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Pharmacokinetics of Bupropion and its Pharmacologically Active Metabolites in Pregnancy

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Pharmacokinetics of Bupropion and its Pharmacologically Active Metabolites in Pregnancy

by

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Dedication

To the memory of my mother

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Bupropion, an antidepressant and anti-smoking medication, is used for treatment of depression during pregnancy, however its efficacy as a smoking cessation aid in pregnancy has not been confirmed. The drug is extensively biotransformed by liver with the formation of pharmacologically active metabolites, namely hydroxybupropion (OHBUP), threo- (TB) and erythrohydrobupropion (EB). CYP2B6 is the primary enzyme catalyzing the formation of OHBUP; the latter is the main plasma metabolite of bupropion and is thought to contribute majorly to pharmacological efficacy of the drug. CYP2C19 enzyme is involved in hydroxylation of bupropion, as well as TB and EB. Pregnancy-associated changes in maternal physiology may alter the pharmacokinetics of bupropion and thus influence the efficacy of bupropion in promoting cessation in pregnant smokers. Therefore, the primary objective of this study was to evaluate the effect of pregnancy on bupropion biodisposition. In addition, we studied the impact of functional genetic variants of *CYP2B6* and *CYP2C19* on bupropion pharmacokinetics in

pregnancy. Our data indicated that the isoform-specific effect of pregnancy on bupropion-metabolizing enzymes and changes in renal function during pregnancy could collectively result in slight increase of bupropion apparent oral clearance; however, no changes in the plasma levels of OHBUP were evident. Further, our data demonstrated that the effect of *CYP2B6*6* (reduced function allele) and *CYP2C19*2* (loss-of-function allele) on bupropion biodisposition in pregnancy are similar to those observed in the non-pregnant state. We also investigated the *in vivo* placental transfer of the drug and its metabolites. For this purpose, the drug and its metabolites were determined in the matching maternal-umbilical cord blood plasma samples collected at delivery from pregnant women treated with bupropion for depression during pregnancy. The levels of OHBUP, TB, and, with a few exceptions, bupropion, in the umbilical cord venous plasma were lower than those in the matching maternal plasma, suggesting limited fetal exposure. In addition, we found that the concentrations of OHBUP and TB in the fetal circulation could be predicted from those in the maternal plasma. Further, bupropion and its metabolites were determined in the amniotic fluid, suggesting additional pathway of fetal exposure to maternally administered bupropion.

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8-OH-dG	8-hydroxydeoxyguanosine
11βHSD	11β-hydroxysteroid dehydrogenase
AKR	aldo-keto reductases
AUC	area under the concentration curve
BCRP	breast cancer resistance protein
BID	twice daily
CAR	constitutive androstane receptors
CL/F_{ss}	apparent steady state oral clearance
CL_R	renal clearance
C_{max}	maximum plasma concentration
CO	carbon mono-oxide
COHb	carboxyhemoglobin
CPD	cigarettes per day
DA, DAT	dopamine (DA) transporters (DAT)
E2	estradiol
EB	erythrohydrobupropion
EM	extensive metabolizer phenotype
ERE	estrogen-response element
ER	α-estrogen receptor
ESI	electrospray ion source
GFR	glomerular filtration rate
HPLC	high performance liquid chromatography
IM	intermediate metabolizer phenotype
IR	immediate release
IS	internal standard
K_m	Michaelis constant
LLE	liquid-liquid extraction method
LLOQ	lower limit of quantification
LOD	lower limit of detection
MRP	multidrug resistance associated proteins
MS	mass spectrometry
nAChRs	nicotinic acetylcholine receptors
NE, NET	norepinephrine (NE) transporter (NET)
NNAL	4-methylnitrosamino-1-(3-pyridyl)-1-butanol
NNK	4-methylnitrosamino-1-(3-pyridyl)-1-butanone
NO	nitric oxide
NRT	nicotine replacement therapy
OCT	organic cation transporters
OHBUP	hydroxybupropion
P-gP	P-glycoprotein
PBREM	phenobarbital-responsive enhancer module
PK	pharmacokinetics

PM	poor metabolizer phenotype
PXR	retinoid X receptor
QC	quality control
QD	once daily
ROS	reactive oxygen species
RSD	relative standard deviation
SIDS	sudden death syndrome
SIM	selective ion monitoring
SNP	nucleotide polymorphisms
SR	sustained release
SSRI	selective serotonin reuptake inhibitors
STDEV	standard deviation
TB	threohydrobupropion
TCA	trichloroacetic acid
TID	three times a day
UGT	uridinediphosphate (UDP)-glucuronosyltransferase
UM	ultrarapid metabolizer phenotype
VMAT2	vesicular monoamine trasnporter-2
V_{max}	maximum velocity
XL	extended release

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1. Bupropion and its use in pregnancy

Bupropion is successfully used as a mono therapy or as an add-on medication in the treatment of a wide spectrum of depressive disorders in different populations including pregnant women (Gulrez, 2012; Dhillon, 2008; Jefferson, 2008). The drug was originally approved in 1989 by the FDA (Berigan TR, 2002) in immediate release (IR) formulation, later on the sustained release formulation (SR) was developed in order to avoid sharp increases in the plasma concentrations of bupropion and thus improve its safety profile. To date, three bioequivalent formulations of the drug are available, namely, IR, SR, and extended release (XL) (Dhillon, 2008).

Bupropion SR under the brand name Zyban, was approved in 1997 as a smoking cessation aid for males and non-regnant females (Raupach et al., 2011). Treatment of depression with bupropion can last for months/years and dose is titrated based in patients' response to the therapy. On the other hand, the typical therapy with bupropion SR to promote cessation from smoking last 7-12 weeks with the dose of the drug, fixed at 150 mg once a day (QD) for 3 days and then 150 mg twice a day (BID) for the rest of the treatment (GlaxoSmithKline, data on file).

The potential benefits of using therapeutic agents during pregnancy should justify the potential risk(s) to the fetus. Depression is a serious condition; although taking an antidepressant during pregnancy might pose risks for the fetus, untreated depression might have negative effect for the mother and consequently have adverse outcomes for the fetus (Kalra et al., 2005). The use of antidepressants during pregnancy, and bupropion

in particular, is consistent with clinical practices. However, bupropion SR is not routinely prescribed to pregnant smokers as a smoking cessation aid due to the lack of data on its safety and efficacy in this population.

1.2. Smoking in pregnancy

Smoking in pregnancy is one of the largest modifiable risk factors for pregnancy-associated morbidities and mortalities. Up to 10% of perinatal deaths, 35% of low-birth weights, and up to 15% of preterm deliveries are linked to maternal smoking during pregnancy (USDHHS, 1990). In addition, several postnatal morbidities are associated with exposure to cigarette smoke *in utero*, such as sudden infant death syndrome (SIDS), respiratory infections, asthma, atopy, and otitis media (Marufu et al., 2015). Children born from mothers who smoked during pregnancy are at higher risk to develop non-Hodgkin's lymphoma, leukemia, and certain types of brain tumors, and these risks are positively correlated with the number of cigarettes smoked per day (CPD) (Stjernfeldt et al., 1986; Heck et al., 2016). Many female smokers quit smoking when they become pregnant; however, 10% of all pregnant women continue smoking throughout pregnancy (Curtin and Matthews, 2016). Although quitting smoking earlier in pregnancy is the most beneficial for maternal and fetal wellbeing, quitting late in pregnancy still has positive effect on the infant's health and birth weight (Fingerhut et al., 1990). The chances of quitting smoking during pregnancy are inversely related to the level of smoking, therefore heavy smokers in particular might benefit from pharmacotherapy to help them achieve cessation (Windsor et al., 2000).

Cigarette smoke is a complex mixture of 7360 chemicals, of which more than 70 were recognized as mutagens or carcinogens (MHNZ, 2000). Nicotine is one of the alkaloids in tobacco leaves and is thought to be a major factor causing dependence from smoking (Wickstrom, 2007). Nicotine is an agonist of nicotinic acetylcholine receptors (nAChRs); it competitively binds to these receptors and triggers dopamine release in the nucleus accumbens, a region in the brain crucial for the reward pathway (Siu and Tyndale, 2007). Data from the perfusion of human placental cotyledon *ex vivo* suggest that nicotine crosses the placenta freely and appears in the fetal circulation (Pastrakuljic et al., 1998; Nekhayeva et al., 2005). *In vivo* data indicate that the levels of nicotine in the amniotic fluid during the second trimester of pregnancy and in fetal serum and placental tissue at birth exceeded those in the corresponding serum of the smoking mothers (Luck et al., 1985). Nicotine, being present in the fetal circulation, can act directly on the fetal nAchRs, which expression and function has been detected in fetal brain as early as 4-6 weeks of gestation (Falk et al., 2003; Hellström-Lindahl et al., 2000). nAchR signaling is important for proper neurodevelopment; perinatal exposure to nicotine can lead to disruption in the formation, survival and differentiation of brain cells (Slotkin et al., 2002) and result in permanent changes in behavioral performance after birth (Pauly et al., 2008; Wickstrom, 2007).

Besides nicotine, other toxic constituents of cigarette smoke are, but not limited to:

a. Carbon mono-oxide (CO). CO binds to hemoglobin at sites that normally bind oxygen; the resulting carboxyhemoglobin (COHb) complex is present in the blood of a smoker at concentrations of 5-10%. Elevated COHb in the blood stimulates the

production of erythrocytes and leads to increased blood viscosity, which is a risk factor for suboptimal placenta perfusion. Moreover, fetal levels of COHb are higher than maternal levels due to higher binding capacity of CO to fetal hemoglobin. Consequently, the impaired mother-to-fetus and fetal blood-to-tissue transfer of oxygen results in chronic cellular hypoxia in the fetus (Dempsey and Benowitz, 2001).

b. Superoxide and reactive oxygen species (ROS). These components of cigarette smoke reduce the availability of nitric oxide (NO); the latter is important for the regulation of vascular tone and tissue perfusion. NO depletion leads to vasoconstriction, which can cause insufficiency in tissue perfusion and blood flow (Tsuchiya et al., 2002)

c. Carcinogens and pro-carcinogens, such as polyaromatic hydrocarbons, aromatic amines, N-nitrosamines (MHNZ, 2000). The latter were shown to cause DNA mutations and are known human carcinogens. Specifically, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a tobacco-specific compound and is one of the most potent carcinogens (Atalla and Maser, 2001; Hecht, 1998). Its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), in a free and conjugated form, has been detected in the amniotic fluid and in the first urine of newborns delivered by mothers who smoked throughout pregnancy (Florek et al., 2011). These results indicate either transplacental transfer of NNK and/or its metabolism by the fetus or transfer of maternal NNAL across placenta (Oncken and Kranzler, 2009; Milunsky et al., 2000).

The placenta is a highly specialized temporary organ that performs the following functions: transfer oxygen and nutrients from the mother to the fetus; synthesis of

hormones, namely peptides and steroids; transfer of fetal waste products to the maternal circulation; immunoprotection to the fetus and protection of the fetus from the maternal immune system. All therapeutic agents that are administered by the mother appear in the fetal circulation to some extent and thus can affect the fetal wellbeing directly through their presence in the fetal circulation, or indirectly, by affecting placental development and function. Similarly, maternal smoking during pregnancy can impact fetal wellbeing directly or via disrupting the normal functions of the placenta. Therefore, the balance of risk and benefits is of a concern when pharmacotherapy for smoking cessation in pregnancy is considered.

1.3. Smoking cessation therapy during pregnancy

To date, behavioral counseling is the only therapy that is routinely offered to pregnant smokers to help achieve cessation from smoking (Oncken and Kranzler, 2009). Varenicline, a highly selective inhibitor of a $\alpha 4\beta 2$ subtype of nAchRs, is not recommended for pregnant smokers due to the lack of data on its efficacy for this population and safety for the fetus (Oncken and Kranzler, 2009). Nicotine replacement therapy (NRT), in a form of a patch, gum, or lozenges, can be used during pregnancy; however the potential fetal toxicity associated with NRT is of concern. Although with the use of NRT by the pregnant woman the fetus is not exposed to the toxic constituents of tobacco smoke, the doses of nicotine delivered to the fetus are higher than those observed with active smoking (Oncken and Kranzler, 2009; Wickstrom, 2007). Moreover, the clearance of nicotine is increased in pregnancy (Dempsey et al., 2002); therefore higher

doses of nicotine might be required for a pregnant smoker to achieve cessation, which result in increase fetal exposure to nicotine.

Bupropion might be a safe and effective pharmacotherapy in helping pregnant women to quit smoking. A pilot double-blind placebo-controlled clinical trial on bupropion as a smoking cessation aid in pregnancy has been concluded recently at the University of Texas Medical Branch at Galveston, TX. The participating pregnant smokers received behavioral counseling and either bupropion or placebo in a course of a therapy to promote smoking cessation. Preliminary results suggest higher quit rate in the bupropion group than in the placebo group (unpublished data). Therefore, bupropion could be an effective pharmacotherapy to assist pregnant smokers in achieving abstinence from smoking.

