in alternate regulatory effects. First, the total level of protein and mRNA in the fetal liver is lower than in the maternal liver, thus perturbations in expression levels may be more marked in the fetus \([250]\). Second, the regulatory mechanisms that mediate transporter and drug-metabolizing enzyme expression \textit{in utero} may not be fully developed, leading to differences in target gene expression in the fetus. Third, \(P.\) \(\textit{berghei}\) ANKA malaria infection induces a pro-inflammatory cytokine microenvironment in the placenta. While it is believed that systemic pro-inflammatory cytokines do not permeate the placenta \([374]\), the placenta itself can produce pro-inflammatory cytokines in response to stress and infection that can be released into the fetal circulation. This could result in a dissimilar inflammatory response in the fetus. Finally, the differences in the circulation and hepatic accumulation of endogenous and exogenous substances within the pups may have contributed to altered regulation of Abcb1a and Abcc1 when compared to the maternal liver, as the influence of parasite-derived bioactive molecules, red blood cell breakdown products (i.e., bilirubin and
bilirubin conjugates), increased bile acids, and hemozoin would not be present. Mechanistically, a potential contributing factor to the upregulation of Abcc1 expression in maternal liver is malaria-induced oxidative stress, which may activate Nrf2 upstream in the Abcc1 transcriptional-regulatory pathway.

Our findings with Cyp3a11 are in agreement with what has been previously observed. In these studies, both bacterial endotoxin and TNF-α administration mediated a dramatic downregulation in fetal hepatic Cyp3a11 at the level of mRNA and protein, which was believed to occur due to PXR repression [355,356]. It is plausible that a comparable mechanism may contribute to the changes we observed in fetal Cyp3a11 and transporter expression during *P. berghei* ANKA malaria infection.

### 4.2.2 Impact on Fetal Brain

The expression of Abcb1a, Abcb1b, and Abcg2 were assessed due to their known role in the extrusion of drugs in the developing fetal blood-brain barrier [375]. To date, the impact of infection and inflammation on the expression of transporters in fetal brain has not been investigated. We observed a modest increase in Abcb1a expression, whereas the expression of Abcb1b and Abcg2 were unchanged.

In maternal brain, we observed significantly increased Abcb1b and Mdr1 protein expression, likely attributable to the localized release of pro-inflammatory cytokines in response to sequestration of *P. berghei* ANKA parasitized erythrocytes in the maternal cerebrovasculature. In contrast, as malaria does not infect the fetus, it is unlikely that *P. berghei* infected erythrocytes would sequester in the fetal cerebrovasculature, thus the overall release of pro-inflammatory cytokines may be different in fetal as opposed to maternal brain.

Also, as discussed above, the production of pro-inflammatory cytokines released by the placenta in response to sequestered placental parasitized erythrocytes would likely distribute throughout the fetal circulation before reaching the fetal brain. This may result in altered levels of pro-inflammatory cytokines in the fetal brain as compared to the maternal brain, and, perhaps, a dampened response. Furthermore, it is possible that the regulators of gene expression in the fetal brain are not fully developed, however this requires further investigation.
4.3 Impact of *P. berghei* ANKA Malaria Infection on Transporter Expression in the Placenta

Placental transporters significantly contribute to the fetal exposure and accumulation of many clinically important xenobiotics. As such, we chose to investigate the impact of *P. berghei* ANKA infection on placental transporters which have been shown to play an important role in drug transport.

*P. berghei* ANKA malaria infection led to significant changes in ABC transporter expression in the placenta. We observed a significant decrease in placental mRNA expression of Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, and Abcg2 at GD19 in *P. berghei* ANKA infected Balb/c dams. A corresponding decrease in the expression of Mdr1 protein was seen in placentae isolated from *P. berghei* infected dams. Although, this is the first report investigating the impact of malaria infection on transporter expression in the placenta, these results are in agreement with those observed in studies in placenta isolated from bacterial endotoxin-treated rodents in addition to *in vitro* studies investigating the impact of pro-inflammatory cytokines on transporter expression in the human placental syncytiotrophoblast [277,364].

