Understanding Pregnancy-induced Changes in the Disposition of Norbuprenorphine, Metformin and Glyburide in Mice and Humans for Optimizing Drug Therapy during Pregnancy

Michael Zecong Liao

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Reading Committee:

Qingcheng Mao, Chair

Mary F. Hebert

Danny D. Shen

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Abstract

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Michael Zecong Liao

Chair of Supervisory Committee:

Qingcheng Mao, PhD. Associate Professor

Pharmaceutics

Pharmacotherapy usage during pregnancy is common and increasing. In the current clinical practice, healthcare providers are increasingly prescribing buprenorphine (BUP) for opioid addiction treatment, as well as metformin, and glyburide for gestation diabetes mellitus (GDM). Due to the dual risks and benefits to the mother and fetus, it is important to understand how pregnancy affects the disposition of these drugs and their metabolites. To understand the effects of P-gp and Bcrp on tissue distribution during pregnancy, and the effects of pregnancy on drug disposition, we administered norbuprenorphine (NBUP), the major active metabolite of BUP, to nonpregnant wild-type (WT), pregnant WT, pregnant P-gp (Mdr1a/1b) knockout (KO), and pregnant P-gp/Bcrp (Mdr1a/1b/Abcg2) KO mice. We have shown that that fetal exposure to NBUP and norbuprenorphine-3-β-D-glucuronide (NBUP-G) in pregnant mice accounts for ~60%

and ~700% of maternal plasma exposure, respectively, suggesting that fetal exposure to the two major active metabolites of BUP is substantial and hence caution should be taken regarding fetal safety with the use of BUP during pregnancy. We have also demonstrated significant differential impact of P-gp on fetal and brain exposure to NBUP, with a much greater role of P-gp in restricting NBUP distribution across the blood brain barrier (BBB) versus the blood placental barrier (BPB). Subsequently, we demonstrated that the systemic clearance (CL) of NBUP in WT pregnant mice was ~2.5-fold higher compared with WT non-pregnant mice. Intrinsic CL of NBUP by glucuronidation in mouse liver microsomes from pregnant mice was ~2-times greater than non-pregnant mice. Targeted LC-MS/MS proteomics quantification revealed that hepatic Ugt1a1 and Ugt2b1 protein levels in the same amount of total liver membrane proteins were significantly increased (~50%) in pregnant mice versus non-pregnant mice. Recently, metformin and glyburide have gained increasing popularity for the GDM management. However, the optimal dosing regimens for these two drugs during pregnancy are unknown. We conducted a multicenter, prospective Phase II, randomized, parallel-design pharmacokinetics study. We analyzed the pharmacokinetics of orally administered steady-state metformin in pregnant women with GDM and non-pregnant women with Type-2-Diabetes Mellitus (T2DM), and the pharmacokinetics of oral glyburide in fed and fasted pregnant subjects with GDM. For the metformin cohort, with both 500 mg and 1000 mg doses, bioavailability, volume of distribution, clearance and renal clearance of metformin were increased during pregnancy. For the glyburide cohort, simultaneous consumption of a standardized meal increased glyburide dose-normalized AUC and decreased glyburide apparent oral volume of distribution β, apparent oral clearance, and half-life. Overall, this body of information provides insights pharmacokinetic and mechanistic understanding of the use of buprenorphine, metformin, and glyburide during

pregnancy, laying the foundation to optimize management of opiate addiction and gestational diabetes mellitus during pregnancy.

Dedication

To the love of my life, Dongmei Tan,

Thank you for your understanding and daily encouragement, my life will be very different without you. I cherish every minute we spend together. Your creativity, optimism, humor, and hard work inspire me to be the best that I can be.

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Chapter 1: Introduction

1.1 Drug use during pregnancy

Illicit and licit drug use during pregnancy is an emerging public health concern. In the United States, the National Survey on Drug Use and Health reported that 4.4 to 5.9% of the pregnant women used illicit drugs during pregnancy 2012 (1), resulting in over 380,000 offspring exposed to illegal substances (2). The most commonly used illicit substances in pregnancy is nicotine, alcohol, marijuana, cocaine, and opiates (3, 4). With the recent national opiate epidemic, opiate abuse by pregnant women increased five-fold between 2000 and 2009 in the US (5, 6). Prescription medication usage is very common for treatment of addiction, hypertension, diabetes, bacterial/viral infections, and epilepsy during pregnancy. According to a study conducted by Mitchell et al. (7) in 2011, more than 90% of pregnant women take at least one medication during pregnancy. A retrospective study of 8 health maintenance organizations reported (8), during the period 1996 through 2000, 152,531 deliveries were identified that met the study criteria. For 98,182 deliveries (64%), a drug other than a vitamin or mineral supplement was prescribed in the 270 days before delivery: 3595 women (2.4%) received a drug from category A; 76,292 women (50.0%) received a drug from category B; 57,604 women (37.8%) received a drug from category C; 7333 women (4.8%) received a drug from category D, and 6976 women (4.6%) received a drug from category X of the United States Food and Drug Administration risk classification system. Overall, 5157 women (3.4%) received a category D drug, and 1653 women (1.1%) received a category X drug after the initial prenatal care visit.(8). Because the old system of pregnancy categories poorly correlated with drug safety, the new FDA Pregnancy and Lactation Labeling Rule removed pregnancy letter categories – A, B, C, D and X and replaced them with drug specific safety information in the pregnancy. Even with supporting

evidence that pregnant women are commonly taking medications during pregnancy, there is a strong resistance to investigate drugs during pregnancy, due to the tragic history of thalidomide in the 1950s. In addition, the current drug approval process in the US only requires drug makers to provide safety information about whether or not the drugs can be used in pregnancy according to the FDA pregnancy guidance, which is mostly based on studies in animals and non-pregnant human subjects. Drug labels are not required to provide dosing recommendations for the pregnant population. Due to the lack of PK/PD information, healthcare providers usually prescribe drugs to pregnant women and determine doses empirically. The empirical treatment and dosing strategies are typically based on standard adult doses, despite the fact that dosing, safety, and efficacy of these drugs were determined in healthy, mostly male, individuals (9). There is a need for understanding the effects of pregnancy on drug disposition to determine dosage strategies in this under-studied population, in hope of increasing efficacy and decreasing adverse impacts of drugs on both the fetus and the mother. Therefore, the main focus of my Ph.D. thesis research is to determine and understand the changes in drug PK during pregnancy including fetal drug exposure in animal models or human subjects. In the following chapters, I will provide relevant information about the drugs abused or used by pregnant women that are related to or have been studied in my Ph.D. thesis projects. I will also briefly describe the rationale behind my thesis projects.

1.2 Opioid Abuse and Adverse Effects

Opioid therapy is regarded as a valuable tool in physicians' armamentarium for control of acute and chronic pain due to various conditions (e.g., back pain, cancer) (10). Currently, marketed prescription opioids include codeine, fentanyl, hydrocodone, hydromorphone, levorphanol, meperidine, methadone, morphine, oxycodone, oxymorphone, pentazocine,

buprenorphine, tapentadol, and tramadol. In addition, a wide variety of opiates is abused, including heroin, methadone, and oxycodone. Based on their abuse potential, opioids are classified by the U. S. Drug Enforcement Agency as schedule I, II, III, or IV substances (11). Opiates can be inhaled, injected, snorted, ingested, or used subcutaneously. Opioid addiction as a result of prescription opioid use during pregnancy accounted for 28% of hospital admissions of pregnant women for treatment of drug abuse between 1992 to 2012(12). During pregnancy, untreated chronic addiction is associated with the lack of prenatal care, increased risk of fetal growth restriction, placental abruption, fetal death, preterm labor, and intrauterine passage of meconium (13). Untreated addiction is associated with sexually transmitted infections, violence, and legal consequences, including the loss of child custody, criminal proceedings, and/or incarceration (14). Two opioid agonists, methadone and buprenorphine, are commonly used for management of opiate addiction in pregnant women (15, 16). Studies have shown that the use buprenorphine for addiction treatment during pregnancy tripled over the last decade (17, 18).

1.3 Opiate Addiction Treatments

Methadone

Methadone is a widely used opioid receptor agonist for pain management and for suppression of opioid-agonist abstinence syndrome in patients who are dependent on opioids (19, 20). There is substantial inter-individual and intra-individual variability in the PK and PD of methadone among men and non-pregnant women (21). Studies have indicated that stereoselective disposition, poly-pharmaceutical use, disease states and co-administration of illicit drugs are sources for inter and intra-individual variability (22-26).

Methadone has a long half-life, on average approximately 24 h. The time for analgesia onset after a single IV bolus is about 10 to 20 min with a duration of 4 to 8 h (27). Methadone is metabolized in the liver and excreted by the kidney. Methadone has low extraction ratio and high oral bioavailability. Methadone is metabolized by CYP3A4, CYP2B6, and is a substrate of P-gp (28, 29). In the kidney, changes in urinary pH may increase methadone excretion. When urinary pH is above 6, renal excretion is responsible for just 4% of the total excreted drug, while in pH below 6, 30% of the total dose is excreted by the kidneys (30).

Two studies demonstrated that methadone clearance increased during pregnancy (31, 32). Since methadone is a substrate of CYP3A4, CYP2B6, and P-gp, and undergoes renal elimination (33, 34), increases in hepatic CYP3A4 and CYP2B6, hepatic P-gp protein expression (see **Chapter 4**) and renal clearance during pregnancy may all lead to the increase in methadone clearance and decrease in plasma concentrations during pregnancy. Indeed, the PK of methadone in pregnant women changes across gestation and significantly differs from the non-pregnant population. For example, the half-life of methadone falls from an average of 22–24 h in non-pregnant women to 8.1 h in pregnant women, a significant decrease in methadone exposure during pregnancy (35). Although methadone is often administered via daily dosing, split dosing (every 12 h) can also be used to account for increased clearance throughout pregnancy. Comparing to pregnant women who use heroin alone, women treated with methadone maintenance therapy have better prenatal care adherence, reduced fetal death rates, and higher infant birth weights (11). The use of methadone during pregnancy has been reported to have adverse effects on the mother and her fetus, although many of these findings are inconsistent and have not established a causal link to methadone use. Poor outcomes include a higher incidence of prematurity, intrauterine growth restriction, decreased fetal activity, and slow fetal heart rate(36).

In some animal species high-dose methadone during pregnancy resulted in congenital anomalies. However, the main concern in humans is neonatal withdrawal.

A newer alternative of methadone for opiate maintenance therapy in pregnancy is buprenorphine (see below), a partial mu-opioid agonist approved in 2002 for medication-assisted treatment of opiate dependence (37).

Buprenorphine

Buprenorphine (BUP) is a semisynthetic thebaine derivative that acts as a mixed partial agonist-antagonist opioid receptor modulator (38). It is 25 to 40 times more potent than morphine. The most common route of administration of BUP is sublingual administration. Sublingual BUP maintenance dosage range is 4-24 mg/day for non-pregnant patients to treat opiate addiction (39-41). BUP is extensively metabolized primarily by CYP3A via *N*-dealkylation to norbuprenorphine (NBUP), and then both BUP and NBUP undergo glucuronidation (41). Multiple CYP450 enzymes are involved in BUP metabolism including CYP2C8, CYP2C9, CYP2C18, CYP2C19, and CYP3A4. CYP3A accounts for 65% of norbuprenorphine formation (42). BUP also undergoes glucuronidation, mainly by UGT1A1, UGT1A3, and UGT2B7 (43).

NBUP is the major active metabolite of BUP with approximately a quarter of BUP's intrinsic analgesic effect (44). Furthermore, NBUP is a μ-opioid, δ-opioid, and nociceptin receptor full agonist and a κ-opioid receptor partial agonist. NBUP produces respiratory depression, and was ten times more potent than buprenorphine in rodents in producing respiratory depression (45, 46) and may be fatal to infants (47). NBUP can be further glucuronidated at the third carbon to norbuprenorphine-3-β-D-glucuronide (NBUP-G), which is also biologically active (44). Concheiro et al. (48) showed that both NBUP and NBUP-G could

be detected in umbilical cord blood samples 5 h after administration of buprenorphine but before delivery with concentrations higher than those of the parent drug, suggesting that the fetus is exposed to substantial concentration of the pharmacologically active metabolites.

NBUP is also a P-gp/ABCB1 substrate, and NBUP-G is a possible BCRP/ABCG2 substrate. P-gp and BCRP are the two most important ABC efflux transporters for drug disposition and have a broad spectrum of substrates, including many drugs routinely used by pregnant women (49-51). P-gp and BCRP are highly expressed on the apical membrane of the liver hepatocyte, the brain endothelium and the placental syncytiotrophoblasts (52-54). Hence, both transporters play a critical role in facilitating biliary elimination and tissue distribution of drugs. For example, it has been shown that P-gp and BCRP can protect the brain and fetus from potential chemical injury by actively expelling drugs, xenobiotics, and harmful substances from the brain or the fetus to the systemic or maternal circulation (55-59). Previous studies have shown that P-gp can effectively transport NBUP but not BUP (44, 60) and that P-gp is a significant determinant of brain NBUP exposure (44). BCRP is known to transport glucuronide conjugates of drugs, xenobiotics, and endogenous substances (61). Given the fact that NBUP is a P-gp substrate, and NBUP-G is possibly a BCRP substrate, it is essential to know whether P-gp or BCRP restricts fetal exposure to NBUP and/or its glucuronide metabolite. Murine knockout models have been developed to interrogate the effects of P-gp and BCRP on drug disposition (62-65). Numerous studies using chemical inhibitors and genetic knockout of P-gp and BCRP have demonstrated the importance of the two transporters in determining brain or fetal distribution of several prototypic substrates, including methadone, loperamide, fentanyl, glyburide, and nitrofurantoin (56, 57, 66-71). In **Chapter 2** we describe the studies showing the

role of P-gp and BCRP in limiting fetal exposure to NBUP and NBUP-G, using wild-type, *Abcb1a*$^{-/-}$/*1b*$^{-/-}$, and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ pregnant mice.

The activity of human CYP3A4, UGT1A1 and UGT2B7 are known to be significantly induced during pregnancy (72-74). In humans, CYP3A activity was shown to be increased approximately 2-fold in the third trimester as measured by 1'-hydroxymidazolam formation clearance (73). This induction can result in a decrease in plasma concentration of drugs cleared by CYP3A. Indeed, studies have reported that the plasma concentrations of several CYP3A substrates (e.g., indinavir and nifedipine) are decreased to subtherapeutic concentrations during pregnancy (75, 76). UGT1A1 and UGT1A4 play a major role in metabolizing labetalol (77) and lamotrigine (78), respectively. NBUP is a substrate of UGT1A1, UGT1A3, UGT2B7 and UGT2B17 (42, 43, 79). Several studies have shown that some UGTs may be induced by pregnancy (74, 80, 81). Studies have demonstrated that UGT1A4 and 1A1 are inducible by pregnancy-related hormones. It has been shown that that 17β-estradiol up-regulates human UGT1A4 via the estrogen receptor α (80), and that progesterone induces human UGT1A1 (81) possibly through the pregnane-X-receptor (82). Whether the pregnancy-related hormones could induce other human UGT isoforms including UGT1A3, UGT2B7 and UGT2B17 are not known. While the disposition of BUP has been extensively studied (83-85) and the dose-normalized plasma AUC of BUP in pregnant women has been shown to be significantly decreased (~2-fold) compared to non-pregnant controls (86), information regarding the disposition of NBUP especially during pregnancy is limited. Given that NBUP is extensively metabolized by UGTs (43, 79), whether pregnancy alters the disposition of NBUP due to induction of glucuronidation warrants investigation. In the studies described in **Chapter 3**, we investigated the PK of NBUP and NBUP-G in animal models, that is, pregnant and non-pregnant mice. We demonstrated that

pregnancy significantly increased the intrinsic clearance of NBUP by increasing glucuronidation and inducing Ugt protein expression in the mouse liver.

Even though pregnancy significantly increased formation clearance of NBUP-G, we also observed that the NBUP-G/NBUP AUC ratio remained relatively unchanged in pregnant and non-pregnant mice. This observation suggests that elimination clearance of NBUP-G is significantly increased as well in pregnant mice as compared to non-pregnant mice. One possible explanation for that observation is that Bcrp protein abundance in the liver could be increased, thereby facilitating biliary elimination of NBUP-G. To test this hypothesis, we quantified and compared Bcrp protein abundance in the mouse livers of pregnant and non-pregnant mice (**Chapter 4**). We recognized that only a few studies have systematically investigated the effects of pregnancy on protein expression or activity of drug transporters in organs vital for drug disposition. Several studies have evaluated changes in mRNA expression for some transporters during pregnancy (87, 88); however, mRNA expression is not always correlated with protein expression or activity. Thus, in **Chapter 4**, we evaluated the effects of pregnancy on protein expression of various drug transporters important for drug/xenobiotic disposition, including Acbc1a, Abcg2, Abcc2, Slco1a1, and other transporters, in the liver, kidney, and brain of mice, using highly sensitive LC-MS/MS-based quantitative proteomics with multiple reaction monitoring (MRM) (89, 90). Knowledge of the changes in drug transporter expression by pregnancy is important for understanding and predicting of pregnancy-induced alternations in drug/xenobiotic PK, efficacy, and safety.

1.4 Oral glucose lowering agents

Gestational diabetes mellitus (GDM) complicates 5-13% of pregnancies in the US (91). GDM is associated with carbohydrate intolerance, resulting in hyperglycemia of variable severity (92). In patients with mild GDM, initial treatment usually includes lifestyle modification and dietary adjustments. Glucose crosses the placenta via glucose transporters. The maternal glucose concentration determines fetal glucose exposure. When maternal glucose concentration is high, the fetus is also exposed to a high concentration of glucose (93). Untreated GDM is associated with increased risks for maternal infections, traumatic deliveries, hypertensive disorders, and preeclampsia as well as neonatal hypoglycemia, respiratory distress syndrome, macrosomia, polyhydramnios, shoulder dystocia and kernicterus (94, 95). Surgical deliveries are much more common in women with GDM. Although GDM resolves after pregnancy, these women have an increased risk of type 2 diabetes mellitus (T2DM) later in life (96, 97). Therefore, patients with inadequate glucose control with lifestyle and dietary adjustments are treated with pharmacotherapy in order to achieve glycemic control.

Insulin

Historically insulin has been the first line of treatment for women with GDM who are unable to achieve glycemic control with diet and exercise. Insulin is available in rapid-, short-, intermediate- and long-acting insulin/insulin analogs. Regular U-100 is synthesized in *E.coli* bacteria. It is usually used before a meal to cover for the carbohydrate intake. Regular U-500 insulin is similar to U-100 but more concentrated. Both U-100 and U-500 have an onset time of 30 minutes; the duration of effect may last from 8 hrs for regular insulin to 24 hrs for glargine insulin (98, 99). Aspart, lispro, and glulisine are very short acting insulin analogs that are produced in yeast or *E. Coli*. Their onset of action is about 10-15 minutes, peaks between 30-90

minutes with a duration of 3-5 hours (100-102). Intermediate and long-acting insulin/insulin analogs include NPH, detemir, glargine and degludec have time to onset of 1 to 2 hours, with duration up to 24 hours (103). However, the national guideline lacks an algorithm for adjusting doses of insulin in pregnancy. Weight-based dosing, weight plus gestational age-based dosing, and a one-dose for all types of dosing have been used. In clinical practice, adjustment can be made every few days until control of blood glucose is attained. The adjustment depends on accurate blood glucose testing, the type of insulin used, and carbohydrate consumption per meal. For GDM, patients that requires a large daily doses of insulin, insulin pump therapy may be appropriate (104). Due to the complexity and high cost of using insulin, oral hypoglycemic agent are being more frequently used to treat GDM.

Oral hypoglycemic agents such as glyburide (second-generation sulfonylurea) and metformin (biguanide) are attractive alternatives to insulin due to their lower cost, ease of administration, and better patient adherence. Most of retrospective and prospective studies suggest similar efficacy and safety of metformin, glyburide and insulin when used in the treatment of women with GDM (105).

Metformin

Metformin, an oral glucose-lowering agent that is frequently utilized in the treatment of GDM, decreases insulin resistance (106-108). Metformin is a small, basic compound with a high unbound fraction (109). It is a substrate of organic cation transporters (OCTs) (110-113) and is almost entirely excreted unchanged in the urine (114). OCT2 (encoded by SCL22A2) has been proposed to play a critical role in the PK of metformin. Metformin is actively transported from the circulation into renal epithelial cells primarily by OCT2, leading to net renal secretion (115). As a strong base with pKa of 11.5, metformin's net tubular secretion is unlikely to be subject to

passive tubular reabsorption (116). Decreased activity of OCT2 in non-pregnant subjects due to genetic differences or drug-drug interactions with inhibitors such as cimetidine results in 30-60% decrease in metformin renal and net renal secretion clearances, and up to 74% increase in the area under the concentration-time curve (AUC) (117-121). Renal excretion of metformin from tubular cells into the urine is mediated through the multidrug and toxin extrusion proteins 1 and 2-K (MATE1/SLC47A1 and MATE2-K/SLC47A2) (122-125). In addition to OCT2, MATE1, and MATE2-K, metformin is also a substrate of OCT1 (SLC22A1) and the plasma membrane monoamine transporter (PMAT). OCT1 is predominantly expressed in the liver but has also been reported to be expressed on the apical side of both the proximal and distal tubules in the kidney (126). PMAT (SLC29A4) is expressed on the apical membrane of intestinal epithelial cells and has been suggested to be involved in intestinal absorption of metformin (127). Thus, pregnancy may alter metformin PK by changing expression or activity of these transporters. Metformin may also cross the placental barrier via active transport. Umbilical cord concentrations of metformin at the time of delivery have been reported to be from 50% of maternal concentrations to exceeding maternal concentrations in some cases (128-130). Our preliminary data suggest that the clearance of metformin is increased during pregnancy (131). In **Chapter 5,** we described a clinical study in which metformin disposition in pregnant women with GDM was investigated and compared with that in non-pregnant women with type 2 diabetes mellitus (T2DM). This study was also powered to compare the PK of metformin in women receiving 500 mg BID and 1000 mg BID.

Glyburide

Glyburide is a second-generation oral sulfonylurea approved for treatment of T2DM and commonly used in the management of GDM. Glyburide stimulates insulin production by the

pancreas (132). Glyburide is a highly protein bound, lipophilic, low molecular weight compound, which undergoes extensive metabolism in the liver and intestine (133, 134). It is metabolized by CYP2C9, CYP3A4, CYP3A5 and CYP2C19 in the adult liver, and CYP3A7 in the fetal liver (135-138). CYP2C9 is an essential enzyme in the metabolism of many sulfonylureas, such as glipizide, glimepiride, and tolbutamide (139). CYP2C9 contributes to 30% of the formation of glyburide metabolites (140). CYP2C9 is a highly polymorphic enzyme. Multiple clinical studies have shown that subjects with decreased CYP2C9 activity (*CYP2C9*3*) have approximately 40% decreased in glyburide oral clearance and a 200-300% increase in plasma AUC (135, 136, 141). Moreover, the allelic variants *CYP2C9*2, *3, *5, *8, *11*, and **13* result in decreased CYP2C9 activity and **6* is a null allele conferring complete loss of CYP2C9 activity (135, 136, 141-146). A previous study has demonstrated that the systemic clearance of glyburide in pregnant women with GDM is significantly increased (~2-fold) compared to non-pregnant women with T2DM, likely due to induction of CYP3A and CYP2C9 during pregnancy (133). Dose adjustments may, therefore, be required in order to maintain the same efficacy of glyburide in pregnant women.

Glyburide forms at least six metabolites in human liver including M1 (4-trans-hydrocyclohexyl glyburide), M2a (4-cis-hydrocyclohexyl glyburide), M2b (3-cis-hydrocyclohexyl glyburide), M3 (3-trans-hydrocyclohexyl glyburide), M4 (2-trans-hydrocyclohexyl glyburide) and M5 (ethylene-hydroxylated glyburide) (147-150). M1 and M2b are pharmacologically active (151, 152). The activities of M4 and M5 are unknown. Previous work reported the M1/glyburide AUC ratio to be 0.26 ± 0.22(133). Glyburide is not significantly cleared by the kidney (133). High lipophilicity enables glyburide to cross the placenta via passive diffusion. Shuster et al. demonstrated that CYP3A7 is substantially expressed in human

fetal livers and mediates metabolism of glyburide primarily to ethylene-hydroxylated glyburide (M5) (153).

Previous studies on the effects of food on glyburide PK have been inconclusive. Prendergast et al. (154) reported that the oral absorption of glyburide is not affected by co-administration of a meal in non-pregnant patients with T2DM. However, Otoom et al. (155) and Marathe et al. (156) reported that food decreased exposure to glyburide in healthy male and healthy non-pregnant female subjects. No previous studies have been reported regarding the effects of food on glyburide PK in pregnant women. In **Chapter 6,** we described a clinical study in which the effects of food, metformin exposure, and genotype on glyburide PK in pregnant women were investigated and compared to those in non-pregnant women with T2DM. Placental transfer of glyburide and its metabolites were also determined in the clinical study.

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Chapter 2: P-gp/ABCB1 Exerts Differential Impacts On Brain and Fetal Exposure to Norbuprenorphine

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2.1 Introduction

Recent statistics indicate that the prevalence of opioid use among pregnant women ranges from 1-2% to as high as 21% in the United States [1], posing a major risk for maternal morbidities and neonatal complications. Methadone and buprenorphine are prescribed to pregnant women to treat opiate addiction [2, 3]. Although the fetus is not the target of treatments, it is exposed to these drugs *de facto* when pregnant women use the medications. Randomized clinical trials comparing methadone and buprenorphine indicate that both medications are effective in preventing relapse to illicit opioids in opioid-dependent patients. Buprenorphine, however, results in less severe neonatal abstinence syndrome than methadone [4].

Buprenorphine (BUP) is a semisynthetic thebaine derivative that acts as a mixed partial agonist-antagonist opioid receptor modulator [5]. BUP undergoes *N*-dealkylation in the liver, forming the major metabolite norbuprenorphine (NBUP). NBUP is pharmacologically active

with ~25% of buprenorphine's intrinsic analgesic effect [6]. Furthermore, NBUP is a μ-opioid, δ-opioid, and nociceptin receptor full agonist, and a κ-opioid receptor partial agonist. NBUP produces respiratory depression and was 10 times more potent than buprenorphine in rodents [7, 8] and may be fatal to infants [9]. NBUP can be further glucuronidated at the third carbon to norbuprenorphine-3-β-D-glucuronide (NBUP-G), which is also biologically active [6]. Concheiro et al. [10] showed that both NBUP and NBUP-G could be detected in umbilical cord blood samples 5 hours after administration of buprenorphine but before delivery with concentrations greater than those of the parent drug, suggesting that the fetus may have been exposed to substantial levels of pharmacologically active metabolites. To better predict fetal exposure and hence fetal safety of NBUP, it is critical that we first understand the mechanisms that control fetal exposure to NBUP and NBUP-G, which are currently still not clear.

P-gp/ABCB1 and BCRP/ABCG2 are the two most important ABC efflux transporters for drug disposition and have a broad spectrum of substrates, including many drugs routinely used by pregnant women [11-13]. P-gp and BCRP are highly expressed on the apical membrane of the liver hepatocyte, the brain endothelium and the placental syncytiotrophoblasts [14-16]. Hence, both transporters restrict penetration across tissue barriers and facilitate biliary elimination of drugs and xenobiotics. For example, it has been shown that P-gp and BCRP can protect the brain and fetus from potential chemical assaults by actively expelling drugs, xenobiotics and harmful substances from the brain or the fetus to the systemic or maternal circulation [17-21]. Previous studies have shown that P-gp can effectively transport NBUP but not BUP [6, 22] and that P-gp is a major determinant of brain NBUP exposure [6]. BCRP is known to transport glucuronide conjugates of drugs, xenobiotics, and endogenous substances [23]. Given the fact NBUP is a P-gp substrate and NBUP-G is possibly a BCRP substrate, it is important to know whether P-gp or

BCRP restricts fetal exposure to NBUP and/or its glucuronide metabolite. Murine knockout models have been developed to interrogate the effects of P-gp and BCRP on drug disposition [24-27]. Numerous studies using chemical inhibition and genetic knockout of P-gp and BCRP have demonstrated the importance of the two transporters in determining brain or fetal distribution of several prototypic substrates, including methadone, loperamide, fentanyl, glyburide, and nitrofurantoin [18, 19, 28-33]. Therefore, the aim of this study was to investigate whether P-gp and BCRP affect maternal pharmacokinetics of NBUP and its metabolite NBUP-G and whether P-gp and BCRP play important roles in restricting fetal penetration of NBUP and NBUP-G, using wild-type, *Abcb1a*$^{-/-}$/*1b*$^{-/-}$, and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ pregnant mice. It has previously been shown that P-gp in the blood-brain barrier (BBB) and the blood-retinal barrier (BRB) of mouse models has dissimilar impacts on tissue exposure to a substrate drug verapamil, with a much greater effect on limiting brain exposure than retinal exposure [34]. Hence, to investigate whether P-gp and BCRP in the BBB and the blood-placental barrier (BPB) display differential roles in restricting brain and fetal drug exposure, we also examined and compared brain and fetal exposure to NBUP and NBUP-G in the same wild-type and transporter knockout dams.

2.2 Materials and Methods

Materials

Norbuprenorphine (NBUP) and norbuprenorphine-3-β-D-glucuronide (NBUP-G) used in animal studies were provided by the National Institute on Drug Abuse (Bethesda, MD). NBUP, norbuprenorphine-d3 (NBUP-d3) and NBUP-G used for analytical calibrations in LC-MS/MS analysis were from Cerilliant (Round Rock, TX). Optima grade or high-performance liquid

chromatography grade methanol, acetonitrile, polyethylene glycol 400, ethanol, formic acid, DMSO, dimethyl sulfoxide and water were from Thermo Fisher Scientific (Waltham, MA) or Acros Organics (Pittsburgh, PA). Isoflurane was purchased from Piramal Healthcare (Mumbai, India) through the University of Washington Medical Center Pharmacy. Synthetic signature peptides for absolute protein quantification by LC-MS/MS were obtained from New England Peptides (Boston, MA). The corresponding stable-isotope-labeled (SIL) peptides were purchased from Thermo Fisher Scientific (Rockford, IL). The protein extraction kit for ProteoExtract native membrane was from Calbiochem (Temecula, CA). Ammonium bicarbonate and sodium deoxycholate were obtained from Thermo Fisher Scientific and MP Biomedicals (Santa Ana, CA), respectively. BCA protein assay and in-solution trypsin digestion kits, iodoacetamide and dithiothreitol were obtained from Pierce Biotechnology (Rockford, IL).

Animals

Wild-type FVB, *Abcb1a*$^{-/-}$/*1b*$^{-/-}$, and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice, 7-10 weeks of age, were purchased from Taconic Farms (Germantown, NY) and cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council. Mice were maintained under 12-hour light/dark cycles, and food was provided ad libitum. Female mice, 7-10 weeks of age, were mated with male mice of the same genotype and the same age overnight using a female to male ratio of 2:1. Gestation day (gd) 1 was defined as the presence of a sperm plug following overnight housing. Progress of pregnancy was monitored by visual inspection and body weight increase. Body weight was recorded on the day of dosing. Pregnant mice used in this study were on gd 15, which approximately corresponds to the late second trimester in humans. On gd 15, high levels of both P-gp and BCRP are expressed in

mouse placenta, and hence gd 15 was chosen in this study [35-37]. This animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington.