1.4. Bupropion: pharmacological properties and mechanism of action

The exact mechanism of action of bupropion is not fully understood. Unlike canonical antidepressants, namely tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRI), bupropion does not act on serotonin transporters (Bondarev et al., 2003; Dwoskin et al., 2006). Bupropion is an inhibitor of norepinephrine (NE) transporter (NET), and also an antagonist of nAChRs with the highest capacity towards the $\alpha 3\beta 4$ isoform of nAChRs (Bondarev et al., 2003; Dwoskin et al., 2006). However, bupropion lacks affinity to the $\alpha 4\beta 2$ isoform of nAChR, which is the most abundant type of nAChR in the brain (Arias, 2009; Bondarev et al., 2003). Further, bupropion is a nonselective inhibitor of dopamine (DA) transporters (DAT), which leads to an increase of dopamine levels in the synaptic cleft (Dwoskin et al., 2006). However, the selective

affinity of the drug to DAT results in a receptor occupancy of less than 22%, which is modest as compared to 80% occupancy of serotonin transporters that occurred in clinically effective doses for select SSRIs (Meyer et al., 2001; Meyer et al., 2002). On the other hand, it was demonstrated that bupropion also stimulates the vesicular monoamine trasnporter-2 (VMAT2) protein, which facilitates the release of DA into the extracellular space (Rau et al., 2005). Bupropion-mediated reduction of DA in the mesolimbic system as well as a decrease in the NE reuptake in locus coeruleus are thought to contribute to the clinical action of the drug as a smoking cessation aid (Richmond and Zwar, 2003). These pathways are associated with reward, craving and nicotine-associated withdrawal (Richmond and Zwar, 2003). As was mentioned above, bupropion exhibits the highest specificity to the α3-containing isoforms of nAChRs, particularly α3β4, which has been implicated in the addiction to nicotine (Arias, 2009; Glick et al., 2002).

Bupropion, which is always administered orally in humans, is rapidly absorbed; the time bupropion reaches maximum concentration in plasma (C_{max}) depends on the drug formulation: for the IR formulation, C_{max} is 2 hours; for the SR, 3 hours; and for XL, 5 hours (Dhillon et al., 2008; Shroeder, 1983). Bupropion is extensively metabolized in the liver and small intestine (Connarn et al., 2015; Shroeder, 1983) with the formation of three major metabolites, namely, hydroxybupropion (OHBUP), threohydrobupropion (TB), and erythrohydrobupropion (EB) (Shroeder, 1983) (Illustration 1). The formation of OHBUP is catalyzed predominantly by the hepatic CYP2B6 enzyme, while TB and EB are formed by 11βHSD1 and carbonyl reductases (Connarn et al., 2015; Molnari et al., 2012). It was reported recently that CYP2C19 also contributes to the hydroxylation of bupropion, and possibly TB and EB (Zhu et al, 2014). The elimination $t_{½}$ for bupropion is

20-21 hours, while the $t_{½}$ for OHBUP, TB and EB are ~20, ~33 and ~37 hours, respectively (Dhillon et al, 2008). Less than 10% of the bupropion dose is excreted in urine as unchanged drug; the rest is in the form of free and conjugated plasma metabolites in addition to a number of other derivatives which result from the metabolism of bupropion (Petsalo et al., 2007).

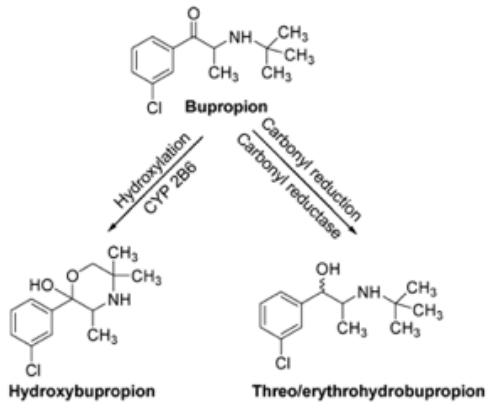

Illustration 1. Structure of bupropion and its metabolites. Modified from Wang et al, 2010.

The plasma metabolites of bupropion, namely, OHBUP, TB and EB, exhibit some of the pharmacological properties of the parent drug. Experiments *in vitro* (DA and NE uptake studies, nAChRs functional tests) and behavioral tests *in vivo* using animal models for depression and smoking cessation suggest that out of all three plasma metabolites, OHBUP is the most potent and is reported to possess ~50% of the pharmacological properties of the parent drug (Bondarev et al., 2003). It has to be noted that bupropion is a chiral compound and is used clinically as a racemic mixture (Dwoskin et al., 2006). Bupropion hydroxylation results in the three enantiomers of OHBUP, namely 2S,3S-OHBUP, 2R,3R-OHBUP and 2S,3R-OHBUP (Illustration 2). *In vitro* studies using synaptosomes of rat cerebral cortex demonstrated that 2S,3S-OHBUP is as potent as bupropion in inhibiting DA and NE uptake (Damaj et al., 2004). Moreover, 2S,3S-

OHBUP, is 4-fold more potent antagonist of the α4β2 isoform of nAChR than the parent drug (Damaj et al., 2004). Other enantiomers of OHBUP, namely 2R,3R-OHBUP and 2S,3R-OHBUP, are less potent than bupropion and 2S,3S-OHBUP. A limited number of studies investigated the relative levels of OHBUP enantiomers in the circulation of those given single or multiple doses of bupropion (Kharasch et al., 2008; Masters et al., 2016; Xu et al., 2007). It was reported that the levels of 2S,3S-OHBUP are lower than those of less potent R-enantiomers of OHBUP after a single dose of bupropion (Kharasch et al., 2008).

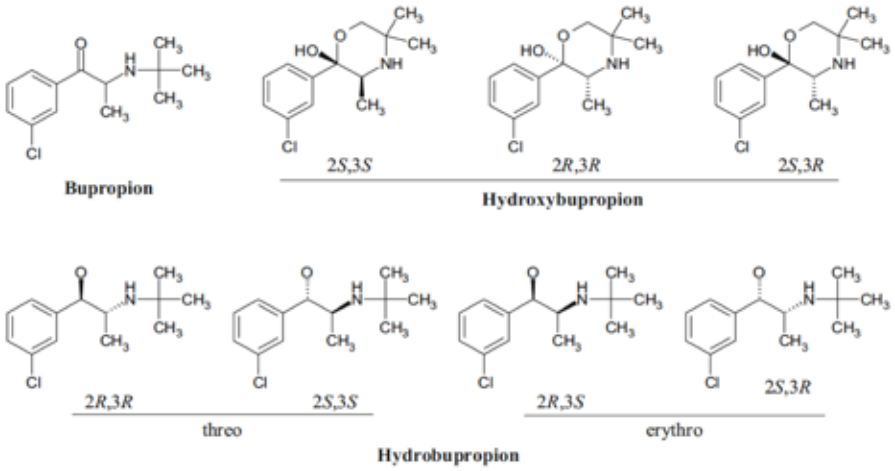

Illustration 2. Structure of bupropion and its metabolites. Modified from Dwoskin et al, 2006

Nevertheless, given that a) the fraction of the free OHBUP (binding 84%) is slightly higher than that of bupropion (binding 80%) (Findlay et al., 1981; GlaxoSmithKlein, 2004; Jefferson et al., 2005); b) the plasma levels of OHBUP exceed that of the parent drug 10 to 100 times (Findlay et al., 1981; Golden et al., 1988); and c) the concentrations of OHBUP and bupropion in cerebrospinal fluid being similar to that of plasma, OHBUP is thought to be a major contributor to the pharmacological efficacy of bupropion. In support of this concept, Zhu et al. (2012) reported the positive

correlation of the levels of OHBUP in plasma and the quit rate among smokers treated with bupropion to achieve cessation (Zhu et al, 2012). Those findings suggest that the levels of OHBUP, but not bupropion, are predictors of cessation outcome, and the therapeutic drug monitoring might be considered to target the minimum 700 ng/mL steady state concentration of OHBUP (Zhu et al., 2012). Moreover, Laib et al. (2014) observed positive correlation of OHBUP levels and the therapeutic effect of bupropion as an antidepressant, in female patients in particular (Laib et al., 2014). Based on those data, the serum threshold level of 860 ng/mL of OHBUP was recommended in treatment of depression with bupropion (Laib et al., 2014).

1.5. Effect of *CYP2B6* genetic polymorphism on bupropion metabolism and pharmacologic activity of the drug in promoting cessation from smoking

The levels of bupropion and OHBUP exhibit high inter-individual variability, which is attributed in part to genetic polymorphism of *CYP2B6*. More than 500 single nucleotide polymorphisms (SNPs) have been identified in the coding regions, non-coding regions and promoter, and the SNPs can occur alone or in combination (Mo et al, 2009; Zenger and Klein, 2013). Schematic representation of the CYP2B6 gene is shown (Illustration 3). Currently there are more than 37 distinct star-alleles listed on the CYPallele website (http://www.cypalleles.ki.se); the most prominent variants in terms of functional impact and frequency are displayed in Illustration 4.

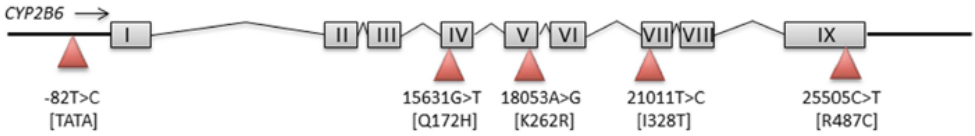

Illustration 3. Schematic representation of the *CYP2B6* gene. Boxes represent *CYP2B6* exons (not to scale) and show the most important SNPs as triangles. SNP, single nucleotide polymorphism. Modified from Zanger and Klein, 2013

The most studied variant allele of *CYP2B6* is *CYP2B6*6*, which corresponds to the combination of allele *4 (785A>G, rs2279343) and *9 (516G>T, rs3745274). *CYP2B6 *6* allele occurs with a frequency ranging from 10% to 60% depending on the population (Zenger and Klein, 2013) and results in the decreased expression and activity of CYP2B6. Specifically, the *6 allele is correlated with the increased amount of the splice variant of *CYP2B6* that lacks the exons 4-6 region, resulting in the deficient transcript (Zenger and Klein, 2013). The impact of the *6 allele on the disposition of CYP2B6 substrates and its clinical relevance has been reported. For example, CYP2B6 catalyzes metabolism of methadone, specifically its (S)-enantiomer; and the *CYP26*6* allele was associated with the higher plasma levels of (S)-methadone (Crettol et al., 2006). Consequently, in the course of methadone therapy, the therapeutic needs of carriers of the *6 allele were met with lower doses of the drug as compared to patients who did not carry this reduced function *CYP2B6* allele (Levran et al., 2013).

Several *CYP2B6* alleles have been implicated in the differences in treatment outcomes with bupropion in smoking cessation therapy. The *CYP2B6*5* (1459C>T) allele, which confers a decrease in expression and activity, has been associated with an increase in craving and relapse rate (Lerman et al., 2002). *CYP2B6*4* (785A>G, rs2279343) results in increased expression and activity, and thus more rapid bupropion

hydroxylation than the wild type (Mo et al, 2009). Tomaz and coworkers (2015) reported that carriers of the *4 allele, "fast metabolizers" of bupropion, had a lower success rate in achieving cessation with bupropion treatment, than those subjects who did not carry this variant allele (Tomaz et al., 2015)

Nucleotides substitution	g.-82T>C	c.785A>G	c.1459C>T	c.516G>T	c.983T>C	Enzyme activity	References
Effect on protein	Promoter	K262R	R487C	Q172H	I328T		
*CYP2B6*1*						Normal	Zanger and Klein, 2013
CYP2B6*4		**X**				**Increased**	Lang et al., 2001; Tomaz et al., 2015
CYP2B6*5			**X**			**Decreased**	Kirchheiner et al., 2003
CYP2B6*6		**X**		**X**		**Decreased**	Levran et al, 2011, Lee et al, 2006,
*CYP2B6*7*		X	X	X		Increased	Mo et al, 2009
*CYP2B6*18*					X	Decreased	Zhou et al, 2012
*CYP2B6*22*	X					Decreased	Zanger and Klein, 2013

Illustration 4. Select genetic polymorphisms of *CYP2B6* and their functional effects on CYP2B6 enzymatic activity. c., cDNA position; g., genomic position are given in bp. CYP2B6 alleles studied in association with smoking cessation are marked in bold. Modified from Zanger and Klein, 2013

Moreover, carriers of the *CYP2B6*6* allele, "slow metabolizers" of bupropion, exhibit higher abstinence rates when treated with bupropion for cessation than wild type carriers of *CYP2B6* (Lee et a., 2007). Of note, expression and activity of DAT, DA receptors, the extent of smoking/nicotine dependence, involvement of CYP2B6 in metabolism of nicotine and other factors could be co-variates in the quit rate of smokers treated with bupropion to aid achieving cessation (Quaak et al., 2009). Nevertheless, it appears that individuals with decreased activity variants of *CYP2B6*, and thus slower bupropion metabolism, are better candidates for smoking cessation therapy with bupropion.

The *CYP2B6*18* (I328T, rs28399499) allele is considered a reduced or null-function variant based on the results obtained *in vitro* from various expression systems

(Zanger and Klein, 2013). The *CYP2B6*18* allele has a frequency of 4-7% and is exclusively found in population of African decent. This allele is associated with reduced plasma levels of OHBUP at steady state (Benowitz et al., 2013). To date there are no data on the association of the *18 allele and the efficacy of bupropion in smoking cessation. However, based on the functional impact of this allele on bupropion hydroxylation, one could speculate that smokers who carry the *18 allele would have a greater response to bupropion therapy in achieving cessation.

1.6. Effect of CYP2C19 genetic polymorphism on bupropion biodisposition

The contribution of CYP2C19 in the hydroxylation of bupropion and its TB and EB metabolites has been reported recently (Chen et al, 2010). Similar to *CYP2B6*, the *CYP2C19* is highly polymorphic, with more than 25 alleles currently listed on the CYPallele website (http://www.cypalleles.ki.se). The most important variants in relation to their functional impact and frequency are illustrated below (Illustrations 5 & 6). The *CYP2C19*2* allele (681G>A; rs4244285) results in the truncated nonfunctional protein due to an aberrant splice site. The frequency of the *CYP2C19*2* variant allele ranges from 15% to 25% depending on the studied population (Scott et al., 2012). The *CYP2C19*3* to **8* variant alleles are all associated with the absent or diminished CYP2C19 activity, but are less frequent than the *2 variant allele. Further, the *CYP2C19*17* (-806C>T, rs12248560) variant allele results in increased expression and activity of the enzyme: the C>T substitution produces consensus binding site in the promoter region leading to the up-regulation of transcription.