The observed decreases in placental transporter expression could significantly increase the fetal exposure to their substrates via reductions to CL$_{Fetal \rightarrow Maternal}$, and this may have potential deleterious consequences on fetal health. Furthermore, beyond their role in drug-disposition, alterations in placental ABC transporter expression may have consequences on fetal health and development. The ABC transporters MDR1, MRP1-3, and BCRP display an astonishing array of endogenous and exogenous substrates, many of which have yet to be characterized. It has been shown that these transporters provide protection to both non-transformed tissues and cancer cells from endogenous and exogenous stress factors, including pro-inflammatory mediators (i.e., TNF-$\alpha$) and hypoxia [376,377]. These transporters have also been shown to regulate intracellular levels of many toxic metabolites including sphingolipids, protoporphyrins, prostaglandins, and ceramides [378,379,380,381]. It is plausible that the decreased expression of ABCB1, ABCG2, and ABCC2 in the apical domain of the placental syncytiotrophoblast could increase the fetal accumulation of a number of physiological substrates that contribute to placental and/or fetal pathology in *P. berghei* ANKA.
malarial infection. Evseenko and colleagues have illustrated this concept where BCRP-silencing resulted in increased syncytiotrophoblast apoptosis following exposure to pro-inflammatory cytokines and endogenous ceramides [382]. Furthermore, this group also illustrated that placental BCRP expression was a predictor of placental syncytiotrophoblast survival and was reduced in idiopathic IUGR which has been linked to increased plasma levels of TNF-α [382]. Interestingly, within our study, BCRP mRNA expression significantly correlated with fetal weight (r = 0.5554; p < 0.05) and it is possible that a comparable mechanism may have contributed to IUGR and low birth weight in our model. However, the pharmacokinetic and physiological implications of our novel findings remain to be determined and provide an area warranting future research focus.

In the placenta, PEs bind chondroitin sulfate A expressed on the syncytiotrophoblast, which mediates sequestration and prevents elimination by the reticuloendothelial system [24,305]. Bioactive products from ruptured PEs within the intervillous space can stimulate resident macrophages in the syncytiotrophoblast and circulating monocytes to promote the release of C5a, TNF-α, IFN-γ, and other pro-inflammatory cytokines and chemokines [9,17,306,307,308]. This in turn can contribute to further macrophage infiltration associated with low birth weight and detrimental outcomes of malaria in pregnancy [309].

TNF-α is one of the few factors associated with malaria in pregnancy that has been shown to mediate placental pathology. An increase in TNF-α and other pro-inflammatory cytokines in cord blood samples has been shown in several studies [317,318,319], and the role of TNF-α as a mediator of fetal loss has been directly demonstrated through TNF-α-inhibition experiments in Plasmodium-infected pregnant mice and non-human primates [320,321,322]. Moreover, TNF-α has been previously shown to downregulate drug transporter expression in the placenta [277,382]. Thus, it is likely that the changes we observed in transporter expression were mediated by pro-inflammatory cytokines.

We also investigated the placental expression of iNOS and HO-1 as these enzymes are often altered in infection and may also contribute to the pathology of malaria infection. We observed a significant decrease in iNOS and a significant increase in HO-1 placental expression, which were correlated to fetal weight. The
placental induction of HO-1 is thought to occur by a TNF-α-mediated process [310].

Increased placental HO-1 expression has been implicated in fetal protection in bacterial endotoxin treatment and TNF-α mediated cytotoxicity in pregnancy [310,311,312]. Importantly, increased HO-1 expression has been shown to lead to placental vasculature relaxation, which may be a critical process in the placental microvasculature where sequestered parasitized erythrocytes have reduced blood flow. This vasculature relaxation may increase blood flow to developing fetuses and contribute to the increased birthweight that we observed in figure 19B. Increased placental iNOS expression has been implicated in fetal loss in infection models in pregnancy [313,314,315,316] and it is possible that the decreased iNOS may have occurred in an attempt to maintain fetal growth and sustain pregnancy. The decreased iNOS and increased HO-1 expression may act in tandem to attempt to maintain fetal growth and sustain pregnancy, however this claim requires further research to substantiate it.

Alternatively, the increased expression of HO-1 in the placenta of *P. berghei* ANKA infection dams may provide a potential mechanistic link for the propensity of primigravid women infected with *P. falciparum* to experience pre-eclampsia and maternal-fetal conflict during pregnancy, which has been previously associated with HO-1 induction [383]. Further investigation of this link is warranted.

### 4.4 Implications for Drug-Disposition

Given that we observed alterations in the expression of many xenobiotic-disposition mechanisms, it is interesting to hypothesize how these changes may affect the transport and disposition of therapeutics or other xenobiotics in the *P. berghei* ANKA animal model. To test this experimentally, a complete pharmacokinetic study would be required. However, the results from this thesis provide a framework for a theoretical discussion of the impact of these alterations on xenobiotic disposition. Let us consider a xenobiotic, X, with the following characteristics: (1) low-extraction ratio, (2) high lipophilicity, (3) orally administered, (4) binds to serum albumin, (5) a BCRP and CYP3A substrate, (6) X-metabolite is conjugated with GSH to a X-GSH metabolite,
and (6) cleared primarily by biliary excretion.

