Malaria Infection Alters the Expression of Hepatobiliary and Placental Drug Transporters in Pregnant Mice

Authorship:
Alex M. Cressman, Chloe R. McDonald, Karlee Silver, Kevin C. Kain, Micheline Piquette-Miller

Author Affiliations:
Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto (AMC, MPM); Sandra Rotman Centre for Global Health, University Health Network-Toronto General Hospital, University of Toronto (CRM, KS, KCK)
DMD #53983

Running Title:

Altered Expression of Transporters in Placental Malaria

Corresponding Author:

Dr. Micheline Piquette-Miller, PhD
Leslie Dan Faculty of Pharmacy
University of Toronto
144 College St.
Toronto, Ontario, Canada, M5S 3M2
Telephone: 1-416-946-3057
Fax: 1-416-978-8511
E-mail: m.piquette.miller@utoronto.ca

Number of Text Pages: 16 (28 in document)

Number of Tables: 1

Number of Figures: 6

Number of References: 58

Number of Words in Abstract: 249

Number of Words in Introduction: 740

Number of Words in Discussion: 1493

List of Nonstandard abbreviations:

ABC, ATP-binding cassette; Pgp, P-glycoprotein; Mdr, multidrug resistance; Mrp, multidrug resistance-associated protein; Bcrp, breast cancer resistance protein; Oatp, organic anion-transporting polypeptide; TNF, tumor necrosis factor; IL, interleukin.
Abstract:

Preventing and treating malaria in pregnancy is a global health priority. However little is known regarding the impact of malaria infection on the maternal and fetal disposition of pharmaceuticals and other xenobiotics. Our objective was to characterize expression of key determinants of drug-disposition in maternal and fetal tissues in a validated murine model of experimental placental malaria. Balb/c mice were infected with *Plasmodium berghei* at mid gestation [gestational day (GD) 13] and maternal, placental, and fetal tissues were collected at GD19. Expression of key ABC drug transporters and Cyp3a11 was examined by qRT-PCR. Western blotting was used to examine the protein expression of Multidrug resistance protein 1 (Mdr1, Abcb1). As compared to controls, placental mRNA expression of *Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, and Abcg2* were significantly down-regulated in the malaria-infected group (*p* < 0.05), as was placental Mdr1 protein (*p* < 0.05). Significantly decreased hepatic expression of *Abcc2, Abcg2* and *Abcb11* and significantly increased expression of *Abcb1b, Abcc1*, and *Abcc3* were seen in malaria-infected dams (*p* < 0.05) in comparison to uninfected controls. The expression of *Abcb1a* and *Abcg2* was significantly decreased in fetal liver of infected dams while levels of *Abcb1b* were increased (*p* < 0.05). Maternal and fetal hepatic expression of *Cyp3a11* was significantly down-regulated in the malaria group (*p* < 0.05). Together, malaria- induced alterations in the expression of transporters and drug-metabolizing enzymes in maternal and fetal tissues may alter the disposition of endogenous and therapeutic substrates, potentially impacting maternal and fetal outcomes.
Introduction:

Each year over 125 million women are at risk of malaria infection during pregnancy. In Sub-Saharan Africa an estimated 25% of all pregnancies are complicated by placental malaria (PM) infection (Dellicour et al., 2010). PM is associated with increased risk of adverse outcomes for both mother and fetus, including an increased risk of anemia, preterm birth, stillbirth and delivery of low birth weight infants. During pregnancy malaria-infected red blood cells accumulate in the placental intervillous blood spaces, resulting in altered placental angiogenesis and vascular flow, reduced nutrient and waste transfer, placental insufficiency, and a chronic localized pro-inflammatory environment (Fried and Duffy, 1996; Matteelli et al., 1997; Miller et al., 2002; Conroy et al., 2013). As such, optimization of pharmacological treatment of malaria in pregnancy is a global health priority. However, little research has been conducted to investigate the determinants of drug-disposition in malaria-infected pregnant populations.

Studies in humans and experimental rodent models have reported malaria-induced alterations in the hepatic metabolism and clearance of numerous drugs, including many that are used in the treatment of malaria (Mihaly et al., 1987; Mansor et al., 1990; Murdoch et al., 1991; Pukrittayakamee et al., 1997). In mice, malaria infection is associated with decreases in the hepatic expression of Cyp3a11 and other drug metabolizing enzymes (DMEs) (De-Oliveira et al., 2006; Carvalho et al., 2009). Cyp3a11 is the murine ortholog of human CYP3A4, which is involved in the metabolism of many antimalarials including quinine (Zhang et al., 1997), chloroquine (Kim et al., 2003), halofantrine (Baune et al., 1999), mefloquine (Fontaine et al., 2000), piperaquine (Lee et al., 2012), artemesinin and artemisinin derivatives (Svensson and Ashton, 1999). Although many of these drugs are
used to treat malaria in pregnant populations, little is known about the impact of malaria infection on CYP3A expression in maternal and fetal tissues.