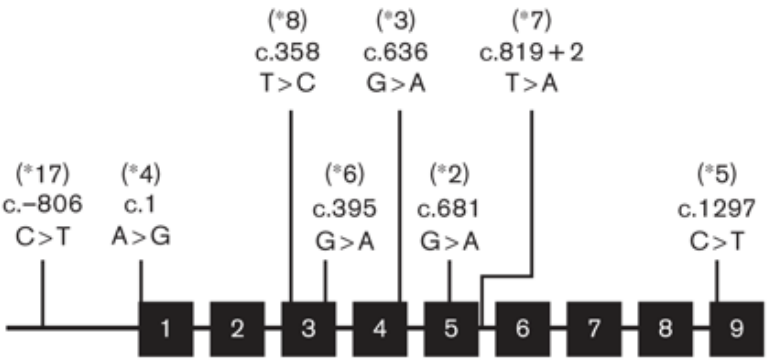

Illustration of the *CYP2C19* gene highlighting the location of selected loss-of-function (**2–*8*) and gain-of-function (**17*) variant alleles. Exons are represented by numbered black boxes (not to scale).

Illustration 5. Schematic representation of *CYP2C19* gene highlighting the location of selected polymorphisms. Boxes represent *CYP2C19* exons (not to scale). SNP, single nucleotide polymorphism. Modified from Scott et al., 2013.

Based on the capacity of CYP2C19 to metabolize substrates, the individuals are classified in four groups, namely, poor metabolizers (PM, carriers of two reduced function alleles), intermediate metabolizers (IM, heterozygotes for a reduced function allele), extensive metabolizers (EM, homozygotes for a wild-type allele) and ultrarapid metabolizers (UM, homo- or heterozygotes for the *17 allele)(Table 2). Of note, the carriers of the *2 loss-of-function and the *17 gain-of function alleles (*2/*17 heterozygote genotype) are classified into the IM group due to the dominating effect of the *2 variant on the net CYP2C19 activity in metabolizing the tested probes (Scott et al, 2013). The role of CYP2C19 metabolizer status in the pharmacokinetics of CYP2C19 substrates (for example, clopidogrel) and its therapeutic implications has been reported (Hulot et al, 2010). In the case of clopidogrel, care providers could consider alternative antiplatelet therapy for individuals with the *2 allele due to higher risk of adverse cardiovascular events with clopidogrel treatment for these patients (Scott et al., 2013).

Likely phenotype of *CYP2C19* gene		Enzymatic activity	Examples of diplotype (most common genotype combinations)
Ultrarapid metabolizer (~5-30% of the population)	**UM**	Increased	*1/*17, *17/*17
Extensive metabolizer (~35-50% of the population)	**EM**	Normal	*1/*1
Intermediate metabolizer (~18-45% of the population)	**IM**	Intermediate	*1/*2, *1/*3, *2/*17
Poor metabolizer (~2-15% of the population)	**PM**	Poor	*2/*2, *2/*3, *3/*3

Illustration 6. Assignment of likely CYP2C19 phenotypes based on genotypes. UM, ultrarapid metabolizer; EM, extensive metabolizer, IM, intermediate metabolizer; PM, poor metabolizer. Modified from Scott et al., 2013

Zhu and coworkers (2014) studied the role of *CYP2C19* polymorphism in the pharmacokinetics of bupropion and its potential effect on the drug's therapeutic efficacy in promoting cessation from smoking. The results demonstrated that *CYP2C19*2* carriers have higher exposure to bupropion, TB and EB, but not OHBUP, as compared to the **1/*1* carriers (Zhu et al., 2014). However, no correlation was observed between the subjects' CYP2C19-metabolizer status and the ability of bupropion to promote cessation from smoking (Zhu et al., 2014).

1.7. Physiological changes in pregnancy and their potential effect on bupropion biodisposition

During pregnancy, maternal physiology undergoes multiple changes that may affect the absorption, distribution, metabolism and excretion of administered medications and thus affect their efficacy and safety (Loebstein et al., 1997). The influence of these changes on a drug's biodisposition depends on the properties of the drug.

In pregnancy, the gastric pH is increased and gastrointestinal emptying time is reduced due to higher progesterone production (Olagunju et al., 2012). The increased gastric pH would lead to a more rapid adsorption of basic drugs such as bupropion

(bupropion pKa =7.9). In pregnancy, body water is increased by 1.5 times; however, it is unlikely to affect bupropion's biodisposition. The drug is lipophilic; therefore, it would preferentially distribute into the tissue over the water compartment (Findlay et al., 1981). Moreover, a pregnancy-associated increase in maternal fat might lead to retardation of bupropion elimination (Costantine, 2014). In pregnancy, the plasma albumin levels decline by 15% and the levels of alpha-1-acid glycoprotein decline by 50% (Olagunju et al., 2012). Bupropion binds primarily to plasma albumin and it is considered a low-binding drug (80%) (Findlay et al., 1981). Therefore, changes in plasma proteins in pregnancy should not influence the fraction of a free drug and thus its pharmacologic efficacy and metabolism. Likewise, decline in plasma proteins in pregnancy should not affect the fraction of a free OHBUP, which binding capacity to plasma proteins is 77% (GlaxoSmithKline, data on file).

The glomerular filtration rate (GFR) is at least 50% higher in pregnancy as compared to the non-pregnant state (Dunlop, 1981); therefore, the clearance of renally excreted medications is accelerated during pregnancy. Regardless, renal elimination is not the primary route of bupropion clearance. Thus, pregnancy-associated changes in the GFR alone should not significantly affect bupropion clearance or the steady state levels of the drug (Benowitz et al., 2013). However, the renal clearance of OHBUP, TB and EB, in a free or conjugated state, could be accelerated in pregnancy due to the higher polarity of these metabolites and the higher fraction eliminated with urine as compared to the parent drug (Dawes and Chowienczyk, 2001).

Bupropion has a high extraction ratio; therefore its clearance is sensitive to changes in hepatic blood flow and the activity of metabolizing enzymes (Rowland and

Tozer, 1995). Therefore, a pregnancy-associated increase in hepatic blood flow could lead to an accelerated clearance of bupropion.

Alterations in the activity of the hepatic metabolizing enzymes during pregnancy have been reported following the results of clinical pharmacokinetic studies. For instance, the activity of CYP2D6 is increased in pregnancy as revealed by the accelerated clearance of CYP2D6 substrates, metoprolol (Ryu et al., 2016) and dextromethorphan (Tracy et al, 2005), while the activity of CYP2C19 is decreased based on the decreased clearance of proguanyl in pregnancy (McCreedy et al, 2003). In addition, the activity of UDP-glucuronosyltransferases (UGT) is increased in pregnancy based on the accelerated elimination of labetalol (Green et al., 1995; Jeong et al., 2008). The exact mechanisms underlying the effect of pregnancy on the activity of the hepatic enzymes and why this effect is isoform-specific is not understood in great detail. However, accumulating evidence from *in vitro* studies suggest the hormonal modulation of gene expression is the primary mechanism leading to the altered activity of drug-metabolizing enzymes in pregnancy (Jeong et al., 2010; Sarlis et al., 2005). During pregnancy, the levels of estradiol and progesterone reach 0.1 and 1 μM at term, respectively, which is 100-fold higher as compared to the non-pregnant state (Cunningham, 2014). The levels of estrone and estriol reach up to 30 nM at term while their pre-pregnancy levels are in a sub-nanomolar range. The hormonal regulation of expression of drug-metabolizing enzymes occurs via canonical pathways (activating nuclear receptors) and non-classical mechanisms (not involving nuclear receptors) and is concentration-dose dependent (Jeong et al., 2010).

The effect of pregnancy on the activity of CYP2B6, the primary enzyme responsible for bupropion hydroxylation, has not been investigated extensively *in vivo*. *In vitro* studies suggest that in low concentrations, estradiol regulates expression of hepatic CYP2B6 via binding to the cognate estrogen receptor ERα (Illustration 7) (Koh et al., 2012). The hormone-receptor complex may either directly bind to the estrogen-response element (ERE) of the gene, or mediate gene expression indirectly by interacting with other transcriptional factors (Bjornstrom and Sjoberg, 2005). However, at high concentrations, estradiol binds to constitutive androstane receptors (CAR), and the complex then associates with phenobarbital-responsive enhancer module (PBREM), a distal promoter enhancer, resulting in the up regulation of CYP2B6 expression (Koh et al., 2012) (Illustration 7).

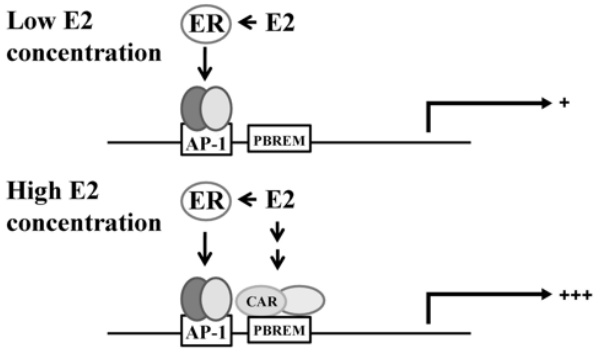

Illustration 7. Proposed model for pregnancy-associated upregulation of *CYP2B6*. E2, estradiol; AP-1, ER, estrogen receptors; CAR, constitutive androstane receptor; PBREM, phenobarbital-responsive enhancer module; AP-1, activator protein 1. Modified from Koh et al., 2012

Moreover, the plasma levels of biologically active, non-bound, cortisol are 3-folds higher in pregnancy as compared to the non-pregnant state (Jeong, 2010). Cortisol binds to glucocorticoid receptors and retinoid X receptor (PXR), which leads to an increase in the expression levels of several genes, including CAR (Pascussi et al., 2000). Therefore,

the synergistic effect of estradiol and cortisol in pregnancy leads to upregulation of CYP2B6. The results of *in vitro* studies using human hepatocytes were extrapolated *in vivo*; thus, the predicted magnitude of increase in the expression of CYP2B6 in pregnancy is 2-3-fold as compared to pre-pregnancy levels (Koh et al., 2012). This estimated 2-3-fold difference was correlated with the 2-fold increase in the clearance of methadone during pregnancy (Dickmann and Isoherranen, 2013; Wolff et al., 2005). Methadone is a substrate of CYP2B6, and the observed alterations in the clearance of methadone in pregnancy were attributed primarily to the upregulation of CYP2B6 (Dickmann and Isoherranen, 2013). Given that CYP2B6 is a primary hepatic enzyme responsible for bupropion hydroxylation, pregnancy-associated changes in CYP2B6 activity might significantly affect the plasma levels of bupropion and its pharmacologically active metabolite, OHBUP, in pregnancy.

As was mentioned above, CYP2C19 also contributes to bupropion, TB and EB hydroxylation (Zhu et al., 2014), and the activity of CYP2C19 in pregnancy is decreased (McCreedy et al, 2003). Several reports suggested that a pregnancy-associated decline in CYP2C19 activity could occur at least in part due to the estrogen-mediated downregulation of CYP2C19 (Laine et al., 2000; Laine et al., 2003). Studies *in vitro* demonstrated that exposure to estrogen at the concentrations consistent with its levels in pregnancy leads to decrease in CYP2C19 transcription. The estrogen-regulated downregulation of CYP2C19 occurs via classic pathway. Specifically, the novel estrogen-responsive element-binding site was identified downstream of the promoter of CYP2C19, and binding of the estrogen-ERα complex to that target region leads to downregulation of CYP2C19 expression (Mwinyi et al., 2010). Although CYP2C19 is

not a primary enzyme that is involved in bupropion metabolism, the pregnancy-associated inhibition of CYP2C19 may affect the levels of the parent drug and also its metabolites, TB and EB, in pregnant women treated with bupropion.

Taken together, it appears that the effect of pregnancy on the pharmacokinetics of bupropion is multifactorial. While the increase in CYP2B6 activity in pregnancy could accelerate bupropion's metabolic clearance, the decline in CYP2C19 activity could, in contrast, reduce bupropion clearance. Altered activity of bupropion-metabolizing hepatic enzymes along with other pregnancy-induced changes in maternal physiology can lead to significant changes in bupropion biodisposition during pregnancy. The altered systemic exposure to the drug and its active metabolite(s) could subsequently influence the efficacy of the drug.

1.8. Fetal exposure to maternally administered medications: the role of the placenta and amniotic fluid.

The placenta is a temporary organ of fetal origin; it plays an essential role in maintaining a healthy pregnancy and ensuring normal development of the fetus. The human placenta is of the hemochorial type, which means that the maternal blood is in direct contact with the fetal tissue (Hutson, 2011). The cotyledon is a functional vascular unit of the placenta; every cotyledon contains a villus tree suspended in the intervillous space. The intervillous space is filled with maternal blood, which is supplied by the spiral arteries and removed by uterine veins. The outer throphoblast layer of the villous tree is syncytiotrophoblast, a polarized single multinucleated layer; the syncytiotrophoblast serves as the rate-limiting barrier for the transfer of endogenous and exogenous

substances (Illustration 8). The transplacental transfer of xenobiotics can occur via passive diffusion, in addition to facilitated diffusion and active uptake and efflux. A variety of membrane transporters are present in the maternal-facing apical (brush border) membrane and fetal-facing basal membrane and were shown to mediate active transport of endogenous and exogenous substances (Illustration 8). These transporters are, but not limited to, P-glycoprotein (P-gP), breast cancer resistance protein (BCRP), multidrug resistance associated proteins (MRP) and organic cation transporters (OCT) (Weier et al., 2008).