In vivo animal studies

NBUP was dissolved in a solution that contained 0.5 % (v/v) dimethyl sulfoxide, 10% (v/v) ethanol, 39.5% (v/v) saline, and 50% (v/v) polyethylene glycol 400 at a concentration of 0.5 mg/ml. Under 2-5% isoflurane anesthesia, pregnant mice on gd 15 were administered 1 mg/kg NBUP by retro-orbital injection (60-70 µl each mouse). The 1 mg/kg dose was selected based on literature data about elicitation of a significant decrease in respiratory rate in mice and achievement of maternal plasma exposure of NBUP in mice that was comparable to that observed in pregnant women in the late second and early third trimesters [6, 38, 39]. Previous pharmacokinetic studies indicated that the half-life of NBUP in rodents was approximately 0.9 hour [40]. We therefore administered NBUP to pregnant mice and collected blood at time points up to 480 min which is longer than 7 half-lives. Thus, at various times (2, 10, 30, 120, 240, 360, and 480 min) after NBUP administration, mice (n = 3 per time point) were sacrificed under anesthesia by cardiac puncture. Maternal blood was collected in heparinized microcentrifuge tubes (BD Bioscience, San Jose, CA) and centrifuged at 1,000 RCF for 10 min at 4ºC. Plasma was collected and stored at -80ºC until use. Maternal liver and brain as well as individual whole fetuses and placentas were collected from pregnant mice immediately following collection of maternal blood. All the tissues were immediately rinsed with ice-cold PBS, snap-frozen in liquid nitrogen, and stored at -80ºC until analysis.

Simultaneous quantification of NBUP and NBUP-G in maternal plasma and tissues by LC-MS/MS

Maternal brain, maternal liver and fetus were homogenized in 1.0 ml of PBS using an Omni Bead Ruptor Homogenizer (Omni International, Kennesaw, GA) (see below). To every 500 µl of maternal plasma, brain/fetus homogenate or maternal liver extract, 1 ml of acetonitrile and 20 µl of internal standard (2 ng/µl NBUP-d3) were added and mixed. Samples were vortexed for 30 s and centrifuged at 24,000 rpm for 10 min at 4ºC. Supernatants were transferred to clean glass tubes and evaporated using nitrogen gas. Maternal liver homogenate was subjected to solid-phase extraction (SPE) with preconditioned Agilent Bond Elut C_8 cartridges (Santa Clara, CA). Each sample was reconstituted with 100 µl of 1% formic acid in acetonitrile and 5 µl per sample were injected for LC-MS/MS analysis. Calibration curves and quality control samples were prepared along with samples by homogenizing blank tissues and adding analytical grade NBUP or NBUP-G at various concentrations.

Quantification of NBUP and NBUP-G in maternal plasma as well as maternal brain, maternal liver and fetal homogenates by LC-MS/MS was as previously described with some modifications [41]. Briefly, quantification of NBUP and NBUP-G was performed on Agilent 1290 ultra-high-performance liquid chromatograph interfaced with an Agilent 6410B triple-quadrupole mass spectrometer (Aglient Technologies, Palo Alto, CA). The mass spectrometer was operated in positive ion electrospray ionization mode. Chromatographic separation was performed on an Agilent Poroshell 120 SB-C_{18} column (2.1 mm × 55 mm, 2.7 µm) (Santa Clara, CA). The injection volume was 5 µl and the column oven temperature was 35ºC. The HPLC mobile phase (operated at the rate of 0.4 ml/min) was (A) 0.1% formic acid and (B) methanol. The gradient was maintained at 30% B from 0 to 4 min, increased to 35% B from 4 to 6 min,

then declined to 30% B from 6 to 6.5 min, and equilibrated at 30% B until 8 min. Under these conditions, retention times of NBUP and NBUP-G were 6.54 min and 1.91 min, respectively. Both Q1 and Q3 quadrupoles and mass spectrometer conditions were optimized for each analyte. The ionization and fragmentation parameters were set as follows: capillary voltage, 2800 V; gas temperature, 350°C; gas flow rate, 10 L/min; nebulizer, 35 psi; fragmentor, 235 V for NBUP-G and 195 V for NBUP; collision energy, 36 V for NBUP-G and 60 V for NBUP. Multiple reaction monitoring transitions for each analyte and internal standard were *m/z* 414.2>83.1 for NBUP, *m/z* 590.3>414.2 for NBUP-G, and *m/z* 417.2>83.1for NBUP-d3. NBUP-d3 was used as an internal standard for both NBUP and NBUP-G.NBUP and NBUP-G in maternal plasma and tissues were quantified using peak area ratios (NBUP or NBUP-G to NBUP-d3) with calibration curves and quality controls prepared using analytical grade NBUP or NBUP-G dispensed in matching blank mouse matrix (plasma or tissues). The lower quantification limits were 0.5 ng/ml and 2.5 ng/ml for NBUP and NBUP-G, respectively, in maternal plasma. The lower quantification limits were 1 ng/g and 5 ng/g for NBUP and NBUP-G, respectively, in all tissues. For NBUP quantification in both plasmas and tissues, intraday and interday variations were 3.7% and 3.8%, respectively. Intraday and interday variations were 2.7% and 5%, respectively, for NBUP-G quantification in plasma and tissues.

Absolute protein quantification of Abcb1a and Abcb1b in maternal brain and placenta by LC-MS/MS

Absolute protein quantification of Abcb1a and Abcb1b was carried out using a surrogate peptide-based LC-MS/MS method as previously described [42]. Two signature peptides unique for Abcb1a and Abcb1b (Table 1) that are not present in any other known proteins were selected for quantification of each transporter based on previously reported criteria [42]. The selected

signature peptide sequences were NTTGALTTR and NSTGSLTTR for murine Abcb1a and Abcb1b, respectively. The corresponding heavy peptides containing labeled [$^{13}C_6$ $^{15}N_2$]-lysine or [$^{13}C_6$ $^{15}N_4$]-arginine were used as internal standards. Prior to protein quantification, maternal brain and placenta tissues were homogenized in extraction buffer I of the ProteoExtract native membrane protein extraction kit (Calbiochem, Temecula, CA) containing protease inhibitor cocktail according to the manufacturer's instruction. The resulting homogenate was centrifuged at 16,000 RCF for 15 min and the supernatant was discarded. The pellet was resuspended in 1 ml of extraction buffer II with protease inhibitor cocktail and incubated with gentle shaking for 30 min at 4°C followed by centrifugation at 16,000 RCF for 15 min at 4°C. The concentration of total isolated membrane proteins in the supernatant was then determined using the BCA protein assay. The supernatant was diluted to protein concentration of 2 µg per µl or lower. Total membrane proteins were isolated through methanol-chloroform precipitation. Isolated total membrane proteins were reduced, denatured, alkylated and digested as per our previously reported protocol [43]. Samples were stored at -80ºC until analysis.

Abcb1a and Abcb1b were quantified using optimized parameters (Table 1) using AB Sciex Triple Quad 6500 system (Framingham, MA) coupled to Waters® Acquity™ UPLC system (Waters, Hertfordshire, UK). A UPLC column (Acquity UPLC® HSS T3 1.8 µm, 2.1 x 100 mm, Waters) with a Security Guard column (C_{18}, 4 mm × 2.0 mm) from Phenomenex (Torrance, CA) was eluted (0.3 mL/min) with a gradient mobile phase consisting of water and acetonitrile (with 0.1 formic acid; see below). The injection volume was 5 µL (~10 µg of total protein). The mobile phase gradient conditions were 97% A (water containing 0.1% v/v formic acid) and 3% B (acetonitrile containing 0.1% v/v formic acid) held for 3 min, followed by four steps of linear gradient of mobile phase B concentration of 3% to 13%, 13% to 25%, 25% to

50% and 50% to 80% over 3-10 min, 10-20 min, 20-24 min and 24.1-25 min, respectively. This was followed by a washing step using 80% mobile phase B for 0.9 min, and re-equilibration for 4.9 min. The parent to product ion transitions for the analyte peptides and their respective SIL peptides were monitored using optimized LC-MS/MS parameters (Table 1) in ESI positive ionization mode.

Pharmacokinetic data analysis

Maternal plasma, maternal brain, maternal liver, and fetal area-under-the-curve (AUC) concentration values over 0 to 480 min were calculated using the trapezoidal method for each combination of genotype, analyte, and tissue. Adopting a method similar to one described previously [44], 95% confidence intervals of the AUC sample distributions were estimated by bootstrapping the concentration-time data for each genotype-analyte-tissue group. Concentration-time points from the genotype-analyte-tissue dataset of interest were randomly sampled with replacement, the AUC was calculated, and the process was repeated for 10,000 iterations. The 95% confidence interval was the range of AUCs that encompassed the 2.5^{th} to the 97.5^{th} percentiles of the sample distribution. Confidence intervals of AUC ratios were also estimated by bootstrapping 10,000 iterations of the paired tissue to plasma AUCs and metabolite to parent AUCs. For comparisons between genotypes, the null distribution was estimated by the following bootstrapping procedure. Concentration-time points from the two genotypes under comparison were combined, randomly sampled with replacement, and assigned to either genotype with 10,000 iterations. The mixed data generated were used to calculate the AUC and AUC ratios for each genotype, and the distribution of the bootstrapped data was considered an estimate of the null distribution. The *p* value for the comparison between genotypes was calculated as the percent of the iterations that the actual sampled data resulted in a more extreme

AUC and AUC ratios comparison than the bootstrapped estimate of the null distribution. Values were corrected for multiple testing using the Bonferroni correction. The differences were considered statistically significant if the *p* values were less than 0.05.

2.3 Results

The knockout of Abcb1a/1b increases systemic exposure to NBUP and NBUP-G.

To assess the roles of P-gp and BCRP in determining maternal plasma exposure to NBUP and NBUP-G, NBUP was administered to wild-type, *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ pregnant mice on gd 15 by retro-orbital injection of 1 mg/kg NBUP. Fig. 1 depicts mean maternal plasma concentration-time profiles of NBUP (Fig. 1a) and NBUP-G (Fig. 1b) over 480 min. Table 2 presents the maternal plasma $AUC_{0\text{-}480min}$ data on NBUP and NBUP-G. Mean maternal plasma concentrations of NBUP and NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice showed similar increases at times up to 240 min compared to those in wild-type mice (Fig. 1). At 360 and 480 min, maternal plasma concentrations of NBUP, but not NBUP-G, were higher in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice compared to those in wild-type mice (Fig. 1). Consequently, maternal plasma $AUC_{0\text{-}480min}$ of NBUP in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice was significantly higher by 1.8- and 2.2-fold, respectively, compared to that in wild-type mice (Table 2). Maternal plasma $AUC_{0\text{-}480min}$ of NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice was also significantly higher by 3.6-and 4.4-fold, respectively (Table 2). No significant differences in maternal plasma AUCs of NBUP and NBUP-G between *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were observed.

In wild-type mice, maternal plasma concentrations of NBUP-G were approximately 4-9 times higher than those of NBUP at each time point over 480 min (Fig. 1), leading to a maternal

plasma NBUP-G/NBUP AUC ratio of 4.6 (Table 2). The knockout of *Abcb1a/1b* or *Abcb1a/1b*/*Abcg2* increased maternal plasma NBUP-G/NBUP AUC ratio ~2-fold compared to wild-type mice (Table 2). This increase in maternal plasma AUC of the metabolite NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice could be due to an increase in hepatic availability of the parent NBUP for access to metabolism in the liver. Thus, we determined concentrations of NBUP in maternal livers over 480 min as shown in Fig. 2 and calculated the maternal liver $AUC_{0\text{-}480min}$. As expected, the maternal liver $AUC_{0\text{-}480min}$ of NBUP in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ or *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice was significantly higher by ~2-fold compared to that in wild-type mice (Table 3). Maternal liver $AUC_{0\text{-}480min}$ of NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ or *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice also significantly increased by ~2 fold (Table 3). There were no significant differences in maternal liver AUCs of NBUP and NBUP-G between *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice.

Maternal brain exposure to NBUP is strongly limited by Abcb1a/1b, but not by Abcg2

We next examined maternal brain exposure to NBUP in wild-type, *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice. As shown in Fig. 3a, maternal brain concentrations of NBUP in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ or *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were approximately 10-100 times higher than those in wild-type mice at all times over 480 min. The maternal brain $AUC_{0\text{-}480min}$ of NBUP in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ or *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice was significantly increased 54-60-fold compared to that in wild-type mice, respectively (Table 4). No significant difference in maternal brain AUC between *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice was observed. Likewise, the maternal brain-to-maternal plasma AUC ratio of NBUP in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ or *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice was significantly increased 25 to 34-fold compared to that in wild-type mice (Table 4). Again,

the maternal brain-to-maternal plasma AUC ratio for NBUP was not significantly different between *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice, suggesting that the knockout of *Abcg2* had no additional effect on maternal brain exposure to NBUP.

We also compared maternal brain exposure to NBUP-G in wild-type, *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice. The maternal brain concentrations of NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were 2-5 times higher than those in wild-type mice (Fig. 3b). Maternal brain concentrations of NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were consistently higher than those in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ mice at most time points over 480 min (Fig. 3b). Consequently, maternal brain $AUC_{0\text{-}480min}$ of NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice was higher by 2.2- and 5.2-fold, respectively, as compared to that in wild-type mice (Table 4), *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice brain exposure was significantly higher than wild-type mice. However, the maternal brain-to-maternal plasma AUC ratio for NBUP-G was not significantly changed by the knockout of *Abcb1a/1b* or *Abcb1a/1b*/*Abcg2*, with the fold-change varying from 0.62 to 1.3.

Abcb1a/1b and Abcg2 do not restrict fetal distribution of NBUP and NBUP-G

Finally, we investigated whether the knockout of *Abcb1a*/*1b and Abcb1a/1b/Abcg2* had any effects on fetal exposure to NBUP and NBUP-G as they did on maternal brain exposure. As shown in Fig. 4, fetal concentrations of NBUP in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were generally higher than those in wild-type mice (Fig. 4a). As a result, fetal $AUC_{0\text{-}480min}$ of NBUP in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice was significantly increased 1.6-fold as compared to that in wild-type mice (Table 5). The fetal-to-maternal plasma AUC ratio of NBUP remained relatively unchanged in wild-type, *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ *and Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice, with AUC ratios of 0.5-0.6. These results indicate that the increase in fetal NBUP exposure in

transporter knockout mice is most likely driven indirectly by the increase in maternal plasma exposure, and not directly by the knockout of placental *Abcb1a*/*1b* or *Abcb1a/1b*/*Abcg2*.

Fetal concentrations of NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were significantly higher than those in wild-type mice at 6 h and 8 h (Fig. 4b). Fetal $AUC_{0\text{-}480min}$ of NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice was significantly higher by 1.7- and 2.0-fold, respectively, as compared to that in wild-type mice (Table 5). The fetal-to-maternal plasma AUC ratios of NBUP-G in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were decreased by ~45% compared to that in wild-type mice. This decrease was due to much greater maternal NBUP-G exposure in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice versus wild-type mice. No significant differences in fetal NBUP-G exposure between *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were observed (Table 5). Notably, no matter whether in wild-type or transporter knockout mice, fetal AUCs of NBUP-G were 54-65 times higher than fetal AUCs of NBUP (Table 5).

P-gp protein levels in maternal brain and placenta were quantitatively determined by targeted proteomics

One possible explanation of the dissimilar impacts of P-gp on maternal brain and fetal exposure to NBUP could be differential P-gp protein expression in the brain and placenta. We therefore quantified absolute amounts of P-gp protein (both Abcb1a and Abcb1b) in maternal mouse brain and placenta tissues by LC-MS/MS proteomic analysis. The protein levels of Abcb1a and Abcb1b per μg of total membranes in whole mouse placenta were comparable to those in whole maternal mouse brain (Fig. 5a). Since only trophoblasts or brain microvessels are the cells that form the BPB and BBB, respectively, we would have to consider the amounts of Abcb1a and Abcb1b in the cells that form tissue barriers, rather than the amounts of the

transporters in whole tissues. Given the percentage of trophoblasts and brain microvessels out of total numbers of cells in the mouse placenta and brain (~50% [45] and ~6% [46] , respectively, on gd 15), the protein levels of Abcb1a and Abcb1b in the BBB and BPB were estimated and expressed as fmol of Abcb1a and/or Abcb1b protein in trophoblasts per placenta or in brain microvessel endothelial cells per maternal brain as follows: [P-gp concentration in total membrane proteins (fmol/μg membrane protein) × yield of membrane protein isolation (μg/g tissue) × mass of tissue per organ (g of tissue per placenta or maternal brain)] divided by the percentage of trophoblasts out of total cells in the placenta (50%) or the percentage of brain microvessel endothelial cells out of total cells in maternal brain (6%). The yield of total membrane protein isolation from the placenta or maternal brain varied between 60 – 100 μg/g tissues. The average mass of tissue per organ was approximately 0.5 g per maternal brain or 0.05 g per placenta on gd 15. Thus, the density of Abcb1a and Abcb1b protein in the BBB per brain was ~70 and ~30 times greater than that in the BPB per placenta, respectively (Fig. 5b). The density of the sum of Abcb1a/1b protein in the BBB per brain was ~50 times higher than that in the BPB per placenta (Fig. 5b). The assumptions we used in this estimation are that 1) the number of cells is proportional to the weight of cells and 2) P-gp is only expressed in brain endothelial cells or placental trophoblasts that form tissue barriers. Indeed, previous studies have shown that P-gp is highly concentrated in endothelial cells of the brain [47] or syncytiotrophoblasts of the placenta [48].

2.4 Discussion

BUP is increasingly used as an alternative to methadone for treatment of opiate addiction in the pregnant population, as BUP exposure has been associated with lower neonatal abstinence syndrome scores [49, 50]. However, BUP exhibits a ceiling respiratory effect and its primary

active metabolites NBUP and NBUP-G are the potent respiratory depressors in young children. Fetal exposure to NBUP and NBUP-G therefore poses significant risks to the developing fetus. To predict fetal exposure and fetal safety of NBUP, it is important that we first understand the mechanisms that control fetal exposure to NBUP and NBUP-G. In this study, we characterized the roles of P-gp/ABCB1 and BCRP/ABCG2 in modulating penetration of NBUP and NBUP-G across the BBB and BPB using transporter knockout mouse models. At present, little is known regarding the impacts of ABC efflux transporters on fetal exposure to opioids.

We found that, after retro-orbital injection which mimics intravenous administration, maternal plasma AUC of NBUP was significantly increased about 2-fold by the knockout of *Abcb1a/1b*, but additional knockout of *Abcg2* had no further effect (Fig. 1a and Table 2). This suggests that P-gp likely facilitates biliary excretion of NBUP, which is consistent with the fact that NBUP is a substrate of P-gp, but not BCRP [22]. Maternal plasma AUC of NBUP-G was also concurrently increased 3-4-fold and the NBUP-G/NBUP maternal plasma AUC ratio was increased ~2-fold, by the knockout of *Abc1b1a/1b* (Table 2). In contrast, additional knockout of *Abcg2* had no significant further effect on systemic NBUP-G exposure (Table 2). This increase in NBUP-G/NBUP maternal plasma AUC ratio may be explained by one or more of the following factors: 1) a decrease in metabolite clearance; 2) an increase in metabolite formation; and 3) an increase in hepatic bioavailability of NBUP for access to metabolism in the liver. To the best of our knowledge, no data is currently available regarding the effects of the knockout of *Abcb1a/1b* on expression of metabolizing enzymes or transporters for NBUP disposition that may result in the first two scenarios. The third scenario would be expected due to lack of canalicular P-gp in *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ mice to efflux norbuprenorphine into the bile. The inefficiency of norbuprenorphine biliary elimination may result in the apparent increase in hepatic

bioavailability. To test this possibility, we analyzed concentrations and exposure of NBUP in maternal mouse livers and noted that maternal liver AUC of NBUP were indeed significantly increased 2.3-fold by the knockout of *Abcb1a/1b* (Table 3), suggesting that the increase in systemic exposure to NBUP-G is likely caused by the increase in amounts of NBUP available for metabolism in the liver. Nevertheless, the possibility that the knockout of *Abcb1a/1b* alters expression of metabolizing enzymes and transporters and ultimately affects NBUP disposition cannot be completely ruled out.

The site of action of NBUP as an opioid receptor agonist is in the brain. Previous studies have shown that P-gp is a major determinant of brain exposure to NBUP [6]. We noted that maternal brain AUC of NBUP was increased 60-fold by the knockout of *Abcb1a/1b*, but additional knockout of *Abcg2* had no further effect (Fig. 3a and Table 4). Likewise, the maternal brain-to-maternal plasma AUC ratio of NBUP was increased 34-fold by the knockout of *Abcb1a/1b* and additional knockout of *Abcg2* did not further increase the AUC ratio (Table 4). These results again support the notion that P-gp plays a pivotal role in restricting maternal brain NBUP exposure, which is consistent with previous studies [6]. We found that maternal brain AUC of NBUP-G was increased ~2-fold by the knockout of *Abcb1a/1b*, but its maternal brain-to-maternal plasma AUC ratio was decreased by 25% (Table 4), suggesting that P-gp is not a determining factor for brain NBUP-G exposure. Additional knockout of *Abcg2* further increased maternal brain AUC of NBUP-G more than ~2-fold over maternal brain NBUP-G exposure in *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ mice (Table 4), indicating that brain penetration of NBUP-G is restricted by *Abcg2*. This is consistent with the notion that NBUP-G is possibly a BCRP, but not P-gp substrate, based upon current understanding of substrate specificity for P-gp and BCRP.

We determined that fetal AUC of NBUP was ~64% of maternal plasma AUC in wild-type mice (Fig. 4 and Table 5); in contract, maternal brain AUC of NBUP accounted for ~20% of maternal plasma AUC (Table 4), suggesting that fetal NBUP exposure is substantial. Caution therefore should be taken regarding fetal safety of NBUP when BUP is used by pregnant women given the fact that maternal plasma concentrations of NBUP could be as high as or even greater than those of BUP in pregnant women [51]. We also found that fetal AUC of NBUP was increased 1.6 fold by the knockout of *Abcb1a/1b or Abcb1a/1b/Abcg2*, but its fetal-to-maternal plasma AUC ratio remained relatively unchanged (Table 5), indicating that the increase in fetal NBUP exposure in *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice is primarily driven by the increase in maternal plasma exposure. Therefore, unlike in the BBB, P-gp in the BPB plays a minor role in restricting fetal NBUP exposure. Recently, Eyal et al. showed that chemical inhibition of P-gp in non-human primate increased maternal brain distribution of [^{11}C]-verapamil ~4-fold, but its fetal liver distribution (as a surrogate of fetal exposure) was increased only ~2-fold [52]. Chapy et al. reported that the knockout of *Abcb1a/1b* increased [^{3}H]-verapamil distribution across the BBB and blood-retinal barrier (BRB) ~10- and ~1.5-fold, respectively [34]. Thus, our results regarding dissimilar impacts of P-gp on NBUP distribution across the BBB and BPB are consistent with previous studies. It appears that the BBB is generally tighter than other tissue barriers such as BPB and BRB.

The dissimilar impacts of P-gp on drug distribution across different tissue barriers may be explained by the following factors. First, this differential effect could be attributable to differences in P-gp protein expression. To test this possibility, we performed absolute protein quantification of P-gp (both Abab1a and Abcb1b) in maternal mouse brain and placenta tissues by LC-MS/MS proteomics. After having taken into account of the percentage of trophoblasts or

brain microvessels out of total cells in mouse placenta or maternal brain, respectively, we found that the density of Abcb1a/1b in the BBB was ~50 times greater than that in the BPB (Fig. 5b). In other words, the BBB expresses much more P-gp than the BPB, thus contributing to the greater impact of P-gp on brain drug exposure versus fetal drug exposure. Second, physical characteristics of tissue barriers could also have an effect. It is a general consensus that the BBB possesses the tightest junction between brain capillary endothelial cells. In contrast, the BPB possesses numerous paracellular junctions which are quite different from the brain endothelial junctions, showing no positional preference (luminal or abluminal) and being interspersed with adherens junctions along the paracellular cleft [53]. These clefts would allow significant passive diffusion of drug molecules across the BPB. Hence, the fraction of drug transported by P-gp across the BPB may be smaller than that across the BBB, resulting in smaller effects of P-gp on drug distribution across the BPB versus the BBB. Finally, other factors such as tissue blood flow rate and expression of other transporters may also play a role. The placenta is a much more perfused organ than the brain, and hence placenta blood flow rate would be expected to facilitate passive diffusion, thus decreasing the impact of transporters. If the knockout of *Abcb1a/1b* alters expression of other efflux or uptake transporters in the BBB and BPB that are able to transport NBUP, this could also affect the role of P-gp in determining fetal or brain exposure. We would like to point out that whether P-gp exerts dissimilar impacts on brain and fetal drug exposure are possibly drug-dependent. Previous studies have shown that the knockout of P-gp increased fetal exposure to paclitaxel ~16-fold [17] and brain exposure ~8-fold [54]. Thus, P-gp could have a comparable or a greater effect on fetal exposure compared to brain exposure to paclitaxel. Nevertheless, since a previous study only analyzed paclitaxel fetal concentrations at single time points [17], definitive comparisons between NBUP and paclitaxel could not be made.

Fetal NBUP-G exposure was ~54 times higher than fetal NBUP exposure in the wild-type mice, and this was not affected by the knockout of *Abcb1a/1b* or *Abcb1a/1b/Abcg2* (Table 5). Given that maternal plasma exposure of NBUP-G was only 5-9 times higher than maternal plasma exposure of NBUP in wild-type and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ or *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice (Table 2), this drastic increase in fetal NBUP-G exposure cannot be explained solely by the increase in maternal NBUP-G exposure. Since fetal NBUP exposure was actually decreased in pregnant mice of all three genotypes compared to maternal NBUP exposure (with a fetal-to-maternal plasma AUC ratio of 0.5-0.6) (Table 5), the drastic increase in fetal NBUP-G exposure is likely caused by metabolism of NBUP in the fetus and/or placenta, rather than maternal plasma driven. Buckley et al. [55] have shown that Ugt1a, Ugt2a, Ugt2b and Ugt3a are expressed in mouse placenta. Although studies of rodent fetal livers showed glucuronidation activity, expression of specific Ugt isoforms in mouse fetal livers has not yet been fully described [56, 57]. In *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice, maternal plasma AUC of NBUP-G was increased 3-4-fold compared to wild-type mice (Table 2); however, fetal AUC of NBUP-G was increased only 1.7-2.0-fold (Table 5), indicating a low membrane permeability of NBUP-G which makes sense as NBUP-G is much more water soluble than its parent NBUP. Unlike in the brain, the additional knockout of *Abcg2* only slightly increased fetal NBUP-G exposure, suggesting that BCRP is not a major determinant for fetal NBUP-G exposure. These results suggest that NBUP metabolism in the fetus and/or placenta could play a significant role in determining fetal exposure to NBUP-G.

2.5 Tables and Figures

Table 2-1: Surrogate peptides of Abcb1a and Abac1b and their MS/MS parameters

Protein name	Surrogate peptide	Peptide type	Parent ion (*m/z*)	Fragment ion (*m/z*)	Declustering potential (V)	Collision energy (V)
Abcb1a	NTTGLTTR	Light	467.75	719.41	60	21
Abcb1a	NTTGLTTR	Light	467.75	618.36	60	21
Abcb1a	NTTGLTTR	Light	467.75	490.3	60	21
Abcb1a	NTTGLTTR	Heavy	472.75	729.42	60	21
Abcb1a	NTTGLTTR	Heavy	472.75	628.37	60	21
Abcb1b	NSTGSLTTR	Light	468.74	634.35	60	21
Abcb1b	NSTGSLTTR	Light	468.74	735.4	60	21
Abcb1b	NSTGSLTTR	Light	468.74	577.33	60	21
Abc11b	NSTGSLTTR	Heavy	473.74	644.36	60	21
Abcb1b	NSTGSLTTR	Heavy	473.74	745.41	60	21

Table 2-2: Maternal plasma AUCs of norbuprenorphine (NBUP) and norbuprenorphine-3-β-D-glucuronide (NBUP-G) after retro-orbital injection (1 mg/kg) to pregnant mice on gd 15 over 480 min. Data are reported as mean (95% confidence interval). The NBUP-G/NBUP AUC ratios were calculated based on molar concentrations of NBUP and NBUP-G in maternal plasma. Differences between the analyte AUCs or the AUC ratios of wild-type and *Abcb1a*$^{-/-}$*/1b*$^{-/-}$or *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ mice were calculated using bootstrapping as described in the Materials and methods section. The *p* values were corrected for multiple testing using the Bonferroni method.

Parameter	Genotype				
	Wild-type	*Abcb1a*$^{-/-}$*/1b*$^{-/-}$	*p*	*Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$	*p*
$AUC_{0\text{-}480min}$ of NBUP (μg×min×mL^{-1})	4.1 [3.6, 5.8]	7.1 [6.2, 9.4]	< 0.005	8.7 [7.1, 11.3]	< 0.005
Fold-change	1.0	1.8		2.2	
$AUC_{0\text{-}480min}$ of NBUP-G (μg×min×mL^{-1})	26.8 [21.8, 34.2]	95.0 [75.5,120.8]	<0.0001	116.7 [84.4, 154.1]	< 0.0001
Fold-change	1.0	3.6		4.4	
NBUP-G/NBUP maternal plasma AUC ratio	4.6 [3.7, 5.8]	9.4 [7.1, 11.3]	0.09	9.4 [6.1, 13.5]	0.3
Fold-change	1.0	2.0		2.0	

Table 2-3: Maternal liver tissue AUCs of norbuprenorphine (NBUP) and norbuprenorphine-3-β-D-glucuronide (NBUP-G) after retro-orbital injection (1 mg/kg) to pregnant mice on gd 15 over 480 min. Data are reported as mean (95% confidence interval). Differences between the analyte AUCs or the AUC ratios of wild-type and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$or *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were calculated using bootstrapping as described in the Materials and methods section. The *p* values were corrected for multiple testing using the Bonferroni method.

Parameter	Genotype				
	Wild-type	*Abcb1a*$^{-/-}$/*1b*$^{-/-}$	*p*	*Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$	*p*
$AUC_{0\text{-}480min}$ of NBUP (µg×min×g^{-1})	4.2 [3.6, 5.1]	9.9 [8.4, 11.8]	< 0.005	8.3 [6.7, 10.2]	< 0.05
Fold-change	1.0	2.3		2.0	
$AUC_{0\text{-}480min}$ of NBUP-G (µg×min×g^{-1})	148.4 [118.9, 179.2]	338.0 [297.2, 376.8]	< 0.0001	252.4 [229.4, 272.8]	<0.0001
Fold-change	1.0	2.3		1.7	

Table 2-4: Maternal brain AUCs of norbuprenorphine (NBUP) and norbuprenorphine-3-β-D-glucuronide (NBUP-G) after retro-orbital injection (1 mg/kg) to pregnant mice on gd 15 over 480 min. Data are reported as mean (95% confidence interval). Differences between the analyte AUCs or the AUC ratios of wild-type and *Abcb1a*$^{-/-}$*/1b*$^{-/-}$or *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ mice were calculated using bootstrapping as described in the Materials and methods section. The *p* values were corrected for multiple testing using the Bonferroni method.

Parameter	Genotype				
	Wild-type	*Abcb1a*$^{-/-}$*/1b*$^{-/-}$	*p*	*Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$	*p*
Brain $AUC_{0\text{-}480min}$ of NBUP (μg×min×g^{-1})	0.8 [0.7, 0.9]	47.3 [39.9 52.4]	< 0.0001	42.8 [37.0, 47.9]	< 0.0001
Fold-change	1.0	60.1		54.4	
Brain/Maternal plasma AUC ratio of NBUP	0.2 [0.1, 0.3]	6.7 [4.7, 8.1]	< 0.01	4.9 [3.2, 6.5]	< 0.01
Fold-change	1.0	34.1		24.5	
Brain $AUC_{0\text{-}480min}$ of NBUP-G (μg×min×g^{-1})	10.7 [8.4, 13.1]	23.6 [20.6, 27.4]	0.8	56.1 [48.1, 66.3]	<0.005
Fold-change	1.0	2.2		5.2	
Brain/Maternal plasma AUC ratio of NBUP-G	0.4 [0.3, 0.5]	0.25 [0.18, 0.37]	0.26	0.48 [0.36, 0.65]	0.9
Fold-change	1.0	0.6		1.2	

Table 2-5: Fetal AUCs of norbuprenorphine (NBUP) and norbuprenorphine-3-β-D-glucuronide (NBUP-G) after retro-orbital injection (1 mg/kg) to pregnant mice on gd 15 over 480 min. Data are reported as mean (95% confidence interval). The NBUP-G/NBUP AUC ratios were calculated based on molar concentrations of NBUP and NBUP-G in the fetus. Differences between the analyte AUCs or the AUC ratios of wild-type and *Abcb1a*$^{-/-}$*/1b*$^{-/-}$or *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ mice were calculated using bootstrapping as described in the Materials and methods section. The *p* values were corrected for multiple testing using the Bonferroni method.