In addition to DMEs, the ATP-Binding Cassette (ABC) drug transporters act as major determinants of maternal and fetal exposure to endogenous and exogenous compounds during pregnancy. The multidrug resistance protein (MDR) 1 (P-glycoprotein; encoded by \textit{ABCB1} in humans and \textit{Abcb1a} and \textit{Abcb1b} in the mouse), multidrug resistance associated proteins (MRP; encoded by \textit{ABCC} genes) and the breast cancer resistance protein (BCRP; encoded by \textit{ABCG2}) are transporters that mediate the ATP-driven efflux of substrates from liver and kidney and limit drug exposure of highly sensitive sites such as the brain and fetal compartment. Within the placenta, these transporters reduce the materno-fetal transfer of their substrates via ATP-driven efflux from the fetal-to-maternal circulation (\textbf{Figure 1}). Their importance in determining fetal xenobiotic exposure has been well established \textit{in vivo} in knockout mouse and \textit{ex vivo} in human placental perfusion models (Vahakangas and Myllynen, 2009; Aye and Keelan, 2013). With regards to therapeutics, MDR1 (Pgp, ABCB1) plays an important role in the pharmacokinetics of quinine (Pussard et al., 2007; Mukonzo et al., 2010), and could transport other antimalarials as the Mdr homologue of \textit{P. falciparum}, PfMDR1, has been shown to extrude a wide variety of antimalarials including amodiaquine, mefloquine, lumefantrine, and artemisinin; thereby contributing to drug resistance (Foote et al., 1989; Wilson et al., 1989; Price et al., 2004; Sisowath et al., 2007; Sa et al., 2009; Chavchich et al., 2010).

The expression of several drug transporters as well as CYP3A are altered in both pregnant and non-pregnant rodent models of bacterial and viral infection and these changes have been associated with altered disposition of their substrates (Cressman et al., 2012). Administration of bacterial endotoxin also impacts the expression of Cyp3a11 in fetal
liver (Xu et al., 2005). Whether these findings extend to malaria infection is unknown. As many clinically important drugs are substrates of the ABC transporters and DMEs, disease-induced changes could have important implications on drug efficacy and toxicity; thereby impacting maternal and fetal outcomes. Hence further investigation on the impact of malaria infection on drug transporters and metabolic enzymes is warranted.

We hypothesized that malaria infection would induce changes in the maternal and fetal expression of drug transporters and Cyp3a11. The impact of malaria was examined in a *Plasmodium berghei* ANKA (PbA) murine model of PM that mimics the pathophysiology and clinical features of human PM (Hviid et al., 2010). To our knowledge, this is the first study to examine the impact of malaria on drug transporters in maternal and fetal tissue.

**Materials and Methods:**

**Plasmodium berghei ANKA Placental Malaria Model:**

Animal protocols were approved by the Toronto University Health Network Animal Care Committee and performed in accordance with the Canadian Council on Animal Care Guidelines. We used a previously validated mouse model of PM that replicates key pathogenic features of human PM, including placental parasite sequestration, placental inflammation, spontaneous abortion and fetal growth restriction (Silver et al., 2010). Eight to ten week old Balb/c female mice were obtained from Jackson Laboratories Inc. (Bar Harbor, Maine, USA) and maintained on a 12-hour dark and 12-hour light cycle with *ad libitum* access to standard rodent chow and water. Females were mated and checked for the presence of a vaginal plug (GD1). Cryopreserved PbA strain malaria (MR4; Manassas, VA, USA) was passaged through a male Balb/c mouse as previously described (Silver et al., 2010). On GD13 dams were inoculated with $10^6$ PbA-infected erythrocytes in RPMI
DMD #53983
(Sigma-Aldrich, Oakville, ON, Canada) via tail-vein injection. Control dams were injected with an equivalent volume of RPMI 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) and is reported as % infected red blood cells. Dams were euthanized by CO2 on GD19. 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 were removed immediately after sacrifice and snap frozen in liquid nitrogen. Uteri were removed and examined for evidence of reabsorptions. Yolk sacs were dissected from uteri and fetuses were removed and weighed. Fetal viability was determined by the pedal withdrawal reflex and placentae and fetal tissue from viable fetuses were removed immediately and snap frozen in liquid nitrogen. Placentae and fetuses were obtained from 5-6 dams per group. Samples were subsequently stored at -80°C until further use.

**Total RNA Extraction and qPCR:**
Methods for RNA isolation, cDNA synthesis, and qRT-PCR have been described previously([Anger et al., 2012](#)). Briefly, RNA was extracted from ~75 mg of snap-frozen tissue using TriZol® reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 2µg of DNAse I treated RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Burlington, ON) according to manufacturer’s instructions. mRNA expression of drug transporters and Cyp3a11 were determined by qPCR using LightCycler® technology with SYBR detection (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 (Table 1). Amplicon sequences were amplified as described elsewhere([Anger et al., 2012](#)). Melt-curve
DMD #53983

analysis was used to ensure primer-specificity. Expression levels of each gene 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 to cyclophilin A mRNA expression. Data are presented as percentage of expression as compared to control ± SEM.