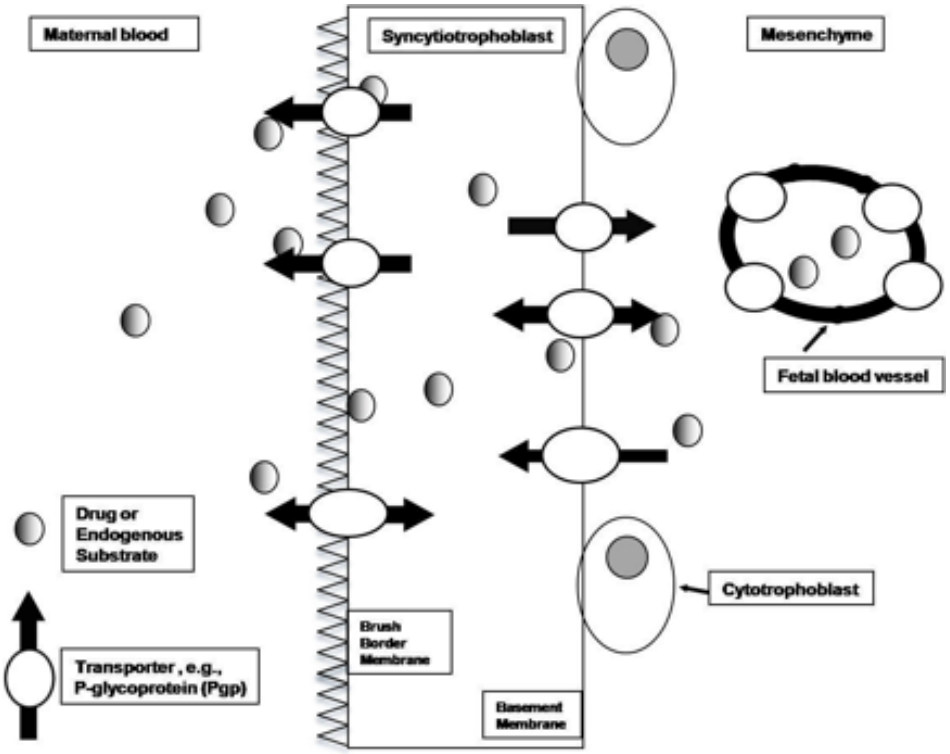

Illustration 8. Schematic representation of fetal-maternal blood interface in human placenta. Modified from Hutson, 2011

The extent of drug transfer across the placenta depends on various factors related to the physicochemical characteristics of the drug and pharmacokinetic properties (Hutson, 2011). Lipid-solubility, low molecular weight (<500 Da) and un-ionized state

are the properties that would favor the free transfer of most compounds across the placenta. The fetal blood has a pH~7.35 and is slightly more acidic than maternal blood (pH=7.4); therefore, basic drugs could be trapped in the fetal circulation due to ionization. Highly lipophilic drugs can be retained by placental tissue; thus, the placenta would represent a drug depot (Johnson et al., 1997). Further, protein binding of a drug in the maternal and fetal circulation will influence the drug transfer across the placenta. The placenta-dependent pharmacokinetic parameters that impact drug transfer include, but are not limited to, expression and activity of placental transporters and placental metabolism. It has been shown that several enzymes, namely cytochrome P450 enzymes, carbonyl-reductases, and enzymes involved in phase II metabolism are expressed and active in placental tissue (Syme et al., 2004). The placental biotransformation of xenobiotics is thought to play a secondary role in limiting the placental passage of drugs (Weier et al., 2008); however, it is important to recognize the factors altering the metabolizing capacity of the placenta and thus affecting fetal exposure to maternally administered therapeutics.

The amniotic fluid is another route of potential fetal exposure to maternally administered medications (Loughhead et al., 2006a). During first trimester, the amniotic fluid composition is very similar to that of fetal plasma (Underwood et al., 2005). With advancing gestation, fetal urine is the major contributor to the amniotic fluid content, along with the secretion of oral, nasal, tracheal and pulmonary fluids (Underwood et al., 2005) (Illustration 9). Transcutaneous absorption of the amniotic fluid by the fetus occurs prior to the fetal skin keratinization, which takes place within weeks 19-25 of gestation (Beall et al., 2007; Underwood et al., 2005). Fetal swallowing begins as early as ~16

weeks of gestation and is the main pathway of amniotic fluid removal (Gilbert, 2006) (Illustration 9).

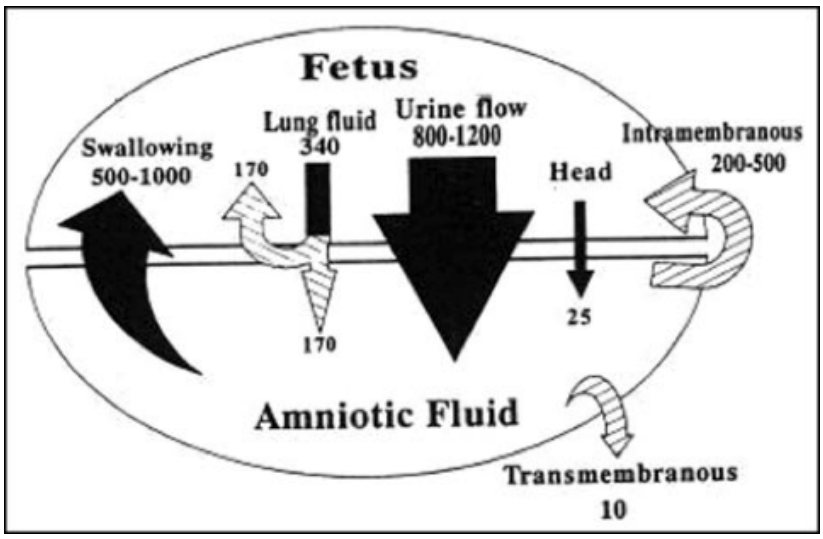

Illustration 9. Schematic diagram of all known pathways for amniotic fluid dynamics in the fetus near term. The solid arrows represent directly measured flows; the hatched arrows represent estimated flows. The numbers represent volume flow in mL/day. Modified from Gilbert, 2006

The umbilical vein carries the blood from the placenta to the fetus; approximately two thirds of this blood is supplied to the portal vein and enters the fetal hepatic circulation (Ring et al., 1999). Several reports indicated that drug metabolizing enzymes are present in fetal liver, although their expression levels and catalytic capacities depend on gestational age and are in general lower than in adult livers (Croom et al., 2009; Ekström et al., 2013; Juchau et al., 1980; Pasanen et al., 1987; Shuster et al., 2014). It is plausible that the fetus is able to biotransform drugs received via umbilical circulation, as well as via intake of the amniotic fluid. Moreover, continuous fetal exposure to maternally administered medications is plausible due to amniotic fluid recirculation (fetal urine excretion and subsequent swallowing) and release of drugs retained by the placental tissue.

1.9. Placental disposition of bupropion *in vitro* and *ex vivo* and its implications

The transfer of bupropion across the placenta was studied using dual perfusion of placental lobule (Earhart et al, 2010). It the course of the perfusion experiment, the drug did not affect the viability of the tissue, it crossed placenta and appeared in the fetal circulation. At the end of a 4-hour placental perfusion, ~50% of the drug was retained by placental tissue, while ~30% remained in the maternal circulation (Earhart et al, 2010). Moreover, the formation of TB and its release into the maternal and fetal circuits of placental perfusion system was observed, although the metabolic activity of placental enzymes under the experimental condition was low (Earhart et al., 2010).

Further, *in vitro* studies demonstrated that bupropion is a substrate of BCRP and P-gp placental efflux transporters (Hemauer et al., 2010). On the other hand, unlike bupropion, its metabolite OHBUP is not a substrate of placental efflux transporters, and does not undergo further metabolism during the perfusion experiments (Hemauer et al, 2010). OHBUP did not adversely affect placental viability during perfusion, however its retention by the placental tissue was lower than that of bupropion due in part to differences in physicochemical properties (Hemauer et al, 2010). In addition, the concentrations of OHBUP in the fetal circuit following perfusion reached ~32% of its initial concentrations in the maternal circuit (Hemauer et al., 2010), which was higher than that of bupropion (20%)(Earhart et al., 2010).

Placental metabolism of bupropion has been studied *in vitro* using subcellular fractions of human placental tissue (Wang et al., 2010). The microsomal fraction displayed the highest activity in bupropion metabolism, indicating that membrane-bound

enzymes are predominantly responsible for the placental biotransformation of the drug (Wang et al., 2010). Placental microsomes metabolized bupropion to OHBUP, TB and EB, with the rate of TB and EB formation being several fold higher than that of OHBUP, and TB was the major bupropion metabolite (Wang et al., 2010). As was mentioned previously (Section 1.4), TB and EB are less pharmacologically potent than the parent drug and OHBUP (Bondarev et al., 2003), suggesting an important role of placental metabolizing enzymes in decreasing fetal exposure to bupropion. Placental 11βHSDs and also aldo-ketoreductates were identified as the carbonyl-reducing enzymes catalyzing the formation of TB and EB, while CYP2B6 and to a lesser extent CYP2C19, were responsible for bupropion hydroxylation in human placenta (Wang et al, 2010).

Taken together, the *ex vivo* and *in vitro* data suggest that human placenta actively regulates the disposition of bupropion (metabolism, efflux transport), and if it is true *in vivo*, the placenta plays an important role in limiting fetal exposure to maternally administered bupropion. In contrast, nicotine is not metabolized by placental tissue and diffused freely across the placenta (Nekhayeva et al., 2005; Pastrakulji et al., 1998; Sastry et al., 1987), which suggests that the accessibility of nicotine to the fetus exceeds that of bupropion. Overall, data suggest that bupropion might have a better safety profile in smoking cessation therapy than NRT. However, OHBUP is present in the circulation of patients treated with bupropion; therefore, fetal exposure to OHBUP in pregnant patients should be taken into consideration.

Data from the placental metabolism of bupropion *in vitro* revealed that the formation of TB and EB from bupropion was significantly higher in microsomes obtained from the placentas of women who smoked during pregnancy as compared to non-smokers

(Wang et al., 2010). These suggest that smoking increases the activity of placental 11βHSDs and aldo-ketoreductases (AKR) that catalyze the formation of TB and EB.

A previous study demonstrated that NNK, a potent carcinogen of cigarette smoke, undergoes reductive metabolism in placenta with the formation of NNAL (Atalla and Maser, 2001). The placental soluble and membrane-bound carbonyl reducing enzymes catalyzing the reaction were characterized using placental subcellular fractions (Atalla and Maser, 2001).

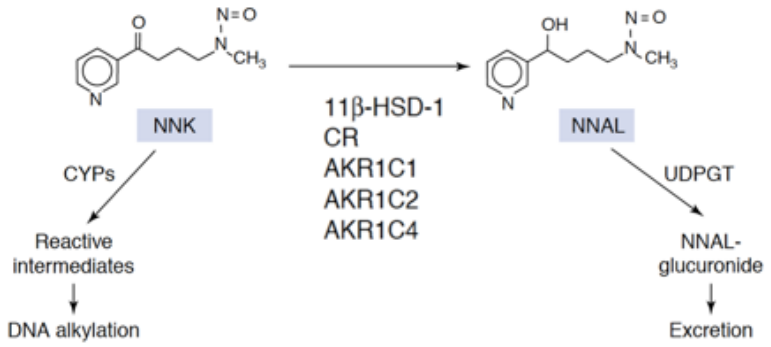

Illustration 10. Simplified scheme of the metabolic pathways of NNK. NNK, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone; NNAL, 4-methylnitrosamino-1-(3-pyridyl)-1-butanol; 11βHSD, 11β-hydroxysteroid dehydrogenase; AKR, aldo-keto reductases; UDPGT, uridinediphosphate-glucuronosyltransferase. Modified from Maser, 2004.

As was mentioned in Section 1.2, NNK is a pro-carcinogen; hydroxylation of NNK by CYP enzymes leads to the formation of the reactive electrophilic species capable to react with purine bases of DNA (Hecht, 1998). Consequently, the resulting 8-hydroxydeoxyguanosine (8-OH-dG) may lead to formation of DNA lesions (Xue et al., 2014). Alternatively, NNK can undergo reduction with the formation of NNAL, which is subsequently conjugated by UGTs for subsequent elimination. Although NNAL is also a substrate for hydroxylation and is a pro-carcinogen, it appears that the reductive metabolism of NNK into NNAL is a crucial step in NNK detoxification (Akopyan and

Bonavida, 2006). It is likely that free and conjugated NNAL detected in the urine from the newborns of smoking mothers originated from the fetal uptake of NNAL following maternal hepatic, pulmonary and placental metabolism of NNK, although the formation of NNAL from NNK by the fetal organs and subsequent NNAL glucuronidation by the fetus are plausible. Taken together, it appears that reductive metabolism of NNK by placental tissue might be a limiting factor in transplacental fetal exposure to the smoking-related carcinogen NNK. Given that the formation of TB and EB for bupropion is increased in placental microsomes derived from placentas of smoking mothers (Wang et al, 2010), the effect of bupropion on the activity of placental carbonyl reductases cannot be ruled out.

1.10. Objectives of the current study

The primary aim of the current study was to investigate the effect of pregnancy on the pharmacokinetics of bupropion and its major plasma metabolites. In addition, we investigated the association of CYP2B6 and CYP2C19 genotypes with the biodisposition of bupropion during pregnancy, irrespective of pregnancy-induced changes.

The second aim was to study whether prolonged exposure to bupropion impacted the capacity of placental carbonyl reducing enzymes to catalyze the formation of NNAL from NNK.

The third aim of the current study was to investigate the *in vivo* transplacental transfer of bupropion and its major pharmacologically active metabolites. In addition, the concentrations of the drug and its metabolites were determined in the amniotic fluid.

CHAPTER 2: PHARMACOKINETICS OF BUPROPION IN PREGNANCY

2.1. Introduction

As a smoking cessation aid in males and non-pregnant females, bupropion SR, fixed at 150 mg BID, is used for a limited period of time, 7-12 weeks. Bupropion is extensively metabolized, and its major product OHBUP is thought to contribute to the drug clinical efficacy for smoking cessation.