Parameter	Genotype				
	Wild-type	Abcb1a/1b$^{-/-}$	p	Abcb1a/1b;Abcg2$^{-/-}$	p
Fetal $AUC_{0\text{-}480min}$ of NBUP (µg×min×g^{-1})	2.5 [2.0, 2.9]	4.0 [3.5, 4.5]	< 0.005	4.1 [3.5, 4.6]	< 0.05
Fold-change	1.0	1.6		1.6	
Fetal/Maternal plasma AUC ratio of NBUP	0.64 [0.40, 0.83]	0.56 [0.39, 0.63]	1.0	0.45 [0.28, 0.60]	0.6
Fold-change	1.0	0.87		0.71	
Fetal $AUC_{0\text{-}480min}$ of NBUP-G (µg×min×g^{-1})	189.2 [159.2, 199.2]	314.5 [266.0, 363.1]	< 0.0001	373.5 [287.6, 485.1]	< 0.0001
Fold-change	1.0	1.7		2.0	
Fetal/Maternal plasma AUC ratio of NBUP-G	7.2 [4.8, 9.2]	3.31 [2.1, 4.6]	0.2	3.2 [2.0, 4.8]	0.2
Fold-change	1.0	0.46		0.45	
NBUP-G/NBUP fetal AUC ratio	53.9 [53.9, 54.6]	55.9 [46.4, 66.3]	0.9	64.6 [50.2, 83.3]	0.9
Fold-change	1.0	1		1.2	

Figure captions

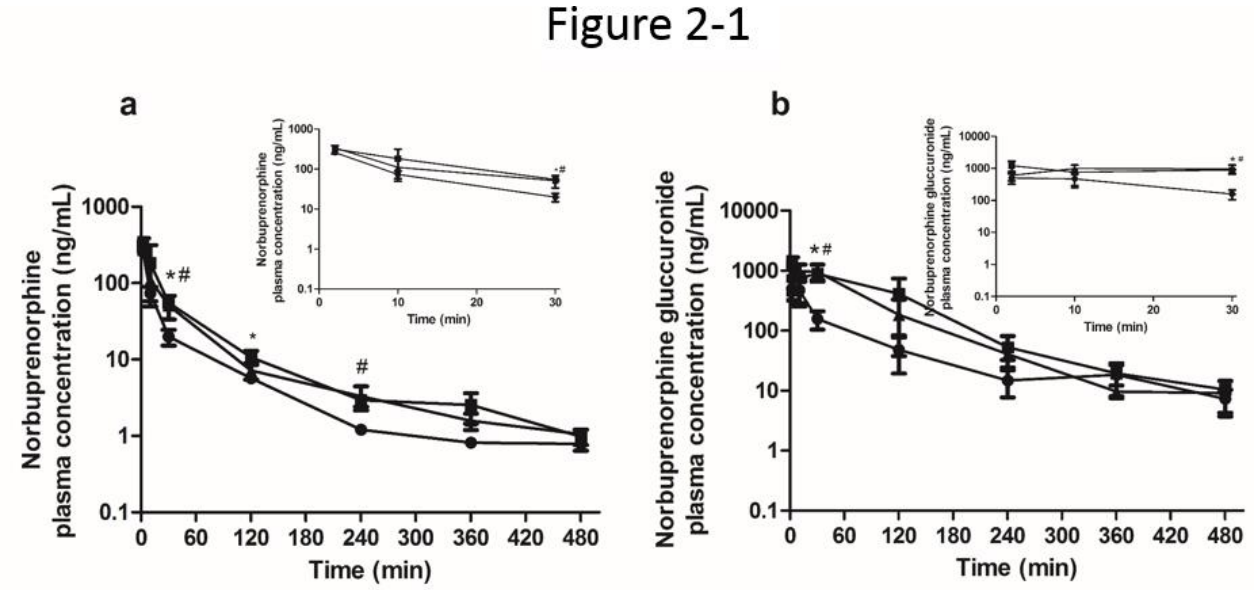

Figure 2-1. Maternal plasma concentration-time profiles. Shown are maternal plasma concentration-time profiles of norbuprenorphine (**a**) and norbuprenorphine-β-D-glucuronide (**b**) over 480 min after retro-orbital injection of 1 mg/kg norbuprenorphine to pregnant wild-type (circle), *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ (triangle) and *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ (square) mice. Data are shown as means ± SD from three mice at each time point. Statistically significant differences between wild-type and *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ or *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ mice were analyzed by ANOVA analysis for multiple comparisons followed by a Bonferroni correction assuming a significance of 0.05. * indicates $p < 0.05$ for *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ mice; # indicates $p < 0.05$ for *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ mice. Inserts show early time points up to 30 min in an expanded format.

Figure 2-2

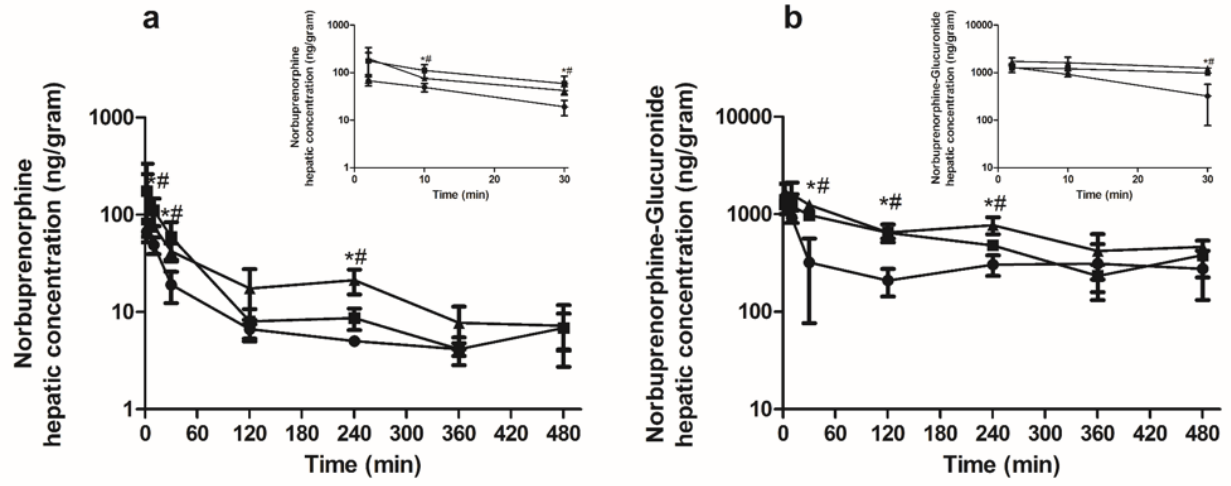

Figure 2-2. Maternal liver concentration-time profiles. Shown are maternal liver concentrations of norbuprenorphine (**a**) and norbuprenorphine-β-D-glucuronide (**b**) over 480 min after retro-orbital injection of 1 mg/kg norbuprenorphine to pregnant wild-type (circle), *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ (triangle) and *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ (square) mice. Data are shown as means ± SD from three mice at each time point. Statistically significant differences between wild-type and *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ or *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ mice were analyzed by ANOVA analysis for multiple comparisons followed by a Bonferroni correction assuming a significance of 0.05. * indicates $p < 0.05$ for *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ mice; # indicates $p < 0.05$ for *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ mice. Inserts show early time points up to 30 min in an expanded format.

Figure 2-3

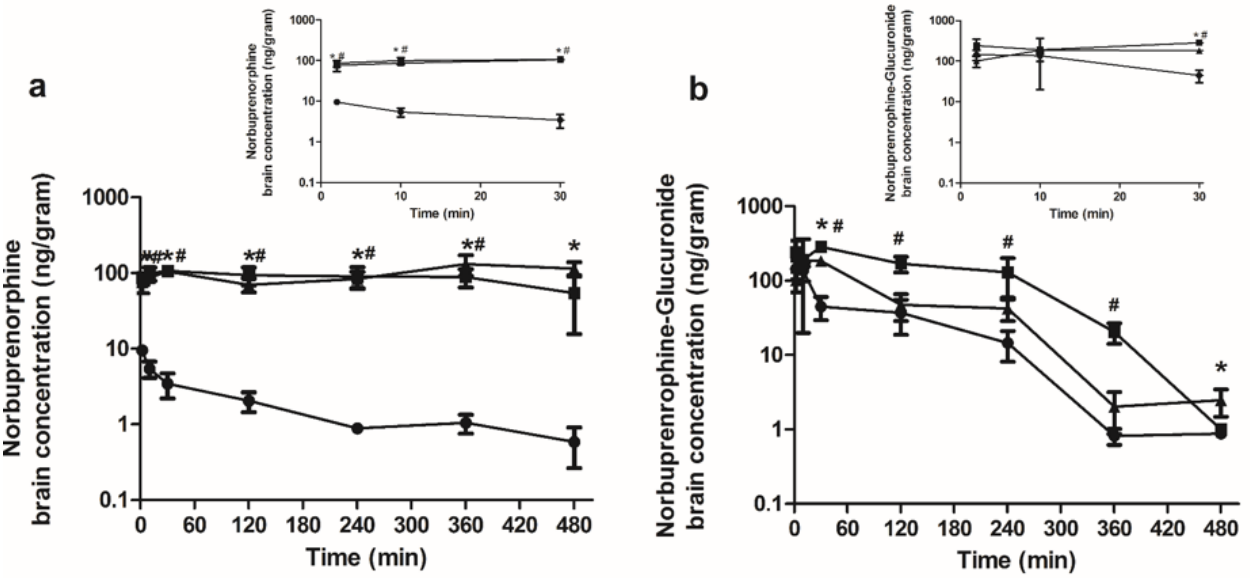

Figure 2-3. Maternal brain concentration-time profiles. Shown are maternal brain concentration-time profiles of norbuprenorphine (**a**) and norbuprenorphine-β-D-glucuronide (**b**) over 480 min after retro-orbital injection of 1 mg/kg norbuprenorphine pregnant wild-type (circle), *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ (triangle) and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ (square) mice. Data are shown as means ± SD from three mice at each time point. Statistically significant differences between wild-type and *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ or *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice were analyzed by ANOVA analysis for multiple comparisons followed by a Bonferroni correction assuming a significance of 0.05. * indicates $p < 0.05$ for *Abcb1a*$^{-/-}$/*1b*$^{-/-}$ mice; # indicates $p < 0.05$ for *Abcb1a*$^{-/-}$/*1b*$^{-/-}$/*Abcg2*$^{-/-}$ mice. Inserts show early time points up to 30 min in an expanded format.

Figure 2-4

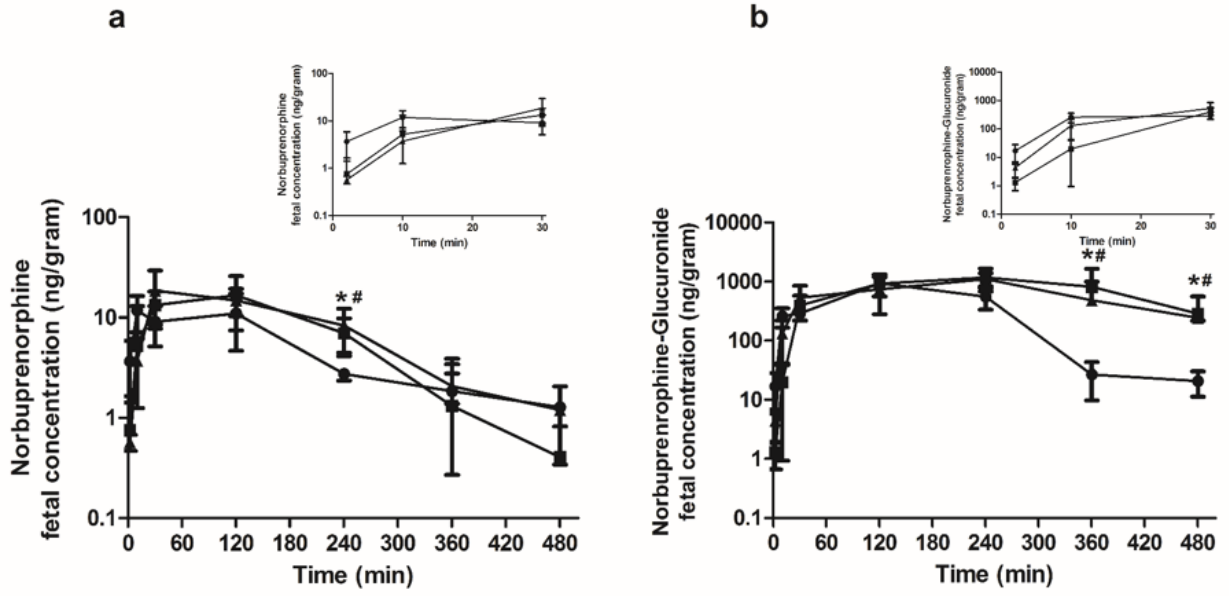

Figure 2-4. Fetal concentration-time profiles. Shown are fetal concentration-time profiles of norbuprenorphine (**a**) and norbuprenorphine-β-D-glucuronide (**b**) over 480 min after retro-orbital injection of 1 mg/kg norbuprenorphine to pregnant wild-type (circle), *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ (triangle) and *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ (square) mice. Data are shown as means ± SD from three mice at each time point. Statistically significant differences between wild-type and *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ or *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ mice were analyzed by ANOVA analysis for multiple comparisons followed by a Bonferroni correction assuming a significance of 0.05. * indicates $p < 0.05$ for *Abcb1a*$^{-/-}$*/1b*$^{-/-}$ mice; # indicates $p < 0.05$ for *Abcb1a*$^{-/-}$*/1b*$^{-/-}$*/Abcg2*$^{-/-}$ mice. Inserts show early time points up to 30 min in an expanded format.

Figure 2-5

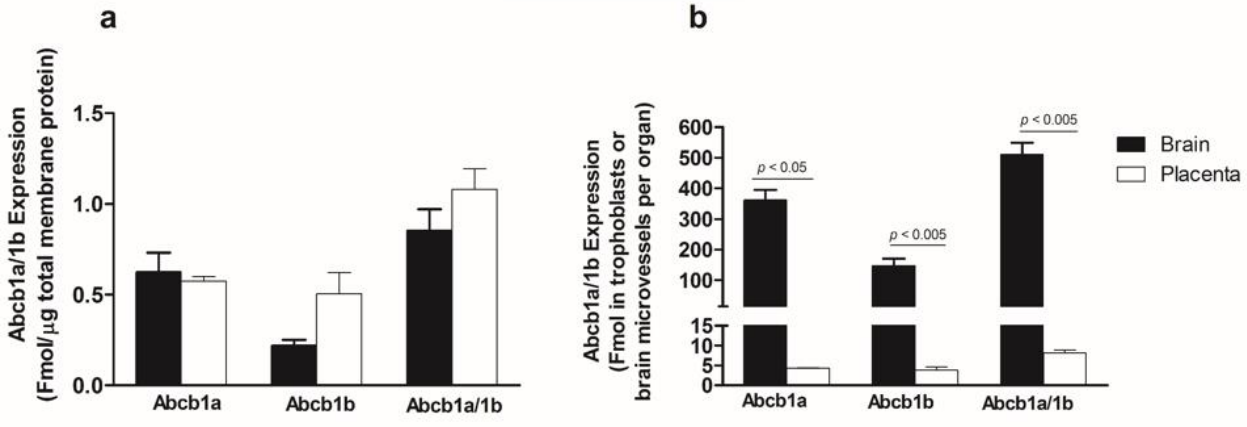

Figure 2-5. Absolute protein quantification of Abcb1a and Abcb1b protein in maternal brain and placenta. Shown are absolute protein quantifications of Abcb1a and Abcb1b in maternal brain (black bar) and placenta (open bar) in total membrane proteins (**a**) or scaled to expression in cells that form the mouse blood-brain and blood-placental barriers (**b**). Data shown are means ± SD in tissues collected from three different mice. Statistically significant differences between maternal brain and placenta were analyzed by unpaired Student *t*-test. Differences with *p* values of < 0.05 were considered statistically significant. Inserts show early time points up to 30 min in an expanded format.

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Chapter 3: Pregnancy Increases Norbuprenorphine Clearance in Mice by Induction of Hepatic Glucuronidation

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3.1 Introduction

Opioid addiction cases in the United States continue to escalate as a top national health crisis. Opioid addiction as a result of prescription opioid use during pregnancy accounted for 28% of admissions of pregnant women to treatments of drug abuse from 1992 to 2012 (1). Untreated substance abuse during pregnancy increases the risk for adverse pregnancy outcomes and fetal anomalies. Due to the dual risk to the mother and fetus, opioid addiction treatment is an increasing priority for this understudied population. Although methadone as a medication-assisted treatment for opioid addiction is the gold standard, recent research supports the use of

buprenorphine (BUP) for opioid treatment during pregnancy. Randomized clinical trials comparing methadone and BUP indicate that both medications are effective in preventing relapse in opioid-dependent patients (2). Moreover, increasing clinical research suggest that the use of BUP results in more favorable maternal and fetal outcome (shorter hospital stay, shorter duration of treatment for neonatal abstinence syndrome) compared to methadone (3).

BUP (Fig. 1) is a semisynthetic μ-opioid receptor agonist and κ-opioid and δ-opioid receptor antagonist. BUP is metabolized by cytochrome P450 3A (CYP3A) through *N*-dealkylation to norbuprenorphine (NBUP) (Fig. 1). NBUP is the major active metabolite of BUP in humans and rodents with ~25% of BUP's intrinsic analgesic activity (4). Both BUP and NBUP can be further metabolized primarily to buprenorphine-3-β-D-glucuronide and norbuprenorphine-3-β-D-glucuronide (NBUP-G, Fig. 1), respectively, by uridine 5'-diphospho-glucuronosyltransferases (UGTs) (5). A mass balance study on BUP revealed that NBUP and NBUP-G exposure accounted for >10% of the total BUP plasma exposure (6). As per FDA Safety Testing of Drug Metabolite Guidance for Industry, human metabolites formed at >10% of total drug-related exposure at steady state could raise safety concerns (7). Given the fact that NBUP can produce respiratory depression 10 times more potent than BUP in rodents (8) and possibly in infants (9), drug-drug interactions with BUP leading to elevated systemic concentrations of NBUP may contribute to an increased risk of neurorespiratory depression. Therefore, in order to optimize BUP's dosing regimen with better efficacy and safety, we need to understand the disposition and pharmacokinetics (PK) of not only BUP but also NBUP. While the disposition of BUP has been extensively studied (10-12) and the dose-normalized plasma AUC of BUP has been shown to be significantly decreased about 2-fold by pregnancy in pregnant women (13), much less is known about NBUP in this regard, particularly during

pregnancy. Pregnancy causes drastic changes in hormone production, such as elevated levels of estrogens, progesterone, cortisol and growth hormones, which may be responsible for altered expression and activity of drug metabolizing enzymes (14). As a result, some UGTs may be upregulated by pregnancy (15-17). Given that NBUP is extensively metabolized by UGTs (18, 19), whether the disposition of NBUP is altered by pregnancy due to induction of glucuronidation warrants investigation. No such studies have been reported so far.

Therefore, in this study, we first determined the PK of NBUP and NBUP-G in pregnant and non-pregnant mice. We then demonstrated whether the intrinsic clearance of NBUP by glucuronidation and Ugt protein expression in the mouse liver were increased by pregnancy. The data obtained will facilitate mechanistic understanding of changes in the disposition of NBUP during pregnancy, which could be important to inform therapeutic strategies in pregnant women for BUP and drugs that are primarily metabolized by UGTs.

3.2 Materials and Methods

Materials

NBUP and NBUP-G used for animal studies were provided by the National Institute on Drug Abuse (Bethesda, MD). NBUP, norbuprenorphine-d3 (NBUP-d3) and NBUP-G used for analytical calibrations in LC–MS/MS assays were from Cerilliant (Round Rock, TX). Optima grade or high-performance liquid chromatography grade methanol, acetonitrile, polyethylene glycol 400, ethanol, formic acid, dimethyl sulfoxide (DMSO) and water were from Thermo Fisher Scientific (Waltham, MA) or Acros Organics (Pittsburgh, PA). Isoflurane was purchased from Piramal Healthcare (Mumbai, India) through the University of Washington Medical Center Pharmacy. UDP-glucuronic acid (UDPGA) and alamethicin (from *Trichoderma viride*) were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic signature peptides for LC-MS/MS

protein quantification were obtained from New England Peptides (Boston, MA). The corresponding stable-isotope-labeled (SIL) peptides were from Thermo Fisher Scientific (Rockford, IL). The ProteoExtract native membrane protein extraction kit was from Calbiochem (Temecula, CA). Ammonium bicarbonate and sodium deoxycholate were from Thermo Fisher Scientific and MP Biomedicals (Santa Ana, CA), respectively. BCA protein assay and in-solution trypsin digestion kits, iodoacetamide and dithiothreitol were obtained from Pierce Biotechnology (Rockford, IL).

Animal studies

This animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington. Wild-type FVB mice, 7-10 weeks of age, were purchased from Taconic Farms (Germantown, NY) and cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council. Mice were maintained under 12-hour light/dark cycles, and food and water were provided ad libitum. Female mice were mated with male mice of the same age overnight using a female to male ratio of 2:1. Gestation day (gd) 1 was defined as the presence of a sperm plug following overnight housing. Increase in body weight was used as an indicator for healthy pregnancy. Body weight was recorded on the day of dosing. Pregnant mice used in this study were on gd 15, which approximately corresponds to the late second trimester in humans. Non-pregnant female mice were compared to pregnant mice throughout this study with respect to the PK of NBUP and its derived glucuronidated metabolite NBUP-G.

The animal study protocol was essentially the same as previously described (20). Briefly, NBUP was dissolved in a solution that contained 0.5 % (v/v) dimethyl sulfoxide, 10% (v/v) ethanol, 39.5% (v/v) saline, and 50% (v/v) polyethylene glycol 400 at a concentration of 0.5

mg/ml. Under 2-5% isoflurane anesthesia, each pregnant or non-pregnant mouse was administered the same dose of 33 μg of NBUP by retro-orbital injection. This dose was selected based on literature data about elicitation of a significant decrease in respiratory rate in mice and the achievement of plasma concentration of NBUP in mice (4) that was comparable to that observed in pregnant women in the late second and early third trimesters (21). Previous pharmacokinetic studies showed that the half-life of NBUP in rodent was approximately 0.9 hour (22). We therefore administered NBUP to pregnant and non-pregnant female mice and collected blood at time points up to 720 min which is longer than 10 half-lives. Thus, at various times (2, 30, 120, 240, 480 and 720 min) after NBUP administration, mice (n = 3 per time point) were sacrificed under anesthesia by cardiac puncture. Blood samples were collected in heparinized microcentrifuge tubes (BD Bioscience, San Jose, CA) and centrifuged at 1,000 × g for 10 min at 4ºC. Plasma was collected and stored at -80ºC until use. At the first two time points, blood samples were collected under the same anesthetic used for the NBUP administration. Liver tissues were collected at the same time points as blood samples. NBUP and NBUP-G in plasma samples were quantified by a validated LC-MS/MS assay as previously described (20).

Plasma protein binding of NBUP

Protein binding of NBUP in mouse plasma was determined by ultrafiltration using Microcon-10 kDa Centrifugal Filter with Ultracel-10 membranes (EMD Millipore Corporation, Billerica, MA) at ~1 μM which is the C_{max} of NBUP in pregnant mice determined in this study. Plasma protein binding by ultrafiltration method was performed as previously described (23). Briefly, NBUP in methanol was aliquoted into 1.5 ml Eppendorf tubes and evaporated to dryness. Plasma from pregnant or non-pregnant mice (gd 15) was added to each tube to a total volume of 220 μl. Samples were briefly vortexed and allowed to equilibrate for 30 min at 37°C.

Two 100-μl aliquots from each sample were transferred to ultrafiltration cartridges, equilibrated for 30 min at 37°C, and centrifuged at 1000 × *g* for 20 min. NBUP in three microliters of filtrate and unfiltered plasma was quantified by LC-MS/MS as previously described (20). The fraction unbound (f_u) of NBUP was calculated as the percentage of the concentration of NBUP in filtrate to the concentration of NBUP in corresponding unfiltered plasma. This plasma protein binding study was repeated with 6 independent experiments with each done in in triplicate.

NBUP-G formation kinetics in mouse liver microsomes from pregnant and non-pregnant mice

Microsomes were prepared from mouse liver tissues isolated from pregnant and non-pregnant mice as previously described (23). Briefly, every 1 g of mouse liver tissue was homogenized in 3 ml of homogenization buffer (50 mM KPi buffer containing 0.25 M sucrose and 1 mM EDTA) using an Omni Bead Ruptor Homogenizer (Omni International). The homogenate was centrifuged at 15,000 × *g* for 30 min at 4°C, and the supernatant was centrifuged at 120,000 × *g* for 70 min at 4°C. Microsomal pellets were re-suspended in the washing buffer (10 mM KPi, 0.1 mM KCl, and 1 mM EDTA, pH 7.4) and then centrifuged at 120,000 × *g* for 70 min at 4°C. Microsomal pellets were finally resuspended in 1 ml of storage buffer (50 mM KPi, 0.25 M sucrose, and 10 mM EDTA, pH 7.4), and stored at −80°C until use. Microsomal protein concentrations were determined using the BCA protein assay.

NBUP-G formation kinetics were determined with substrate (NBUP) concentrations up to 400 μM. Microsomes were diluted to a final concentration of 1 mg/ml of protein in 100 μl of 0.1 M Tris/HCl buffer (pH7.4) containing 8 mM $MgCl_2$. The microsomes were activated by adding alamethicin at a concentration of 25 μg/mg microsomal protein and left on ice for 15 min. The NBUP solutions and microsomes were then mixed and pre-incubated for 5 min at 37°C, and the

reaction was initiated by adding 2 mM UDPGA. Concentrations of NBUP used in the microsome incubations were selected based on the K_m value (~144 μM) of UGTs for NBUP (18). Incubations without UDPGA were used as negative controls. Our preliminarily experiments indicated that the NBUP-G formation was linear up to 10 min. Therefore, an incubation time of 10 min was used for all subsequent microsome incubations. All reactions were stopped by the addition of 100 μl of ice-cold methanol. The kinetic experiments were repeated independently three times, each with triplicate determinations at each substrate concentration. After the addition of 20 μl NBUP-d_3 (internal standard, 5 ng/μl), samples were briefly vortexed and centrifuged at 20,800 × *g* for 10 min at 4°C. The supernatant was transferred to a 96-well plate and subjected to LC-MS/MS as previously described (20) and NBUP-G was quantified. K_m and V_{max} of NBUP-G formation kinetics were estimated by fitting the Michaelis-Menten equation to the experimental data with nonlinear regression using the GraphPad Prism software (GraphPad Prism 5.01, La Jolla, CA). The intrinsic clearance CL_{int} was calculated by dividing the V_{max} by the K_m (V_{max}/K_m).

Protein quantification of Ugts in pregnant and non-pregnant mouse livers by LC-MS/MS

Protein quantification of murine Ugt isoforms (Ugt1a1, Ugt1a6, Ugt1a8, Ugt1a10, Ugt2b1, and Ugt2b35) in pregnant and non-pregnant mouse livers was carried out using a surrogate peptide-based LC-MS/MS method as previously described (24). One or two signature peptides unique for the Ugt isoforms (Table 1) were selected for quantification of each enzyme based on previously reported criteria (24). The corresponding heavy peptides containing labeled [$^{13}C_6$ $^{15}N_2$]-lysine or [$^{13}C_6$ $^{15}N_4$]-arginine were used as internal standards (Table 1). Prior to protein quantification, liver tissues from pregnant and non-pregnant mice were homogenized in

extraction buffer I of the ProteoExtract native membrane protein extraction kit containing protease inhibitor cocktail according to the manufacturer's instruction. The resulting homogenate was centrifuged at 16,000 × g for 15 min and the supernatant was discarded. The pellet was resuspended in 1 ml of extraction buffer II with protease inhibitor cocktail and incubated with gentle shaking for 30 min at 4°C, followed by centrifugation at 16,000 × g for 15 min at 4°C. The concentration of total isolated membrane proteins in the supernatant was then determined using the BCA protein assay. The supernatant was diluted to protein concentration of 2 µg per µl. The total membrane proteins were reduced, denatured, alkylated, desalted and digested as per our previously reported protocol (25). Twenty µl of heavy peptide internal standard cocktail (prepared in acetonitrile:water, 80:20 (v/v) with 0.5% formic acid) and 10 µl of acetonitrile:water 80:20 (v/v) containing 0.1% formic acid was added to each sample. After mixing and centrifugation at 4000 × g (4°C) for 5 min, samples were transferred to LC-MS autosampler vials and stored at -20ºC until analysis.

The Ugts were quantified using optimized parameters (Table 1) on the AB Sciex Triple Quad 6500 system (Framingham, MA) coupled to Waters® Acquity™ UPLC system, M-Class (Waters, Hertfordshire, UK). The volume for sample injection was 5 µL that contained ~10 µg of total protein. The UPLC column (Acquity UPLC® HSS T3 1.8 µm, 2.1 x 100 mm, Waters) with a Security Guard column (C_{18}, 4 mm × 2.0 mm) from Phenomenex (Torrance, CA) was eluted (0.3 mL/min) with a gradient mobile phase consisting of water and acetonitrile (with 0.1 formic acid; see below). The mobile phase gradient conditions were 97% A (water containing 0.1% v/v formic acid) and 3% B (acetonitrile containing 0.1% v/v formic acid) held for 3 min, followed by four steps of linear gradient of mobile phase B of 3% to 13%, 13% to 25%, 25% to 50% and 50% to 80% over 3-10 min, 10-20 min, 20-24 min and 24.1-25 min, respectively. This was

followed by a washing step using 80% mobile phase B for 0.9 min, and re-equilibration for 4.9 min. The parent to product ion transitions for the analyte peptides and their respective SIL peptides were monitored using optimized LC-MS/MS parameters (Table 1) in ESI positive ionization mode. For data analysis, peak response of multiple reaction monitoring (MRM) transition from each peptide was averaged and the area ratio of analyte peptide versus internal standard (IS) peptide was obtained. Relative protein expression of individual Ugt was presented as the peak area ratio of analyte peptide over that of the internal standard (IS) peptide and, then the ratio was normalized to the corresponding ratio of respective Ugt in the pooled liver tissue homogenates. Pooled tissue homogenates were pooled individual liver samples from the same experiment. Normalization to internal standard was necessary to reduce variations between sample preparations. Normalization to pooled tissue homogenates was used as batch to batch quality controls. The experiments were repeated with 3 independent mouse livers from each group (pregnant and non-pregnant mice) with the same amount of total membrane protein for trypsin digestion and LC-MS/MS analysis. Relative expression of individual Ugt in total membrane protein extracts was scaled to the whole liver using the following method: Relative expression of a Ugt isoform in the whole mouse liver = relative protein expression of the Ugt per μg of total membrane protein used in tryptic digestion × yield of protein isolation (μg/g liver tissue) × the weight of a whole mouse liver (g) isolated from respective mouse.