**Detection of Mdr1 Protein Expression by Western Blot:**

Given the dissimilar changes seen in the expression of the *Abcb1a* and *Abcb1b* mRNA in several tissues and the fact that both of these mRNA encode for the Mdr1 protein in mice, we further investigated the impact of malaria on the protein expression of Mdr1. Methods for protein isolation and Western blotting have been described previously (Anger et al., 2012). Briefly, protein samples were isolated from 300 mg of snap-frozen tissue homogenized in lysis buffer containing dithiothrietol (1 mM; DTT; Sigma-Aldrich, Oakville, ON), phenylmethylsulfonyl fluoride (0.5 mM; PMSF; BioShop Canada Inc., Burlington, ON,) and 1X protease-inhibitor cocktail (Sigma-Aldrich, Oakville, ON,) at 4°C. Homogenates were centrifuged at 18,000 x g for 15 minutes at 4°C and the supernatant isolated. Protein concentrations were measured using the Bradford assay. Protein samples containing 50 µg of isolated protein were separated via 10% SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories Canada, Ltd., Mississauga, ON). PVDF membranes were then blocked in 5% non-fat dry milk in Tris-Buffered Saline + 0.1% Tween 20 (TBST) and incubated with an anti-Mdr1 (C219) mouse monoclonal antibody (1:500, 1mg/mL mC219 clone, ID Labs Biotechnology, Inc., London, ON) overnight at 4°C. After a series of washes with TBST, membranes were incubated with an anti-mouse horseradish peroxidase-labeled secondary antibody (1:5,000; goat-anti
DMD #53983
mouse Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 hour in 2% non-fat milk TBST. Bound immunoreactive proteins were detected using an ECL Plus chemiluminescence kit (Amersham Biosciences, Baie d’Urfé, QC, Canada) and visualized using the Alpha Innotech FluorChem imaging system (San Leandro, CA, USA. The optical density (OD) of each Mdr1 band was determined using ImageJ (Software version 1.44) for Macintosh. To confirm equivalent protein loading, each blot was stained with Amido Black (0.03% Napthol Blue Black in 3% acetic acid) (BioShop Canada Inc., Burlington, ON) and allowed to air-dry 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. Unfortunately, due to low expression and limited quantity of fetal tissues, we were unable to assess Mdr1 expression in fetal liver.

Total Cellular Antioxidant Assay:
Total cellular antioxidant capacity of hepatic lysates from infected and control dams were measured using the Cayman antioxidant assay kit (Cayman Chemical Company, Ann Arbor, MI) which evaluates the combined effect of all antioxidants present in tissue. The colorimetric antioxidant assay was conducted according to the manufacturer’s protocol. Briefly, ~150 mg of maternal liver was homogenized on ice in 1 mL of homogenization buffer (5mM potassium phosphate, pH 7.4 containing 0.9% NaCl and 0.1% glucose) and centrifuged at 12,000 x g for 15 minutes at 4°C and supernatant collected and stored on ice until use. Protein concentrations were measured using the Bradford assay. Whole cell lysate samples (5-10mg/mL) and kit reagents were added to a 96-well plate and absorbance of sample was determined using a UV-Vis spectrophotometer at 750nm following manufacturer’s protocol. Results were then calculated and reported as µmol of
total antioxidant levels relative to the Trolox standard, normalized to total hepatic protein content.

**Serum Chemistry Analysis:**

Total bile acid concentrations were analyzed in plasma samples obtained from infected and control dams at a certified GLP laboratory (IDEXX laboratories, Inc., Markham, ON) using current standard methods of the International Federation of Clinical Chemistry. Total bile acid levels were also compared in 100 mg liver homogenate samples of infected and control dams using the Mouse Total Bile Acids Assay Kit (Crystal Chem Inc., Downers Grove, IL) following manufacturer’s protocol.

**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 were completed using Student’s unpaired two-tailed t-test. 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. Gene and protein expression results are presented as mean % control ± SEM.

**Results:**

**Maternal and Fetal Parameters:**

Given the established impact of malaria infection on maternal and fetal weight and splenomegaly in humans, we confirmed whether these changes were present in this study.
Increasing peripheral parasitemia was seen from GD16 to GD19 from 2.37% ± 0.38% to 49.88% ± 2.67% at GD16 and GD19, respectively. A corresponding decrease in maternal body weight was observed with PbA-infected dams weighing 7.53g ± 1.21g less at GD19, relative to uninfected dams (p < 0.05). No significant differences were seen on GD13 or GD16. Spleen weight was significantly higher in PbA-infected dams relative to uninfected control dams (208.1mg ± 13.0mg vs. 102.2mg ± 8.9mg; p < 0.001). Fetal weight was also significantly lower in fetuses obtained from PbA-infected dams, relative to those from uninfected control dams (748.50mg ± 40.38mg vs. 1082.00mg ± 44.71mg, respectively; p < 0.001).