First, X would be highly-absorbed from the intestine and enter the hepatic portal vein. As it passes through the liver, CL_{hepatic} would be altered due to the decreased drug-metabolizing processes, such as decreased Cyp3a expression. In addition, biliary excretion of X would be decreased via the lower Bcrp, and through reduced bile flow due to decreased Bsep expression. A caveat here is that the f_{iu} of X would likely increase due to the decreased serum albumin that has been observed in patients infected with *P. falciparum*, and via the hemodilution effects of pregnancy on the concentration of serum albumin [384,385]. This would alter the amount of free X available for hepatic and non-hepatic clearance processes. Let us assume that the CL_{intrinsic} decreases to a greater magnitude than f_{iu} for this low-extraction ratio drug and as such, plays a greater role in determining overall CL_{hepatic}. In addition to alterations in the transport of X, it would be likely that X-GSH transport from the liver would be increased due to the dramatic increases in Abcc1 and Abcc3 in the basolateral domain.

As a consequence of reduced first pass CL_{hepatic}, more of X would enter the systemic circulation upon first-pass through the liver. Once X reaches the systemic circulation, it is then available for distribution into tissue sites, with transport processes either promoting or limiting distribution. At the level of the maternal brain, we would not expect to see any differences in brain penetration of X given the lack of change in Abcg2 expression. At the kidney, if X-GSH is renally cleared, it is plausible that its renal excretion would be increased given previous findings demonstrating increased renal and hepatic Mrp1, 3, and 4 in response to compromised hepatobiliary clearance [125,126,127,128,129,130,131].

At the level of the placenta, given the purported increases in % unbound drug due to decreases in albumin, more free drug would be available for placental permeation. The altered placental Bcrp and Mrp1-3 expression would likely result in the developing fetus being exposed to an increased amount of X and X-GSH. Further, based on the changes in fetal Cyp3a and transporter expression, CL_{fetal} may also be reduced.

Importantly, in applying these findings in a pharmacokinetics study, one would also need to consider the potential for *P. berghei* ANKA malaria-induced alterations in other phase II processes (e.g., changes to the expression of GSTs). In addition, the
pattern of distribution of the metabolites of X could be studied, along with transporter-mediated uptake processes.

4.5 Impact of C5aR Silencing on *P. Berghei* ANKA Infection and the Expression of Drug Transporters and Cyp3a11 in Maternal Liver and the Placenta

Our second hypothesis was that silencing or inactivation of the complement factor 5a (C5a) innate immune pathway during malaria infection would modulate the expression of transporters (MDR1, MRP1, BCRP, BSEP) and drug-metabolizing enzymes (CYP3A11). Testing this hypothesis using the *P. berghei* ANKA animal model revealed novel insights into the role of C5aR activation in both the expression of placental and hepatic transporters, as well as in malaria pathogenesis and fetal outcomes. We investigated if C5aR activation had any impact on fetal weight and/or fetal viability by comparing these measures between infected wildtype Balb/c dams and C5aR−/− dams on a Balb/c background. We noted a significant rescue of fetal body weight in infected C5aR−/− Balb/c dams, suggesting involvement of C5aR signaling in the pathogenesis of placental malaria. This may have been due to the reduction of C5a mediated pro-inflammatory cytokine and chemokine secretion by immune cells or via the reduced input of C5a to the endothelium of the placental microvasculature.

4.5.1 Maternal Liver

We next examined the expression of transporters and Cyp3a11 in the maternal liver of infected and uninfected C5aR−/− dams, and compared this expression profile to that observed in infected and uninfected wildtype dams. As expected, we observed a significant impact of malaria infection on the expression of Cyp3a11 and hepatic transporters in C5aR−/− dams. As was observed with the wildtype dams, malaria infection significantly decreased the expression of Cyp3a11 and Abcb11, increased the expression of Abcc1 and Abcb1b, and resulted in no change in the expression of Abcb1a in C5aR−/− dams.
When comparing these results to the changes observed in infected wildtype dams, we saw a significant increase in the hepatic expression of Abcb1b in infected C5aR−/− dams. Given that Abcb1a, and not Abcb1b, is believed to drive the expression of Mdr1 protein in the liver, our data suggest that C5aR activation does not play a major role in the regulation of hepatic transporter expression during P. berghei ANKA malaria infection. In an in vitro model of malaria, pfGPI and C5a have been shown to promote pro-inflammatory cytokine release by monocytes [17]. Interestingly, pfGPI and C5a were found to act synergistically to enhance cytokine release, and inhibition of C5aR reduced release of pro-inflammatory cytokines to levels observed in monocytes treated with pfGPI alone.