As was described in the Section 1.7, physiological changes occurring in pregnancy can alter the pharmacokinetics of administered medications. Consequently, an adjustment of a dose and regimens may be required for a variety of drugs to avoid excessive dosing and/or maintain the therapeutic concentrations. For example, pregnancy-associated upregulation of CYP2B6 contributes to an increase in the clearance of methadone, thus the need for the increase of methadone dose is not uncommon in pregnant patients treated for opiate addiction (Bogen et al., 2013; Shiu et al., 2012). Another example is an anti-convulsant lamotrigine: induction of certain UGT enzymes and changes in renal function, both associated with pregnancy, accelerate the excretion of lamotrigine in pregnant patients (Ohman et al., 2008; Franco et al., 2008). Therefore, care providers can recommend increasing of the dose of lamotrigine during pregnancy following specific guideline with the systematic monitoring of the plasma level of the drug (Sabers et al., 2012).

Pregnant women were historically excluded from the pharmacokinetic studies due to ethical, legal and practical considerations (Ke et al., 2014). However, opportunistic

pharmacokinetic studies are conducted on pregnant women and typically enroll pregnant subjects who chronically receive the studied medication as part of the clinical care. The use of bupropion for treatment of depression during pregnancy is acceptable in clinical practice. Thus, the opportunistic studies on bupropion in pregnancy would not impose additional risks to the mother and the fetus.

The aim of this work was to study the effect of pregnancy on the pharmacokinetics of bupropion. The results will help to evaluate the necessity of the dose adjustment of bupropion in promoting smoking cessation during pregnancy. We hypothesized that the hydroxylation of bupropion to OHBUP is increased in pregnancy due to upregulation of CYP2B6. Thus, it is plausible that the systemic exposure to OHBUP is higher in pregnancy as compared to the non-pregnant state. On the other hand, the downregulation of CYP2C19 due to pregnancy could in turn increase the systemic exposure to the parent drug, bupropion. Moreover, pregnancy associated changes in the GFR and the isoform-specific effect of pregnancy on the activity of UGT enzymes could also affect the biodisposition of bupropion in pregnancy.

The second aim of this study was to examine whether the genetic polymorphism of CYP2B6 and CYP2C19 affect the pharmacokinetics of bupropion in pregnancy. We investigated whether the predicted metabolic phenotypes of CYP2B6 and CYP2C19 in pregnancy are similar to those in the non-pregnant state. Genetic polymorphisms of CYP2B6 and CYP2C19 could potentially serve as markers for individualized bupropion pharmacotherapy for smoking cessation in pregnancy.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

Chemicals were purchased from the following companies: bupropion (BUP), triprolidine hydrochloride, phenacetin, and β-Glucuronidase from Helix pomatia, ammonium acetate, from Sigma-Aldrich (St. Louis, MO); OHBUP, TB, EB, and the deuterium labeled internal standards (IS) BUP-d9, OHBUP-d6, EB-d9 and TB-d9, from Toronto Research Chemicals Inc. (North York, Canada); LC/MS-grade methanol, LC/MS-grade acetonitrile, methylene chloride, formic acid, acetic acid, trichloroacetic acid (TCA), potassium phosphate mono- and dibasic from Fisher Scientific (Fair Lawn, NJ).

2.2.2. Subjects

This was an opportunistic study conducted following the protocol approved by the Institutional Review Board of the University of Texas Medical Branch (UTMB). Eligible participants were pregnant women 18 years of age or older taking bupropion for treatment of depression as prescribed by their regular care provider. An essential criterion for eligibility was the willingness to participate in the study during pregnancy and postpartum. Exclusion criteria were: hematocrit of less than 28%, current or prior conditions indicating clinically significant alterations in hepatic, renal or gastrointestinal functions, multiple pregnancies, cardiac disease and major fetal structural/chromosomal abnormalities. Eligible subjects were enrolled during early pregnancy (10-14 weeks of gestation), mid-pregnancy (22-26 weeks of gestation), and late pregnancy (34-38 weeks of gestation). The subjects enrolled postpartum during lactation period were reached for

additional participation in the study post-lactation. Thus, each subject was eligible to participate in the study three times during pregnancy and one or two times postpartum. The postpartum non-/post-lactating period represented a non-pregnant control for each individual.

All procedures involving human subjects were conducted according to the declaration of Helsinki and its actual amended version the International Conference on Harmonization-Good Clinical Practice (ICH-GCP) guidelines. All subjects were compensated for participation.

2.2.3. Bupropion dosing regimen

The subjects were taking bupropion for treatment of depression as prescribed by their regular care provider without regard of the study. The recruited pregnant women were receiving the following formulations and dosage of bupropion: IR formulation, 100 mg TID (Mylan); SR, 150 mg QD (TEVA, Actavis) and 150 mg BID (Actavis, GSK, Watson lab), and XL, 300 mg QD (Actavis, Zydus).

Each subject used the same formulation and dose of bupropion during all study days. The study provided the drug for the participants treated with bupropion SR 150 mg BID (GSK) for 3 days prior to each pharmacokinetic (PK) study visit; the remainder subjects used their own stock of the medication. Each subject completed a 4-day dosing calendar of administration times of bupropion; pill count was used for verification with the dispensed study drug. Subjects using their own medication were instructed to bring their supply of bupropion to the PK study appointment for dosing.

2.2.4. Samples collection

On the day of the PK study, serial blood samples were collected prior to dosing (0 hours) and at the time points post-dosing as follows: 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 hours, and truncated to reflect the dosing interval. The subjects were discharged home overnight prior to the 24-hour post dose blood draw next morning. All blood samples were collected in the BD Vacutaner® heparinized tubes, and plasma was separated immediately by centrifugation. Urine samples were collected every 2 to 6 hours within the same dose interval. Subjects discharged home overnight were instructed to collect all nocturnal urine void (the collection vessels were provided), and submit the output upon return to the clinic in the morning. All urine volumes were measured and recorded. Blood or buffy coat was collected from each subject for genotyping. All samples were stored at -80°C until analysis. The samples were analyzed using the respective methods as described below.

2.2.5. Quantitative determination of bupropion and its three major metabolites in plasma and urine

2.2.5.1. Quantification of bupropion, OHBUP, TB and EB in plasma

Instrumental and analytical conditions

The analysis of bupropion, OHBUP, TB and EB in plasma were conducted simultaneously using an Agilent HPLC 1200 series system coupled with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The detailed

description of the HPLC system and analytical conditions were reported previously (Wang et al, 2012).

Preparation of stock and working standard solutions

For analysis of plasma samples, the working standard solutions of BUP and its metabolites were prepared in 30% methanol in the following serial dilutions: BUP, 2.5-2500 ng/mL; OHBUP, 20-20 X 103 ng/mL; TB, 12-1200 ng/mL; and EB, 10-10 X 103 ng/mL. The stock solutions for IS was prepared in 30% methanol at the final concentrations of 80 ng/mL for each of BUP-d9, OHBUP-d6, EB-d9, and TB-d9. The solutions were stored at 4°C.

Calibration standards and quality control samples

Calibration standards for plasma samples were prepared by adding 5 µl of working standard solution into a 50 µl blank plasma. The calibration standards were prepared in the following concentrations: BUP, 0.25-250 ng/mL; OHBUP, 2-2000 ng/mL; TB, 1.2-1200 ng/mL; and EB, 1-1000 ng/mL. 10 µl of IS solution was added to each of the calibration sample. Quality control (QC) samples were prepared at low, medium and high concentration levels as well as low limit of quantification (LLOQ).

Preparation of plasma samples for analysis

10 µl of IS solution was spiked with the 50 µl of plasma sample. Then, 90 µl of 5%, w/v of TCA was added (final 3%, w/v), samples were vortexed, followed by addition of 1000 µl of acetonitrile. Each sample solution was vortexed for 1 min and centrifuged for 10 min at 12,000 x g. The supernatant was transferred to another tube and dried at 40°C under a stream of nitrogen. The residues were reconstituted in 150 µl of initial mobile phase and 10 µl was injected into the HPLC system for analysis by LC-MS/MS.

2.2.5.2. Analysis of urine sample

Instrumental and analytical conditions

The analysis of bupropion, OHBUP, TB and EB in urine was performed using Waters HPLC system coupled with a Waters EMD 1000 single quadrupole mass spectrometer (Waters, Milford, MA). The HPLC system consisted of Waters 1525 binary HPLC pump and a 717-plus autosampler controlled by Empower™ 2 chromatography Data Software (Waters, Milford, MA). Separation of the analytes was achieved using Waters Symmetry C18 column (150 mm × 4.6 mm, 5 μm) connected to a Phenomenex C18 guard column (4 mm × 3.0 mm) by isocratic elution of the mobile phase at a rate of 1.0 mL/min. For the analysis of OHBUP, TB and EB, the mobile phase consisted of 40% methanol and 60% of 10mM ammonium acetate buffer with 0.02% of acetic acid. For the analysis of BUP, the mobile phase consisted of 25% methanol and 75% of deionized water/formic acid (0.04% v/v).

The analytes were detected using mass spectrometry (MS). The mass spectrometer (Waters EMD 1000 single-quadrupole; Milford MA) was supplied with an electrospray ion source (ESI) operated in positive mode. The MS parameters were as follows: capillary voltage, 2.2 kV; cone voltage, 40 V; source temperature, 95°C; desolvation temperature, 350 °C; desolvation gas flow rate, 450 L/h; cone gas flow rate, 100 L/h. The analytes were monitored by selective ion monitoring (SIM) at m/z 184 for BUP, m/z 179 for triprolidine hydrochloride (IS for BUP); m/z 238 for OHBUP, m/z 168 for TB and EB, m/z 180 for phenacetin (IS for OHBUP, TB and EB).

Preparation of stock and working standard solutions

All stock solutions were prepared in 30 % methanol.

For detection and quantification of OHBUP, TB and EB in urine, the working standard solutions were prepared in the following ranges: OHBUP, 50-20 X 104 ng/mL; TB, 50-10 X 104 ng/mL; EB, 10-20 X 104 ng/mL. The corresponding IS, phenacetin, was prepared at the final concentration of 1 μg/ml.

For detection and quantification of BUP in urine, the stock solutions of BUP ranged from 2.5 to 20 X 103 ng/mL. The corresponding IS, triprolidine hydrochloride, was prepared at the final concentration of 1 μg/ml.

All solutions were stored at 4°C.

2.2.5.3. Quantitative determination of free- and conjugated OHBUP, TB and EB in urine

Calibration standards and quality control samples

Calibration curves for OHBUP, TB and EB standards were constructed by combining of 10 μl of standard working solution with 10 μl of blank urine. The final concentrations of the analytes were as follows: OHBUP, 50-10 X 104 ng/mL; TB, 50-10 X 104 ng/mL; EB, 10-2 X 104 ng/mL. 10 μl of IS (phenacetine) working solution was added to each sample. Corresponding QC samples were prepared for each of the curves at high, middle, low concentration levels and LLOQ.

Preparation of urine samples for OHBUP, TB and EB analysis

To quantify OHBUP, TB and EB in urine, the samples were processed in the presence or absence of β-glucuronidase.

For analysis of non-conjugated OHBUP, TB and EB, 10 μl of IS was added to 10 μl of a urine sample mixed with 90 μl of 0.1 M phosphate-buffered saline, pH=5. Then,

40 µl of 40%, w/v of TCA was added, sample vortexed, followed by addition of 500 µl of acetonitrile. Sample was vortexed for 1 min and centrifuged for 10 min at 12,000 x g. The supernatant was transferred to another tube and dried at 40°C under a stream of nitrogen. The dried residue was reconstituted in 200 µl of the initial mobile phase; an aliquot of 50 µl of a sample was injected into HPLC system for analysis by a single-quad mass spectrometer.

The concentrations of the glucuronidated OHBUP, TB and EB in the urine samples was computed as difference from the free (non-conjugated) and the total drug.

For analysis of total OHBUP, TB and EB, 10 µl of IS was added to 10 µl of urine sample mixed with 90 µl of 0.1 M phosphate-buffered saline, pH=5, containing 200 units of β-glucuronidase, the mixture was incubated at 37°C overnight. The reaction was stopped by adding 40 µl of 40%, w/v of TCA, and each sample was processed as described above.

2.2.5.4. Quantitative determination of bupropion in urine

Calibration standards and quality control samples

Calibration curves for BUP standards were prepared as follows: 10 µl of standard working solutions were combined with 100 µl of blank urine. BUP final concentrations range was 0.25-2 X 103 ng/mL. 10 µl of IS (triprolidine hydrochloride) working solution was added to each sample. QC samples were prepared for high, middle, low concentration levels and LLOQ.

Preparation of urine samples for bupropion analysis

10 µl of IS was added to 100 µl of urine, then each sample was acidified with 40 µl of 40% w/v TCA and vortexed. Each sample was extracted with 1000 µl of methylene chloride as follows: methylene chloride added, sample vortexed for 10 min and centrifuged at 12000 x g for 10 min, organic layer transferred to a tube. Extraction was repeated, the organic layers combined and evaporated to dryness at 40°C under a stream of nitrogen. The residue reconstituted in 200 µl of the initial mobile phase containing 8%TCA, and 50 µl was injected into the HPLC system for LC-MS analysis.

2.2.5.5. Validation of the methods for quantification of bupropion, OHBUP, TB and EB in plasma and urine

The LC-MS and LC-MS/MS methods for analysis of bupropion and its metabolites were partially validated for specificity, matrix effect, linearity, sensitivity, precision and accuracy following the US Food and Drug Administration guideline (FDA, 2011). The corresponding QCs at low, middle and high concentration levels were used to assess matrix factor, precision, accuracy, and stability. The corresponding LLOQ samples were also included in testing selected validation parameters.