**Increased Hepatic Inflammation and Oxidative Stress:**

We examined mRNA expression of hepatic inducible Nitric Oxide Synthase (iNOS) and Heme Oxygenase-1 (HO-1) as these enzymes are both induced by hepatic inflammation and have been shown to play a protective role in host-response to malaria infection (Taylor et al., 1998; Seixas et al., 2009; Sass et al., 2012). As depicted in Figure 2A, we identified pronounced increases in the hepatic expression of both iNOS and HO-1 in the maternal liver of PbA-infected dams, relative to uninfected control dams (p < 0.001). We also observed a significant decrease in the total cellular antioxidant capacity of the livers of infected dams relative to uninfected control dams (p < 0.001) (Figure 2B).

**Hepatic Cyp3a11 and Drug Transporters in Maternal Liver:**

Given that hepatic metabolism and transport processes influence the pharmacokinetics of xenobiotics and their metabolites within the maternal circulation and that these processes are known to be modulated by infection and inflammation, we examined whether malaria infection would alter their expression. Expression of Cyp3a11 was significantly decreased (p < 0.001) in the livers of infected dams relative to uninfected control dams (Figure 3A).
Significant decreases in the expression of the canalicular transporters Abcc2, Abcg2, Abcb11 were seen in the livers of infected dams, where expression levels ranged from 11% - 54% compared to controls (Figure 3A). A dramatic increase in mRNA expression of Abcb1b (p < 0.001) was seen in the infected group while Abcb1a was unaffected. Hepatic protein expression of the gene product of Abcb1a and Abcb1b, Multidrug resistance protein 1 (Mdr1), was not significantly different between infected and control dams (Figure 4).

Dramatic increases in the expression of the basolateral transporters Abcc1 and Abcc3 were observed in the livers of infected dams (p < 0.001) (Figure 3A). We also found a significant decrease in the expression of the organic anion transporter Slco2b1 (40% ± 33%; p < 0.01), whereas the expression of Slco10a1 (also known as the Na⁺-dependent taurocholate transporter, NTCP), an important transporter involved in hepatic bile salt uptake was not significantly changed relative to control dams (80% ± 30%; p > 0.05).

**Malaria Infection Alters Total Plasma Bile Acid Levels:**
Since jaundice, hyperbilirubinemia and hepatic cholestasis are seen in severe malaria and as bilirubin and bile acids are substrates for many of the same transporters (Treeprasertsuk et al., 2010), we investigated the functional consequence of the observed PbA induced changes in the maternal hepatic expression of the bile transporters by examining the levels of endogenous serum bile acids. The Bile Salt Export Transporter (BSEP; ABCB11) is a critical determinant of bile acid homeostasis in liver and facilitating hepatobiliary clearance of lipophilic substrates. We hypothesized that the decreased expression of canalicular Abcb11 would result in decreased efflux of bile salts into bile resulting in a compensatory increased transport into the maternal circulation by Abcc1 and Abcc3 at the basolateral domain (Figure 3A). These processes in turn would result in significantly increased serum bile acids. As illustrated in Figure 3B, we observed a significant and dramatic increase of
~80-fold in total serum bile acid concentrations in PbA infected dams relative to uninfected control dams (382.0 ± 152.3 µmol/L vs. 4.833 ± 0.1667 µmol/L, respectively). Relative total bile acid levels remained increased in the livers of PbA infected dams (195 ± 32 % of control values, p<0.05).

**Drug Transporter Expression changes in Maternal Brain:**

The expression of *Abcb1a, Abcb1b,* and *Abcg2* were assessed in whole brain homogenates of pregnant dams due to the important protective role they play in the blood-brain barrier. A significant increase in *Abcb1b* was observed in infected dams relative to control dams (158% ± 22%; p < 0.01). We observed a corresponding significant increase in the protein expression of Mdr1 in brain isolated from PbA infected dams as compared to controls (p < 0.05; **Figure 4**). No significant differences were observed in the expression of *Abcb1a* or *Abcg2*.

**Gene 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. We observed significant 2- to 3.5-fold increases in the expression of *Abcb1a* (350% ± 101%; p < 0.05) and *Abcb1b* (226% ± 99%; p < 0.05) in infected as compared to control dams. Likewise, malaria infection was associated with a significantly higher renal protein expression of Mdr1 (p < 0.05, **Figure 4**).