Pharmacokinetic data analysis

The area under concentration-time curve ($AUC_{0-\infty}$) values of plasma NBUP and NBUP-G were calculated using the linear trapezoidal method for both pregnant and non-pregnant mice. Since plasma concentrations of NBUP at 720 min were below the limit of quantification, data at 720 min were not used in the AUC calculation. Therefore, $AUC_{0-\infty}$ was estimated by

extrapolating from the time points at 120, 240 and 480 min. The AUC from 480 min to infinity accounted for less than 15% of total $AUC_{0-\infty}$. Because NBUP in plasma was sampled in different animals at each time point (one-point sampling), we performed the PK data analysis using a population-based bootstrap method as previously described previously (23). $AUC_{0-\infty}$ and clearance (CL) were calculated. CL was calculated by dose divided by $AUC_{0-\infty}$. The 95% confidence intervals of the sample distributions were estimated by bootstrapping the concentration-time data for each group with modification (26). Briefly, concentration-time data points from each dataset of interest were randomly sampled with replacement using the R program. This process was repeated 10,000 times to create 10,000 pseudo concentration-time profiles. The 95% confidence interval was the range of AUCs that encompassed the 2.5^{th} to the 97.5^{th} percentiles of the sample distribution. For each profile, concentrations at each time point were averaged and the PK parameters were calculated. Confidence intervals of metabolite/parent $AUC_{0-\infty}$ ratios were also estimated by bootstrapping 10,000 iterations of the paired metabolite to parent AUC ratios.

Statistical analysis

Quantitative assays were performed in triplicates and repeated at least three times. Data are presented as mean ± SD of three independent experiments. The 95% confidence intervals around each estimated PK parameters were determined using the non-parametric bootstrap method (23). Two-side *p* values were calculated using permutation tests with 10,000 replications (23). For comparisons of data in microsome incubations and proteomic studies, statistically significant differences were determined by unpaired Student's *t*-test. Differences with *p* values of < 0.05 were considered statistically significant.

3.3 Results

Pregnancy increased systemic clearance of NBUP

To demonstrate how pregnancy affects the disposition of NBUP, NBUP was administered to pregnant (gd 15) and non-pregnant mice by retro-orbital injection with the same dose of NBUP (33 μg) to each mouse. Maternal plasma concentrations of NBUP (Fig. 2A and Fig. 2C) and NBUP-G (Fig. 2B and Fig. 2D) in pregnant mice were significantly lower than those in non-pregnant mice. As a result, the plasma $AUC_{0-\infty}$ of NBUP in pregnant mice was ~2.5 times lower than that in non-pregnant mice ($p < 0.05$) (Table 2). The corresponding systemic CL of NBUP in pregnant mice was increased ~2.5-fold compared to that in non-pregnant mice ($p < 0.05$) (Table 2). Likewise, the plasma $AUC_{0-\infty}$ of NBUP-G significantly decreased ~2.4-fold in pregnant mice compared to non-pregnant mice (Table 2). The NBUP-G/NBUP $AUC_{0-\infty}$ ratio and plasma protein binding (f_u) of NBUP were not significantly affected by pregnancy (Table 2). We noted that the plasma concentrations of NBUP-G, even at early time points (e.g., 2 min), were ~10 times greater than those of the parent compound NBUP in both pregnant and non-pregnant mice (Fig. 2), suggesting that NBUP was rapidly and extensively metabolized to NBUP-G in mice. This finding is in agreement with human studies showing that the maximum plasma concentrations of NBUP-G were 4-10 times higher than those of NBUP or BUP after oral administration of BUP (27). Thus, in both humans and mice, NBUP-G is the major circulating metabolite after administration of either BUP or NBUP.

Pregnancy increased hepatic glucuronidation of NBUP

UGT-mediated metabolism is the primary elimination pathway of NBUP (18, 28). The significantly increased CL of NBUP in pregnant mice could be the result of increased Ugt-mediated hepatic metabolism of NBUP. To test this hypothesis, NBUP-G formation by

glucuronidation in microsomes prepared from pregnant and non-pregnant mouse livers was performed with NBUP at concentrations up to 400 μM. Fig. 3 shows a representative NBUP-G formation kinetic profile with liver microsomes isolated from non-pregnant (Fig. 3A) and pregnant (Fig. 3B) mice. No glucuronidation of NBUP was observed in the absence of UDPGA (data not shown). We found that K_m values of NBUP-G formation in pregnant and non-pregnant mice were not significantly changed (21.9 ± 2.5 μM with pregnant mice vs. 25.2 ± 4.5 μM with non-pregnant mice); however, the V_{max} of pregnant mice was significantly increased compared to non-pregnant mice (3,261 ± 155 pmol/mg protein/min vs. 2,322 ± 174 pmol/mg protein/min) (Fig. 3A and Fig. 3B). Thus, pregnancy significantly increased the microsomal CL_{int} of NBUP-G formation ~1.6-fold compared to non-pregnant controls (150 ± 21 μL/(min × mg protein) vs. 94 ± 16 μL/(min × mg protein)) (Fig. 3C).

Pregnancy induced Ugt expression in mouse liver quantified by proteomic LC-MS/MS

To confirm whether the increased hepatic CL_{int} of NBUP by glucuronidation in pregnant mice is due to induction of Ugt expression and which Ugts are induced by pregnancy, six mouse Ugt isoforms (Ugt1a1, Ugt1a6, Ugt1a8, Ugt1a10, Ugt2b1, and Ugt2b35) were selected based upon their homology to human UGT isoforms that have been shown to metabolize NBUP (29) and their moderate to high basal expression in female mouse livers (30). Due to the lack of selective antibodies and probe substrates for individual mouse Ugt isoforms, the use of immunoblotting or measurement of glucuronidation activity to demonstrate pregnancy-induced changes in expression or activity of individual mouse Ugts is not feasible. Moreover, LC-MS/MS based proteomics possesses superior accuracy, sensitivity, and reproducibility in quantification of proteins compared to conventional immunoblotting. Since we were primarily

interested in pregnancy-induced changes in Ugt expression, only relative (not absolute) hepatic protein expression of individual Ugt isoforms between pregnant and non-pregnant mice was analyzed in this study.

Since 160 μg of total membrane proteins were used in each tryptic digestion, the data shown in Fig. 4 demonstrate changes in protein levels of individual Ugts quantified in the same amount of total membrane proteins used in tryptic digestion. Thus, Ugt1a1 (Fig. 4A) and Ugt2b1 (Fig. 4B) protein expression in pregnant mice were significantly increased by approximately 50% compared to non-pregnant mice. Pregnancy did not significantly affect protein expression of Ugt1a6, Ugt1a8, Ugt1a10, and Ugt2b35 (Fig. 4C-4F).

The yield of total membrane protein isolation and liver weight may be altered by pregnancy, affecting the total amount of Ugts in the whole liver. The yield of total membrane protein isolation from mouse liver was 0.08 ± 0.01 and 0.10 ± 0.02 μg per 1 mg of liver tissue for non-pregnant and pregnant mice, respectively. The liver weight was 1.3 ± 0.1 and 2.1 ± 0.1 g for non-pregnant and pregnant mice, respectively. After scaling the data of Fig. 4 to the whole liver, the protein levels of Ugt1a1, Ugt1a10, Ugt2b1, and Ugt2b35 were significantly induced approximately 2-2.5-fold by pregnancy (Fig. 5A-5D). Pregnancy did not significantly affect the protein levels of Ugt1a6 and Ugt1a8 in the whole liver (Fig. 5E and 5F).

3.4 Discussion

The present study demonstrates that pregnancy significantly increases the systemic clearance of NBUP in a mouse model, which could be due to pregnancy-induced changes in factors important for NBUP disposition such as enhanced biliary excretion, induced hepatic metabolism, decreased plasma protein binding, or increased renal excretion. NBUP is a substrate of the efflux transporter P-glycoprotein and Ugts in the liver, and can be cleared by the kidney

(4, 20, 31). To date, there has been no evidence suggesting that pregnancy induces P-glycoprotein in the liver. The contribution of altered plasma protein binding can be ruled out as we showed that pregnancy did not significantly change plasma protein binding of NBUP in mice. Thus, the increase in NBUP clearance could be due to the combination of increased Ugt-mediated hepatic metabolism and increased renal excretion. Kacinko et al. (31) showed that the amount of NBUP-G excreted in urine was 6 times the amount of NBUP after administration of BUP and the cumulative NBUP excreted in urine was minimal, suggesting that renal clearance of NBUP plays a minor role in the overall disposition of NBUP. However, since pregnancy is known to increase renal clearance of drugs (Jeyabalan and Conrad, 2007), increased renal clearance of NBUP in pregnant mice versus non-pregnant mice cannot be ruled out. On the other hand, the data of NBUP-G formation in mouse liver microsomes suggest that induction of Ugt-mediated hepatic metabolism of NBUP is likely the primary cause of the increased systemic CL of NBUP during pregnancy. We did perform *in vitro*-to-*in vivo* scaling in an attempt to predict *in vivo* intrinsic CL of NBUP based on the *in vitro* microsomal CL_{int} of NBUP (data not shown). Consistent with literature data (32, 33), the Ugt-mediated CL_{int} of NBUP from microsome incubations under predicted the i*n vivo* intrinsic CL by nearly 20-fold. Reasons for the under prediction have been well documented. It is conceivable that the drug entry into the alamethicin-permeabilized membrane active sites is different from *in vivo* microenvironment. Other confounders include non-specific binding, inappropriate kinetic modeling, and failure to account for transporters and extrahepatic metabolism.

UGT1A1 and UGT1A4 play a major role in metabolizing labetalol (34) and lamotrigine (35), respectively. BUP is a substrate of UGT1A1, UGT1A3, UGT2B7 and UGT2B17 (18, 19, 28). Our results are in line with clinical studies showing that the apparent oral clearance of these

medications is significantly increased during pregnancy compared to postpartum or non-pregnant women (13, 36, 37). An increase in the clearance of drugs can significantly decrease systemic drug concentrations and may lead to suboptimal therapeutic outcomes. It is thus critical to investigate if the increase in the clearance of these drugs during pregnancy is due to induction of UGT-mediated hepatic metabolism. Results of this study suggest that the mouse model could possibly be suitable for such studies. Nevertheless, caution should be taken when extrapolating data from mice to humans due to the lack of sufficient knowledge about species differences in functions, expression and tissue distribution of individual UGT/Ugt isoforms between humans and mice (38). Indeed, we showed in this study that the K_m values of NBUP-G formation by Ugts in mouse liver were 6-7 times lower than the K_m of human UGTs reported in a previous study (Chang and Moody, 2009).

We found that protein expression of Ugt1a1 and Ugt2b1 in total membrane proteins isolated form the mouse liver was significantly induced by pregnancy (Fig. 4A and Fig. 4B). Induction of protein expression of mouse Ugts by pregnancy is consistent with mouse liver microsome incubation analysis, which showed that pregnancy did not affect the K_m of NBUP-G formation, but significantly increased the V_{max}. It has been shown that that 17β-estradiol may up-regulate human UGT1A4 via the estrogen receptor α (15), and that progesterone induces human UGT1A1 (16) possibly through the pregnane-X-receptor (39). Whether other human UGT isoforms including UGT1A3, UGT2B7 and UGT2B17 could be induced by the pregnancy-related hormones is not known. The induction of UGT1A expression by pregnancy-specific hormones is in line with our protein quantification data. However, our protein quantification data are not consistent with other published studies for murine Ugt1a1 (17, 39). Wen et al. (17) demonstrated that on gestation day 14, hepatic protein expression of Ugt1a1 in pregnant mice

determined by immunoblotting was not significantly altered compared to that in non-pregnant mice. Chen et al. (39) also showed by immunoblotting that hepatic Ugt 1a1 in mice was not induced by pregnancy. Changes in protein levels of Ugt1a1 in the previous studies could possibly have gone unnoticed given the low selectivity and sensitivity of immunoblotting in detecting changes in protein expression compared to the highly sensitive LC-MS/MS technique used in this study.

We noted that protein levels of Ugt1a1 and Ugt2b1 in total membrane protein extracts shown in Fig. 4 were increased by ~50% in pregnant mice versus non-pregnant mice. This 50% increase cannot fully explain the 2-fold increase in the systemic CL of NBUP. We figured that the increase in Ugt protein levels in total membrane protein extracts do not fully reflect the changes in the amount of Ugts expressed in the whole liver due to concomitant increase in membrane protein content and liver weight during pregnancy. Indeed, after scaling to the whole liver with consideration of the yield of membrane protein isolation and the change in liver weight during pregnancy, we found that the whole liver content of Ugt1a1, Ugt1a10, Ugt2b1 and Ugt2b35 increased 2-2.5-fold by pregnancy (Fig. 5). It appears that the increase in hepatic Ugt content can now explain the ~2-fold increase in the systemic CL of NBUP in pregnant mice versus non-pregnant mice. It is important to note that even though relative protein expression of some Ugts (Ugt1a10 and Ugt2b35) in total membrane proteins was not changed by pregnancy, the amount protein of these Ugts in the whole liver were still significantly increased by pregnancy due to increase in protein content and liver weight in pregnant mice versus non-pregnant mice. This should be taken into account in interpretation of pregnancy-induced PK changes for drugs metabolized by UGTs.

We showed that the plasma concentrations of NBUP-G were several times higher than those of NBUP over time in both pregnant and non-pregnant mice (Fig. 2), suggesting that the systemic clearance of NBUP is much faster than that of NBUP-G. Furthermore, even though hepatic glucuronidation of NBUP is significantly induced by pregnancy, the $AUC_{metabolite}/AUC_{parent}$ ratio remained relatively unchanged in pregnant and non-pregnant mice (Table 2). The metabolite/parent AUC ratio is dependent on both the formation clearance (CL_f) and elimination clearance of the metabolite (CL_m). The relatively unchanged metabolite/parent AUC ratio indicates that the systemic elimination clearance of NBUP-G is increased in pregnant mice versus non-pregnant mice. The increased elimination clearance of NBUP-G in pregnant mice could be due to increased expression of Bcrp in mouse liver of pregnant mice compared to non-pregnant mice. We have previously shown that protein levels of Bcrp in the mouse liver increase ~3-fold in pregnant mice on gd 15 versus non-pregnant mice (40). Our most recent study suggested that NBUP-G is likely a substrate of mouse Bcrp (20). Hence, biliary elimination of NBUP-G could be facilitated due to increased Bcrp expression in the liver of pregnant mice. NBUP-G is also partly eliminated via renal clearance (31). Studies have shown that glomerular filtration rate (GFR) is increased by ~50% during the first trimester and continues to increase throughout pregnancy (41). Hence, an increase in GFR during pregnancy may also contribute to the increased elimination of NBUP-G in pregnant mice. We realize that, to fully explain the PK changes of NBUP-G, it is necessary to directly investigate the mechanisms and kinetics of NBUP-G clearance in mice. This would be a topic of investigation in future studies.

3.5 Tables and Figures

Table 3-1. Surrogate peptides of Ugts and their MS/MS parameters

Protein name	Surrogate peptide	Peptide type	Parent ion (*m/z*)	Fragment ion (*m/z*)	Declustering Potential (V)	Collision Energy (V)
Ugt1a1	EGSFYTLR	Light	486.743	699.382	66.6	26.4
Ugt1a1	EGSFYTLR	Light	486.743	552.314	66.6	26.4
Ugt1a1	EGSFYTLR	Heavy	491.747	796.423	66.6	26.4
Ugt1a1	EGSFYTLR	Heavy	491.747	709.391	66.6	26.4
Ugt1a1	EGSFYTLR	Heavy	491.747	562.322	66.6	26.4
Ugt1a1	TAFNQDSFLLR	Light	656.338	992.516	79	32.5
Ugt1a1	TAFNQDSFLLR	Light	656.338	878.473	79	32.5
Ugt1a1	TAFNQDSFLLR	Light	656.338	750.414	79	32.5
Ugt1a1	TAFNQDSFLLR	Heavy	661.342	888.481	79	32.5
Ugt1a1	TAFNQDSFLLR	Heavy	661.342	562.262	79	32.5
Ugt1a6	DSATLSFLR	Light	505.269	736.435	68	27
Ugt1a6	DSATLSFLR	Light	505.269	635.388	68	27
Ugt1a6	DSATLSFLR	Light	505.269	522.303	68	27
Ugt1a6	DSATLSFLR	Heavy	510.273	746.443	68	27
Ugt1a6	DSATLSFLR	Heavy	510.273	645.396	68	27
Ugt1a6	DSATLSFLR	Heavy	510.273	532.312	68	27
Ugt1a8	LVEYLK	Light	382.731	651.371	59	22.6
Ugt1a8	LVEYLK	Light	382.731	552.303	59	22.6
Ugt1a8	LVEYLK	Light	382.731	423.26	59	22.6
Ugt1a8	LVEYLK	Heavy	386.738	560.317	59	22.6
Ugt1a8	LVEYLK	Heavy	386.738	431.274	59	22.6
Ugt1a8	IPQTVLWR	Light	506.801	899.51	68.1	27.1
Ugt1a8	IPQTVLWR	Light	506.801	674.398	68.1	27.1
Ugt1a8	IPQTVLWR	Light	506.801	573.351	68.1	27.1
Ugt1a8	IPQTVLWR	Heavy	511.805	812.465	68.1	27.1
Ugt1a8	IPQTVLWR	Heavy	511.805	684.407	68.1	27.1
Ugt1a8	IPQTVLWR	Heavy	511.805	583.359	68.1	27.1
Ugt1a10	YTGTRPSNLAK	Light	604.325	785.463	75.2	30.6
Ugt1a10	YTGTRPSNLAK	Light	604.325	629.362	75.2	30.6
Ugt1a10	YTGTRPSNLAK	Light	604.325	579.289	75.2	30.6
Ugt1a10	YTGTRPSNLAK	Heavy	608.332	894.525	75.2	30.6
Ugt1a10	YTGTRPSNLAK	Heavy	608.332	793.477	75.2	30.6
Ugt1a10	YTGTRPSNLAK	Heavy	608.332	637.376	75.2	30.6
Ugt2b1	TPLVYSLR	Light	474.779	750.451	65.7	25.9
Ugt2b1	TPLVYSLR	Light	474.779	637.367	65.7	25.9
Ugt2b1	TPLVYSLR	Light	474.779	538.298	65.7	25.9

Ugt2b1	TPLVYSLR	Heavy	479.783	760.459	65.7	25.9
Ugt2b1	TPLVYSLR	Heavy	479.783	647.375	65.7	25.9
Ugt2b1	TPLVYSLR	Heavy	479.783	548.307	65.7	25.9
Ugt2b35	IILDELVQR	Light	549.83	759.4	71.2	28.7
Ugt2b35	IILDELVQR	Light	549.83	644.373	71.2	28.7
Ugt2b35	IILDELVQR	Light	549.83	515.33	71.2	28.7
Ugt2b35	IILDELVQR	Heavy	554.834	769.408	71.2	28.7
Ugt2b35	IILDELVQR	Heavy	554.834	654.381	71.2	28.7
Ugt2b35	IILDELVQR	Heavy	554.834	525.338	71.2	28.7

Table 3-2. **The maternal plasma exposure (AUC), systemic clearance (CL), and plasma fraction unbound (f_u) of norbuprenorphine (NBUP) and norbuprenorphine-3-β-D-glucuronide (NBUP-G) in pregnant and non-pregnant mice**. Data shown are means (95% confidence interval). The NBUP-G/NBUP plasma AUC ratios were calculated based on molar concentrations of NBUP and NBUP-G in the maternal plasma. Differences between the pharmacokinetic parameters were calculated by the non-parametric bootstrap method as described in "Materials and Methods". Differences in the PK parameters between pregnant and non-pregnant mice with *p* values of < 0.05 were considered statistically significant.

PK Parameter			
	Pregnant	**Non-pregnant**	***p***
$AUC_{0-\infty}$ of NBUP (μg × min × mL^{-1})	4.2 [3.0, 6.5]	10.8 [6.0, 16.1]	< 0.05
$AUC_{0-\infty}$ of NBUP (μM × min)	10.2 [7.3, 15.7]	26.1 [14.5, 38.9]	< 0.05
CL of NBUP (mL × min^{-1})	7.8 [5.1, 10.7]	3.1 [2.0, 5.5]	< 0.05
$AUC_{0-\infty}$of NBUP-G (μg × min × mL^{-1})	27.9 [23.0, 40.8]	66.2 [43.2, 101.5]	< 0.05
$AUC_{0-\infty}$ of NBUP-G (μM × min)	57.0 [47.0, 83.3]	135.2 [88.2, 207.3]	< 0.05
NBUP-G/NBUP plasma AUC Ratio	5.6 [5.3, 6.5]	5.2 [5.0, 6.0]	> 0.05
f_u of NBUP (%)	11.1 [8.8, 13.4]	17.2 [13.8, 20.6]	> 0.05

Figure legends

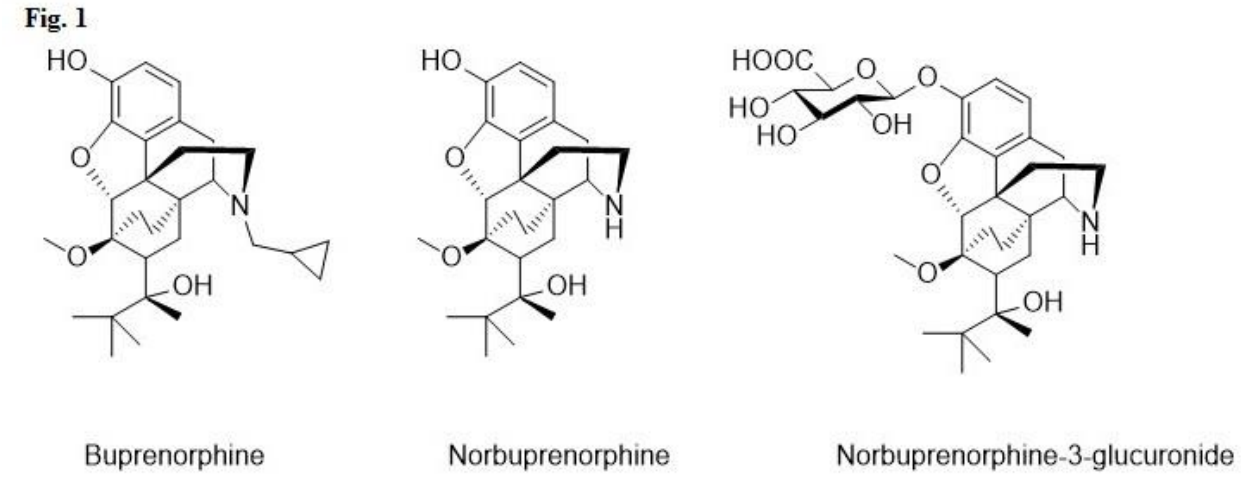

Figure 1. Chemical structures of buprenorphine, norbuprenorphine, and norbuprenorphine-3-β-D-glucuronide.

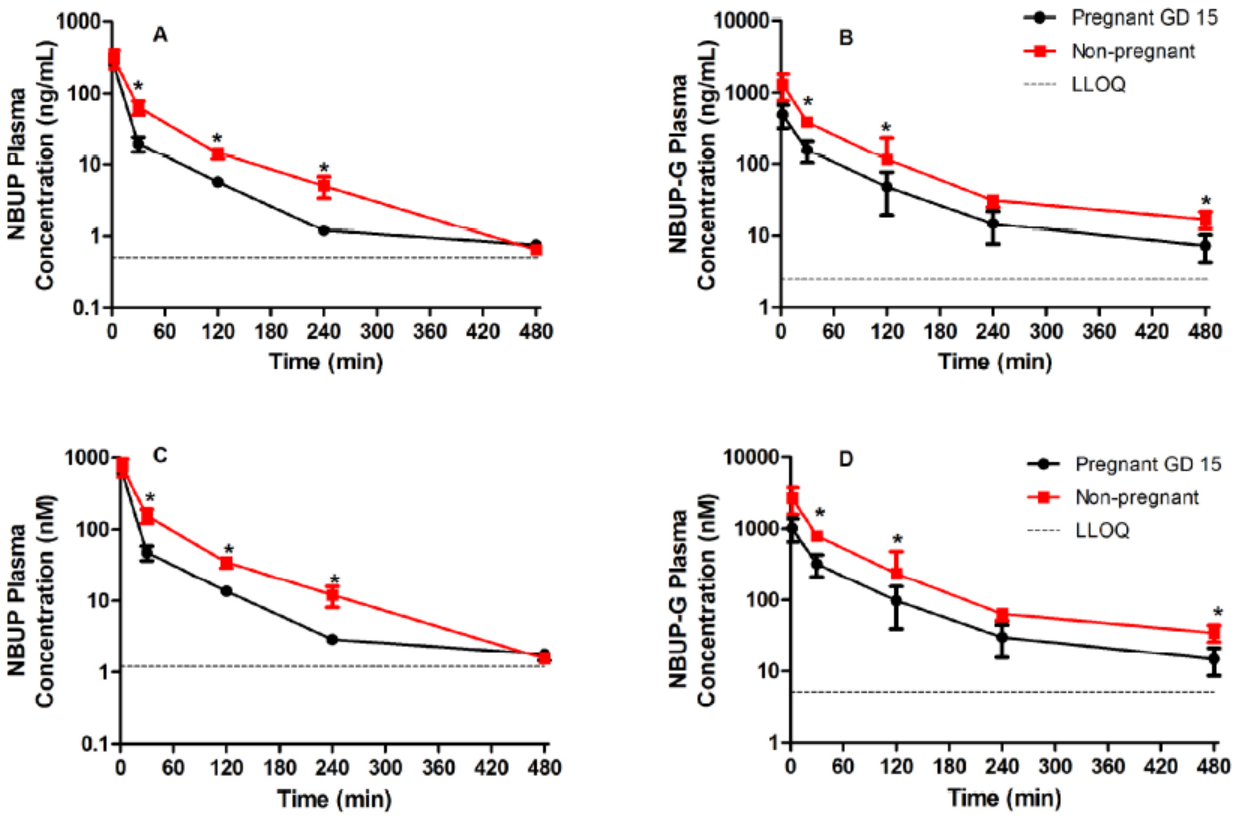

Figure 2. The plasma concentration-time profiles of norbuprenorphine (NBUP) and norbuprenorphine-3-β-D-glucuronide (NBUP-G) in pregnant and non-pregnant mice. Shown are the plasma concentration concentration-time profiles of NBUP (**A**) and NBUP-G (**B**) in ng/ml concentrations and of NBUP (**C**) and NBUP-G (**D**) in nM concentrations, over 480 min after retro-orbital injection of 33 μg of NBUP to each of pregnant (circles) and non-pregnant (squares) mice. Data are shown as means ± SD from three mice at each time point. Statistically significant differences between pregnant and non-pregnant mice were analyzed by the non-parametric bootstrap method, with *p* values of < 0.05. Dash lines represent lower limits of quantification (LLOQ).

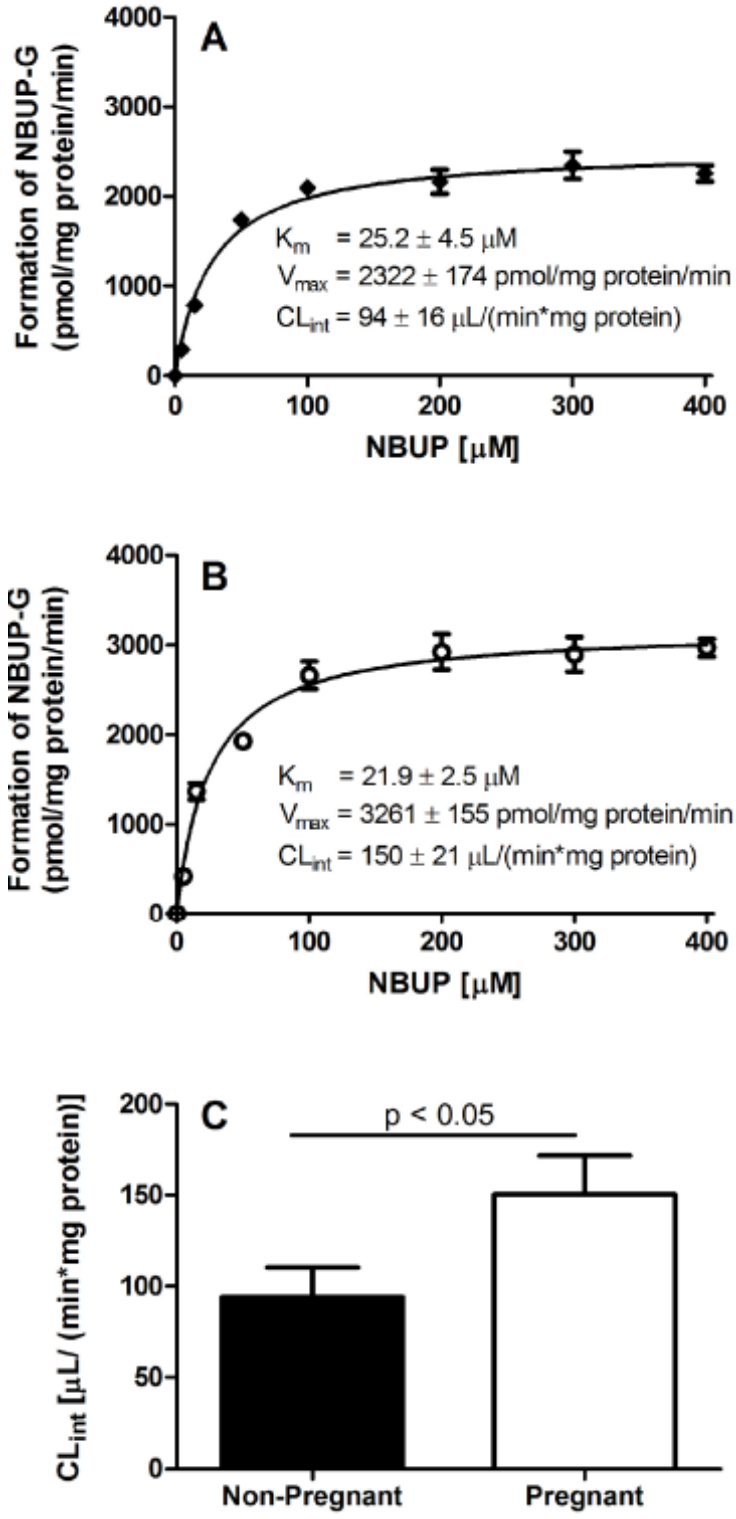

Figure 3. Norbuprenorphine-3-β-D-glucuronide (NBUP-G) formation kinetics in mouse liver microsomes isolated from pregnant and non-pregnant mice. A representative

NBUP-G formation kinetic profile over substrate (NBUP) concentrations up to 400 µM in mouse liver microsomes isolated from non-pregnant and pregnant mice is shown in panels **A** and **B**, respectively. Shown are means of SD of a representative kinetic experiment with triplicate determinations at each substrate concentration. The kinetic experiments were repeated independently three times, each with triplicate determinations at each substrate concentration, and similar results were obtained. The K_m, V_{max}, CL_{int} were calculated with all experimental data obtained. **C** depicts CL_{int} of NBUP-G formation in pregnant (open bar) and non-pregnant (filled bar) mice. Statistically significant differences between pregnant and non-pregnant mice were analyzed by Student's *t*-test, with *p* values of < 0.05.