Given that malaria infection activates a number of inflammatory signalling pathways through the action of pfGPI and other parasite bioactive products on TLR2, 4, and 9, it is possible that a number of pathways contribute to the observed changes in Abcb1a, Abcb1b, Abcc1, Abcb11 and Cyp3a11 expression in the maternal liver. Therefore, silencing of C5aR activation does not negate the overall inflammation-mediated changes in these genes. As such, the findings reported do not support a major contributory role for C5aR activation in the regulation of target genes in maternal liver.

Conversely, it is possible that the comparable expression of Abcc1 and Abcb11 following C5aR gene knockout may be due to other contributory factors. For instance, hepatic oxidative stress or hepatic accumulation of ruptured red blood cells, bilirubin, and/or parasite derived bioactive products such as hemozoin may alter the expression of these transporters. The mechanism by which these changes occurred remains to be elucidated.

### 4.5.2 Placenta

While the activation of the complement cascade represents a crucial step in the innate immune response to malaria infection, complement signaling in the placenta is thought to cause adverse fetal outcomes during pregnancy, via the potentiation of inflammatory cytokine release and inhibition of angiogenesis [10,11,17]. Unpublished data from the laboratory of Dr. Kevin C. Kain suggests that global knockout of the C5a receptor in pregnant rodents improves fetal outcomes (e.g. birth weight and viability)
during *P. berghei* ANKA malaria infection [35]. Since several transcription factors (e.g. NF-κB) known to regulate the expression of placental drug-metabolizing enzymes and drug transporters are located downstream of C5a receptor activation, it remains to be determined whether C5a receptor activation is critical in the regulation of drug transporters in malaria [190,323,324,325,326,327].

Given the potential for C5aR to regulate gene expression via downstream signaling of C5aR activation, we asked if the expression of placental transporters in infected and control C5aR+/− dams differed when compared to their expression in infected and control wildtype dams. In infected C5aR+/− dams, we observed significant decreases in the placental expression of Abcc2 and Abcg2, with no change observed in the expression of Abcb1a or Abcb1b, when compared to uninfected C5aR+/− mice. When comparing these results to the changes observed in infected wildtype Balb/c dams, some notable differences were revealed. We observed a significantly higher placental expression of both Abcb1a and Abcg2 in C5aR+/− infected dams, above that which was observed in the wildtype *P. berghei* ANKA infected dams. Although malaria infection imposed a downregulation of Abcb1a and Abcb1b in placenta of wildtype infected dams, placental expression of Abcb1a and Abcb1b was unaffected by malaria in C5aR+/− dams.

The increased placental Abcb1a and Abcg2 transporter expression in the C5aR+/− dams during malaria infection may implicate that placental transporters play an important protective role during *P. berghei* ANKA malaria infection. This is comparable to what has been observed with BCRP as a placental survival factor in idiopathic IUGR [382], and in the correlation we observed between BCRP expression and fetal weight. However, given the lack of temporal relationship in this study and the *a priori* decision to study transporter expression in the placenta of viable fetuses, such questions remain unanswered.

From the current evidence available, it is likely that the significant pro-inflammatory placental microenvironment mediated by *P. berghei* ANKA mediated the decreased expression of BCRP and other transporters, and transporter expression may be acting as a proxy for placental inflammation and placental pathology. Furthermore, the expression of Abcc2 and Abcg2 in infected C5aR+/− dams remained significantly lower than that observed in the uninfected C5aR+/− dams. This indicates that other
regulatory mechanisms, such as TLR activation, may also be responsible for the regulation of these transporters.

Overall, it is plausible that C5aR gene knockout dampens the placental pro-inflammatory microenvironment, and that this resulted in the modest changes in the expression of Abcb1a, Abcb1b, and Abcg2 transporter expression. The mechanism governing these changes remains to be elucidated.

4.6 Strengths of this work

4.6.1 Novelty

To the best of our knowledge, no study currently exists where the impact of malaria infection on the maternofetal expression of genes which impact drug disposition is examined. To date, the impact of malaria infection on drug transporter expression in either pregnant or non-pregnant rodents has not been elucidated. Moreover, there are few reports investigating the impact of inflammatory disorders or infection on fetal expression of ABC transporters and Cyp3a11.