**Drug Transporter Expression in the Placenta:**

Given the critical role of placental drug transporters in fetal drug accumulation and their function as a protective barrier limiting fetal exposure to toxic xenobiotics, we investigated the impact of malaria infection on the placental expression of several ABC efflux drug transporters. As compared to controls, we observed significant decreases in the placental
mRNA expression of *Abcb1a, Abcb1b, Abcc1, Abcc2, Abcc3, and Abcg2* in malaria infected dams (p < 0.05) (**Figure 5**). A significant decrease in the protein expression of Mdr1 was seen in placenta isolated from infected dams as compared to uninfected controls (p < 0.05; **Figure 4**).

**Expression of Hepatic Cyp3a11 and Drug transporter expression in Fetal Liver:**

Given that fetal hepatic metabolism and transport processes may influence levels of xenobiotics and their metabolites within the fetal compartment, we examined whether malaria infection would alter their expression. As depicted in **Figure 6**, we observed significant changes in the expression of Cyp3a11 and drug transporters in the fetal liver. Significant 40 – 70% decreases in expression of fetal hepatic Cyp3a11, *Abcb1a, Abcg2,* and *Abcb11* was seen in pups isolated from infected dams as compared to uninfected controls, whereas the expression of *Abcb1b* was significantly increased (p < 0.01). Contrary to changes observed in the maternal liver, the expression of fetal liver *Abcc1* (151.94% ± 62.00% of control; p = 0.097) was not altered.

**Discussion:**

In this study we demonstrated that malaria imposes significant changes in the expression of Cyp3a11 and transporters including Mdr1, Mrp1, Mrp3, Bsep, and Bcrp in maternal and fetal tissue of pregnant mice. This was associated with functional changes in the transport of bile acid substrates as plasma bile acid concentrations were dramatically increased in the infected dams. Evidence of inflammation and oxidative stress were also seen in maternal liver.

A substantial decrease in the expression of Cyp3a11 was seen in livers obtained from malaria infected dams. The down-regulation we observed likely stems from
inflammation associated with parasitic infection. Our data demonstrating increases in HO-1 and iNOS mRNA as well as decreased antioxidant levels support this. This is in agreement with a body of literature illustrating infection and inflammation-mediated downregulation of hepatic CYP3A in rodents and humans (Cressman et al., 2012). Previous reports also indicate reduced expression of Cyp3a11 in non-pregnant rodent models of malaria (De-Oliveira et al., 2006; Carvalho et al., 2009). Inflammation-mediated suppression of CYP3A4 activity has been shown to impart clinically important changes in patients (Morgan et al., 2008).

Cyp3a11 was also down-regulated in fetal livers of pups from malaria infected dams. Although little is known about the impact of maternal disease on fetal gene expression, it was previously reported that endotoxin administration elicited a reduction in expression of Cyp3a11 in maternal and fetal livers on GD17 (Xu et al., 2005). While expression of Cyp3a11 in fetal liver is much lower than that of adults, levels increase rapidly from GD16 to post-gestation, therefore infection-mediated downregulation of gene expression could impact ontogenic changes and hepatic function in the fetus and neonate. As so many of the antimalarial drugs are metabolized by CYP3A, inflammation-mediated changes could have important implications for metabolic capacity of both mother and developing offspring.

In the malaria-infected dams we also observed pronounced changes in the hepatic expression of several transporters that are involved in the transport of bile and bilirubin and this was associated with a dramatic eighty-fold increase in serum levels of bile acids. This could have important consequences on fetal outcomes as increased levels of bilirubin and bile acids within maternal plasma are clinical features associated with poor pregnancy outcomes including premature birth, fetal distress and stillbirths (Uneke, 2007);(Lammert et al., 2000). Expression of Abcb11, the key transporter responsible for secretion of bile salts
DMD #53983

into bile, was decreased by nearly 10-fold in the infected animals resulting in increased hepatic accumulation of bile acids. Observed increases in basolateral expression of Abcc3 and Abcc1, likely served to shuttle excess bile acids from hepatocytes into maternal blood. The changes in expression of these transporters and increased serum bile acids are consistent with other animal models of inflammation-induced cholestasis (Vos et al., 1998; McGillicuddy et al., 2009; Yang et al., 2009; Kosters and Karpen, 2010). It is believed that increased expression of basolateral efflux transporters is a homeostatic mechanism evoked to protect hepatocytes (Wagner et al., 2009; Keppler, 2011). Decreases in Abcb11 are also frequently associated with downregulation of uptake transporters such as Slco10a1 (Ntcp) in cholestasis. While we did not detect significant changes in Slco10a1, expression of Slco2b1, which is involved in sodium-independent bile acid uptake was significantly downregulated.