The matrix factor was defined as a ratio of the analyte peak area of a post-extracted sample over the peak area of a pure standard X 100%. The recovery was defined as a ratio of the analyte peak area of an extracted sample over the peak area of the post-extracted sample X 100%. The precision and accuracy were evaluated on 3 validation days and should be within 85% to 115% of added concentrations. A variability of the matrix effect, precision and accuracy was measured by relative standard deviation (RSD) and should be <15% (with <20% for LLOQ concentrations) (Viswanathan et al.,

2007). The stability of BUP and its metabolites was accessed for each of the low, middle and high concentrations in triplicates as follows: room temperature (RT) stability, freeze-thaw stability (three cycles, -80°C) and 37°C stability (4 hours, water bath, OHBUP, TB and EB only). For RT stability, each QC sample (low, middle and high concentrations) was spiked with plasma or urine according to the corresponding protocol, left on the bench at room temperature for 4 hours; then, corresponding IS was added and each sample was processed as described above. For the 37°C stability assessment, the sample was incubated overnight at 37°C, and processed accordingly. To evaluate the freezing-thawing stability of bupropion and its metabolites, the corresponding QC samples were mixed following the protocol, frozen at -80°C and defrosted at room temperature, the freezing-thawing cycle was repeated three times, then samples was extracted following the corresponding procedure.

2.2.6. *CYP2B6* and *CYP2C19* genotyping

Genomic DNA was isolated from the whole blood or buffy coat using the Puregene Blood Core Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. DNA concentration was determined by DeNovix DS-11 FX Spectrophotometer (DeNovix, Wilmington, Delaware, USA). Subjects were genotyped for 5 non-synonymous SNPs that result in the 7 common *CYP2B6* variant alleles, namely *CYP2B6**2 (64C>T), *CYP2B6**3 (777C>A), *CYP2B6**4 (785A>G), *CYP2B6**5 (1459C>T), *CYP2B6**6 (516G>T and 785A>G), and *CYP2B6**7 (516G>T, 785A>G and 1459C>T). SNPs were identified as described by Fokina et al., (2016b).

*CYP2C19**2 (681G>A; rs4244285) *CYP2C19**3 (636G>A; rs4986893) and *CYP2C19**17 (-806C>T; rs12248560) were determined using TaqMan based assays following the previously reported method (Zhu et al., 2014) as described by Fokina et al., (2016b).

2.2.7. Data analysis

The PK parameters were derived using non-compartmental analysis (Kinetica software version 5.0, Thermo Scientific, Waltham, MA). Individual maximum plasma concentrations (C_{max}) for the drug and OHBUP were derived from the visual inspection of the respective concentration-time curves. The area under the plasma concentration-time curve for a dose interval at steady state (AUC_{ss}) was computed to assess the exposure to the drug and its metabolites. For several participants in this study the collection of plasma samples was terminated prior to an end of the respective dosing intervals, therefore the remainder plasma concentration values were extrapolated from the best fit curve, and the AUC_{ss} were calculated as a sum of AUC_{0-n} and $AUC_{n-\tau}$ where n is the last measured time point. The apparent steady state oral clearance (CL/F_{ss}) of bupropion was estimated as dose/AUC_{ss} with and without normalization to the actual body weight (kg). OHBUP/BUP metabolic ratio in plasma was calculated as a ratio of the AUC_{ss} for OHBUP over that of bupropion and corrected for a molecular weight difference. OHBUP/BUP metabolic ratio was used to measure the activity of CYP2B6 in the hydroxylation of bupropion to OHBUP. TB/BUP and EB/BUP metabolic ratios were computed similarly to estimate the metabolic clearance of bupropion via reductive pathway(s). The renal clearance (CL_R) of bupropion and its metabolites was calculated as ((urine concentration, ng/mL)/(average

plasma concentration, ng/ml))X((urine volume, mL)/(dose interval, hrs). The molar percentage of bupropion dose excreted in a form of unchanged drug and free and conjugated OHBUP, TB, and EB metabolites was calculated as (total excreted, mg)/(dose, mg)*100, corrected for the molecular weight difference. Creatinine clearance was computed using the following Cockcroft-Gault formula: 0.85*((140-age)/serum creatinine [mg/dL])*(pre-pregnancy weight [kg] /72).

2.2.9. Statistical analysis

Data are presented as average values (mean) ± standard deviation (STDEV). Data were analyzed using non-parametric methods as follows: pairwise comparisons, Wilcoxon signed rank test (IBM SPSS Statistics, version 23); independent samples comparisons, Mann-Whitney-U test (IBM SPSS Statistics, version 23); comparisions across pregnancy and postpartum from individuals treated with bupropion SR 150 mg BID, Skilling-Mack test (XLSTAT, version 2015.1.03).

P values < .05 were deemed statistically significant. *P* values above .05 but below .10 were deemed as nearly significant.

2.3. RESULTS

2.3.1. Validation of the LC-MS and LC-MS/MS methods for quantitative determination of bupropion and its major metabolites in plasma and urine. Optimization of the analytical and experimental procedures.

Method validation for the quantitative determination of bupropion, OHBUP, TB and EB in plasma

The specificity, selectivity and stability of this LC-MS/MS method were established previously (Wang et al., 2012). For analysis of the plasma samples, the TCA content was adjusted to 3% (w/v) to achieve a stable and reproducible retention time and a proper chromatographic separation of the analytes (Figures 1 A-D).

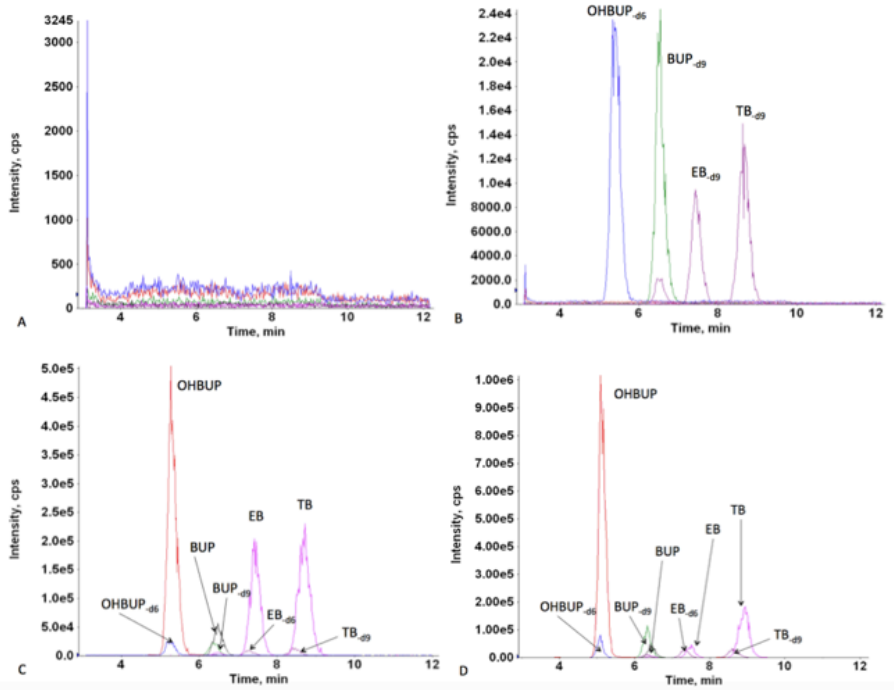

Figure 1. The representative MRM chromatograms for the following samples: (A) blank plasma; (B) blank plasma spiked with internal standards; (C) blank plasma spiked with OHBUP (400 ng/ml final), EB (200 ng/ml), TB(240 ng/ml), BUP (50 ng/ml), and internal standards; (D) plasma samples from a study subject. OHBUP, hydroxybupropion; EB, erythohydrobupropion; TB, threohydrobupropion; MRM, multiple reaction monitoring

The matrix effect for bupropion and its major metabolites ranged from 94.2% and 114.5% with RSD ≤ 4.6%, while the extraction recovery was between 91.1% and 112.5% with RSD ≤ 4.3%. The calibration curves were fit using weighed (1/y2 or 1/y) least-

squares linear regression analysis. The linearity was achieved for the following ranged of the analytes: BUP, 0.5-250 ng/mL; OHBUP, 2-2000 ng/mL; TB, 2.8-1200 ng/mL; EB, 2-1000 ng/mL. The LLOQ and lower limits of detection (LOD) concentrations were: BUP, 0.5 ng/mL and 0.125 ng/mL; OHBUP, 2 ng/mL and 0.5 ng/mL; TB, 2.8 ng/mL and 0.5 ng/mL; EB, 2 ng/mL and 0.5 ng/ml. The intra-day accuracy for the LLOQ and the low, middle and high concentrations were between 92.4% and 100.1% with RSD ≤ 6.93%, while the inter-day accuracy ranged from 94.7% to 101.7% with RSD ≤ 5.76%.

Method validation for the quantitative determination of OHBUP, TB and EB in urine

The specificity of the method was established previously (Wang et al., 2010; Wang et al., 2011). The selectivity was achieved by comparing the SIM chromatograms of six different blank urine samples (from individuals not exposed to bupropion) (Figures 2 A-C). The matrix effect for OHBUP, TB and EB ranged from 85.8% to 94.2% with RSD ≤ 9.1%. The matrix effect for IS was 87.9 with RSD=11.7%. The extraction recovery of OHBUP, TB and EB ranged from 72.5% to 101.6%, with RSD ≤ 15.2%; the extraction recovery for IS was 103.2% with RSD=12.6%. The calibration curves were fit using weighed (1/y2) least-squares linear regression analysis. The constructed calibration curves exhibited linearity within the following ranges: OHBUP, 50-20 X 104 ng/mL; TB, 50-10 X 104 ng/mL; EB, 10-20 X 104 ng/mL. The LLOQ and LOD concentrations were: OHBUP, 50 ng/mL and 20 ng/mL; TB, 50 ng/mL and 20 ng/mL; EB, 20 ng/mL and 10 ng/ml, respectively. The inter-day accuracy for the low, middle and high concentrations

ranged from 93.7% to 110.3% with RSD ≤ 8.5%, while for the LLOQ it ranged from 90.1% to 99.8% with RSD ≤ 15.7%.

The accuracy after 3 freeze-thaw cycles ranged from 89.0% to 119.3%, with RSD ≤ 5.9%. The accuracy after the RT stability test ranged from 87.7% to 108.3%, with RSD ≤ 8.3%, while after the 37°C stability test it ranged from 89.9% to 104.2%, with RSD ≤ 7.4%.

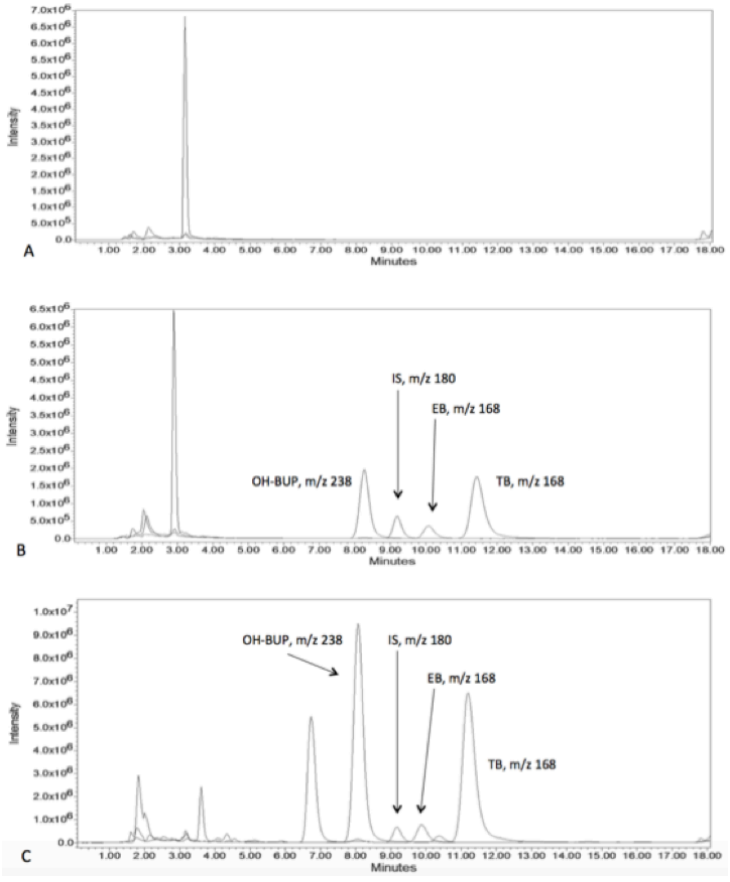

Figure 2. The representative SIM chromatograms for the following samples (A) blank urine; (B) blank urine spiked with OHBUP (200 ng/ml final), EB (40 ng/ml), TB(200 ng/ml), and IS; (C) urine sample from a study subject. IS, internal standard (phenacetin); OHBUP, hydroxybupropion; EB, erythohydrobupropion; TB, threohydrobupropion; SIM, single ion monitoring

The enzymatic hydrolysis of conjugated OHBUP, TB and EB

To optimize the reaction condition for the enzymatic hydrolysis of bupropion urinary metabolites, selected urine samples from 3 subjects were incubated with β-glucuronidase as described in Section 2.2.5.3 for 2, 4, 6, 8, 10, 12, and 16 hrs. We determined that 10 hours is a sufficient time for the maximum yield of de-conjugated OHBUP, TB and EB. No loss or degradation of IS was observed with the incubation up to 16 hours. The incubation was also conducted in the absence of the β-glucuronidase; the determined concentration of OHBUP was within 93 % - 134% of that of 0 time incubation, while concentrations of TB and EB were within 97% - 119% of 0 time incubation. The results indicated that no degradation for OHBUP, TB and EB occurred in a course of a few-hour incubation at 37°C.

Method validation for the quantitative determination of bupropion in urine

LC-MS method development

Samples preparation and liquid chromatographic conditions

The method used to quantify OHBUP, TB and EB in urine was suitable for quantitative determination of bupropion. However, under the LC-MS conditions bupropion eluted at 27 minutes, resulting in a very broad peak and a long time for analysis of each sample. Moreover, a large difference in the concentrations of bupropion and its metabolites in urine did not allow simultaneous analysis of the drug and its metabolites. Thus, we anticipated that for a urine sample of 10 µl the levels of bupropion would be below detection limit in many urine samples. Therefore, the chromatographic

condition including the mobile phase composition was adjusted to optimize bupropion peak resolution and retention time. Moreover, the sample volume was increased to 100 µl to quantitatively determine bupropion in a vast majority of the samples.