To date, the use of models of bacterial infection (LPS injection), viral infection (poly I:C injection), and exposure to exogenous pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ, and TNF-α) comprised the bulk of literature in this field. Here, we present a logical progression from the current literature investigating inflammation-mediated changes in drug-disposition. We provide proof-of-concept for the future use of other animal models with viable infectious agents to study alterations in drug-disposition mechanisms.

4.6.1.1 Examined an array of drug transporters and Cyp3a11 expression, all selected a priori

We examined the expression of an array of ABC efflux and SLC uptake transporters, in addition to the drug-metabolizing enzyme Cyp3a11, which have all been previously shown to play a role in the disposition of a number of clinically important therapeutics and other xenobiotics. We elected to examine the mRNA expression of Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, Abcg2, Abcb11, Slco10a1/Ntcp,
Slco2b1/Oatp2b1, and Cyp3a11 in the liver a priori and a number of these transporters were also examined in other tissues such as the kidney, brain, and the placenta, in order to provide a holistic overview of how malaria infection may impact drug-disposition mechanisms in pregnancy.

4.6.2 Utilized a well-established animal model of malaria in pregnancy

For ethical and logistical reasons, drug-disposition mechanisms are difficult to study in human pregnancy [reviewed in 386]. Tissue biopsy samples from pregnant women are not readily available, precluding the feasibility of studying the effects of obstetrical complications on the tissue expression of drug transporters and metabolic enzymes. In addition, while placental samples are obtainable at term and can be used to estimate the impact of disease on materno-fetal drug disposition mechanisms, an in-depth understanding of the impact of disease on fetal drug disposition mechanisms is impossible.

Although general drug-disposition studies have been conducted in pregnant women, limitations in study design do not allow for the consequences of obstetrical complications to be identified. Studies such as these would require healthy pregnant controls as a comparator group, and this is ethically impermissible. For these reasons, we utilized a mouse model to address our objectives and study hypotheses.

In general, valid animal models are highly predictive and should capture many aspects of the disease being modeled. Thus, an adequate animal model of malaria in pregnancy should capture many of the clinical and pathophysiological characteristics of P. falciparum malaria infection in human pregnancy. These include placental sequestration of PEs via binding to the placental vasculature, mononuclear cell infiltration, placental inflammation, changes to placental villous architecture, as well as parity-related effects such as reduction of symptomology in subsequent pregnancies, low-birth weight and IUGR, premature birth, and reduced fetal viability [9,24,329,334,387,388,389].

The P. berghei ANKA Balb/c animal model of malaria in pregnancy used in this study recapitulates several clinical outcomes that make human malaria in pregnancy a global health priority. These include spontaneous abortion, fetal growth restriction, and preterm delivery [36,329,334]. As such, the P. berghei ANKA Balb/c model is
considered to be the “gold standard” for malaria research in pregnancy [328]. This model allowed for the ability to examine the expression of an array of drug transporters and metabolic enzymes in a number of maternal, fetal, and placental tissues. Furthermore, it allowed control with the timing of infection, the inoculum level, the timing of data collection, and the opportunity to examine the contribution of specific pathways to study outcomes using modified mouse strains, as completed with our work in C5aR−/− mice. Collectively these attributes make the P. berghei ANKA in pregnancy model a powerful tool to help understand the impact of malaria in pregnancy on drug-disposition mechanisms.

### 4.6.3 Investigated the contribution of the C5aR signaling pathway in drug transporter and Cyp3a11 gene regulation

Our experiments in C5aR−/− mice provide insight into the potential role of C5aR in mediating transporter and drug-metabolizing enzyme regulation. The role of C5a in facilitating the pathogenesis of numerous infections has been well-characterized in the literature [339,390], and, more recently, has been illustrated in models of placental and cerebral malaria [18,35]. Laboratory investigation has illustrated that C5a potentiates the production and release of pro-inflammatory cytokines by pfGPI in peripheral blood mononuclear cells, and that blockade of C5a attenuates this phenomenon [17,18]. Within the context of our study, we concluded that this attenuation of pro-inflammatory cytokine release might have contributed to a diminution of malaria-induced changes in transporter expression. Results from this work could be applied to study the role of C5aR in the regulation of transporters in other models of infection and inflammation.