ABCB1/MDR1 is involved in absorption, distribution, and clearance of many clinically important drugs. This transporter has been shown to transport quinine (Pussard et al., 2007; Mukonzo et al., 2010), and may play a role in transport of other antimalarial drugs as discussed earlier. In maternal liver, mRNA levels of Abcb1b but not Abcb1a were increased; however protein levels of Mdr1 were unchanged. Previous studies in rodent models of infection and inflammation have also shown an induction of Abcb1b mRNA. However, the contribution of Abcb1b, relative to Abcb1a, to hepatic Mdr1 protein expression is currently debated. To this point, decreased hepatic Mdr1 protein expression, corresponding to decreased Abcb1a mRNA levels, has been shown in endotoxin-treated rats, despite an observed 10-fold increase in mRNA levels of Abcb1b (Wang et al., 2005). We observed decreased Abcb1a expression and increased Abcb1b expression in fetal livers from infected dams. This suggests that drug transporter regulatory mechanisms occur
in fetal tissues and that these tissues are also subject to inflammation-mediated changes in
gene expression. While expression of Abcb1a, Abcb1b is much lower in fetal liver than that
of adult levels (Sharma et al., 2013), levels are thought to increase rapidly post-gestation
(Lee et al., 2011), therefore inflammation-mediated alterations in gene expression could
impact ontogenic changes and potential function in fetal and neonatal livers.

Interestingly, we found increased expression of Abcb1b in brains of infected dams,
which was associated with increased protein expression of Mdr1. Changes in Mdr1
expression at the blood-brain barrier impact the CNS exposure and effects of its substrates.
Therefore, malaria may be associated with a reduced accumulation of Mdr1 substrates in
brain. Our findings may provide a mechanism to a previous study investigating mefloquine
brain permeation in malaria-infected rodents. Farinotti observed a 2-fold decrease in
concentrations of mefloquine (a Mdr1 substrate) in the brains of mice infected with malaria,
compared to controls (de Lagerie et al., 2009). It is plausible that malaria-induced changes
in expression of Mdr1 in brain contributed to these altered brain concentrations.

Conversely, a decreased expression of Abcb1a and Mdr1 has been reported in the brains
of endotoxin-treated rats. These divergent results may stem from potential differences in
models and chronicity of infection. Inflammatory stimuli such as endotoxin, viruses and
malaria exert effects on the immune system through unique mechanisms. Endotoxin is a
characteristic TLR4 ligand (Koga and Mor, 2010) and may down-regulate gene expression
via the activation of NF-κB downstream. The viral mimetic, poly I:C activates the immune
system through TLR2 and TLR3 pathways. In contrast, P. falciparum
glycosylphosphatidylinositol and the malaria pigment, hemozoin, have been shown to
activate TLR2, TLR4, and TLR9 (Coban et al., 2005; Trinchieri and Sher, 2007; Erdman et
DMD #53983 al., 2008), and this broad TLR activation may result in a unique effect on gene regulation and consequent protein expression.

Drug transporters in kidney are known to impact the pharmacokinetics and renal clearance of numerous drugs and the expression of these transporters is mutable due to pathophysiology (Cressman et al., 2012). Indeed, within our study, we found a significant increase in expression of Abcb1a and Abcb1b in infected as compared to control dams. Likewise, malaria infection was associated with a significantly higher renal protein expression of Mdr1. Others have also reported infection or inflammation-mediated increases in the mRNA and protein expression of Mdr1 in kidney (Hartmann et al., 2005; Heemskerk et al., 2010). Increased renal expression of Mdr1 in endotoxin treated mice was associated with increased renal elimination of the Mdr1 substrate, doxorubicin (Hartmann et al., 2005). It is plausible that malaria-imposed changes could impact renal clearance of antimalarials and other substrates.

One of our key objectives was to examine expression of transporters in placenta as these proteins are important in fetal protection. In addition to the decreased expression of Abcb1a and Abcb1b resulting in a corresponding reduction in immunodetectable levels of Mdr1 levels, we observed decreased expression of several other transporters including Abcc1-3 and Abcg2. These proteins are also important in transport of drugs, conjugated-drug metabolites, and endogenous substrates (e.g., bile acids) (Cressman et al., 2012). Previous studies in models of bacterial and viral infection have also reported similar changes (Petrovic et al., 2008; Petrovic and Piquette-Miller, 2010). In endotoxin-treated rats, decreased placental expression of Abcg2 and Abcb1 was associated with increases in fetal accumulation of the Abcg2 and Abcb1 substrates, glyburide and sestamibi (Petrovic et al., 2008; Wang et al., 2005). The implications of our findings suggest that comparable
changes in drug-disposition may occur in malaria infection and may contribute to increased fetal exposure to endogenous and exogenous compounds.

Changes in fetal expression of transporters were similar to the changes seen in the maternal liver. This may suggest that comparable regulatory mechanisms are occurring in fetal and maternal liver and also suggests that transporter regulatory pathways are intact in the fetal liver. Therefore fetal exposure to potentially toxic substances, such as the elevated concentrations of bile acids observed in the malaria infected dams, may be further altered due to infection-mediated changes to transporters and metabolizing enzymes in placental and fetal tissues. Very little is known about the expression of transporters and DMEs in fetal tissues, and even less about how disease-states impact the expression of these proteins. Nevertheless, this field is currently evolving and it will be important to investigate how these alterations impact the in vivo disposition of their substrates. Whether these changes persist in the developing fetus and offspring and the consequences is an important question and currently being examined in ongoing studies.