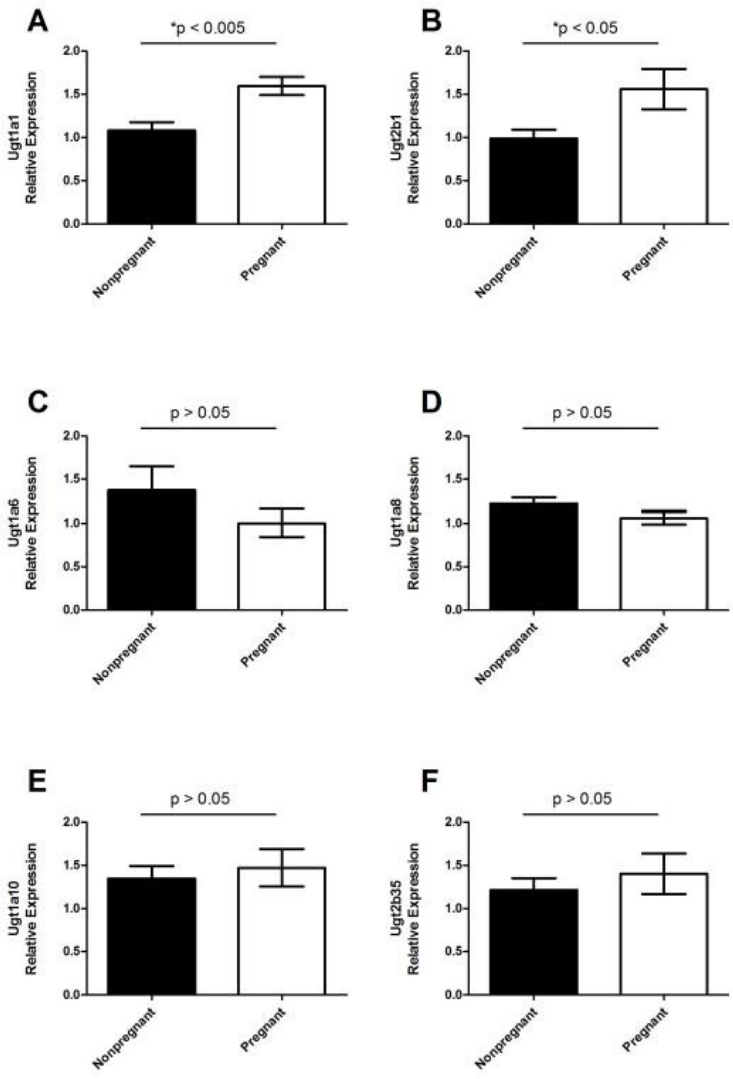

Figure 4. Relative protein expression of Ugts in hepatic tissues of pregnant and non-pregnant mice. Relative protein expression of mouse Ugt1a1 (**A**), Ugt2b1 (**B**), Ugt1a6 (**C**), Ugt1a8 (**D**), Ugt1a10 (**E**), Ugt2b35 (**F**) in isolated total membrane proteins of mouse livers from pregnant (open bars) and non-pregnant (filled bars) mice was quantified by LC-MS/MS. The protein expression levels were normalized to protein expression of respective Ugt in pooled tissue homogenates which were used as quality controls. Data shown are means ± SD from three independent liver tissues per group. Statistically significant differences between pregnant and non-pregnant mice were analyzed by Student's *t*-test, with *p* values of < 0.05.

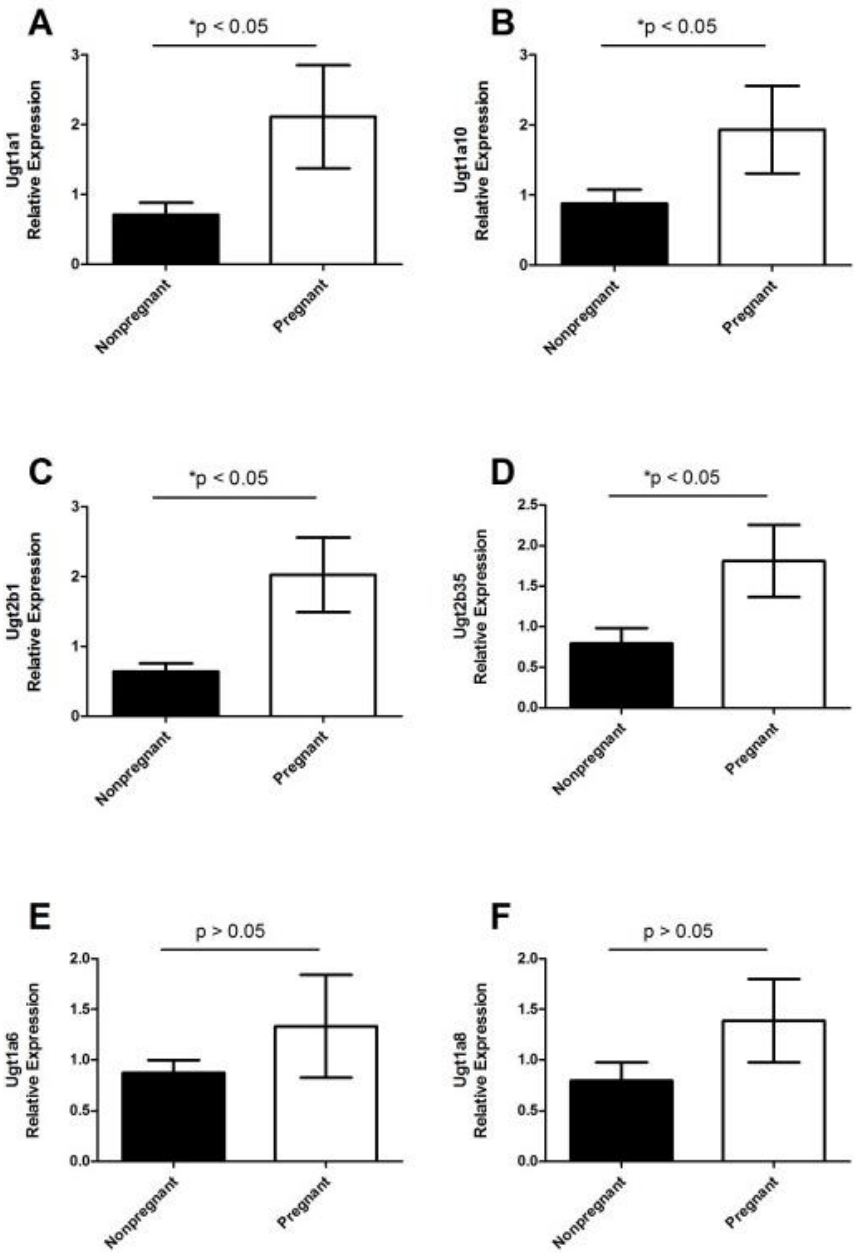

Figure 5. Relative protein expression of Ugts in hepatic tissues of pregnant and non-pregnant mice after scaling to the whole liver. Relative protein expression of mouse Ugt1a1 (**A**), Ugt2b1 (**B**), Ugt1a6 (**C**), Ugt1a8 (**D**), Ugt1a10 (**E**), Ugt2b35 (**F**) after scaling to the whole liver of pregnant (open bars) and non-pregnant (filled bars) mice. Data shown are means ± SD from three independent liver tissues per group. Statistically significant differences between pregnant and non-pregnant mice were analyzed by Student's *t*-test, with *p* values of < 0.05.

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Chapter 4: Quantitative Proteomics Reveals Changes in Transporter Protein Abundance in Liver, Kidney and Brain of Mice by Pregnancy

The work presented in this chapter is submitted

to Drug Metabolism and Disposition

4.1 Introduction

Pregnant women undergo extensive physiological changes such as increase in production of pregnancy-related hormones which are essential for maintenance of normal pregnancy and proper fetal growth and development (1, 2). Such changes can also affect drug disposition in pregnant women by altering expression of drug disposition enzymes and transporters (3). While changes in expression or activity of drug-metabolizing enzymes (i.e. CYPs, UGTs) by pregnancy have been extensively studied (4-8), few studies have systematically investigated the effects of pregnancy on protein expression or activity of drug transporters in organs important for drug disposition, such as the liver, kidney and brain. Several studies have evaluated changes in mRNA expression for some transporters during pregnancy (9, 10); however, mRNA expression is not always correlated with protein expression or activity. To the best of our knowledge, few previous studies have offered a comprehensive evaluation of the effects of pregnancy on protein abundance of a variety of drug transporters important for drug disposition simultaneously in multiple organs. In the present study, we chose the pregnant mouse as the animal model to conduct such analyses as we have previously shown that the pharmacokinetic (PK) changes of

several drugs in pregnant women can be replicated in the pregnant mouse model (5, 7, 11, 12). Knowledge about the changes in drug transporter abundance by pregnancy is important for understanding and predicting of pregnancy-induced alternations in drug/xenobiotic PK, efficacy and safety.

Thus, the goal of this study was to quantitatively examine the effects of pregnancy on protein abundance of various drug transporters important for drug/xenobiotic disposition, including Abcb1a, Abcg2, Abcc2, Slco1a1, and other transporters, in the liver, kidney and brain of mice. Using the highly sensitive LC-MS/MS-based quantitative proteomics with multiple reaction monitoring (MRM) (13, 14), we observed tissue-specific effects of pregnancy on protein abundance of various drug transporters in pregnant mice.

4.2 Materials and Methods

Materials

Optima grade or high-performance liquid chromatography grade methanol, acetonitrile, and water were from Thermo Fisher Scientific (Waltham, MA) or Acros Organics (Pittsburgh, PA). Isoflurane was purchased from Piramal Healthcare (Mumbai, India) through the University of Washington Medical Center Pharmacy. Synthetic heavy signature peptides for LC-MS/MS-based proteomics were obtained from Pierce Biotechnology (Rockford, IL). The corresponding stable-isotope-labeled (SIL) peptides were from Thermo Fisher Scientific (Rockford, IL). The ProteoExtract native membrane protein extraction kit was from Calbiochem (Temecula, CA). Ammonium bicarbonate and sodium deoxycholate were from Thermo Fisher Scientific and MP Biomedicals (Santa Ana, CA), respectively. BCA protein assay and in-solution trypsin digestion kits, iodoacetamide and dithiothreitol were obtained from Pierce Biotechnology (Rockford, IL).

Animal studies

This animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington. Wild-type FVB mice, 7-10 weeks of age, were purchased from Taconic Farms (Germantown, NY) and cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council. Mice were maintained under 12-h light/dark cycles, and food and water were provided *ad libitum*. Female mice, 7-10 weeks of age, were mated with male mice of the same age overnight using a female to male ratio of 2:1. The first day of gestation (gd 1) was defined as the presence of a sperm plug following overnight housing. On gd 15 which approximately corresponds to the late second trimester in human gestation, body weight was recorded, and pregnant mice were sacrificed under anesthesia (isoflurane) by cardiac puncture. Maternal tissues (liver, kidney and brain) were immediately collected, rinsed with phosphate-buffered saline, weighed, snap-frozen in liquid N_2, and stored at -80^{o}C until use. The liver, kidney and brain tissues were also collected in the same way from non-pregnant mice of matching age.

Protein quantification of transporters in pregnant and non-pregnant mouse brains, livers, and kidneys by LC-MS/MS

Quantification of changes in protein levels of Abcb1a (a P-gp isoform), Abcb1b (a P-gp isoform), Abcg2 (Bcrp), Abcb11 (Bsep), Abcc2 (Mrp2), Abcc3 (Mrp3), Abcc4 (Mrp4), Slco1a1 (Oatp1a1), Slco1a4 (Oatp1a4), Slco2b1 (Oatp2b1) and Slc22a3 (Oct3), as well as the plasma membrane marker, Na^+/K^+-ATPase, in the liver, kidney and brain tissues of pregnant and non-pregnant mice was carried out using a surrogate peptide-based LC-MS/MS method as previously described (7, 15, 16). One or two signature peptides unique for the transporters used to quantify each transporter were shown in supplemental Table S1. The corresponding heavy peptides

containing labeled [$^{13}C_6$ $^{15}N_2$]-lysine or [$^{13}C_6$ $^{15}N_4$]-arginine were used as internal standards (Table S1). Total membrane proteins were isolated from tissue homogenates, and total membrane proteins were then reduced, denatured, alkylated, desalted and digested as previously described (7, 14). Bovine serum albumin (BSA) was added to each sample before trypsin digestion (17). Twenty microliters of heavy peptide internal standard cocktail (prepared in acetonitrile:water, 80:20 (v/v) with 0.5% formic acid and 10 µl of acetonitrile:water (80:20 (v/v) containing 0.1% formic acid) was added to each sample. After mixing and centrifugation at 4000 × g (4°C) for 5 min, samples were transferred to LC-MS autosampler vials and stored at -20ºC until analysis.

The chromatographic conditions and instruments used were the same as previously described (7, 16). The parent to product ion transitions for the analyte peptides and their respective SIL peptides were monitored using optimized LC-MS/MS parameters in ESI positive ionization mode (Table S1). Data analysis was similar to previously described (7). Peak response of MRM transition from each peptide was averaged and the area ratio of analyte peptide to internal standard (IS) peptide was obtained. Relative protein levels of individual transporters were presented as the peak area ratio of analyte peptide over that of the IS peptide and, then the ratio was normalized to BSA and the corresponding ratio of respective transporters in the pooled tissue homogenates. Pooled tissue homogenates were prepared by pooling individual liver, kidney, and brain samples from the same experiment. Normalization to BSA and internal standard was necessary to reduce variations and correct for ion suppression between sample preparations (17). Normalization to pooled tissue homogenates was used as batch-to-batch quality controls. All experiments were repeated with three independent mouse liver, kidney or brain tissues from each group (pregnant and non-pregnant mice) using the same amount of total

membrane protein for trypsin digestion and LC-MS/MS analysis. Relative protein levels of individual transporters in total membrane protein extracts (expressed as relative protein levels per μg of total membrane proteins used for trypsin digestion) were then scaled to the whole organ as follows. Relative protein abundance of a transporter in a whole mouse tissue (liver, kidney or brain) = relative protein abundance of the transporter per μg of total membrane proteins × yield of total membrane protein isolation (μg/g tissue) × the weight of the whole mouse tissue (g) isolated from respective mouse.

Statistical analysis

All data were presented as means of SD from three independent mouse tissues, with each tissue sample analyzed in 3 replicates. Statistical analysis by unpaired Student's *t*-test was performed using the GraphPad Prism version 5 software (La Jolla, CA). Differences between pregnant and non-pregnant groups with p values of < 0.05 were considered statistically significant.

4.3 Results and Discussion

In this study, relative protein levels of 12 drug transporters known to be important for drug disposition in the liver, kidney and brain of pregnant and non-pregnant FVB mice have been compared. Several previous studies have reported protein expression of some of these transporters in pregnant mice, but with much less sensitive and non-specific immunoblotting (18-20). In the present study, we used highly sensitive and selective LC-MS/MS proteomics to simultaneously analyze the impact of pregnancy on protein abundance of these transporters in mice. Of the 12 drug transporters, all transporters were detectable and 9 were quantifiable in the liver (Table 1). However, only 6 transporters were quantifiable, 3 detectable and 3 undetectable

in the kidney; 2 quantifiable, 6 detectable and 4 undetectable in the brain. Therefore, it appears that drug transporters generally have higher levels of protein abundance in the liver than in the kidney and brain, with most of the transporters quantifiable in the liver, but only a few are quantifiable in the kidney or brain (Table 1). Notably, Abcb1a, but not Abcb1b, could be quantified in the liver, suggesting that Abcb1a is the major murine P-gp isoform in the liver. Slo22a3 (Oct3) could be detected, but was not quantifiable in all the three tissues.

When the protein levels of quantifiable transporters were expressed as relative protein levels per µg of total membrane proteins, we found that pregnancy significantly decreased protein levels of Abcc2, Abcc3, and Slco1a4 by ~24%, ~72%, ~70%, respectively (Fig. 1), which were similar to prior reports with protein (Abcc2 and Abcc3) or mRNA (*Abcc3* and *Slco1a4*) expression data (10, 20-22). On the other hand, protein levels of Abcg2, Abcb11, Abcb1a, Abcc4, Slco1a1 and Slco1b2 in the liver did not significantly differ between pregnant and non-pregnant mice (Fig. 1), which are consistent with previous studies with immunoblotting of these transporters (20, 21, 23). Na^+/K^+-ATPase was significantly decreased by ~32% in pregnant mice versus non-pregnant controls (Fig. 1), suggesting that Na^+/K^+-ATPase is not an appropriate internal control for assessment of the effects of pregnancy on transporter protein expression in the liver.

We noticed that the mean yields of total membrane protein isolation from mouse livers were 0.08 ± 0.01 µg and 0.10 ± 0.02 µg per 1 mg of liver tissue for non-pregnant and pregnant mice, respectively. The mean liver tissue weights were 1.3 ± 0.1 g and 2.1 ± 0.1 g per liver per mouse for non-pregnant and pregnant mice, respectively. Hence, this increase in liver weight in pregnant mice versus non-pregnant mice suggests that the amounts of transporter protein in the whole liver can be increased even if there is no difference in protein levels of transporters per µg

of total membrane proteins. Indeed, after scaling to the whole liver, protein levels of Abcg2, Abcb11, Abcb1a, Abcc2, Abcc4, Slco1a1 and Slco1b2 were approximately 50-100% higher in pregnant mice compared to non-pregnant mice (Fig. S1). The protein levels of only Abcc3 and Slco1a4 were decreased by ~40% in pregnant mice (Fig. S1), which is because the increase in liver weight does not compensate the drastic decrease in hepatic Abcc3 and Slco1a4 protein abundance normalized to the amounts of total membrane proteins. Pregnancy did not affect protein levels of Na^+/K^+-ATPase in the whole liver (Fig. S1). A previous study reported no effects of pregnancy on the protein levels of mouse P-gp in hepatic S-9 fractions detected by immunoblotting (23), similar to what we showed in Fig. 1F. However, the relative amounts of Abcb1a protein in the whole liver of pregnant mice were increased by ~50% compare to non-pregnant mice (Fig. S1). Therefore, caution should be taken when we use protein abundance data to explain changes in drug disposition *in vivo*. Based on our findings, the protein levels of transporters scaled to the whole liver are recommend and would be more reliable to explain changes in *in vivo* PK. We recently showed that the systemic exposure of norbuprenorphine-β-D-glucuronide, a possible Abcg2 substrate, was significantly decreased in pregnant mice compared to non-pregnant controls, even though hepatic metabolic formation of norbuprenorphine-β-D-glucuronide was significantly increased (7). The increase in hepatic Acbg2 protein levels in the whole liver (Fig. S1) may have increased biliary excretion of norbuprenorphine-β-D-glucuronide to the bile, thus facilitating norbuprenorphine-β-D-glucuronide elimination in pregnant mice. The increase in protein levels of Abcc2 and Abcb11 in the whole liver may explain the alteration of bile acid pathway by pregnancy (20), and a compensatory effect for down-regulation of hepatic Abcc3 (24).

Among the transporters quantifiable in the kidney, we found that pregnancy significantly decreased the protein levels per µg total membrane proteins of Abcg2, Abcc2 and Slco2b1 by approximately 40-50% (Fig. 2A-C). The protein levels of Slco1a4, Abcb1a, Abcc3 and Na^+/K^+-ATPase in the kidney were not significantly affected by pregnancy (Fig. 2D-G). Overall, these results are consistent with previous studies. For example, Abcc2 protein expression in mouse kidney was shown earlier to be significantly decreased; and Abcc3 protein expression in mouse kidney was not changed, by pregnancy (18). Likewise, previous studies also showed that the protein levels of mouse P-gp in the kidney were not significantly altered by pregnancy (18, 23). The mRNA levels of *Slco2b1* in mouse kidney were not significantly affected by pregnancy (18), which is not consistent with the protein data of this study for Slco2b1 (Fig. 2C). Likewise, we previously showed that *Abcb1a* mRNA in mouse kidney was significantly decreased by pregnancy, but in this study, we found no significant changes in Abcb1a protein (10). This is likely due to the fact that mRNA expression is not always correlated with protein abundance. Of note, the protein levels of Abcg2 in the kidney were previously shown to be not significantly affected by pregnancy (18); however, we found a 43% reduction (Fig. 2A). We observed that the kidney weights of pregnant mice were ~23-25% larger than those of non-pregnant mice, which is similar to a previous report (25). The yields of total membrane protein isolation from mouse kidneys did not significantly differ between pregnant and non-pregnant mice (data not shown). After scaling to the whole kidney, the protein levels of all the transporters quantifiable in the kidney were not significantly altered by pregnancy (Fig. 2S).

Among 12 transporters analyzed, only two (Abcg2 and Abcb1a) and the internal control Na^+/K^+-ATPase in the brain were quantifiable (Fig. 3), indicating that mouse Abcg2 and Abcb1a are the major transporters in the brain. Indeed, Abcg2 and Abcb1a are two major efflux

transporters in the blood-brain barrier (26). Since pregnancy did not significantly change brain weight (data not shown), we found that pregnancy had no significant effects on protein levels of Abcg2 and Abcb1a in the brain, no matter whether based on normalization to total membrane proteins or after scaling to the whole brain (Fig. 3 and Fig. S3).

4.4 Tables and Figures

Table 4-1. Summary of the patterns of pregnancy-induced changes in protein abundance of transporters analyzed in this study

Changes in transporter protein abundance per μg of total membrane proteins			
	Liver	Kidney	Brain
Abcb1a	↔	↔	↔
Abcb1b	NQ	NQ	NQ
Abcb11	↔	ND	ND
Abcg2	↔	↓	↔
Abcc2	↓	↓	ND
Abcc3	↓	↔	ND
Abcc4	↔	NQ	NQ
Slc22a3	NQ	NQ	NQ
Slco1a1	↔	ND	NQ
Slco1a4	↓	↔	NQ
Slco1b2	↔	ND	ND
Slco2b1	NQ	↓	NQ
Na^+/K^+-ATPase	↓	↔	↔
Changes in transporter protein abundance after scaling to the whole organ			
	Liver	Kidney	Brain
Abcb1a	↑	↔	↔
Abcb1b	NQ	NQ	NQ
Abcb11	↑	ND	ND
Abcg2	↑	↔	↔
Abcc2	↑	↔	ND
Abcc3	↓	↔	ND
Abcc4	↑	NQ	NQ
Slc22a3	NQ	NQ	NQ
Slco1a1	↑	ND	NQ
Slco1a4	↓	↔	NQ
Slco1b2	↑	ND	ND
Slco2b1	NQ	↔	NQ
Na^+/K^+-ATPase	↔	↔	↔

Statistically significant increase or decrease in protein levels of transporters in tissues of pregnant mice compared to non-pregnant controls are designated as ↑ or ↓, respectively. No significant changes are denoted as ↔. NQ indicates transporters at levels detectable, but below the quantification limit. ND indicates transporters at levels below the detection limit.

Figure legends

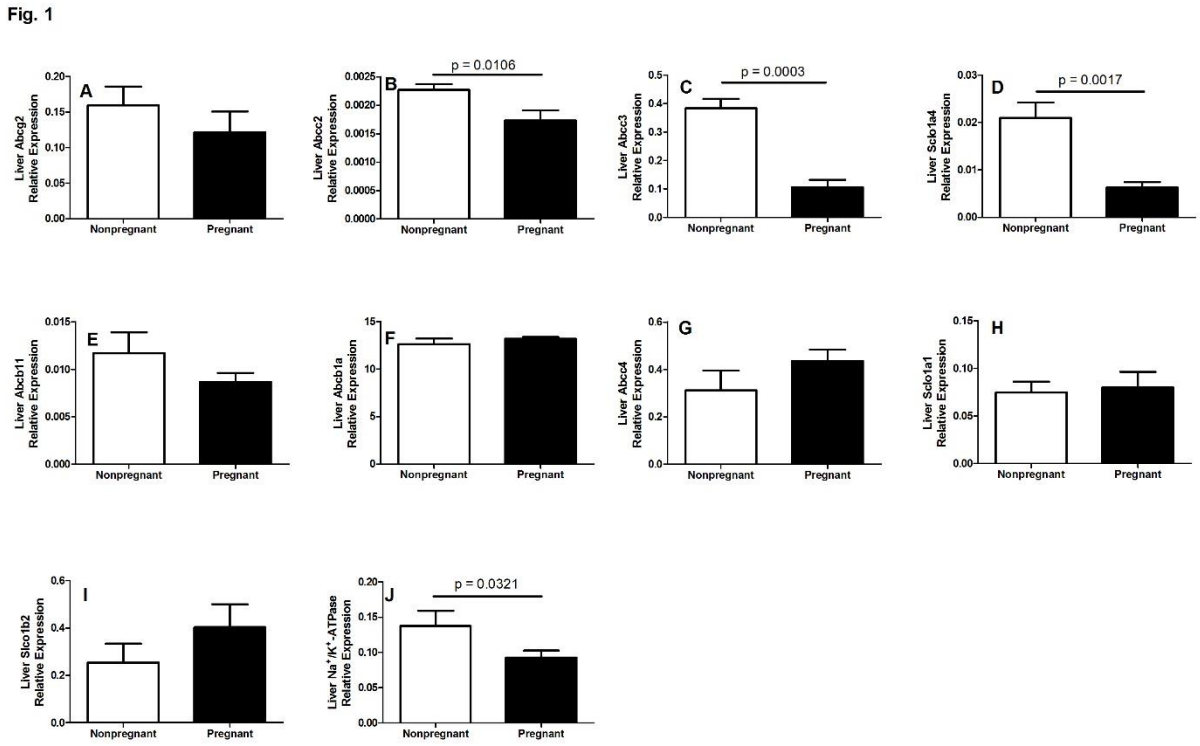


Figure 1. Relative protein abundance of transporters in the liver of pregnant and non-pregnant mice in total membrane proteins. Relative protein levels of Abcg2 (**A**), Abcc2 (**B**), Abcc3 (**C**), Slco1a4 (**D**), Abcb11 (**E**), Abcb1a (**F**), Abcc4 (**G**), Slco1a1 (**H**), Slco1b2 (**I**), and Na^+/K^+-ATPase (**J**) in isolated total membrane proteins of mouse liver from pregnant (filled bars) and non-pregnant (open bars) mice. Data shown are means ± SD from three independent liver tissues per group (pregnant and non-pregnant).

Fig. 2

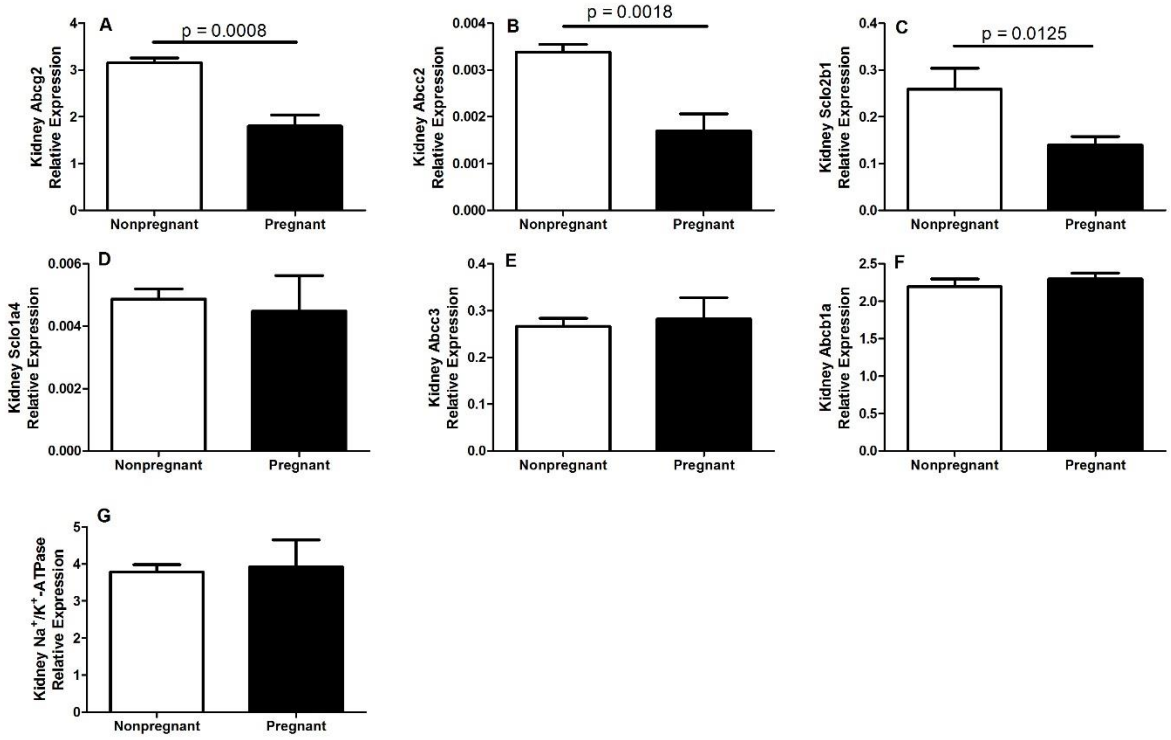


Figure 2. Relative protein abundance of transporters in the kidney of pregnant and non-pregnant mice in total membrane proteins. Relative protein levels of Abcg2 (**A**), Abcc2 (**B**), Slco2b1 (**C**), Slco1a4 (**D**), Abcc3 (**E**), Abcb1a (**F**), and Na^+/K^+-ATPase (**G**) in isolated total membrane proteins of mouse kidney from pregnant (filled bars) and non-pregnant (open bars) mice. Data shown are means ± SD from three independent kidney tissues per group (pregnant and non-pregnant).

Fig. 3

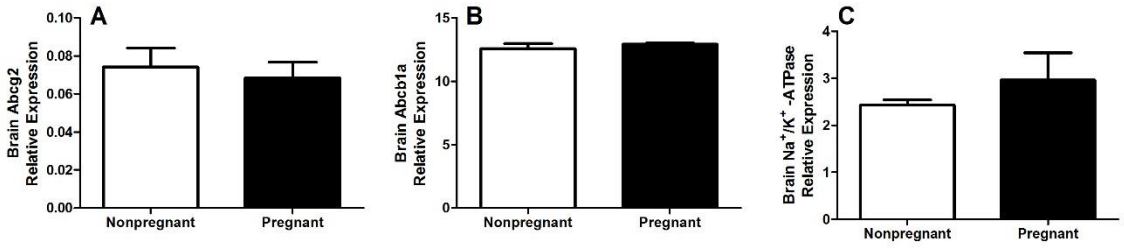
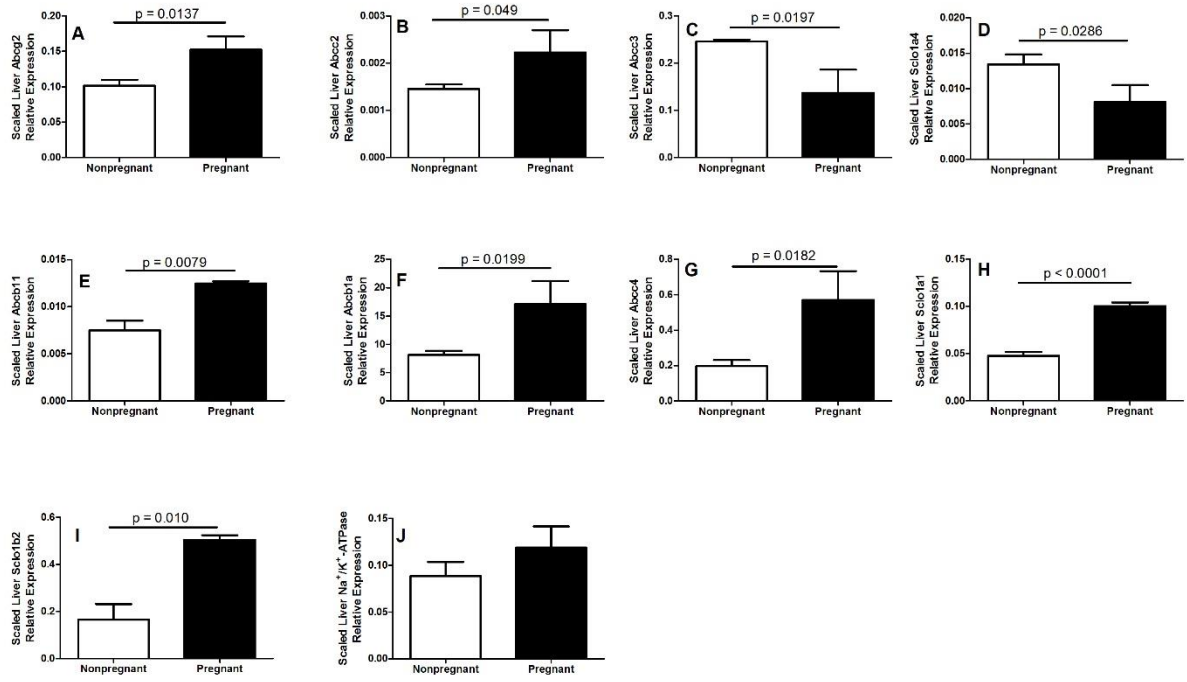


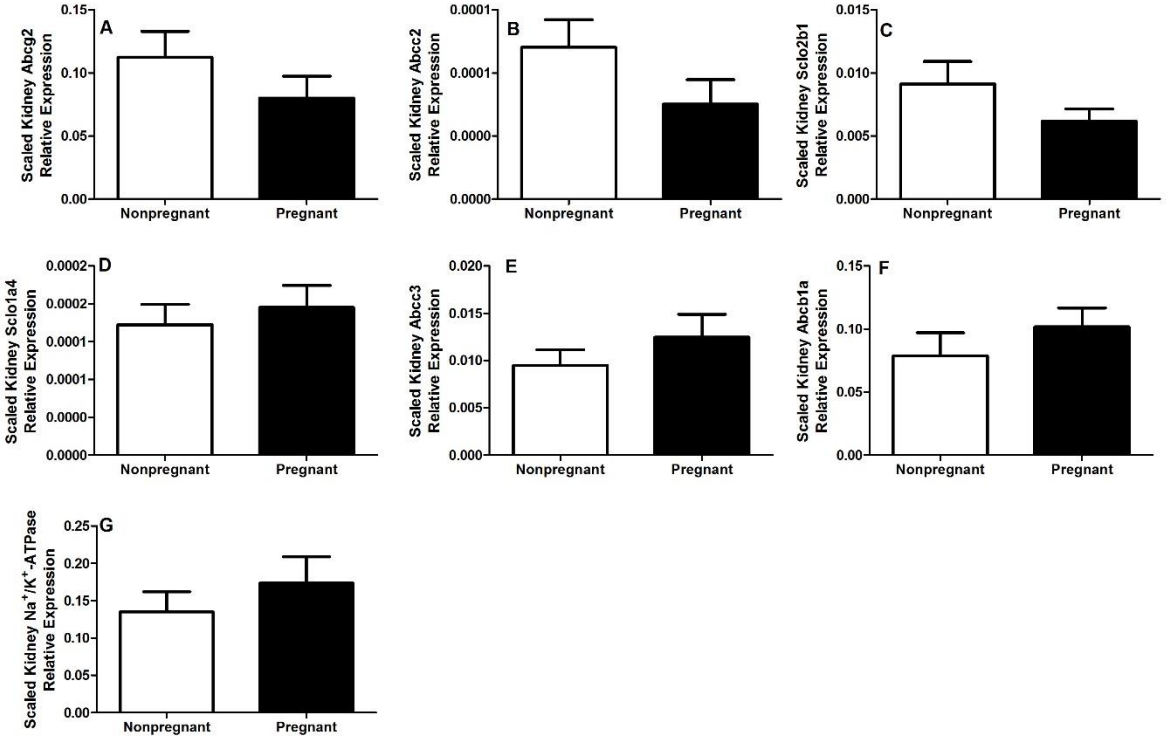
Figure 3. Relative protein abundance of transporters in the brain of pregnant and non-pregnant mice in total membrane proteins. Relative protein levels of Abcg2 (**A**), Abcb1a (**B**), and Na^+/K^+-ATPase (**C**) in isolated total membrane proteins of mouse brain from pregnant (filled bars) and non-pregnant (open bars) mice. Data shown are means ± SD from three independent brain tissues per group (pregnant and non-pregnant).