### 4.7 Limitations of this Work

A major limitation of this work is that this is an animal model of malaria infection in pregnancy. An inherent issue in any animal model investigating drug transporter and drug metabolizing enzyme expression is that the regulation of genes may be different than what occurs in humans. Furthermore, P. berghei ANKA is a model of P. falciparum infection, and as such, this model displays subtle differences in disease pathology when compared to malaria infection in human pregnancy [extensively reviewed in 328].
Nevertheless, this model represented the best available option to answer our important study questions.

4.7.1 *P. berghei* ANKA model may not apply to malaria infection in high-transmission regions

There are a number of limitations to the work presented. Firstly, the *P. berghei* ANKA animal model is a model of severe placental malaria in pregnancy where peripheral parasitemia rose to >40% by GD19. In Africa and areas where malaria is endemic and highly-transmitted, due to pre-pregnancy malaria exposure, women often present to the clinic with negligible parasite in their blood smear and are paucisymptomatic or asymptomatic. Upon birth, accumulation of sequestered *P. falciparum* infected erythrocytes is often observed in the placenta. This phenomenon remains one of the major challenges of diagnosis and treatment of malaria infection in pregnancy. As such, our findings may be most applicable to primigravid women that are exposed to malaria for their first time in low-transmission regions as these women often experience severe symptoms of malaria (i.e., coma, hypoglycemia, and respiratory distress), due to their lack of pre-pregnancy exposure. In designing a animal study that best captures malaria infection in high-transmission regions, it may be more appropriate to expose rodents to *P. berghei* ANKA malaria prepartum and to allow for the development of immunity to infection prior to commencement of the study. Alternatively, rodents could be exposed to *P. berghei* ANKA in a previous pregnancy and then the impact of infection in subsequent pregnancies could be studied. This would allow for development of antibodies to sequestered parasites and may more appropriately model malaria pathogenesis in high-transmission areas such as Africa and Southeast Asia.

4.7.2 *P. berghei* ANKA model does not include all stages of the malaria life cycle

Furthermore, the *P. berghei* ANKA animal model also excludes a large portion of the malaria life cycle. In this work, $10^6$ *P. berghei* ANKA infected erythrocytes were injected directly into the tail vein of pregnant Balb/c dams on GD13. As depicted in figure 1 (Chapter 1.1), the pathogenesis and life cycle of *P. falciparum* is far more
complex and begins with the bite of an Anopheles mosquito followed by the migration of sporozoites to the liver. Following this, the sporozoite replicates and matures and is then released into the blood to infect erythrocytes. The employed *P. berghei* ANKA model circumvents this extra-erythrocytic phase of malaria infection and results in a more immediate and rapid development of disease pathology. The consequences of this on the observed changes within this thesis are difficult to interpret.

4.7.3 The impact of *P. berghei* ANKA malaria infection on transporter and drug metabolizing enzyme expression was not investigated at different time-points

Another limitation of our work is that we examined transporter and drug metabolizing enzyme expression at only one-time point and only in late gestation, (i.e., GD19 or 6-days post infection). It is in our opinion that including additional time points may give a more holistic view of drug-disposition mechanisms over the course of malaria infection. In a recent study by Silver and colleagues, fetal weight, an indicator of placental pathology and inflammation, did not begin to decrease in *P. berghei* ANKA infected mice infected on GD13 until after GD18 [36]. It would be interesting to examine whether transporter expression in the liver or placenta changes at an earlier time-point in gestation (e.g., mid-gestation), and if these changes predict the degree of fetal outcomes as disease progresses.

4.7.4 The mechanism of drug transporter and Cyp3a11 expression regulation remains to be determined

While the purpose of our study was to characterize the panel of expression changes in the selected transporters and Cyp3a11, no mechanistic data for these changes are presented. This was beyond the scope of our work and would provide a direction for future research. Furthermore, we only examined the protein expression of Mdr1 given its well-characterized role in hepatic and extra-hepatic drug disposition. It would be interesting to examine the protein expression of other relevant transporters and to assess if the levels of mRNA and protein correlate.
4.8 Future Directions and Recommendations for Future Research

This thesis describes a set of experiments that investigated the impact of maternal *P. berghei* ANKA malaria infection on maternal, fetal, and placental genes involved in drug-disposition. The results and potential implications of the gene expression changes identified within this thesis provide an interesting model for investigating the impact of altered hepatic and placental transporter expression on maternal and fetal disposition of their substrates. Given that this field is both novel and lacks any substantive focus to date, future work is warranted.