The protein precipitation method provided a good recovery for bupropion, however the co-extracted OHBUP, TB and EB from 100 µl urine sample could overload the column and LC-MS system. Therefore, we decided to develop a liquid-liquid extraction method (LLE) to purify bupropion from the urine samples. Several LLE conditions were tested, in which the QC urine samples were acidified with TCA (8% final concentration, w/v) or alkalized with 1M Na_2CO_3 followed by extraction with either methylene chloride, chloroform, or ethyl acetate. Extraction of the acidified QC samples with methylene chloride produced >90% recovery, which was sufficient for the quantitative determination of bupropion in urine at LLOQ levels. The extraction recovery of Triprolidine hydrochloride was >90% and was stable at this condition, thus this compound was selected as an IS for bupropion. TCA serves as an ion-pairing agent in chromatographic method and affects the retention time and elution time reproducibility, therefore it was added to the sample reconstitution solution (8% final concentration, w/v) to achieve the suitable and reproducible retention time of bupropion and the IS. The chromatographic conditions were modified to the following: 25% methanol and 75% of 0.04% formic acid aqueous solution, v/v. This allowed adjusting the retention time of bupropion to 11.3 min, while achieving the separation of the analytes from endogenous peaks.

Method validation for the LC-MS condition

The selectivity was achieved by comparing the SIM chromatogram of six different urine samples obtained from individuals not exposed to bupropion (Figure 3A-C). The method was validated for the low, middle and high concentrations of QCs, namely 4 ng/ml, 40 ngml, and 400 ng/ml. The matrix effect for bupropion ranged from 96.9% to 103.4% with RSD ≤ 4.1%. The matrix effect of the IS was 83.2 with RSD=7.1%. The extraction recovery of bupropion ranged from 96.5% to 101.0% with RSD ≤ 7.2%, the extraction recovery of the IS was 118.9% with RSD=6.2%. The calibration curves were constructed using weighed (1/y2) least-squares linear regression analysis and exhibited linearity within 4 ng/mL- 800 ng/mL of bupropion. The LLOQ and LOD concentrations were 0.5 ng/mL and 4 ng/mL, respectively. The inter-day accuracy for the low, middle and high concentrations of bupropion ranged from 93.7 to 10.3.3 with RSD ≤ 7.7. For the LLOQ, the inter-day accuracy was 93.7% with RSD=7.7%.

The QCs of bupropion were tested for stability; after the 3 freeze-thaw cycles, the accuracy of bupropion concentration ranged from 101.1% to 103.1% with RSD being ≤ 5%; the room temperature stability test resulted in the accuracy of bupropion ranged from 99.9% to 102 %, with RSD ≤ 3.6%.

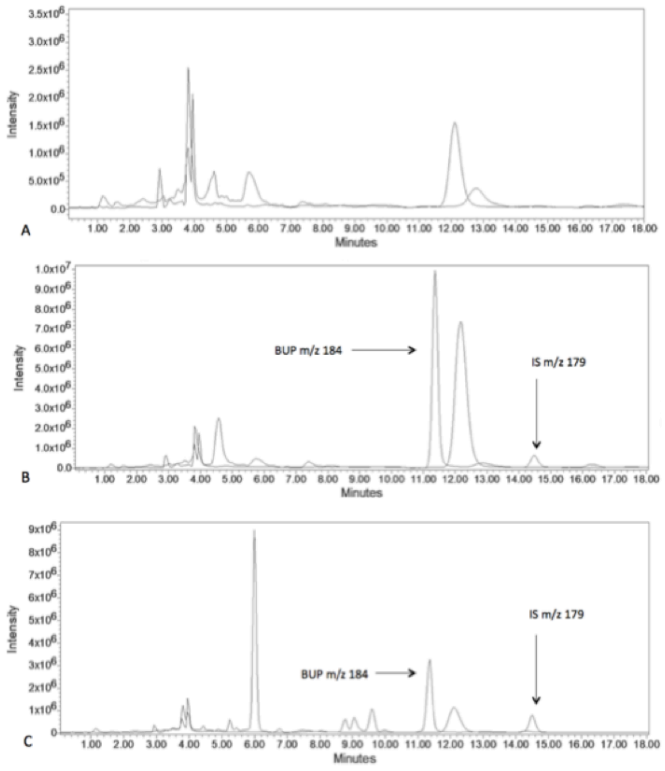

Figure 3. Representative chromatograms for the following samples (A) blank urine; (B) blank urine spiked with bupropion 100 ng/ml and IS; (C) subject's sample; IS, internal standard (triprolidine hydrochloride); BUP, bupropion

2.3.2. Subjects

Twenty-nine pregnant women volunteered to participate in this opportunistic study, of which one was subsequently excluded from the analysis due to deviation from the study protocol. The characteristics of the remaining participants are shown in the Table 1. At the time of enrollment, the subjects had a mean age of 29.2 ± 6.9 (21-39) years, a mean gestational age of 27.5 ± 8.5 (13.1-38.0) weeks/days, and a mean body weight 86.8 ± 24.6 kg (50.4 to 168.8 kg). The race and ethnicity of the subjects were as follows: white/non-Hispanic, n=16 (57%), white/Hispanic, n=9 (36%), black/Hispanic, n=1 (3.6%), and black/non-Hispanic, n=1 (3.6%). Eleven subjects were self-reported

regular smokers at the time of enrollment, two of which reported quitting smoking later on in the study. Due to no effect of cigarette smoking on the pharmacokinetics of bupropion and its metabolites (Hsyu et al., 1997), the data were analyzed irrespective of the subjects' smoking status. Five subjects (18%) were enrolled during the early window of pregnancy, 11 (39%) during the mid-pregnancy, and 12 (43%) during the late pregnancy window. Nine subjects (32%) participated in one PK visit, 12 (43%) completed two PK visits, 6 (21%) completed three PK visits, and 1 subject completed four PK visits. The bupropion dose and formulation were as follows: bupropion SR 150 mg BID, n=16; bupropion SR 150 mg QD, n=5; bupropion IR 100 mg TID, n=3; and bupropion XL 300 mg QD, n=2.

Insufficient number of subjects precluded paired comparisons between the early or mid-pregnancy and postpartum periods. One participant was enrolled in the study during two pregnancies, therefore was logged twice (subject 7 and 8). Consequently, the PK parameters estimated postpartum after the subject's first pregnancy were used in paired analysis with the data obtained during second pregnancy. Urine collection was not completed for several subjects (Table 1); therefore the related PK parameters were not derived.

For several subjects, the full analysis of SNPs of *CYP2C19* and *CYP2B6* could not be completed due to insufficient DNA samples (Table 1); therefore these subjects were excluded from genotyping analysis. None of the participants reported abusing alcohol or being treated with medications known to be inducers or inhibitors of CYP2C19 and CYP2B6 enzymes.

2.3.3. Pharmacokinetics of bupropion and its metabolites during pregnancy and postpartum

Paired comparisons of estimated PK parameters of bupropion and its metabolites were performed for mid-pregnancy against late pregnancy, and late pregnancy against postpartum lactating and postpartum non-/post-lactating (Table 2). Individual paired comparisons (mid-pregnancy against late pregnancy and late pregnancy against non-/post-lactating) for select PK parameters are shown in Figure 4. Insufficient number of subjects precluded paired comparisons between early or mid-pregnancy and postpartum periods. Table 3 shows the PK parameters across all gestational windows and postpartum for subjects receiving bupropion SR 150 mg BID.

Paired analysis revealed that C_{max} of bupropion in postpartum lactating period was 182 ± 68 ng/mL, n=6, and was higher than that of late pregnancy (111 ± 29 ng/mL, n=6, $P < .05$) (Table 2). However, no difference in C_{max} of bupropion was observed in the analysis across gestation (Table 3). In addition, data suggested no effect of pregnancy or lactation on C_{max} of OHBUP.

Pairwise comparisons revealed that the average value for bupropion dose normalized to actual weight in late pregnancy was lower than those in mid-pregnancy (1.70 ± 0.39 vs 1.85 ± 0.47, n=8, $P < .05$, Table 2) and non-/post-lactating period (1.74 ± 0.26 vs 1.92 ± 0.31, n=7, $P < .05$, Table 2). However, pairwise comparisons did not reveal any difference in the mean AUC_{ss} for bupropion and CL/F_{ss} between the groups (Table 2, Figure 4B). On the other hand, no difference in dose per kg was observed across gestation and postpartum in subjects receiving the drug in a dose of 150 mg BID (Table 3). However, analysis across gestation revealed that the AUC_{ss} of bupropion

trended lower during pregnancy than postpartum lactating and non-/post-lactating periods, with the difference being nearly significant in late pregnancy as compared with postpartum lactating (556 ± 206 ng*h/ml, n=13, vs 758 ± 153 ng*h/ml, n=5, *P* =.083). Consequently, a similar trend was observed with the bupropion CL/F_{ss} estimated values, with and without normalization to weight. In late pregnancy, the CL/F_{ss} mean values were 313 ± 130 L/h and 3.79 ± 1.86 L/h/kg and were higher than those in postpartum lactating period (n=5, 207 ± 56 L/h and 2.60 ± 0.70 L/h/kg, *P* =.083, Table 2).

Neither pairwise comparisons, nor analysis across gestation revealed any differences in the mean values of the AUC_{ss} of OHBUP. However, paired comparisons revealed that the OHBUP/BUP metabolic ratio in late pregnancy was higher than that in postpartum non-/post-lactating period (n=7, 17.6 ± 11.4 vs 12.4 ± 9.0, *P* = .093, Table 2, Figure 4).

Further, paired analysis revealed that the mean value for TB AUC_{ss} in mid-pregnancy was higher than that of late pregnancy (4843 ± 3196 ng*h/ml vs 3911 ± 2896 ng*h/ml, *P* = .068, n=8, Table 2, Figure 4). No difference in TB AUC_{ss} was observed in paired comparisons of late pregnancy vs lactating and non-/post-lactating periods (Table 2) as well as in comparisons across gestation (Table 3, Figure 4). The mean value for TB/BUP metabolic ration in late pregnancy trended higher than that in non-/post-partum period as revealed by paired analysis (6.85 ± 3.74 vs 5.27 ± 3.77, *P* = .093, n=7, Table 2, Figure 4). In addition, analysis across gestation demonstrated that the mean value for TB/BUP metabolic ratio in late pregnancy were slightly higher that that of lactating period (8.16 ± 4.04, n=13, vs 4.79 ± 4.75, n=5, *P* = .083, Table 3).

Pairwise comparisons demonstrated that the mean value for EB AUC_{ss} in mid-pregnancy exceeded that of late pregnancy (759 ± 447 ng*h/ml vs 541 ± 370 ng*h/ml, n=8, $P < .05$, Table 2); moreover, the EB/BUP metabolic ratio in mid-pregnancy was higher than that in late pregnancy group (1.33 ± 0.65 vs 1.06 ± 0.57, n=8, $P < .05$). Likewise, in comparison across gestation, the mean value for EB/BUP metabolic ration in mid-pregnancy exceeded that of late pregnancy (1.52 ± 0.63, n=9, vs 1.22 ± 0.50, n=13, $P < .05$, Table 3), although no difference was observed in the corresponding values for EB AUC_{ss} (Table 3).

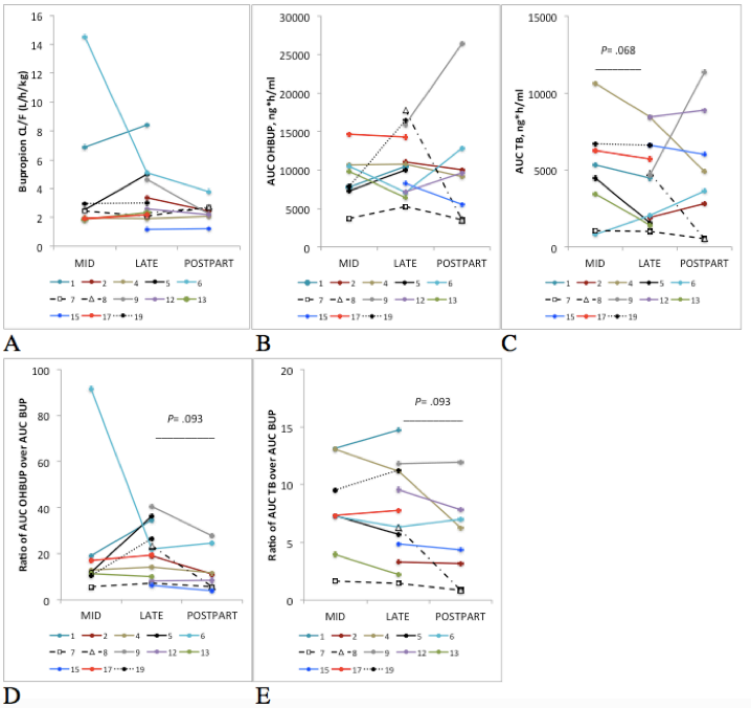

Figure 4. Individual paired analysis for select pharmacokinetic parameters for bupropion during mid-pregnancy compared to late pregnancy; and late pregnancy compared to postpartum (non-/post-lactation). AUC_{ss}, area under the curve at steady state; BUP, bupropion; CL/F, apparent oral clearance; OHBUP, hydroxybupropion; TB, threohydrobupropion; postpart, postpartum non-/postlactating. Mid-pregnancy, 22-26 weeks of gestation; Late pregnancy, 34-38 weeks of gestation