Fig. S1



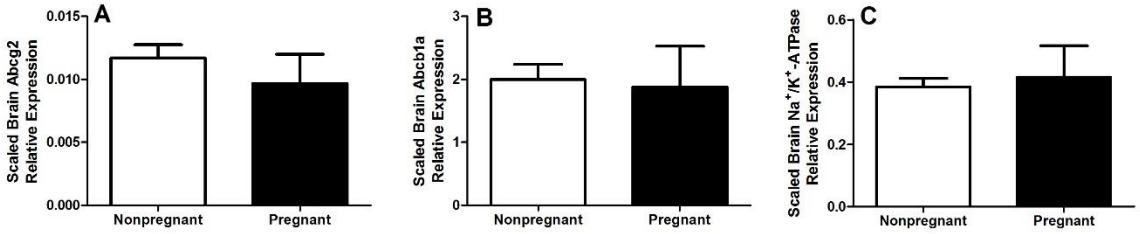
Supplemental Figure S1. Relative protein abundance of transporters in the liver of pregnant and non-pregnant mice after scaling to the whole liver. Relative protein levels of Abcg2 (**A**), Abcc2 (**B**), Abcc3 (**C**), Slco1a4 (**D**), Abcb11 (**E**), Abcb1a (**F**), Abcc4 (**G**), Slco1a1 (**H**), Slco1b2 (**I**), and Na^+/K^+-ATPase (**J**) in isolated total membrane proteins of mouse liver from pregnant (filled bars) and non-pregnant (open bars) mice. Data shown are means ± SD from three independent liver tissues per group (pregnant and non-pregnant).

Fig. S2



Supplemental Figure S2. Relative protein abundance of transporters in the kidney of pregnant and non-pregnant mice after scaling to the whole kidney. Relative protein levels of Abcg2 (**A**), Abcc2 (**B**), Slco2b1 (**C**), Slco1a4 (**D**), Abcc3 (**E**), Abcb1a (**F**), and Na^+/K^+-ATPase (**G**) in isolated total membrane proteins of mouse kidney from pregnant (filled bars) and non-pregnant (open bars) mice. Data shown are means ± SD from three independent kidney tissues per group (pregnant and non-pregnant).

Fig. S3



Supplemental Figure S3. Relative protein abundance of transporters in the brain of pregnant and non-pregnant mice after scaling to the whole brain. Relative protein levels of Abcg2 (**A**), Abcb1a (**B**), and Na^+/K^+-ATPase (**C**) in isolated total membrane proteins of mouse brain from pregnant (filled bars) and non-pregnant (open bars) mice. Data shown are means ± SD from three independent brain tissues per group (pregnant and non-pregnant).

Supplemental Table S1. Surrogate peptides of transporters and their MS/MS parameters

Protein name	Surrogate peptide	Peptide type	Parent ion (*m/z*)	Fragment ion (*m/z*)	Declustering Potential (V)	Collision Energy (V)
Abcb1a	NTTGALTTR	light	467.751	561.335	65.2	25.7
Abcb1a	NTTGALTTR	light	467.751	490.298	65.2	25.7
Abcb1a	NTTGALTTR	light	467.8	719.1	65.2	25.7
Abcb1a	NTTGALTTR	heavy	472.755	628.4	65.2	25.7
Abcb1a	NTTGALTTR	heavy	472.755	729.4	65.2	25.7
Abcb1b	AGAVAEEVLAAIR	light	635.362	971.552	35	21
Abcb1b	AGAVAEEVLAAIR	light	635.362	430.277	35	21
Abcb1b	AGAVAEEVLAAIR	heavy	640.366	981.56	35	21
Abcb1b	AGAVAEEVLAAIR	heavy	640.366	440.286	35	21
Abcb1b	IATEAIENFR	light	582.306	979.484	35	15
Abcb1b	IATEAIENFR	light	582.306	678.357	35	15
Abcb1b	IATEAIENFR	light	582.306	490.246	35	15
Abcb1b	IATEAIENFR	heavy	587.311	688.365	35	15
Abcb1b	IATEAIENFR	heavy	587.311	495.25	35	15
Abcg2	ENLQFSAALR	light	574.807	792.436	73	29.6
Abcg2	ENLQFSAALR	light	574.807	664.378	73	29.6
Abcg2	ENLQFSAALR	heavy	579.811	802.445	73	29.6
Abcg2	ENLQFSAALR	heavy	579.811	674.386	73	29.6
Abcb11	STALQLIQR	light	515.306	841.525	68.7	22.4
Abcb11	STALQLIQR	light	515.306	529.346	68.7	22.4
Abcb11	STALQLIQR	heavy	520.31	851.534	68.7	27.4
Abcb11	STALQLIQR	heavy	520.31	780.497	68.7	27.4
Abcb11	STALQLIQR	heavy	520.31	667.413	68.7	27.4
Abcc2	LTIIPQDPILFSGNLR	light	899.017	1016.59	96.7	41.2
Abcc2	LTIIPQDPILFSGNLR	light	899.017	806.452	96.7	41.2
Abcc2	LTIIPQDPILFSGNLR	light	899.017	693.368	96.7	41.2
Abcc2	LTIIPQDPILFSGNLR	heavy	904.021	1026.6	96.7	41.2
Abcc2	LTIIPQDPILFSGNLR	heavy	904.021	929.544	96.7	41.2
Abcc2	LTIIPQDPILFSGNLR	heavy	904.021	816.46	96.7	41.2
Abcc3	FYVATSR	light	422.222	533.304	61.9	24.1
Abcc3	FYVATSR	light	422.222	434.236	61.9	24.1
Abcc3	FYVATSR	light	427.226	543.312	61.9	24.1
Abcc3	FYVATSR	heavy	427.226	706.4	61.9	24.1
Abcc3	LLEAWQK	light	444.253	661.33	53.5	19.9
Abcc3	LLEAWQK	light	444.253	532.288	53.5	19.9
Abcc3	LLEAWQK	light	444.253	461.251	53.5	19.9
Abcc3	LLEAWQK	heavy	448.26	669.345	53.5	19.9
Abcc3	LLEAWQK	heavy	448.26	540.302	53.5	19.9
Abcc3	LLEAWQK	heavy	448.26	469.265	53.5	19.9

Abcc4	GTYTEFLK	light	479.748	637.356	66.1	26.1
Abcc4	GTYTEFLK	light	479.748	536.308	66.1	26.1
Abcc4	GTYTEFLK	heavy	483.755	645.37	66.1	26.1
Abcc4	GTYTEFLK	heavy	483.755	544.322	66.1	26.1
Abcc4	LNTIIDSDR	light	523.777	718.373	69.3	27.7
Abcc4	LNTIIDSDR	light	523.777	605.289	69.3	27.7
Abcc4	LNTIIDSDR	heavy	528.782	728.381	69.3	27.7
Abcc4	LNTIIDSDR	heavy	528.782	615.297	69.3	27.7
Na^+/K^+-ATPase	AAVPDAVGK	light	414.235	685.388	61.3	18.8
Na^+/K^+-ATPase	AAVPDAVGK	light	414.235	586.32	61.3	18.8
Na^+/K^+-ATPase	AAVPDAVGK	light	414.235	489.267	61.3	18.8
Na^+/K^+-ATPase	AAVPDAVGK	heavy	418.242	693.402	61.3	23.8
Na^+/K^+-ATPase	AAVPDAVGK	heavy	418.242	594.334	61.3	23.8
Na^+/K^+-ATPase	AAVPDAVGK	heavy	418.242	497.281	61.3	23.8
Na^+/K^+-ATPase	VDNSSLTGESEPQTR	light	810.379	1004.46	90.2	33
Na^+/K^+-ATPase	VDNSSLTGESEPQTR	light	810.379	903.417	90.2	33
Na^+/K^+-ATPase	VDNSSLTGESEPQTR	light	810.379	846.395	90.2	33
Na^+/K^+-ATPase	VDNSSLTGESEPQTR	heavy	815.383	1014.47	90.2	38
Na^+/K^+-ATPase	VDNSSLTGESEPQTR	heavy	815.383	913.425	90.2	38
Na^+/K^+-ATPase	VDNSSLTGESEPQTR	heavy	815.383	856.403	90.2	38
Slco1a1	GVQHPLYGEK	light	564.296	843.436	72.3	24.2
Slco1a1	GVQHPLYGEK	light	564.296	706.377	72.3	24.2
Slco1a1	GVQHPLYGEK	light	564.296	609.324	72.3	24.2
Slco1a1	GVQHPLYGEK	heavy	568.303	851.45	72.3	29.2
Slco1a1	GVQHPLYGEK	heavy	568.303	714.391	72.3	29.2
Slco1a1	GVQHPLYGEK	heavy	568.303	617.338	72.3	29.2
Slco1a1	EVATHGVR	light	439.74	650.361	62.8	24.5
Slco1a4	EVATHGVR	light	439.74	579.324	62.8	24.5
Slco1a4	EVATHGVR	light	439.74	478.276	62.8	24.5
Slco1a4	EVATHGVR	light	439.74	341.217	62.8	24.5
Slco1a4	EVATHGVR	heavy	434.735	640.353	62.8	24.5
Slco1a4	EVATHGVR	heavy	434.735	569.315	62.8	24.5
Slco1a4	EVATHGVR	heavy	434.735	468.268	62.8	24.5

Slco1a4	EVATHGVR	heavy	434.735	538.262	62.8	24.5
Slco1b2	SVQPELK	light	400.729	614.351	60.3	23.3
Slco1b2	SVQPELK	light	400.729	486.292	60.3	23.3
Slco1b2	SVQPELK	heavy	404.736	622.365	60.3	23.3
Slco1b2	SVQPELK	heavy	404.736	494.306	60.3	23.3
Sclo2b1	LYVDIDR	light	447.24	617.325	63.7	25
Sclo2b1	LYVDIDR	light	447.24	518.257	63.7	25
Sclo2b1	LYVDIDR	heavy	452.244	790.397	63.7	25
Sclo2b1	LYVDIDR	heavy	452.244	627.334	63.7	25
Sclo2b1	FIGLQFFFK	light	573.821	588.318	72.9	29.5
Sclo2b1	FIGLQFFFK	light	573.821	441.25	72.9	29.5
Sclo2b1	FIGLQFFFK	light	573.821	500.287	72.9	29.5
Sclo2b1	FIGLQFFFK	heavy	577.828	837.475	72.9	29.5
Sclo2b1	FIGLQFFFK	heavy	577.828	724.391	72.9	29.5
Slc22a3	FLQGVFGK	light	448.255	635.351	63.8	25
Slc22a3	FLQGVFGK	light	448.255	507.293	63.8	25
Slc22a3	FLQGVFGK	light	448.255	450.271	63.8	25
Slc22a3	FLQGVFGK	heavy	452.263	643.365	63.8	25
Slc22a3	FLQGVFGK	heavy	452.263	515.307	63.8	25
Slc22a3	FLQGVFGK	heavy	452.263	458.285	63.8	25

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Chapter 5: Effects of Pregnancy on the Pharmacokinetics of Metformin

The work presented in this chapter is under preparation for submission to Drug Metabolism and Disposition

5.1 Introduction

Gestational diabetes mellitus (GDM) complicates 5-13% of pregnancies in the U.S. (1). GDM is associated with carbohydrate intolerance resulting in hyperglycemia of variable severity (2). GDM is associated with increased risk for maternal infections, traumatic deliveries, hypertensive disorders, and preeclampsia as well as neonatal hypoglycemia, respiratory distress syndrome, macrosomia, polyhydramnios, shoulder dystocia and kernicterus (3, 4). Surgical deliveries are much more common in women with GDM. Although GDM resolves after pregnancy, these women have an increased risk of type 2 diabetes mellitus (T2DM) later in life (5, 6).

Historically, insulin has been the mainstay of GDM pharmacological treatment. However, due to ease of administration, lower cost, and comparable efficacy, oral hypoglycemic agents are now commonly used in the management of GDM. Metformin, an oral anti-hyperglycemic biguanide frequently utilized in the treatment of GDM, decreases insulin resistance (7-9). Metformin is a small, basic compound with a high unbound fraction (10). In addition, it is a substrate for organic cation transporters (OCTs) (11-14) and almost entirely excreted unchanged in the urine (15). OCT2 (encoded by SCL22A2) has been proposed to play a critical role in the pharmacokinetics (PK) of metformin. Metformin is actively transported from the peritubular capillaries into renal epithelial cells primarily by OCT2 leading to net renal

secretion (16). As a strong base with pKa of 11.5, metformin's net tubular secretion is not likely to be subject to passive tubular reabsorption (17). Decreased activity of OCT2 in non-pregnant subjects due to genetic differences or drug-drug interactions with inhibitors such as cimetidine, results in 30-60% decrease in metformin renal and net renal secretion clearances, and up to 74% increase in area under the concentration-time curve (AUC) (18-22). Renal excretion of metformin from tubular cells into the urine is mediated through the multidrug and toxin extrusion proteins 1 and 2-K (MATE1/SLC47A1 and MATE2-K/SLC47A2) (23-26). In addition to OCT2, MATE1 and MATE2-K, metformin is also a substrate of OCT1 (SLC22A1) and the plasma membrane monoamine transporter (PMAT). OCT1 is predominantly expressed in the liver, but has also been reported to be expressed on the apical side of both the proximal and distal tubules in the kidney (27). PMAT (SLC29A4) is expressed on the apical membrane of intestinal epithelial cells and has been suggested to be involved in intestinal absorption of metformin (28). PMAT is expressed on the apical membranes of renal epithelial cells and may use luminal proton gradient to drive organic cation reabsorption in the kidney. Finally, metformin crosses the placenta via active transport. Umbilical cord concentrations of metformin at the time of delivery have been reported to exceeding maternal concentrations in some cases by 50% (29-31).

Preliminary data suggest that the pharmacokinetics of metformin are altered by pregnancy (32). The main objective of this study was to evaluate the effects of pregnancy on the PK of metformin.

5.2 Materials and Methods

This was a multicenter, prospective, randomized phase II PK study (clinicaltrials.gov identifier NCT01329016). We examined the time course of orally administered steady-state metformin in the plasma of pregnant women with GDM receiving either metformin mono-

therapy (n=24) or in combination with glyburide (n=30), as well as metformin mono-therapy in non-pregnant women with T2DM (n=24). The study was approved by the institutional review boards at the University of Washington, Madigan Army Medical Center, University of Texas Medical Branch in Galveston, University of Pittsburgh, Indiana University, University of Utah Health Care, University of Alabama at Birmingham and RTI International and conducted in accordance with their guidelines. All subjects gave written informed consent.

Subjects

GDM: Women, 18-45 years of age, with singleton pregnancies were included after receiving a diagnosis of GDM based on either a 1-hour glucose tolerance test (50 Gm) > 185 mg/dL, 2-hour oral glucose tolerance test (75 Gm) with 1 or more values meeting or exceeding the International Association of Diabetes and Pregnancy Study Groups Consensus Panel Guidelines (33) or 3-hour oral glucose tolerance test (100 Gm) with 2 or more values meeting or exceeding Carpenter and Coustan designations (34) and failure to achieve glycemic control with dietary therapy. Women were excluded if they were taking medications expected to interact with metformin or alter blood glucose concentrations or had any of the following: serum creatinine > 1.2 mg/dL, hematocrit < 28%, allergy to metformin, significant hepatic disease, congestive heart failure, history of MI, moderate to severe pulmonary disease, adrenal insufficiency or pituitary insufficiency.

T2DM: Non-pregnant female subjects with T2DM, 18-45 years of age, who were receiving metformin were included in the study. Thirteen non-pregnant subjects were included based on the above criteria. In addition, we included 11 female, non-pregnant 24-44 year old subjects with T2DM from another similar study in which subjects received the same formulation of metformin and underwent the same steady-state metformin pharmacokinetic sample

collections described below (32). Subjects were excluded for serum creatinine > 1.2 mg/dL and hematocrit < 28%.

Dosing regimen

Subjects with GDM were randomized after diagnosis, but prior to 33 weeks gestation, to metformin mono-therapy, glyburide mono-therapy or metformin and glyburide combination therapy. Results from the glyburide mono-therapy group are not reported in this paper. Metformin dosage was initiated at 500 or 1000 mg orally twice daily and titrated based on clinical need. Metformin immediate-release tablets were provided by the investigators for treatment of subjects with GDM and for the 3 days prior to the PK study for non-pregnant subjects with T2DM. Subjects with T2DM were not randomized to treatment. Rather, they received metformin for therapeutic reasons and dosages were titrated without regard to the study. Subjects recorded on a calendar the time each metformin dose was taken for the 3 days prior to the pharmacokinetic study day and pill counts were performed to assess adherence. Except for clear liquids, subjects fasted for ≥ 5 hours prior to study drug administration on the PK study day. Metformin was administered simultaneously with the initiation of a standardized meal containing 2 slices of whole-wheat toast, 2 teaspoons margarine, and 240 mL Boost Plus® consumed within 10 minutes.

Sample Collection

On the day of the metformin PK study, serial blood samples were collected: pre-dose, then 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 hours post-dose, truncated to the dosing interval, for measurement of metformin plasma concentrations. Urine was collected in 4-hour intervals as follows: pre-dose, 0-4 hours, 4-8 hours and 8-12 hours post-dosing, truncated to the dosing interval. When possible, maternal as well as umbilical cord venous and arterial blood samples

were collected at the time of delivery for measurement of plasma metformin concentrations. Blood samples were collected in heparinized tubes and plasma was isolated by centrifugation and stored at -80^{o} C until analysis. Urine was refrigerated until completion of the collection interval, then stored at -80^{o} C until analysis.

Plasma and Urine Metformin Analysis

Metformin plasma and urine concentrations were measured utilizing a validated LC-MS/MS assay as previously described (35). The lower limits of quantitation were 4.95 ng/mL for plasma and 30 µg/mL for urine. For plasma, the coefficients of variation for this method were 2.6-11.9% for intra-day and 2.1-6.4% for inter-day and accuracy was 96-100%. For urine, the coefficient of variation for this method was <14% for intra- and inter-day and accuracy was 94-105%.

Genotyping

DNA was isolated from whole blood, and genotypes were determined using validated TaqMan assays. Maternal and umbilical cord samples were assayed for OCT1: SLC22A1 (rs622342); OCT2: SLC22A2c.808G>T polymorphism (rs316019); MATE1: SLC47A1 (rs2289668), and (rs8065082); MATE2-K: -130G>A polymorphism (rs12943590); as well as PMAT (rs2685753) and (rs6971788).

Steady State Pharmacokinetics Analysis

Steady-state metformin PK parameters were estimated using standard noncompartmental techniques. Maximum concentration (C_{max}) and time to maximum concentration (T_{max}) were determined from the measured concentrations. Area under the concentration-time curve (AUC) was estimated using the linear trapezoidal rule. When concentrations fell below the lower limit of quantification for the assay prior to the end of the dosing interval, the concentration at the end

of the dosing interval was estimated by extrapolating the predicted concentration based on the regression line from the time of the last measurable concentration (C_{last}) using $C_{end\ of\ dosing\ interval} = C_{last} \bullet e^{-kt}$, where k was the elimination rate constant and t was the time between C_{last} and the end of the dosing interval. The elimination rate constant (k) was determined by log-linear regression of the terminal slope. Apparent oral clearance (CL/F) was estimated by CL/F = dose/AUC. Apparent oral volume of distribution (V_β/F) was estimated by $V_\beta/F = (CL/F)/k_{elim}$. Half-life ($T_{1/2}$) was estimated by $T_{1/2} = \ln(2)/k$. Renal clearance (CL_R) was determined by Ae/AUC, where Ae = amount of metformin excreted in the urine unchanged. Metformin net renal secretion clearance (CL_{sec}) was estimated by $CL_{sec} = CL_R - (fu \times CrCL)$, where fu was the unbound fraction of metformin in plasma and was assumed to be 1 and CrCL was creatinine clearance (17). Creatinine clearance was estimated by CrCL = [(Uv)(UCr)]/[(SCr)(time)], where Uv = urine volume, UCr = urine creatinine and SCr = serum creatinine. The percent of dose recovered in the urine was determined by Ae/dose X 100. Metformin is not metabolized, has negligible biliary excretion, and is eliminated exclusively by the kidneys. Therefore, for metformin, it is acceptable to estimate bioavailability (F) by F = Ae/dose (36-40). V_β and CL were estimated by $V_\beta = (V_\beta/F) \times F$ and $CL = (CL/F) \times F$. Pharmacokinetic parameters were adjusted based on actual body weights.

Statistical Analysis

Mann-Whitney test was used to compare PK parameters for mono-therapy vs combination therapy for 500 mg and 1000 mg doses separately, compare all 500 mg to all 1000 mg doses, and compare PK parameters in pregnant to non-pregnant subjects for 500 mg and 1000 mg doses separately. Results are reported as means ± standard deviations, for the above analyses. In addition, all data were combined (all subjects and all doses), and the effects of

pregnancy were assessed using the linear mixed effect model controlling for random effects of dose and weight of individuals and then compared by ANOVA. Genotypes for drug transporters were tested by one-way ANOVA or Mann-Whitney test.These results are reported as mean ± standard error of the mean. $P \leq 0.05$ was considered significant.

5.3 Results

Fifty-seven subjects with GDM at 26-38 weeks gestation (27 White, 19 Hispanic/Latina, 1 Native American, 8 Black, and 2 Asian) and 24 non-pregnant subjects with T2DM (8 White, 9 Hispanic/Latina, 1 Native American, 5 Black, and 1 Asian) participated in this study. Metformin daily doses ranged from 1000-2000 mg/day in 2 divided doses, with the exception of 4 subjects who were taking metformin 3 times per day. We were unable to determine half-life and apparent oral volume of distribution in 3 subjects with GDM and 1 subject with T2DM due to termination of sample collection prior to reaching the terminal elimination phase.

Figure 1 depicts mean steady-state metformin concentration-time profiles in pregnant women with GDM and in non-pregnant women with T2DM receiving 500 mg orally twice daily. Estimated steady-state metformin PK parameters for pregnant women with GDM and non-pregnant women with T2DM are reported in Table 1. For both 500 mg and 1000 mg doses respectively, pregnancy significantly increased metformin bioavailability ($P < 0.01$, $P < 0.01$), V_β ($P < 0.001$, $P < 0.005$), weight-normalized V_β ($P < 0.005$, $P < 0.005$), CL ($P < 0.01$, $P < 0.05$), weight-normalized CL ($P < 0.01$, $P < 0.05$), percent of dose excreted unchanged in urine ($P < 0.01$, $P < 0.01$), CL_R, ($P < 0.01$, $P < 0.05$), weight-normalized CL_R, ($P < 0.01$, $P < 0.05$) as well as creatinine clearance ($P < 0.001$, $P < 0.001$). Pregnancy also significantly increased metformin Tmax ($P < 0.01$), V_β/F ($P < 0.05$), CL/F ($P < 0.05$) and weight-normalized CL_{sec} ($P < 0.05$) for women receiving 500 mg. However, V_β/F, CL/F and CL_{sec} were not significantly different in

pregnant subjects when compared to non-pregnant subjects for those women receiving 1000 mg doses. Half-life of metformin for both 500 mg and 1000 mg was not significantly altered during pregnancy.

All data were combined (all subjects and all doses), and the effects of pregnancy were assessed using the linear mixed effects model controlling for random effects of dose and subjects' weight and then compared by ANOVA. Multiple PK parameters were found to be significantly altered by pregnancy. The presence of the pregnancy factor corresponded to a significant increase of 0.16 ± 0.04 for bioavailability (χ^2 = 12.1, P = 0.0005), 174.24 ± 51 mL/min (χ^2 = 9.57, P = 0.002) for clearance, 77 ± 18 L (χ^2 = 15.36, P = $9*10^{-5}$) for volume of distribution and 193 ± 69 mL/min for renal clearance (χ^2 = 7.35, P = 0.006) of metformin. Pregnancy also significantly increased creatinine clearance by 71 ± 14 mL/min (χ^2 = 22.2, P = $2*10^{-6}$). There was a trend toward an increase in metformin renal secretion clearance during pregnancy (121 ± 63 mL/min, χ^2 = 3.55, P = 0.06). When using the linear mixed effect model controlling for random effects, pregnancy did not significantly alter half-life, time to maximum concentration, apparent oral volume of distribution or apparent oral clearance.

As expected, Figure 2 shows that mean steady-state metformin oral concentration time profiles are lower in pregnant women with GDM receiving 500 mg twice daily vs. 1000 mg twice daily. Table 2 reports metformin PK parameters during pregnancy comparing 500 mg to 1000 mg dose. Doubling the dose increased Vβ/F by 22% (368 ± 133 L versus 450 ± 136 L, P < 0.02), weight adjusted Vβ/F by 25% (4.0 ± 1.7 L/kg versus 5.0 ± 1.6 L/kg, P < 0.006), CL/F by 29% (1085 ± 328mL/min versus 1405 ± 384mL/min, P < 0.02) and weight adjusted CL/F by 33% (11.7 ± 3.8mL/min/kg versus 15.6 ± 4.5mL/min/kg, P < 0.02). Half-life, bioavailability, Cmax, CL, V, percent of dose excreted unchanged in urine, CLR and CLsec were not

significantly changed. Co-administration of metformin with glyburide did not alter the estimated metformin PK parameters (data not shown).

In both pregnant women with GDM and non-pregnant women with T2DM, metformin renal clearance had a moderate correlation with creatinine clearance (Fig. 3a: $r = 0.67$, $P < 0.0001$) and an excellent correlation with renal secretion clearance (Fig 3b: $r = 0.96$, $P < 0.0001$). Metformin renal secretion clearance had a weaker correlation with creatinine clearance ($r = 0.41$, $P < 0.001$)) (data not shown). Metformin apparent oral clearance poorly correlated with CrCL ($r = 0.18$, $P = 0.06$) (data not shown).

Out of 38 umbilical cord plasma samples collected, 14 arterial and 11 venous, metformin concentrations were below the lower limit of quantification. Mean duration from last maternal dose to time of sample collection was 19 ± 7.8 hours (range: 5.5 to 37.3 hours). The mean measurable metformin plasma concentrations in the umbilical cord arterial and venous samples were 290 ± 233 ng/mL (range: 13 to 910 ng/mL) and 331 ± 257 ng/mL (range: 11 to 1070 ng/mL), respectively. The mean maternal metformin plasma concentration was 262 ± 231 ng/mL (range: 6 to 1020 ng/mL). The mean metformin umbilical cord arterial-to-venous plasma concentration ratio was 1.0 ± 0.1. The mean metformin umbilical cord arterial-to-maternal plasma concentration ratio was 1.5 ± 0.5 and umbilical cord venous-to-maternal concentration ratio was 1.4 ± 0.5. Figure 4 depicts the relationship between time post-last dose and metformin umbilical cord-to-maternal plasma concentration ratios ($r = 0.3$, $P = 0.1$).

Genotype for various relevant metformin drug transporters were determined. OCT1: SLC22A1 (rs622342); OCT2: SLC22A2c.808G>T polymorphism (rs316019); MATE1: SLC47A1 (rs2289668), and (rs8065082); MATE2-K: -130G>A polymorphism (rs12943590); as well as PMAT (rs2685753) and (rs6971788) were not

significantly associated with metformin AUC, CL/F, CL, CL_{sec}, bioavailability, or umbilical cord-to-maternal plasma concentration ratio.

5.4 Discussion

Pregnancy has been reported to alter the PK of many drugs, including metformin (41-43). PK changes during pregnancy are challenging to predict. Pregnancy alters gastric emptying, intestinal transit, organ blood flow, blood volume, protein binding, body composition, drug metabolizing enzymes and drug transporters expressions, which can influence the overall drug exposure, clearance, and volume of distribution (41, 43). The current study confirms the changes in metformin PK during pregnancy and offers additional mechanistic insight as to the cause.

Metformin is eliminated almost exclusively by the kidneys. It is not metabolized and biliary excretion is negligible in human (15, 39, 40). Therefore, the ratio of Ae/dose is a reasonable estimate of bioavailability (36-38, 44). We found that metformin bioavailability was significantly higher in pregnant women with GDM compared to non-pregnant women with T2DM. The increase in absorption may be due to slowed gastrointestinal (GI) motility during pregnancy. As reported previously, the increase in progesterone during pregnancy prolongs small intestine transit time, which can increase absorption of metformin (42, 45-47). Higher metformin bioavailability was also observed in healthy non-pregnant individuals taking propantheline, a drug that slows GI motility (45).

The higher bioavailability of metformin during pregnancy may also be due to up regulation of the active uptake transporter processes in the intestine. Currently, the molecular mechanism underlying metformin intestinal absorption is not well understood. Studies in the Caco-2 monolayer culture model suggest that metformin uptake may involve multiple transporters including OCT1 and PMAT (48). Nothing is known about potential changes in the

expression of these transporters during pregnancy. Although documentation of increased xenobiotic absorption during pregnancy is limited, increased intestinal absorption of dietary micronutrients is well documented. In human and animal pregnancy, there is a 2-fold increase in the intestinal absorption of calcium, mediated by increases in 1,25-dihydroxyvitamin D and other mechanisms (49). Intestinal absorption of dietary iron also increases during the second and third trimesters of pregnancy to accommodate for the need to expand red blood cell mass (50). Absorption of dietary zinc and vitamin B12 also increase during pregnancy (51-53).