4.8.1 Investigate the impact of *P. berghei* ANKA malaria infection on Protein Expression of ABC/SLC Transporters and CYP3A

We examined the protein expression of Mdr1 in maternal brain, maternal kidney, maternal liver, and placenta a priori given its well-characterized role in mediating hepatic and extra-hepatic drug disposition. It would be interesting to examine the protein expression of the other transporters and Cyp3a11 which were found to be altered in this study and to assess if the levels of mRNA and protein correlate. It is possible that post-transcriptional or post-translational regulatory mechanisms could result in differences to what was observed at the level of mRNA expression.

4.8.2 Investigate the impact of *P. berghei* ANKA malaria infection on maternofetal drug-disposition in a comprehensive pharmacokinetic study

A comprehensive drug-disposition study using the *P. berghei* ANKA malaria model and an antimalarial medication (e.g. quinine, a substrate of CYP3A and MDR1) would provide additional insights and functional consequences to support the conclusions of this work. Furthermore, the disposition of other drugs (e.g. various components of highly active antiretroviral therapy such as lopinavir/ritonavir) [391] or environmental xenobiotics that women infected with *P. falciparum* malaria are commonly exposed to would be an interesting avenue to investigate. Women in Africa often consume foods contaminated with high levels of Aflatoxin B$_1$ (AFB$_1$), and AFB$_1$ is
a BCRP and CYP3A substrate that has been shown to cross the placenta [145,392]. Furthermore, exposure to AFB₁ in utero is associated with reduced birth-weight and decreased growth in the first year of life [393,394]. Reductions in placental BCRP expression in the context of malaria infection may further exacerbate these effects.

4.8.3 Artemisinin-Mediated Drug Transporter and Drug Metabolizing Enzyme Induction in Pregnancy

It has been shown that the artemisinins, as part of the Artemisinin combination therapy used to treat malaria, induce the expression of MDR1, MRP2, and a variety of other xenobiotic-metabolizing and xenobiotic-transporting genes via PXR/CAR-mediated activation [107,108]. This effect has not been investigated in the context of pregnancy nor has the impact of artemisinin exposure been investigated in extrahepatic tissues such as the placenta, maternal kidney, and maternal brain. Given that ACT is often given to pregnant women in the 2nd and 3rd trimesters of pregnancy, it is plausible that the auto-induction of MDR1 and MRP2 and other xenobiotic clearance mechanisms may exacerbate the increased clearance and decreased plasma AUC of these medications in pregnancy. From a maternofetal drug disposition perspective, ACT may increase placental transporter expression and reduce fetal → maternal disposition of drugs and other xenobiotic.

4.8.4 Mechanistic Studies – Role of Nuclear Receptors in Altering Xenobiotic-Disposition Mechanisms

As discussed in chapter 1, a variety of nuclear receptors are responsible for the expression of the ABC efflux transporters, SLC uptake transporters, and drug-metabolizing enzymes. Many of these ligand activated nuclear receptors share aspects of their signaling pathways, precluding the ability to dissect the contribution of each nuclear receptor to inflammation-mediated transporter changes in wildtype mice. The use of genetic knockout mouse models has provided a means of better understanding how the role of each nuclear receptor contributes to transporter and drug metabolizing enzyme expression, in addition to how each nuclear receptor may play a role in disease pathology. For instance, the farnesoid X receptor (FXR) regulates MRP2, BSEP, NTCP, OATP1B1 and OATP1B3 expression [227,228], as well as the
expression of CYP3A4, SULT2A1 and various UGTs [204,229,230,231,232], and also plays a role in regulating the immune response. Specifically, increased FXR-expression and activation attenuates intestinal and hepatic immune-mediated pathologies [395,396,397]. In considering the changes observed in MRP2, BSEP, and CYP3A11 expression, it is possible that FXR activation may modulate P. berghei ANKA-mediated pathology and transporter changes. This could be investigated using an FXR−/− null-mouse or treating mice with an FXR-specific ligand throughout disease progression.

Furthermore, an array of gene knockouts such as PXR−/−, CAR−/−, or NRF2−/− mice could be utilized to parse apart the individual contribution of each nuclear receptor to transporter expression, drug-metabolizing enzyme expression, and disease pathology. In line with this, a recent study has illustrated that Nrf2 activation plays a role in modulating CD36 expression, the major receptor mediating non-opsonized phagocytosis of P. falciparum-parasitized erythrocytes by macrophages [234]. In addition, it was recently discovered that Nrf2 plays a role in regulating the innate immune response and survival in experimental sepsis [235].