**Conclusions:**

Herein we demonstrate that malaria infection alters expression of a number of drug transporters and Cyp3a11 in maternal and fetal tissues during pregnancy. If these findings translate to malaria infection in human pregnancy, altered maternofetal disposition and clearance of endogenous and exogenous substrates may have important clinical consequences.
Authorship Contributions:

Participated in research design: Cressman, Piquette-Miller, Silver, McDonald, Kain,

Conducted experiments: Cressman, Silver, McDonald

Performed data analysis: Cressman, Piquette-Miller

Wrote or contributed to the writing of the manuscript: Cressman, Piquette-Miller, Silver, McDonald, Kain,
References:


Fried M and Duffy PE (1996) Adherence of Plasmodium falciparum to chondroitin sulfate A in the

P-glycoprotein expression on disposition of doxorubicin in mice. Drug Metab Dispos 33:820-828.

Heemskerk S, Peters JG, Louisse J, Sagar S, Russel FG, and Masereeuw R (2010) Regulation of P-
glycoprotein in renal proximal tubule epithelial cells by LPS and TNF-alpha. J Biomed Biotechnol
2010:525180.


Kim KA, Park JY, Lee JS, and Lim S (2003) Cytochrome P450 2C8 and CYP3A4/5 are involved in

Koga K and Mor G (2010) Toll-like receptors at the maternal-fetal interface in normal pregnancy and

Liver Dis 30:186-194.

Lammert F, Marschall HU, Glantz A, and Matern S (2000) Intrahepatic cholestasis of pregnancy:

metabolizing enzyme and transporter gene expression through the life stages of the mouse. PLoS One
6:e24381.

of piperaquine is primarily mediated by CYP3A4. Xenobiotica 42:1088-1095.

infection on the disposition of quinine and quinidine in the rat isolated perfused liver preparation. J
Pharm Pharmacol 42:428-432.

91:803-810.

McGillicuddy FC, de la Llera Moya M, Hinkle CC, Joshi MR, Chiquoine EH, Billheimer JT, Rothblat
119:1135-1145.

elimination of pyrimethamine during malaria infection. Studies in the isolated perfused rat liver. Biochem
Pharmacol 36:2827-2829.
DMD #53983


Footnotes:

This work was supported by Grand Challenges in Global Health: Preventing Preterm Birth Initiative [Grant No. 12003]; the Canadian Institutes of Health Research (CIHR) [Grants No. MOP-115160, 13721 and 57688]; Canada Research Chair and the Natural Science and Engineering Research Council (NSERC) and Ontario Graduate Scholarships.

Contributed equally to this manuscript:

Co-first authors: Cressman and McDonald

Co-senior authors: Piquette-Miller and Kain

Author e-mail addresses:

Alex Cressman, MSc: alex.cressman@mail.utoronto.ca
Chloe McDonald, MSc: chloe.mcdonald@mail.utoronto.ca
Dr. Karlee Silver, PhD: karlee.silver@grandchallenges.ca
Dr. Kevin Kain, MD, FRCPC: kevin.kain@uhn.ca
Dr. Micheline Piquette-Miller, PhD: m.piquette.miller@utoronto.ca
Legends for Figures:

FIGURE 1: Schematic depiction of ABC Drug Transporters in Liver and Placenta.  (A) The basolateral (sinusoidal) membranes of hepatocytes express the multidrug resistance-associated proteins MRP1 (ABCC1) and MRP3 (ABCC3). The canalicular membranes express the multidrug resistance protein (MDR1, ABCB1), the breast cancer resistance protein (BCRP, ABCG2), MRP2 (ABCC2) and the bile salt export pump (BSEP, ABCB11).  (B), the syncytiotrophoblast layer of the placenta is comprised of multinucleated (N) cells that express MDR1, BCRP and MRP2 on the apical membrane and MRP1 and MRP3 on the basolateral membrane.  The apical membranes of fetal capillary endothelial cells express MRP1 and MRP3.

FIGURE 2: Effect of malaria (PbA infected) on (A) Hepatic mRNA expression of iNOS and HO-1 in maternal liver. Expression was normalized to cyclophilin A and are presented as a percentage of controls ± SEM and (B) Total hepatic cellular antioxidant capacity in maternal liver. Antioxidant activity was normalized to total hepatic protein as described in methods. N = 6 dams/group.  * p < 0.05; ** p < 0.01;  *** p <0.001

FIGURE 3: Effect of malaria (PbA infected) on (A) Hepatic mRNA expression in maternal liver. Expression was normalized to cyclophilin A and are presented as a percentage of controls ± SEM.  (B) Total bile acid levels in maternal serum. N = 6 dams/group.  * p < 0.05; ** p < 0.01;  *** p <0.001