Our study demonstrates that metformin clearance and weight-adjusted clearance were significantly higher during pregnancy. This is driven by changes in metformin renal clearance. Renal clearance was significantly higher during pregnancy and consistent with that previously reported by Eyal et al. (32). The pregnancy related changes in renal clearance appear to be largely influenced by increased active renal transport. Figure 3b illustrates the strong correlation between metformin renal clearance and net renal secretion clearance ($r = 0.96$, $P < 0.0001$). This result is not unexpected because net renal secretion clearance makes up approximately 2/3 of metformin renal clearance, thereby leading to an excellent correlation. Metformin active renal tubular transport has been reported to be mediated by organic cation transporters and multidrug and toxin extrusion proteins. (54-59). Metformin is a substrate for multiple OCTs, including OCT1, OCT2, OCT3, PMAT, and MATEs. In humans, OCT2 and MATEs play a major role in metformin renal clearance (26, 54-59). Several studies *in vitro* and in animals suggest that Oct2 expression and activity in the kidney are regulated by steroidal hormones (48, 60, 61). However, little is known about OCT2 regulation in human pregnancy.

Metformin renal clearance is also influenced by renal filtration. Figure 3a demonstrates the relationship between metformin renal clearance and creatinine clearance ($r = 0.41$, $P <$

0.001). The correlation between metformin renal clearance and creatinine clearance is not as strong as that seen with net renal secretion clearance likely due to the major role active transport plays in metformin renal clearance. Previous work reported as much as a 40% increase in glomerular filtration rate and 10-35% increase in renal blood flow during pregnancy (56). This is consistent with the higher creatinine clearance seen during pregnancy in this study ($P < 0.00002$), as well as the increase in metformin renal clearance.

In contrast to the PK changes reported in this study, Charles et al. (62) utilizing a population PK approach, found no change in metformin clearance in late pregnancy compared to non-pregnant patients. They utilized sparse sampling, with a median of 2 plasma samples per patient in the third trimester of pregnancy. They also fixed their bioavailability at 0.5. Difference in study design and modeling analysis likely contributed to the discrepancy in results.

Apparent oral clearance was significantly higher in pregnant women receiving a 500 mg dose. This will result in lower systemic metformin exposure than what might be expected for a non-pregnant woman with GDM, necessitating an increased dose for the desired pharmacological effect. However, this was not the case with women receiving a 1000 mg dose. In a pilot study, Eyal et al. (32) found no significant change in apparent oral clearance during pregnancy at dosage ranged from 500 to 3000 mg/day. The higher metformin bioavailability during pregnancy offset the increase in metformin CL leading to no significant change in CL/F (ie, dose-normalized AUC) in patients receiving 1000 mg of metformin. The offsetting also occurred with 500 mg dose, however, the increase in clearance outpaced the increase in bioavailability.

It has been previously reported that low functional OCT1 was associated with lower metformin apparent oral clearance (63). Decreased activity of OCT2 has been reported to

decrease metformin renal and renal secretion clearances 30-60% and increase area under the concentration-time curve (AUC) up to 74% (57-59). It has also been reported that MATE1 and MATE2K are important for renal excretion (23-26) and PMAT for reabsorption of metformin (64). In contrast to previous pharmacogenetics studies in non-pregnant subjects described above that demonstrated differences in metformin PK based on genotype (n ranging from 26 to 208), we found no significant association between OCT1, OCT2, MATE1, MATE2-K, or PMAT genotype and metformin AUC, CL/F, CL, renal secretion CL, bioavailability, or umbilical cord to maternal plasma concentration ratio in our 77 pregnant women. Similarly, Duong et al. (65) found no significant relationship between OCTs, MATEs, and PMAT gene variant and apparent oral clearance or AUC with 57 subjects. The limited number of patients in this study and the potential confounding factor of pregnancy may have masked a genetic effect.

Metformin volume of distribution significantly increased during pregnancy ($P<0.001$). The presence of the fetus and placenta, as well as, the increase in total body water, can increase the volume of distribution of hydrophilic drugs such as metformin and is likely the reason for the higher metformin volume of distribution during pregnancy. Apparent oral volume of distribution significantly increased in pregnant women receiving the 500 mg dose; however, the higher bioavailability during pregnancy offset the higher volume of distribution leading to no significant change in apparent oral volume of distribution for pregnant women taking 1000 mg. The apparent oral volume of distribution in our study was similar to that reported by Eyal et al. (32) during late pregnancy (432 ± 168 L), but lower than that reported by de Oliveira Baraldi et al. (66) (551 L; range 385-1173 L). In addition, the weight-normalized apparent oral volume of distribution was comparable to that previously reported during late pregnancy by Eyal et al. (32) (4.0 ± 1.5 L/kg). Lastly, the apparent oral volume of distribution in our non-pregnant T2DM

patients was similar to that reported by Sambol et al. (67) in non-pregnant healthy volunteers (367 ± 42 L).

Our current study enabled us to evaluate the PK of both 500 and 1000 mg doses during pregnancy. Increasing the metformin dose from 500 to 1000 mg during pregnancy increased the CL/F and V_{β}/F ($P < 0.02$, $P < 0.02$, respectively). This observation appears to be driven primarily by the trend toward higher bioavailability with the 500 mg dose and minor differences in clearance and volume. A decrease in bioavailability with increasing dose was also observed in the non-pregnant population reported elsewhere (44). Tucker et al. (44) reported that the bioavailability of a 1500 mg dose of metformin was 9-24% lower than that of a 500 mg dose (n=4). Sambol et al. (38) found that an 850 mg dose of metformin had a bioavailability that was 12% lower than that of a 500 mg dose. These data are consistent with metformin having capacity limited absorption. Although not reaching significance, our current study found a trend towards a lower metformin bioavailability with 1000 mg than with 500 mg ($P = 0.1$) doses during pregnancy. Metformin is highly water-soluble, therefore solubility is unlikely a factor in bioavailability dose-disproportionality. In addition to its water solubility, metformin is largely ionized in the GI tract, which limits its permeability. Active transport processes may be involved in metformin absorption. Higher doses of metformin may saturate the uptake transporters in the enterocytes, which would lead to decreased absorption and bioavailability with higher doses (68).

In our study, metformin umbilical cord-to-maternal plasma concentration ratios range from 0.7 to 2.8. This is consistent with previous reports of metformin umbilical cord serum concentrations being comparable with or exceeding maternal concentrations (32, 66, 69). Given metformin's water solubility, it does not easily diffuse across membranes. These data taken

together suggest active transport of metformin from the maternal to the fetal side. Metformin is a substrate for the serotonin transporter and OCT3 (48, 70), which are expressed in the human placenta. In addition, our finding that the metformin plasma umbilical cord arterial to venous concentration ratio centered around 1 ($r = 0.1$) is consistent with insignificant fetal extraction of metformin and similar to the results reported by de Oliveira Baraldi et al. (66).

5.5 Tables and Figures

Table 5-1. Estimated metformin steady-state pharmacokinetic parameters for pregnant women with GDM and non-pregnant women with T2DM separated by dose.

	500 mg Pregnant (n = 39)	500 mg Non-Pregnant (n = 9)	*P value*	1000 mg Pregnant (n = 15)	1000 mg Non-Pregnant (n = 14)	*P value*
Metformin						
F	0.64 ± 0.2	0.49 ± 0.08	< 0.01	0.53 ± 0.11	0.37 ± 0.14	< 0.01
Half-life (hr)	4.2 ± 1.1	4.0 ± 0.8	0.4	4.3 ± 0.7	3.7 ± 0.6	0.2
Tmax (hr)	2.6 ± 1.3	2.1 ± 0.5	< 0.01	2.4 ± 1.1	2.2 ± 1.3	0.7
V_B/F (L)	368 ± 133	276 ± 52	< 0.05	450 ± 136	486 ± 207	0.6
V_B/F (L/kg)	4.00 ± 1.68	3.19 ± 1.10	0.08	4.99 ± 1.64	5.00 ± 2.07	0.9
V_B(L)	242 ± 138	134 ± 23	< 0.001	234 ± 66	163 ± 48	< 0.005
V_B (L/kg)	2.6 ± 1.6	1.5 ± 0.3	< 0.005	2.6 ± 0.9	1.7 ± 0.6	< 0.005
CL/F (mL/min)	1085 ± 328	854 ± 230	< 0.05	1405 ± 384	1678 ± 574	0.4
CL/F (mL/min/kg)	11.7 ± 3.8	9.5 ± 2.4	0.05	15.6 ± 4.5	17.5 ± 6.5	0.3
CL (mL/min)	712 ± 346	421 ± 136	< 0.01	723 ± 142	567 ± 153	< 0.05
CL (mL/min/kg)	7.6 ± 4.0	4.6 ± 1.1	< 0.01	8.1 ± 2.0	6.1 ± 2.4	< 0.05
Percent of dose recovered in the urine unchanged (%)	64 ± 20	49 ± 8	< 0.01	53 ± 11	37 ± 14	< 0.01
CL_R (ml/min)	731 ± 339	444 ± 121	< 0.01	758 ± 179	572 ± 153	< 0.05
CL_R (ml/min/kg)	7.9 ± 3.9	4.9 ± 1.3	< 0.01	8.5 ± 2.5	6.1 ± 2.4	< 0.05
CrCL (ml/min)	233 ± 68	148 ± 41	< 0.001	233 ± 46	166 ± 25	< 0.001
CL_{sec} (ml/min)	497 ± 312	296 ±97	0.06	530 ± 168	406 ± 143	0.1
CL_{sec} (ml/min/kg)	5.4 ± 3.6	3.3 ± 1.1	< 0.05	5.9 ± 2.3	4.3 ± 2.0	0.2

Results are reported as means ± S.D. Mann-Whitney test was used to compare PK parameters for pregnant and non-pregnant women receiving either 500 mg or 1000 mg doses. $P < 0.05$ was considered significant.

Table 5-2. Estimated metformin steady-state pharmacokinetic parameters for all pregnant subjects (metformin mono-therapy and combination therapy) receiving 500 mg and 1000 mg doses twice daily

	500 mg (n = 39)	1000 mg (n = 15)	*P value*
Metformin			
Half-life (hr)	4.2 ± 1.1	4.3 ± 0.7	0.4
Tmax (hr)	2.6 ± 1.3	2.4 ± 1.1	0.8
Cmax (ug/mL)	1.2 ± 0.5	1.8 ± 0.5	
$AUC_{(0\text{-}12)}$ (ug*hr/mL)	7.8 ± 2.5	12.1 ± 3.4	
V_B/F (L)	368 ± 133	450 ±136	< 0.02
V_B/F (L/kg)	4.0 ± 1.7	5.0 ± 1.6	< 0.006
V_B(L)	242 ± 138	234 ± 66	0.3
V_B (L/kg)	2.6 ± 1.6	2.6 ± 0.9	0.2
CL/F (mL/min)	1085 ± 328	1405 ± 384	< 0.02
CL/F (mL/min/kg)	11.7 ± 3.8	15.6 ± 4.5	< 0.02
CL (mL/min)	712 ± 346	723 ± 142	0.6
CL (mL/min/kg)	7.6 ± 4.0	8.1 ± 2.0	0.3
F	0.64 ± 0.20	0.53 ± 0.11	0.1
Percent dose recovered in the urine unchanged (%)	64 ± 20	53 ± 11	0.1
CL_R (ml/min)	731 ± 339	758 ± 179	0.1
CL_R (ml/min/kg)	7.9 ± 3.9	8.5 ± 2.5	0.06
CrCL (ml/min)	233 ± 68	233 ± 46	0.6
CL_{sec} (ml/min)	497 ± 312	530 ± 168	0.06
CL_{sec} (ml/min/kg)	5.4 ± 3.6	5.9 ± 2.3	0.06

Results are reported as means ± S.D. Mann-Whitney test was used to compare PK parameters for all 500 mg dose vs all 1000 mg doses. $P < 0.05$ was considered significant.

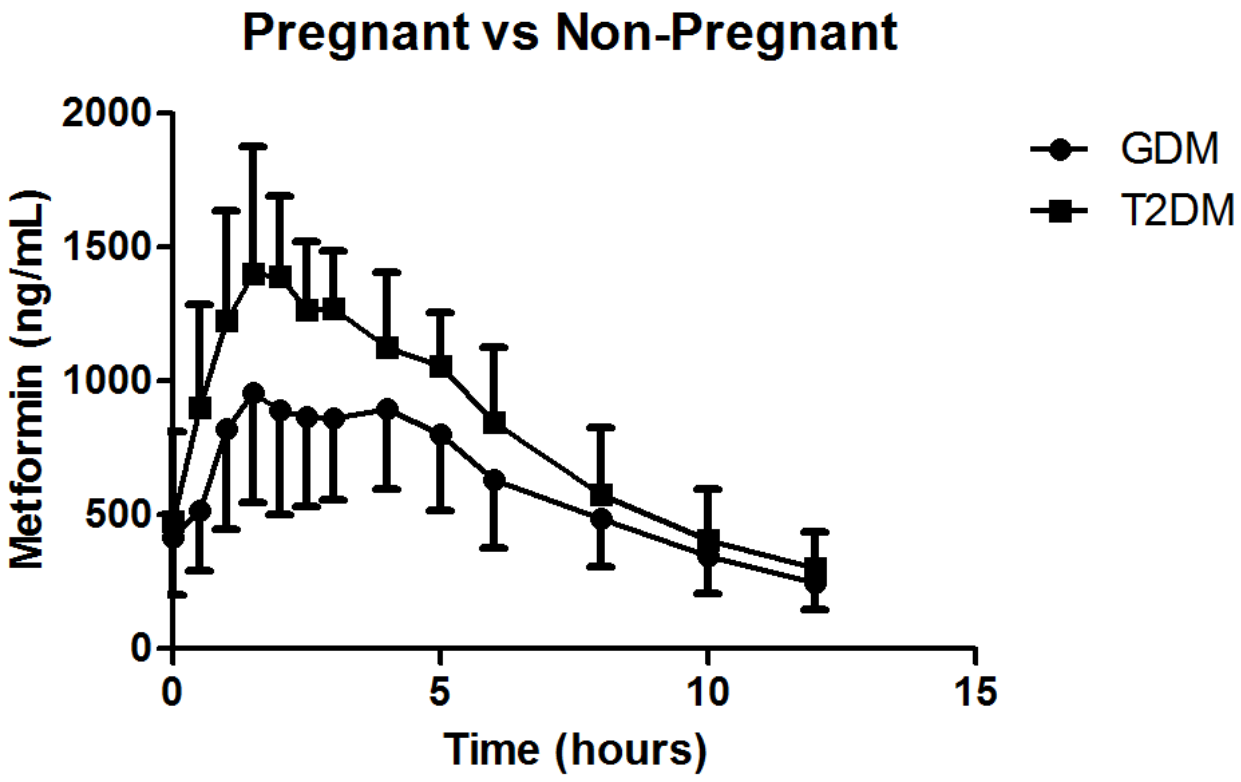

Fig. 1. Mean (error bar S.D.) steady-state metformin (500 mg orally twice daily) in pregnant women with gestation diabetes mellitus (n = 39) and non-pregnant women with type 2 diabetes mellitus (n = 9). Circles indicate concentrations in women with gestational diabetes mellitus and squares indicate concentrations in women with type 2 diabetes mellitus.

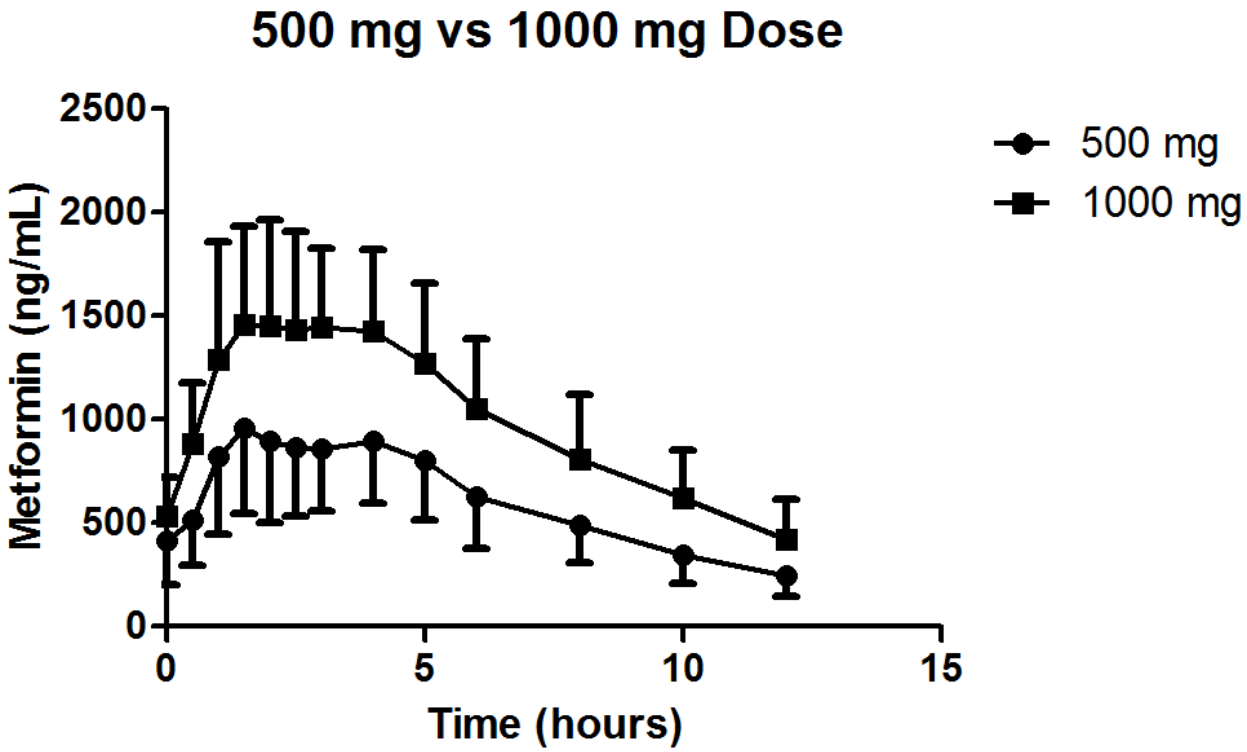

Fig 2. Mean (error bar S.D) steady-state metformin concentration-time curves for pregnant women receiving 500 mg orally twice daily (n= 39) and 1000 mg orally twice daily (n= 15). Circles indicate concentration with the 500 mg cohort and squares indicate concentration with the 1000 mg cohort.

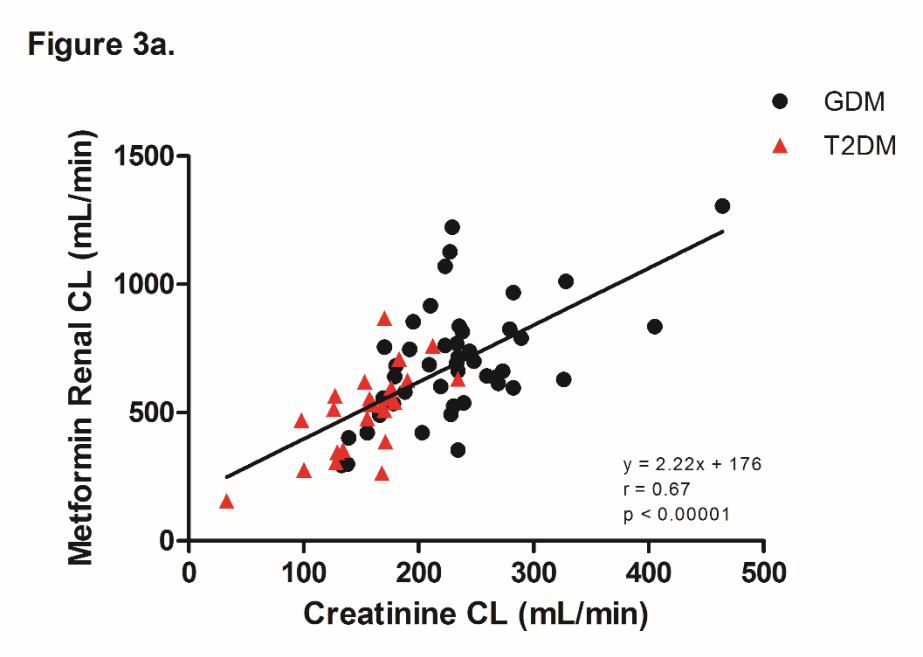

Fig 3a. Correlation between creatinine clearance and metformin renal clearance in pregnant women with GDM (solid circles) and non-pregnant women with T2DM (solid triangles) taking metformin 500 mg and 1000 mg orally twice daily.

Figure 3b.

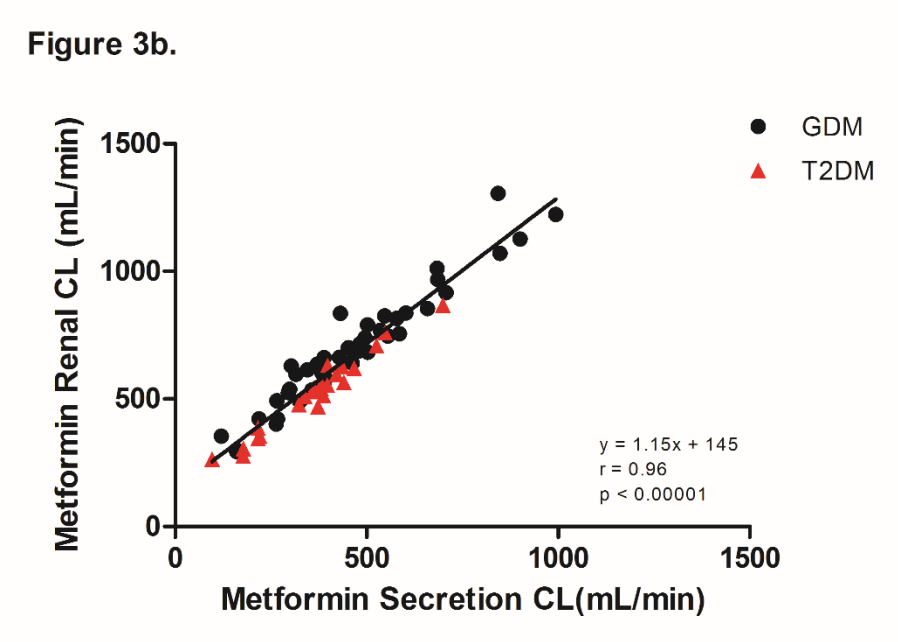

Fig 3b. Correlation between metformin renal clearance and renal secretion clearance in pregnant women with GDM (solid circles) and non-pregnant women with T2DM (solid triangles) taking metformin 500 mg and 1000 mg orally twice daily (n=68).

Figure 4.

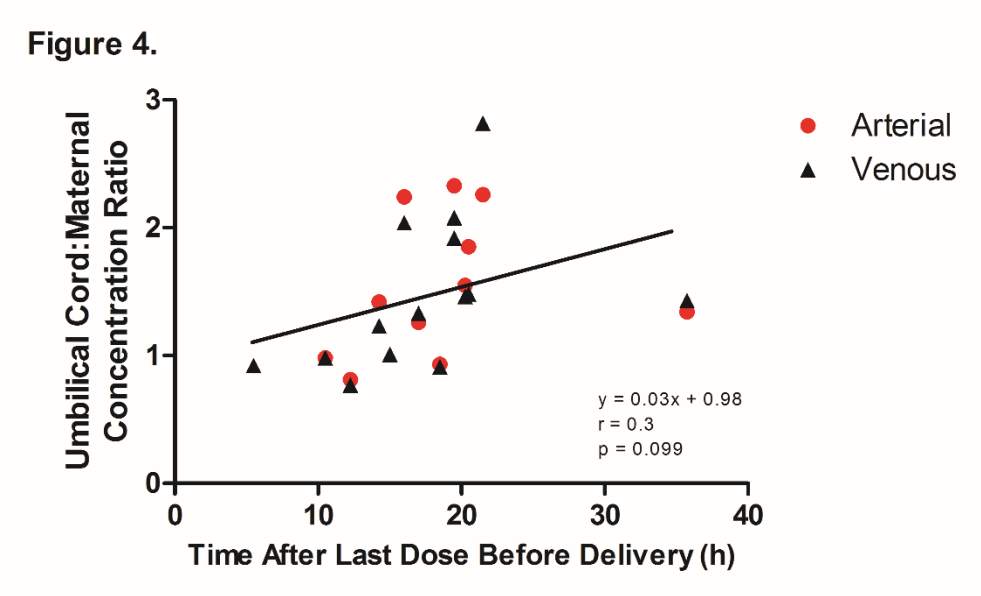

Fig. 4. Ratios of umbilical cord arterial and venous plasma concentrations to maternal concentrations as a function of duration between the last maternal metformin dose and the time of sample collection. Solid circles represent metformin umbilical cord arterial: maternal plasma concentration ratios. Solid triangles represent metformin umbilical cord venous: maternal plasma concentration ratios.

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Chapter 6: Effects of Food, Metformin, and Genetics on Steady-State Glyburide Pharmacokinetics in Pregnant Women with Gestational Diabetes Mellitus

The work presented in this chapter is under preparation for submission

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6.1 Introduction

Glyburide is a second-generation oral sulfonylurea commonly used in the management of gestational diabetes mellitus (GDM). It is approved by the FDA for the treatment of type 2 diabetes mellitus (T2DM) and stimulates insulin production by the pancreas [1]. Glyburide is a highly bound, lipophilic, low molecular weight compound, which undergoes extensive metabolism in the liver and intestine [2, 3]. It is metabolized by cytochrome P450 2C9 (CYP2C9), 3A4 (CYP3A4), 3A5 (CYP3A5) and 2C19 (CYP2C19) in the adult liver, and 3A7 (CYP3A7) in the fetal liver [4-7]. CYP2C9 is an important enzyme in the metabolism of many sulfonylureas, such as glipizide, glimepiride and tolbutaminde [8]. The CYP2C9 enzyme contributes to 30% of the formation of glyburide metabolites [9] and the gene encoding it is subject to multiple polymorphisms. Multiple clinical studies have shown that subjects with *CYP2C9*3* demonstrate approximately 40% decrease in oral clearance of glyburide and 200-300% increase in plasma AUC [4, 5, 10]. Moreover, the allelic variants *CYP2C9*2, *3, *5, *8, *11,* and **13* result in decreased CYP2C9 activity and **6* is a null allele conferring complete loss of CYP2C9 activity [4, 5, 10-15].

CYP3A contributes to more than 50% of glyburide total metabolite formation [9]. Concomitant use of glyburide with clarithromycin has been associated with severe hypoglycemia [16-18]. Both CYP3A4 and 3A5 metabolize glyburide and the genes encoding them are also polymorphic[19]. *CYP3A4*1B, *1G, *2, *3, *5, *6,* and **22* alleles result in decreased CYP3A4 activity [20-24]. The *CYP3A4*20* is a null allele resulting in no CYP3A4 activity [24]. In the case of *CYP3A5, *3, *5, *7* are null alleles [25, 26].

In vitro study results indicate that glyburide is also a substrate for CYP2C19 [6]. *CYP2C19* variants **5 and *8,* result in decreased activity [27, 28]. The *CYP2C19 *2,*3,*4,*5, and *6* have no enzymatic activity [15, 29]. However, a previously published study reported that *CYP2C19* polymorphisms do not affect the pharmacokinetics of glyburide [10].

Glyburide forms at least six metabolites in human liver including M1 (4-trans-hydrocyclohexyl glyburide), M2a (4-cis-hydrocyclohexyl glyburide), M2b (3-cis-hydrocyclohexyl glyburide), M3 (3-trans-hydrocyclohexyl glyburide), M4 (2-trans-hydrocyclohexyl glyburide) and M5 (ethylene-hydroxylated glyburide) [9, 19, 30, 31]. M1 and M2b are pharmacologically active [32, 33]. The activities of M4 and M5 are unknown. Previous work reported the M1/glyburide AUC ratio to be 0.26 ± 0.22[2]. Glyburide is not significantly cleared by the kidney [2]. High lipophilicity enables glyburide to cross the placenta via passive diffusion. Shuster et al. demonstrated that CYP3A7 is substantially expressed in human fetal livers and mediates metabolism of glyburide primarily to ethylene-hydroxylated glyburide (M5) [34].

In vitro and *in vivo* rodent studies suggested that glyburide is transported by both P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) [35, 36]. Human placental perfusion studies and human placental membrane vesicle studies demonstrate that BCRP plays a

major role in the efflux of glyburide across the placenta, potentially limiting fetal exposure but not preventing it [37, 38].

Previously published results on the effect of food on glyburide pharmacokinetics have been inconclusive. Prendergast [39] observed that absorption of glyburide does not seem to be affected by co-administration of a meal in non-pregnant patients with Type II diabetes mellitus. However, Otoom et al. [40] and Marathe et al. [41] reported that food decreased exposure to glyburide in healthy male and healthy non-pregnant female subjects. Given glyburide's low aqueous solubility, its absorption is likely to be dissolution rate limited. The food effect would be dependent upon the formulation of glyburide. The purpose of this study was to examine the effects of food, metformin and genetics on glyburide pharmacokinetics in pregnant women and describe the placental transfer of glyburide and its metabolites.

6.2 Materials and Methods

This is a pharmacokinetic study done as secondary analysis of 2 studies. The fed subjects come from a multicenter, prospective Phase II randomized, parallel-design study to evaluated the pharmacodynamics of glyburide mono-therapy, metformin mono-therapy and combination therapy with glyburide and metformin in patients with gestational diabetes mellitus. The primary endpoint and full study design will be published elsewhere. The fasted subjects come from a previously described multicenter study evaluating glyburide pharmacokinetics in women with gestational diabetes mellitus [2]. In this analysis, we examined the time course of orally administered steady-state glyburide in the plasma of fed pregnant women with gestational diabetes mellitus receiving either glyburide alone or in combination with metformin and compared them with fasted pregnant women with gestational diabetes mellitus receiving glyburide monotherapy. Both studies were approved by the institutional review board at each of

the centers and conducted in accordance with their guidelines. All subjects gave written informed consent.

Subjects

The fed subjects included 57 women, 18-45 years of age, with singleton pregnancies and a clinical diagnosis of GDM according to standard criteria [42], [43]. Fed subjects were excluded if they were taking medications expected to interact with glyburide or for serum creatinine > 1.2 mg/dL, hematocrit < 28%, allergy to glyburide, significant hepatic disease, congestive heart failure, history of myocardial infarction, moderate to severe pulmonary disease, adrenal insufficiency or pituitary insufficiency. The fasted subjects included 40 women, 26-38 years of age, with singleton pregnancies and clinical diagnosis of GDM. Subjects were excluded from that study if they were receiving drugs known to interact with glyburide and for hematocrit < 28%. Both groups received the same formulation of glyburide, and underwent the same steady-state glyburide pharmacokinetic sample collections described below [2].

Dosing and Diet

The 57 subjects in the fed-group were randomized to receive glyburide alone (n=29), metformin alone (n=24), or glyburide and metformin in combination (n=28) for treatment of GDM. Data from the metformin alone group is not included in this publication. Glyburide was administered orally starting with 2.5 mg twice daily and titrated based on clinical need. Glyburide immediate release tablets were provided by investigators throughout treatment. Subjects recorded on a calendar the time they took each glyburide dose for the 3 days prior to the pharmacokinetic study day and pill counts were performed to assess adherence. Women randomized to also take metformin began at a dose of 1000 or 2000 mg a day and were titrated based on clinical need, according to a treatment algorithm. Except for clear liquids, subjects

fasted for ≥ 5 hours prior to study drug administration on the pharmacokinetic study day. Subjects received a standardized meal simultaneously with glyburide administration. The standardized meal consisted of 2 slices of whole-wheat toast, 2 teaspoons margarine, and 240 mL Boost Plus® consumed within 10 minutes.

Forty subjects in the fasted group received glyburide monotherapy and remained fasted until 1 hour post-dosing, at which time they received the above described standardized meal [2].