In addition, it would be interesting to investigate the impact of individual transporters on disease pathology through utilizing selective transporter knockout mice, such as the BCRP (Abcg2−/−) or MDR1 (Mdr1a/Mdr1b−/−) knockout in this mouse model of malaria infection. Given the purported role of BCRP as a placental survival factor [382] and the decreased expression of BCRP in our model that correlated with fetal weight, it would be interesting to investigate if BCRP knockout alters fetal outcomes. This would provide additional evidence for a physiological role for BCRP (or other transporters) beyond its role as a xenobiotic transporter at epithelial barriers.

4.8.5 Translational Research in the Human Placenta Infected with P. falciparum malaria – A Placental Perfusion and Transporter Expression Study

To compliment these preclinical findings, a study investigating the impact of P. falciparum infection on transporter expression in the human placenta would be valuable. This type of study would be subject to minimal ethical restrictions because term placental samples are obtainable and often discarded at birth, and can be used to estimate the impact of disease on placental pathology and transplacental drug transfer,
as exemplified by two recent studies [154,398]. A hypothetical study could 1) assess for the degree of placental *P. falciparum* infected erythrocyte sequestration, 2) determine the impact of *P. falciparum* infection on the placental microvasculature, 3) quantify placental inflammation via qPCR or cytokine microarray, and 4) could use blood biomarkers from patients as indicators of systemic inflammation. These *P. falciparum* infected placentae could also be used in placental perfusion studies [239] to investigate the impact of *P. falciparum* infection on the placental transfer of relevant antimalarial drugs, other commonly used therapeutics in malaria-infected populations (e.g. antiretrovirals and antibiotics), or other xenobiotics present in the diet in malaria-infected populations (e.g the BCRP substrate AFB1). Incorporating gene and protein expression profiling into placental perfusion studies would allow for the relationship between placental drug transporter expression and the placental transfer of substrate drugs in human pregnancies to be investigated. Together, these studies would reveal novel avenues of research if the effects described within this thesis were applicable to human pregnancies.

### 4.9 Conclusions

Every year, an estimated 85 million pregnancies occur in areas of *P. falciparum* transmission, with almost 55 million pregnancies occurring in regions with stable year-round malaria transmission [22]. Pregnant women are at an increased risk for adverse outcomes from malaria infection due to immunological changes that occur in pregnancy [23], in addition to the emergence and selection of parasites with the ability to bind to placental chondroitin sulfate-A (CSA) and to sequester in the placental intervillous space [24]. Given the necessity to safely and effectively treat pregnant women infected with malaria, research investigating the pharmacokinetic processes that impact the materno-fetal disposition of antimalarial drugs and other xenobiotics is warranted. To investigate these processes, a *P. berghei* ANKA animal model was used.

My primary hypothesis was that *P. berghei* ANKA infection would cause changes in the expression of drug transporters and drug metabolizing enzymes that would ultimately contribute to the potential for altered maternal and fetal drug exposure. Key findings herein demonstrate reduced expression of a number of transporters in the
placenta of *P. berghei* ANKA infected pregnant dams relative to uninfected pregnant dams. We also observed alterations in the expression of many drug efflux and uptake transporters and the drug-metabolizing enzyme Cyp3a11 in the maternal liver, maternal kidney, maternal brain, and fetal liver.

My second hypothesis was that C5aR gene knockout would attenuate the malaria-induced changes to the expression of a number of hepatic and placental transporters. We observed significantly higher placental expression of both Abcb1a and Abcg2 in C5aR−/− infected dams when compared to the changes observed in the wildtype Balb/c dams. Also, the expression of Abcb1a and Abcb1b in the placentae of C5aR−/− infected dams was similar to that in uninfected C5aR−/− dams, a differential expression to what was observed in wildtype animals.

It remains to be determined whether the changes in placental transporter expression will lead to alterations in maternofetal disposition of drugs or other xenobiotics. Furthermore, a mechanistic understanding of the phenomena reported within this thesis remains elusive, however it is highly plausible that pro-inflammatory cytokine environments in target organs contributed to the observed changes in transporter and drug-metabolizing enzyme expression.

Taken together, this thesis demonstrates that *P. berghei* ANKA malaria infection alters the expression of a number of genes involved in xenobiotic-disposition mechanisms in pregnancy. If these findings translate to *P. falciparum* infection in human pregnancy, the systemic clearance and maternofetal disposition of a number of clinically-important therapeutics and other xenobiotics may be altered.
References


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