FIGURE 4: Effect of malaria (PbA infected) on the protein expression of Mdr1 (ABCB1) in maternal tissues. Protein levels were determined by Western blotting and normalized to total protein staining as described in Methods. Results are presented as a percentage of control ± SEM. N = 4 dams/group .  * p < 0.05

FIGURE 5: Effect of malaria (PbA infected) on the mRNA expression of transporters in placenta on GD19. Placental mRNA expression was normalized to cyclophilin A and are presented as a percentage of controls ± SEM N = 10 placentae from 5 dams/group.  * p < 0.05; ** p < 0.01;  *** p <0.001
FIGURE 6: Effect of malaria (PbA infected) in pregnant dams on hepatic mRNA expression in fetal liver. Expression was normalized to cyclophilin A and are presented as a percentage of controls ± SEM. N = 6 fetuses isolated from 6 dams/group. * p < 0.05; ** p < 0.01; *** p < 0.001
### TABLE 1: Sequences of oligonucleotide primers used for qRT-PCR analysis.

<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><strong>Normalizing Gene</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CycA</td>
<td>NM_008907.1</td>
<td>GGAGATGGCACAGGAGGAA</td>
<td>GCCCGTAGTGCTTCAGCTT</td>
</tr>
<tr>
<td><strong>ATP-Binding Cassette Transporters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abcb1a</td>
<td>NM_011076.2</td>
<td>GGAGCTGCTGGTCCCATCTTCCA</td>
<td>GACCTCACGTGTCTCTACCTCCCG</td>
</tr>
<tr>
<td>Abcb1b</td>
<td>NM_011075.2</td>
<td>AGGCGCGCTGCTCTCATCTTCTGGA</td>
<td>CATCACACCTACGTGCCACCT</td>
</tr>
<tr>
<td>Abcc1</td>
<td>NM_008576.3</td>
<td>TGAGTGTCAGAAGTGAGGAG</td>
<td>ACCCGCGTGTAGTCATTAT</td>
</tr>
<tr>
<td>Abcc2</td>
<td>NM_013806.2</td>
<td>GGATAATGAGGCCGCCTGAGGGG</td>
<td>CCGGCCGATACCGACCTTGA</td>
</tr>
<tr>
<td>Abcc3</td>
<td>NM_029600.3</td>
<td>CCCACAGCTGCTCAGTATACCTG</td>
<td>GATCAATGTCTGCAGTGAGGG</td>
</tr>
<tr>
<td>Abcg2</td>
<td>NM_011920.3</td>
<td>ATGGAGCACCTACACCTGCCCA</td>
<td>GTCAGGGTGCCCCATACACAGT</td>
</tr>
<tr>
<td>Abcb11</td>
<td>NM_021022.3</td>
<td>CCCCTCGAAGGCATGGTGA</td>
<td>ACCAGGCGGATGTTCTGCGA</td>
</tr>
<tr>
<td><strong>Cytochrome P450 Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>NM_007818.3</td>
<td>AGGGAAGCATGGAGGACATCACA</td>
<td>TGCTAGGAGCACCAGCTTTCCA</td>
</tr>
<tr>
<td><strong>Solute Carrier Transporters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slco10a1</td>
<td>NM_001177561.1</td>
<td>CCCTACGTCTCAAGGCAGCCA</td>
<td>GGCATCAGGAAGGAGGTAGCCA</td>
</tr>
<tr>
<td>Slco2b1</td>
<td>NM_001252530.1</td>
<td>CCTAACGCCAACGCAAGAC</td>
<td>ACTCCTGCACAGACCAAAAA</td>
</tr>
<tr>
<td><strong>Disease-Relevant Genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HO-1</td>
<td>NM_010442.2</td>
<td>CTGGAGCATAGGCCGACC</td>
<td>AGACAATCTCTGGGGCAGTCT</td>
</tr>
<tr>
<td>iNOS</td>
<td>NM_010927.3</td>
<td>CACCTTGAGGTTCAACCG</td>
<td>ACCACTCGTACTTGGGATGC</td>
</tr>
</tbody>
</table>
Figure 1

(A) Hepatic Sinusoid

ABCC1
ABCG2
ABCB11
ABCC2
ABCC3
ABCB1

(B) Maternal Blood

Syncytiotrophoblast

ABCC1
ABCC3

Fetal Mesenchyme
Figure 2

A

Cyclophilin Ratio (% Control)

Control

PB A. Infected

***

B

Total Cellular Antioxidant Level (μM)

***

MOS

HC-1
Figure 5

The bar chart shows the cyclophilin ratio (% control) for various ABC transporters in both control and PbA infected conditions. The chart includes the following transporters:

- Abcb1a
- Abcb1b
- Abcc1
- Abcc2
- Abcc3
- Abcg2

The chart indicates significant differences between control and PbA infected conditions, with asterisks (*) indicating statistical significance. The y-axis represents the cyclophilin ratio in percentage, and the x-axis lists the ABC transporters.