Sample Collection

On the day of the glyburide pharmacokinetic study, serial blood samples were collected: pre-dose, then 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 hours post-dose, truncated to the dosing interval, for measurement of glyburide and metabolite plasma concentrations. When possible, maternal as well as umbilical cord venous and arterial blood samples were collected at the time of delivery for measurement of plasma glyburide and metabolite concentrations. Blood samples were collected into heparinized tubes and plasma was stored at -80^{o} C until analysis. All urine was collected in 4-hour intervals as follows: pre-dose, 0-4 hours, 4-8 hours and 8-12 hours post-dosing, truncated to the dosing interval. Urine was refrigerated until completion of the collection interval, then stored at -80^{o} C until analysis.

Plasma and Urine Glyburide Analysis

Glyburide plasma and urine concentrations from fed-subjects were measured utilizing a validated LC-MS/MS assay as previously described [44]. The lower limit of quantification (LLOQ) in plasma for glyburide was 1.02 ng/mL, and LLOQ for its metabolites were 0.102 ng/mL for M1; 0.109 ng/mL for M2a; 0.101 ng/mL for M2b; 0.100 ng/mL for M3 and 0.113 ng/mL for M4. In urine, LLOQ were 0.0594 ng/mL for glyburide; 0.989 ng/mL for M1; 0.984 ng/mL for M2a; 1.02 ng/mL for M2b; 1.00 ng/mL for M3 and 0.984 ng/mL for M4. At LLOQ

concentrations in plasma and urine, the relative deviation was < 20% and < 16% for intra-day and inter-day, and the accuracy ranged from 81% to 120% for plasma, and from 89% to 105% for urine. Since the M5 standard is not commercially available, the identification of M5 in samples was based on MS fragmentation and retention time. The M5 semi-quantitative analysis and reporting are adapted from Zhang et al. [44] using peak area ratios of M5 to internal standard glyburide-d_{11}. Glyburide plasma and urine concentrations from fasted subjects were measured utilizing a validated LC-MS assay as previously described with CV% <15% and LOQ 0.25 ng/mL [45].

Genotyping

DNA was isolated from whole blood, maternal and umbilical cord, of fed-subjects. Validated TaqMan assays were utilized to determine genotypes for: P-glycoprotein (P-gp) (*ABCB1* SNP rs1045642, rs1128503, rs2032582); breast cancer resistance protein (BCRP) (*ABCG2* SNP rs2032582); CYP2C9 (*CYP2C9*1, CYP2C9*2* SNP rs1799853, *CYP2C9*3* rs1057910, *CYP2C9*5* rs28371686, *CYP2C9*6* rs9332131); CYP3A4 (*CYP3A4*1*, *CYP3A4*1B* SNP rs2740574, *CYP3A4*1G* SNP rs2242480*, CYP3A4*22* SNP rs35599367); CYP3A5 (*CYP3A5*1*, *CYP3A5*3* rs776746, *CYP3A5*6* rs10264272, *CYP3A5*7* rs41303343); and CYP3A7 (*CYP3A7*1*, *CYP3A7*1C* SNP rs11568826, *CYP3A7*1E* SNP rs28451617, *CYP3A7*2* SNP rs2257401))(48).

Steady-state Pharmacokinetic Analysis

Steady-state glyburide pharmacokinetic parameters were estimated using standard non-compartmental techniques. Maximum concentration (C_{max}) and time to maximum concentration (T_{max}) were determined from the measured concentrations. AUC_{0-T} was estimated using linear trapezoidal rule. AUC_{0-T} and C_{max} were dose-normalized to 1 mg glyburide dose by dividing

AUC_{0-T} and C_{max} by the actual dose the subject received for each dosing interval (i.e. AUC_{0-T} /Dose and C_{max}/Dose). When concentrations fell below the lower limit of quantification for the assay prior to the end of the dosing interval, the concentration at the end of the dosing interval was estimated by extrapolating the predicted concentration based on the regression line from the time of the last measurable concentration (C_{last}) using $C_{end\ of\ dosing\ interval} = C_{last} \bullet e^{-k_{elim}t}$, where k_{elim} was the elimination rate constant and t was the time between C_{last} and the end of the dosing interval. The elimination rate constant (k_{elim}) was determined by log-linear regression of the terminal slope. Apparent oral clearance (CL/F) was estimated by CL/F = dose/AUC_{0-T}. Apparent oral volume of distribution (V_{β}/F) was estimated by $V_{\beta}/F = (CL/F)/k_{elim}$. Half-life ($T_{1/2}$) was estimated by $T_{1/2} = \ln(2)/k_{elim}$. Metabolite to maternal AUC ratio was determined by ratio = AUC of metabolite / AUC of glyburide. Creatinine clearance was estimated by CrCL = [(Uv)(UCr)]/[(SCr)(time)], where Uv = urine volume, UCr = urine creatinine and SCr = serum creatinine.

Statistical Analysis

Mann-Whitney test was used to compare pharmacokinetic parameters for fasting vs fed states as well as for comparing glyburide alone vs glyburide with metformin combination treatment. Genotypes for drug metabolizing enzymes and transporters were tested by one-way ANOVA or Mann-Whitney test. Results are reported as means ± standard deviation, unless otherwise indicated. $P < 0.05$ was considered significant.

6.3 Results

97 women with GDM (26-28 weeks of gestation) participated in this study (38 White, 28 Hispanic/Latina, 1 Native American, 25 Black, and 5 Asian). Subjects' mean glyburide doses

were 6.8 mg/day for the fasting group and 7.6 mg/day for the fed group in divided doses.

Figure 1 depicts mean steady-state glyburide concentration-time profiles in pregnant women with GDM receiving their glyburide dose in fasting or fed conditions. Estimated steady-state glyburide pharmacokinetic parameters are reported in Table 1. Overall, administration of glyburide with food significantly increased average glyburide dose-normalized AUC by 32% ($P < 0.02$) and decreased apparent oral clearance by 28% ($P < 0.003$), weight-adjusted apparent oral clearance by 25% ($P < 0.003$), apparent oral volume of distribution β by 48% ($P < 0.0001$), weight-normalized apparent oral volume of distribution β by 46% ($P < 0.0001$) and half-life by 19% ($P < 0.005$). T_{max}, and dose-normalized C_{max} were not significantly altered by food. However, food broadened the peak in the concentration time profile, which was not captured by T_{max}, and C_{max}.

Steady-state glyburide half-life, time to maximum concentration, dose-normalized maximum concentration, dose-normalized AUC, apparent oral volume of distribution, weight-normalized apparent oral volume of distribution, apparent oral clearance, and weight-normalized apparent oral clearance were not significantly different for subjects receiving glyburide monotherapy and those receiving combination therapy with metformin, data not shown. In addition, the dose-normalized amount of metabolite excreted over the dosing interval for M1, M2a, M2b, M3, and M4 were not significantly different in presence or absence of metformin. However, the dose-normalized amount of M5 excreted over one dosing interval was higher in the monotherapy group than in the combination therapy group (0.025 ± 0.020 % versus 0.008 ± 0.007 %, $P < 0.0001$). Metabolite time to maximum concentration, dose-normalized maximum concentration, dose-normalized AUC, and metabolite to parent AUC ratio for M1, M2a, M2b, M3, M4, and M5 were not significantly different for glyburide mono-therapy compared with

glyburide in combination with metformin. Creatinine clearance was slightly higher in the combination therapy group (220 ± 103 mL/min versus 229 ± 62 mL/min, $P < 0.05$).

Out of 64 venous and arterial umbilical cord samples, 15 (23.4%) umbilical arterial and 19 (29.7%) umbilical venous plasma glyburide concentration were below the limit of quantification. Subjects took their last glyburide dose on an average of 24.8 ± 13.0 hours prior to delivery (range: 10-53 hours). Table 2 includes average umbilical cord arterial, umbilical cord venous and maternal venous concentrations and ratios. Figure 2 depicts the relationship between time post last maternal dose and umbilical cord to maternal concentration ratios for glyburide, M1 ($r^2 = 0.67$, $P = 0.04$), and M5 ($r^2 = 0.07$, $P = 0.05$).

Figure 3 depicts the relationship between *CYP3A5* genotype and glyburide apparent oral clearance. *CYP3A5*1*3* and *CYP3A5*3*3* have lower apparent oral clearances than *CYP3A5*1*1* ($P = 0.009$, utilizing ANOVA) during pregnancy. No significant differences were seen with *P-gp, BCRP* or *CYP2C9* genotypes and dose-normalized AUC, half-life, T_{max}, dose-normalized maximum concentration, apparent oral clearance, or weight-normalized apparent oral clearance. In addition, there were no significant differences found for *CYP3A4* except for *CYP3A4 *1*1B* ($n = 9$, $p < 0.001$), which appeared to have a lower apparent oral clearance than *CYP3A4*1*1* ($n = 18$). However, this same effect on apparent oral clearance was not found with *CYP3A4*1B*1B* homozygous subjects (data not shown). Fetal *P-gp, BCRP, CYP2C9, CYP3A4, CYP3A5,* and *CYP3A7* genotypes did not significantly impact umbilical cord to maternal concentration ratios or umbilical cord arterial to venous concentration ratios for glyburide or its metabolites.

6.4 Discussion

In the present study, we found that glyburide AUC was significantly higher when given simultaneously with food ($P < 0.02$) as compared to delaying the meal until 1 hour post-dosing due to a decrease in apparent oral clearance. Both apparent oral clearance (28% decrease) and apparent oral volume of distribution (48% decrease) were lower in the fed state, suggesting that the effect of food is likely attributed to an increase in bioavailability. In contrast, Marathe et al. [41] reported that food did not appear to affect the pharmacokinetics of glyburide in non-obese, non-pregnant volunteers ($n = 24$). The influence of food on the absorption of a particular drug is dependent on the extent of the physiological changes produced in the gastrointestinal tract and the physical and chemical interactions between the components in food, the drug, enzymes and transporters [47]. Contributing factors for the increase in drug absorption could include an increase in dissolution, increase in lipoprotein binding and /or a decrease in first-pass metabolism of glyburide, with simultaneous food consumption with the drug. A significant increase in AUC has been reported with lipophilic drugs such as propranolol and simultaneous consumption of food [48-50]. In addition, studies have shown that the composition of a meal (fat, protein, carbohydrates) can alter the oral bioavailability of cancer chemotherapy [51]. Cyclosporine, a highly lipophilic drug, has been reported to have an increase in area under the concentration-time curve when administered with a high-fat meal [52]. Another study demonstrated that a high-fat meal markedly increased cyclosporine oral bioavailability as well as increased cyclosporine clearance and volume of distribution when cyclosporine was administered intravenously [53]. Manidipine, another highly lipophilic drug, has been reported to have an increased bioavailability in the fed state, which has been attributed to its high lipophilicity and the dissolution effect produced by food and bile secretions [54]. There is also the possibility that

absorption is increased by higher binding of glyburide to lipoproteins taken up into blood by the intestinal lymphatic system. An increase in binding to lipoproteins is consistent with the significant decrease in the apparent oral volume of distribution ($P < 0.0001$).

Another possible mechanism by which the bioavailability of glyburide might be increased is if food lowers the first-pass metabolism of glyburide [55]. Some studies in humans have shown that splanchnic blood flow is increased from 1200 to 1600 mL/min/m^2 in mean blood flow across subjects during the first hour after a meal [56]. Based on the Q_{gut} model [57], an increase in blood flow may increase the intestinal bioavailability of the drug ($F_g = Q_{gut} / (Q_{gut} + (fu_{gut} * Cl_{int,gut}))$ where $Q_{gut} = CL_{permeability} * Q_{enterocyte} / (Q_{enterocyte} + CL_{permeability})$ in which F_g is the bioavailability across the gut, Q is blood flow, fu is the fraction unbound, CL_{int} is the intrinsic clearance, $CL_{permeability}$ is the drug permeability clearance) [58]. Although a decrease in first-pass metabolism is a possibility, it seems unlikely given the effects of food on hepatic clearance of drugs like cyclosporine.

In addition to the effect of food on the pharmacokinetics of glyburide, we have explored the effects of metformin on glyburide pharmacokinetics. Since there are fairly high treatment failure rates for both glyburide and metformin monotherapies, combination drug therapy is being considered for the management of GDM. Therefore, evaluating the potential effects of metformin on glyburide pharmacokinetics is warranted [59, 60]. We observed no significant effect of metformin on glyburide or its metabolites' pharmacokinetics, likely due to different clearance pathways for metformin and glyburide.

Glyburide undergoes metabolism via multiple drug metabolizing enzymes. *In vitro* metabolism studies using human liver microsomes or recombinant systems have demonstrated that CYP3A4 is the major pathway for glyburide metabolism and CYP2C9, CYP2C8, CYP3A5

and CYP2C19 play minor roles [5, 9, 19]. Cavallari and Limdi et al. reported that the *CYP2C9* variant, *CYP2C9*3*, exhibits lower catalytic activity towards glyburide than wild-type *CYP2C9*1* [4, 5]. Kirchheriner et al. reported that the oral clearance of glyburide in the *CYP2C9*3/*3* subjects (n = 3) was ~40% of that in *CYP2C9*1/*1* subjects (n = 4) [4]. In contrast, in the patients with *CYP2C9* variants that we tested (**2, *3, *5*), we observed no significant changes in glyburide pharmacokinetic parameters. The expected decrease in oral glyburide clearance with CYP2C9 variants (**2, *3, *5*) might have been masked by CYP3A and CYP2C9 enzyme induction that occurs during pregnancy [11, 13, 14, 21] or by insufficient sample size.

CYP3A5, constitutes up to 50% of the total hepatic CYP3A content in persons expressing CYP3A5 [22, 61]. *CYP3A5* gene mutation within intron 3 creates an alternative splicing and truncation of translated protein, resulting in low protein content and absence of protein activity [25]. Our study found that the apparent oral clearance was significantly higher in the 5 subjects with *CYP3A5*1*1* than in the 51 subjects with **3* variant allele. Similarly, multiple other studies have reported decreased elimination of drugs (e.g. tacrolimus, carbamazepine) in patients with the *CYP3A5 *3* variant as compared to those with the **1* allele [62].

We found that the average glyburide umbilical cord-to-maternal plasma concentration ratio at the time of delivery was 0.97 ± 0.54 (range: 0.24 to 1.51), similar to that previously reported (0.7 ± 0.4) [2]. The efflux transporters such as P-gp, BCRP, and multidrug resistance proteins (MRPs) are highly expressed on the apical membrane of the placental syncytiotrophoblast facing the maternal side [63-65]. Glyburide has been shown to be a substrate for P-gp, BCRP and MRPs *in vitro* and in rodents [35, 37, 66]. In the BCRP knock-out mouse model, fetal glyburide concentrations were much higher than in the wild-type fetuses

[35]. Although in theory, the efflux transporters could protect the fetus by expelling drugs, xenobiotics and their metabolites from the fetal compartment to the maternal circulation. With the average ratio from this study being 0.97, this does not appear to be the case for glyburide in humans.

The impact of glyburide metabolites on fetal safety requires further study. M1 umbilical cord-to-maternal plasma concentration ratio was 0.65 ± 0.24 ranging from 0.24 to 1.51. M1 is as potent as glyburide in lowering the concentration of glucose [67, 68]. Zharikova et al. [9] reported that human placental microsomes convert 4% of glyburide to M1. Shuster et al. [28] reported that fetal liver microsomes converts 1% of glyburide to M1 compared to 10% in adult human liver microsomes. The mean umbilical cord arterial-to-umbilical cord venous concentration ratio was 1.04 ± 0.04 for M1. The concentration ratio indicates no substantial extraction by the fetus, assuming there is no asymmetric transport across the placenta. The umbilical cord-to-maternal plasma concentration ratio for M2a, M2b, M3, and M4 could not be determined because some concentrations were below LLOQ.

M5 umbilical cord-to-maternal plasma concentration ratio was 14.4 ± 10.4, ranging from 0.1 to 35.3. Further research is needed to characterize the pharmacologic activity of M5. Zharikova et al. [9] reported that human placental microsomes converted ~87% of glyburide to the M5 metabolite, and the rest to other metabolites. Shuster et al. [34] reported that human fetal liver microsomes converted ~ 97% of glyburide to the M5 metabolite, and adult human liver microsomes converted ~65% of glyburide to the M5 metabolite. The high M5 umbilical cord-to-maternal plasma concentration ratio may be a result of both placental drug-metabolizing enzymes and fetal liver enzymes converting glyburide predominantly to M5, although the main contributor appears to be the placenta. The much higher M5 concentration in the umbilical cord

could be significant if M5 turns out to be active.

6.5 Tables and Figures

Table 1. Estimated oral glyburide steady-state pharmacokinetic parameters in pregnant women with gestational diabetes mellitus receiving twice daily dosing in fasting and fed states.

	Fasting (n = 40)	Fed (n = 57)	*P value*
Glyburide			
$Dose_{(0\text{-}12)}$ (mg)	3.4 ± 2.4	3.8 ± 2.4	0.4
$GLYAUC_{(0\text{-}12)}$/Dose (ng*hr/mL/mg)	72 ± 27	94 ± 42	< 0.02
Half-life (hr)	3.6 ± 1.7	2.9 ± 0.9	< 0.005
V_B/F (L)	93 ± 99	48 ± 20	< 0.0001
V_B/F (L/kg)	0.96 ± 0.88	0.52 ± 0.25	< 0.0001
CL/F (L/hr)	17.2 ± 11.5	12.3 ± 4.6	< 0.003
CL/F (mL/hr/kg)	175 ± 90	132 ± 56	< 0.003
Tmax (hr)	2.9 ± 1.5	2.9 ± 1.6	0.5
Cmax/Dose (ng/mL/mg)	15.5 ± 7.8	18.2 ± 8.9	0.2

Results are reported as means ± S.D. Mann-Whitney test was used to compare PK parameters for fasting vs fed states. $P < 0.05$ was considered significant.

Table 2. Mean umbilical cord arterial, umbilical cord venous and maternal venous concentrations and ratios.

	Glyburide	M1	M2a	M2b	M3	M4	M5
Umbilical cord arterial conc. (ng/mL)	6.4 ± 7.9 (1.1-28.4)	2.0 ± 1.6 (0.8-5.6)	<LLOQ	0.9*	<LLOQ	<LLOQ	0.2 ± 0.5 (0.1-1.6)
Umbilical cord venous conc. (ng/mL)	6.8 ± 7.0 (1.2-29.6)	2.1 ± 2.4 (0.6-9.8)	<LLOQ	1.2 ± 1.2 (0.1-2.9)	<LLOQ	<LLOQ	0.3 ± 0.4 (0.2-1.4)
Maternal venous conc. (ng/mL)	16.0 ± 26.6 (1.2-124)	4.8 ± 5.4 (0.7-23.4)	1.1 ± 1.0 (0.2-3.59)	2.3 ± 1.7 (0.4-5.84)	<LLOQ	<LLOQ	0.03 ± 0.05 (0.03-0.3)
Cord arterial-to-venous conc. ratio	1.03 ± 0.04	1.04 ± 0.04	ND	ND	ND	ND	1.14 ± 0.2
Cord venous-to-maternal conc. ratio	0.65 ± 0.24	ND	ND	ND	ND	ND	15.5 ± 11.0
Cord arterial-to-maternal conc. ratio	0.83 ± 0.55	0.53 ± 0.15	ND	ND	ND	ND	12.9 ± 9.4

Results are reported as means ± S.D. (range).
* Only one sample quantifiable
LLOQ: M2a < 0.109 ng/mL, M3 < 0.100 ng/mL; and M4 < 0.113 ng/mL
ND=Not determined, conc.=concentration, LLOQ=lower limit of quantification

Fig. 6-1.

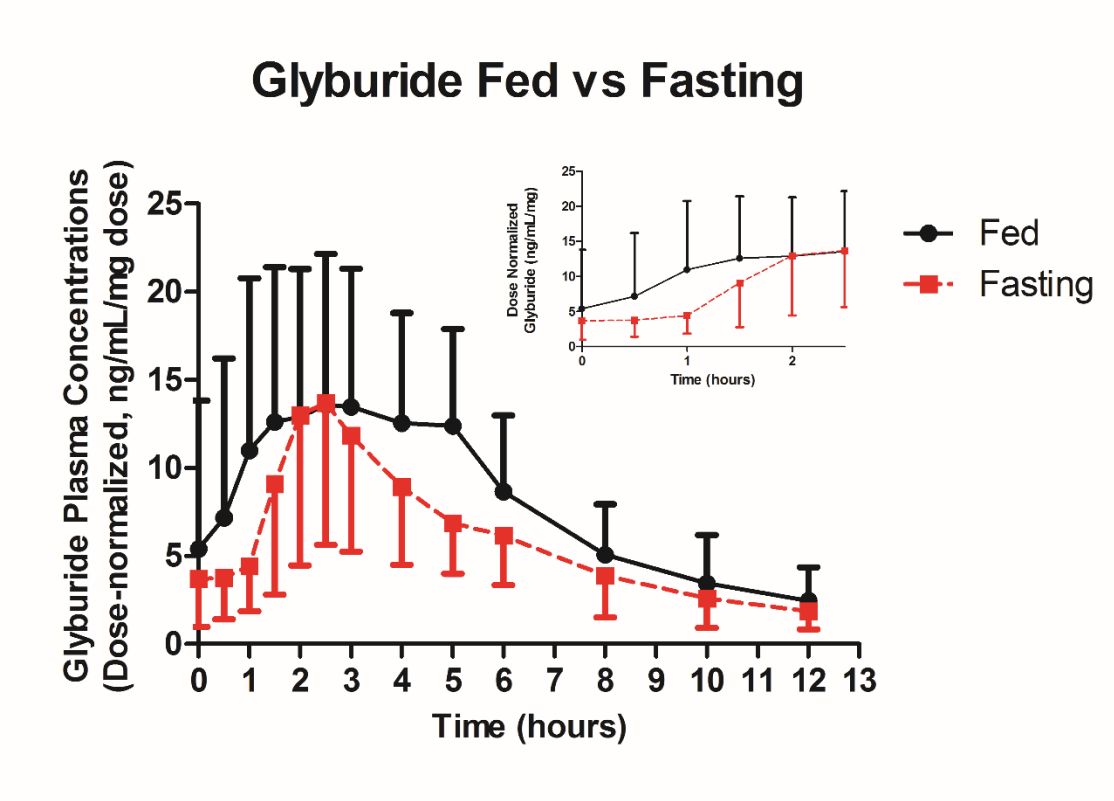

Fig. 6-1. Mean (error bar S.D) steady-state glyburide concentration-time profiles in pregnant women with gestational diabetes mellitus receiving twice daily dosing in fasting (n = 40) and fed states (n = 57). Black circles indicate concentrations in fed condition and red squares indicate concentrations in fasting condition.

Fig. 6-2.

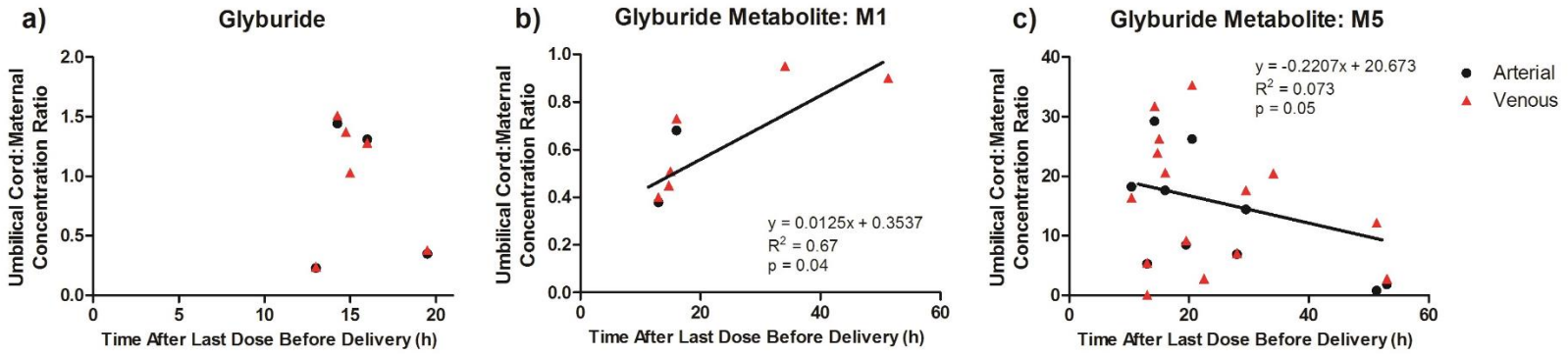

Fig. 6-2. Umbilical cord arterial and venous plasma concentration to maternal concentration ratios for glyburide (a), M1a (b) and M5 (c) as function of duration between the last maternal glyburide dose and time of sample collection. Solid circles represent umbilical cord arterial: maternal plasma concentration ratios. Solid triangles represent umbilical cord venous: maternal plasma concentration ratios.

Fig. 6-3.

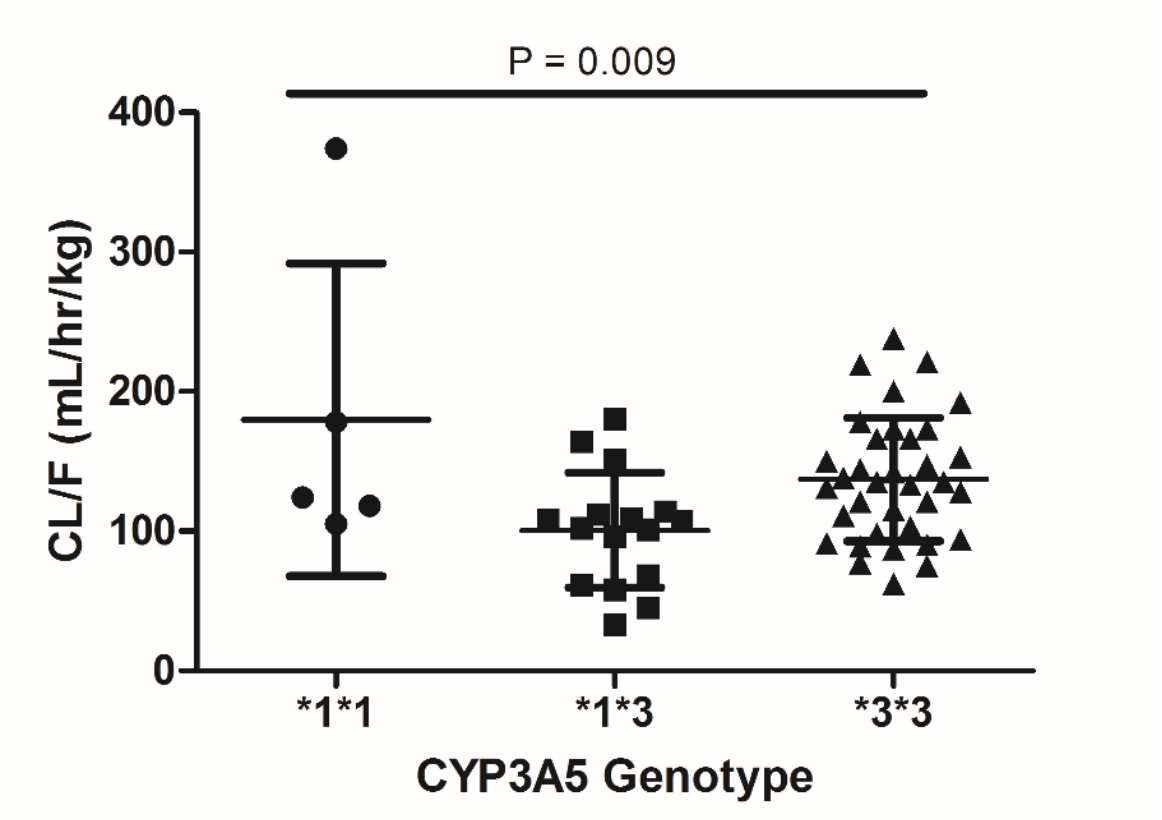

Figure 6-3. Mean and S.D. of weighted normalized clearance of glyburide and CYP3A5 genotypes.

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Chapter 7: Conclusion

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Liao et al., in *Drug Metabolism and Disposition* November 2017.

7.1 Chapter 2: P-gp/ABCB1 Exerts Differential Impacts On Brain and Fetal Exposure to Norbuprenorphine

In the present study, we have shown that fetal exposure to NBUP and NBUP-G in pregnant mice accounts for ~60% and ~700% of maternal plasma exposure, respectively, suggesting that fetal exposure to the two major active metabolites of BUP is substantial and hence caution should be taken regarding fetal safety of the use of BUP during pregnancy. We have also demonstrated significant differential impacts of P-gp on fetal and brain exposure to NBUP, with a much greater role of P-gp in restricting NBUP distribution across the BBB versus

the BPB. This dissimilar effect may be attributable, at least in part, to the difference in P-gp protein expression in the tissue barriers, with much greater amounts of P-gp at the organ level in the BBB than in the BPB. NBUP metabolism in the fetus and/or placenta likely plays a very important role in determining fetal exposure to its metabolite NBUP-G. Finally, based upon discussion above, whether P-gp plays a more significant role in determining brain exposure than fetal exposure to drugs or vice versa could possibly be drug-dependent.

7.2 Chapter 3: Pregnancy Increases Norbuprenorphine Clearance in Mice by Induction of Hepatic Glucuronidation

This study demonstrates that pregnancy induces total protein content of several Ugts in mice, leading to increased systemic clearance of NBUP. Our proteomic analysis revealed that hepatic Ugts are differentially regulated by pregnancy; in particular, Ugt1a1 and Ugt2b1 were significantly induced. How such a change in NBUP disposition may affect overall disposition and hence the efficacy and safety of the parent BUP during pregnancy warrants further investigation. The plasma AUC of BUP has been shown to be significantly decreased during pregnancy (13), and dose adjustments may be required to maintain efficacy in pregnant women. A major concern of such dose adjustments is the toxicity (e.g., neonatal respiratory depression) of the major circulating metabolite NBUP. Results of this study suggest that at the same dose, the toxicity of NBUP may be reduced by its increased clearance during pregnancy.

7.2 Chapter 4: Quantitative Proteomics Reveals Changes in Transporter Protein Abundance in Liver, Kidney and Brain of Mice by Pregnancy

Our quantitative proteomics analysis revealed significant pregnancy-induced changes in protein abundance of major drug/xenobiotic transporters in mice. These changes mostly occurred in the liver, particularly after scaling to the whole liver, followed by the kidney, with little changes in the brain. It is important to note that the increase in organ weight during pregnancy also contributed to the increase in the amounts of protein of transporters in the whole organ (e.g. the liver) even if the protein levels of transporters after normalization to total membrane proteins isolated from the organ were not changed (e.g., Abcg2 in the liver) or even decreased (e.g., Abcc2 in the liver). These findings will enhance our understanding of the impact of drug/xenobiotic transporters on drug/xenobiotic disposition and tissue exposure during pregnancy. If the same changes in transporter protein abundance occur in humans, our results suggest that transporter-mediated hepatic disposition of drugs and xenobiotics is most likely to be affected by pregnancy.

7.3 Chapter 5: Effects of Pregnancy on the Pharmacokinetics of Metformin

Pregnancy significantly increases the bioavailability, clearance, renal clearance and volume of distribution of metformin. With lower doses, pregnancy also increases the apparent oral clearance of metformin suggesting that higher initial doses might benefit pregnant women. However, with higher doses, the changes in bioavailability during pregnancy offset the changes in clearance and volume leading to no significant change in apparent oral clearance and systemic exposure. In addition, we have demonstrated the non-linearity of metformin pharmacokinetics during pregnancy, with a higher apparent oral clearance as metformin doses increase. This

finding appears to be primarily driven by the trend toward lower bioavailability with higher metformin doses.

7.4 Chapter 6: Effects of Food, Metformin, and Genetics on Steady-State Glyburide Pharmacokinetics in Pregnant Women with Gestational Diabetes Mellitus

The simultaneous consumption of food with glyburide during pregnancy significantly increases glyburide AUC and decreases glyburide half-life, apparent oral volume of distribution, and apparent oral clearance. We have demonstrated that co-administration of metformin does not change glyburide or its metabolites (M1, M2a, M2b, M3, M4, and M5) pharmacokinetics during pregnancy. In addition, this study is the first to simultaneously characterize *in vivo* fetal exposure to M1, M2a, M2b, M3, M4, and M5 at the time of delivery in humans.