Table 1. Demographics and bupropion dosing

subjects	Race/ ethnicity	Genotype		At the time of initial enrollment		Early pregnancy		Mid-pregnancy		Late pregnancy		Lactation		Post-/non-lactating	
						n=5		n=13		n=21		n=8		n=8	
		CYP2B6	*CYP2C19*	Age, years	Smoking	Weight kg	Dose, mg	Weight kg	Dose, mg	Weight kg	Dose, mg	Weight kg	Dose, mg	Weight kg	Dose, mg
1	wt/non-hisp	*1/*1	*1/*2	32	Yes	53.1	150*BID	53.9	150*BID	59.0	150*BID				
2	wt/non-hisp	*1/*1	*1/*1	24	Yes	76.2	150*BID			77.6	150*BID	68.0	150*BID	69.4	150*BID
3	wt/non-hisp	*1/*6	*1/*1	39	Yes	70.8	150*BID					76.2	150*BID		
4	wt/hisp	*1/*6	*1/*2	33	No			93.8	150*BID	105.2	150*BID			94.3	150*BID[a]
5	wt/non-hisp	*6/*6	*1/*17	38	Yes			97.1	150*BID	109.6	150*BID				
6	wt/hisp	*1/*1	*1/*2	38	Yes			90.2	150*BID[a]	90.9	150*BID			76.1	150*BID
7	wt/hisp	*1/*1	*1/*1	24	No			95.3	150*BID	99.1	150*BID				
8[b]	wt/hisp	*1/*1	*1/*1	23	No					85.8	150*BID	89.4	150*BID	88.9	150*BID
9	wt/hisp	*1/*9	*2/*17	20	No					82.6	150*BID			70.8	150*BID
10	wt/hisp	*1/*6	*1/*1	36	No					61.7	100*TID	59.9	100*TID		
11	wt/hisp	*1/*1	*1/*1	32	No					95.0	100*TID	89.8	100*TID		
12	wt/hisp	*6/*6	*1/*2	38	No					66.6	150*BID			60.8	150*BID
13	wt/non-hisp	ND	ND	23	No			93.0	150*QD	100	150*QD[a]			101.5	150*BID[a]
14	wt/hisp	ND	*1/*1	22	No	74.4	150*QD	77.1	150*QD			81.6	150*BID		
15	wt/non-hisp	*4/*4	*1/*1	21	Yes					94.8	150*QD			89.4	150*QD
16	wt/non-hisp	ND	ND	25	Yes					89.1	150*BID	85.5	150*BID		
17	wt/non-hisp	*1/*1	*1/*2	39	No			93.0	150*QD	94.8	150*QD[a]				
18	wt/non-hisp	*1/*1	*1/*1	27	No					66.7	150*QD	55.7	150*QD		
19	wt/non-hisp	*1/*6	*2/*2	22	Yes			63.1	150*BID[a]	74.4	150*BID[a]				
20	bl/hisp	*1/*6	*1/*1	40	No	98.1	150*BID								
21	wt/non-hisp	*1/*1	*1/*1	27	Yes					75.3	150*BID				
22	wt/non-hisp	*1/*6	*1/*1	25	Yes					95.6	150*BID				
23	wt/non-hisp	*1/*5	*1/*1	37	Yes			84.1	150*BID						
24	wt/hisp	*1/*6	*1/*1	21	No			63.7	100*TID						
25	wt/non-hisp	*1/*1	*1/*17	28	No					168.8	300*QD				
26	wt/non-hisp	*1/*1	*1/*1	32	No					117.7	300*QD[a]				
27	wt/non-hisp	*1/*1	*17/*17	22	Yes			50.3	150*BID						
28	bl/non-hisp	ND	*1/*17	30	Yes			135.2	150*BID[a]						

[a] Incomplete urine collection; [b] Same subject as subject #7, different pregnancy

ND, not determined; QD, once a day; BID, twice daily; TID, trice daily; wt, white; bl, black; hisp, Hispanic; non-hisp, non-Hispanic; early pregnancy, 10-14 weeks of gestation; mid-pregnancy, 22-26 weeks of gestation; late pregnancy, 34-38 weeks of gestation.

Modified from Eokina et al., 2016b

Table 2. Paired estimated pharmacokinetic parameters for bupropion during mid-pregnancy compared to late pregnancy; and late pregnancy compared to lactation and non/post-lactation postpartum periods

	Parameter	Mid-pregnancy (n=8)	Late pregnancy (n=8)	Late pregnancy (n=6)	Lactation (n=6)	Late pregnancy (n=7)	Non-/post-lactation (n=7)
BUP	dose/weight (mg/kg)	1.85 ± 0.47 *	1.70 ± 0.39	1.71 ± 0.40	1.85 ± 0.54	1.74 ± 0.26 *	1.92 ± 0.31
	AUC_{ss} BUP (ng*h/ml)	640 ± 263	554 ± 214	569 ± 208	664 ± 209	722 ± 320	865 ± 293
	C_{max} (ng/ml)	108 ± 57	114 ± 50	111 ± 29 *	182 ± 68	126 ± 44	131 ± 40
	CL/F_{ss} (L/h)	359 ± 389	321 ± 152	263 ± 107	225 ± 115	248 ± 117	191 ± 61
	CL/F_{ss} (L/h/kg)	4.37 ± 4.41	3.74 ± 2.29	3.30 ± 0.99	3.29 ± 2.42	2.88 ±1.37	2.41 ± 0.73
OHBUP	AUC_{ss} OHBUP (ng*h/ml)	9008 ± 3191	10092 ± 3865	9904 ± 4290	9483 ± 1842	10424 ± 4439	10075 ± 7388
	C_{max} (ng/ml)	748 ± 215	866 ± 368	1027 ± 367	1025 ± 265	957 ± 455	930 ± 743
	OHBUP/BUP M.R.	22.5 ± 28.1	21.3 ± 10.7	19.1 ± 8.4	15.7 ± 6.5	17.6 ± 11.4 #	12.4 ± 9.0
TB	AUC_{ss} TB (ng*h/ml)	4843 ± 3196 #	3911 ± 2896	3006 ± 1198	2524 ± 773	4740 ± 2926	4829 ± 3832
	TB/BUP M.R.	7.91 ± 4.01	7.58 ± 4.63	6.05 ± 3.29	4.18 ± 1.82	6.85 ± 3.74 #	5.27 ± 3.77
EB	AUC_{ss} EB (ng*h/ml)	759 ± 447 *	541 ± 370	488 ± 270	569 ± 201	707 ± 440	979 ± 695
	EB/BUP M.R.	1.33 ± 0.65 *	1.06 ± 0.57	0.94 ± 0.51	0.94 ± 0.42	1.01 ±0.53	1.08 ± 0.70

Data presented as mean ± standard deviation
AUC_{ss}, area under the curve at steady state; BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; EB, erythrohydrobupropion; M.R., metabolic ratio, defined as the ratio of AUCs, corrected for molecular weight
Mid-pregnancy, 22-26 weeks of gestation; Late pregnancy, 34-38 weeks of gestation
*$P < .05$
# $P < .10$ but above .05
Modified from Fokina et al., 2016b

Table 3. Pharmacokinetic parameters of bupropion and its metabolites during pregnancy and postpartum for subjects receiving treatment with bupropion SR 150 mg BID

	Parameter	Early pregnancy (n=4)	Mid-pregnancy (n=9)	Late pregnancy (n=13)	Lactation (n=5)	Non-/post-lactation (n=7)
BUP	dose/weight (mg/kg)	2.11 ± 0.54	1.94 ± 0.63	1.81 ± 0.34	1.89 ± 0.21	1.98 ± 0.40
	AUC_{ss} BUP (ng*h/ml)	546 ± 130	586 ± 232	556 ± 206 #^{LACT}	758 ± 153	790 ± 218
	C_{max} (ng/ml)	110 ± 32	96 ± 32	102 ± 33	168 ± 69	122 ± 42
	CL/F_{ss} (L/h)	291 ± 89	365 ± 359	313 ± 130 #^{LACT}	207 ± 56	203 ± 55
	CL/F_{ss} (L/h/kg)	4.31 ± 2.50	4.66 ± 4.14	3.79 ± 1.86 #^{LACT}	2.60 ± 0.70	2.53 ± 0.59
OHBUP	AUC_{ss} OHBUP (ng*h/ml)	10341 ± 3925	8826 ± 3039	10814 ± 3974	11673 ± 3343	10745 ± 7708
	C_{max} (ng/ml)	1073 ± 462	866 ± 271	1044 ± 356	1151 ± 272	1017 ± 756
	OHBUP/ BUP M.R.	21.6 ± 9.2	24.9 ± 28.8	24.1 ± 12.0	18.4 ± 11.0	14.7 ± 9.7
TB	AUC_{ss} TB (ng*h/ml)	4293 ± 1568	4574 ± 3003	4059 ± 2513	2787 ± 1778	4767 ± 3987
	TB/BUP M.R.	9.10 ± 4.05	8.55 ± 3.78	8.16 ± 4.04 #^{LACT}	4.79 ± 4.75	6.05 ± 4.14
EB	AUC_{ss} EB (ng*h/ml)	798 ± 206	749 ± 413	604 ± 346	718 ± 528	934 ± 693
	EB/BUP M.R.	1.71 ± 0.75	1.52 ± 0.63*^{LATE}	1.22 ± 0.50	1.26 ± 1.37	1.22 ± 0.76

Data presented as mean ± standard deviation.
BID, twice a day; AUCss, area under the curve at steady state; BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; EB, erythrohydrobupropion; M.R., metabolic ratio, defined as the ratio of AUCs, corrected for molecular weight
Early pregnancy, 10-14 weeks of gestation; mid-pregnancy, 22-26 weeks of gestation; late pregnancy, 34-38 weeks of gestation
*$P < .05$
# $P < .10$ but above .05
LATE, compared to late pregnancy; LACT, compared to lactation period
Modified from Fokina et al., 2016b

Table 4. Urinary excretion of bupropion and its metabolites over a dose interval. Paired analysis: mid-pregnancy versus late pregnancy, and late pregnancy versus postpartum lactating and postpartum non-/post-lactating periods

Parameter		Mid-pregnancy (n=4)	Late pregnancy (n=4)	Late pregnancy (n=6)	Lactation (n=6)	Late pregnancy (n=7)	Non-/post-lactation (n=7)
[a]Creatinine clearance	(mL/min)	185 ± 45 *	166 ± 33	183 ± 54 *	128 ± 41	188 ± 39 *	139 ± 24
Renal clearance	CL_R BUP (mL/min)	23.1 ± 12.5	9.06 ± 5.80	23.2 ± 24.4	66.1 ± 101	8.01 ± 6.26	10.4 ± 7.41
	CL_R OHBUP (mL/min)	3.77 ± 3.19	1.34 ± 0.22	4.29 ± 4.54	4.60 ± 4.24	1.14 ± 0.76	1.33 ± 0.60
	CL_R TB (mL/min)	72.1 ± 48.3	34.6 ± 12 6	75.0 ± 62.2	68.3 ± 37.8	28.5 ± 19.1	30.5 ± 17.4
	CL_R EB (mL/min)	50.9 ± 40.3	20.4 ± 7.13	43.3 ± 41.0	38.6 ± 22.0	15.9 ± 11.9	17.4 ± 9.48
% of dose recovered as	BUP	0.59 ± 0.24	0.25 ± 0.24	0.67 ± 0.78	1.41 ± 1.24	0.25 ± 0.20	0.38 ± 0.24
	OHBUP-free	1.20 ± 1.03	0.53 ± 0.17	1.86 ± 2.06	2.28 ± 2.43	0.50 ± 0.43	0.55 ± 0.51
	OHBUP-glucuronide	7.97 ± 4.47#	11.69 ± 8.10	18.2 ± 20.0 *	6.47 ± 2.61	10.0 ± 6.66 *	5.88 ± 6.64
	TB-free	15.9 ± 11.1#	6.37 ± 6.71	12.5 ± 11.9	9.66 ± 7.07	5.74 ± 5.16	5.60 ± 5.14
	TB-glucuronide	1.07 ± 0.73	0.82 ± 0.83	3.63 ± 2.49 *	0.71 ± 0.54	2.57 ± 1.65 *	1.19 ± 1.37
	EB-free	1.71 ± 1.54#	0.47 ± 0.48	0.98 ± 0.83	1.18 ± 0.84	0.42 ± 0.35	0.66 ± 0.63
	EB-glucuronide	2.55 ± 1.93	4.00 ± 3.88	0.67 ± 0.48	0.36 ± 0.25	0.49 ± 0.34	0.45 ± 0.41

Data presented as mean ± standard deviation
BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; EB. Erythrohydrobupropion; CLR, renal clearance
Mid-pregnancy, 22-26 weeks of gestation; late pregnancy, 34-38 weeks of gestation
[a] The number of subjects in paired analysis of estimated renal creatinine clearance was the same as in Table 2.
*$P < .05$
# $P < .10$ but above .05

Modified from Fokina et al., 2016b

Table 5. Urinary excretion of bupropion and its metabolites during pregnancy and postpartum for subjects receiving treatment with bupropion SR 150 mg BID

	Parameter	Early pregnancy (n=4)	Mid-pregnancy (n=6)	Late pregnancy (n=12)	Lactation (n=5)	Non-/post-lactation (n=6)
[a]Creatinine clearance	(mL/min)	144 ± 32	180 ± 48	180 ± 43 *CR, # LACT	136 ± 40	145 ± 29
Renal clearance	CL_R BUP (mL/min)	16.9 ± 17.1	20.9 ± 11.2*LATE	8.67 ± 7.95	37.7 ± 43.6	10.5 ± 8.25
	CL_R OHBUP (mL/min)	3.05 ± 2.44	4.09 ± 2.79	1.24 ± 0.64	2.24 ± 1.79	1.70 ± 1.33
	CL_R TB (mL/min)	56.3 ± 51.1	64.5 ± 40.1	30.6 ± 16.9	54.3 ± 44.0	47.1 ± 56.0
	CL_R EB (mL/min)	41.0 ± 39.8	43.0 ± 34.5	17.2 ± 9.29	29.1 ± 23.3	23.1 ± 20.7
% of dose recovered as	BUP	0.61 ± 0.56	0.50 ± 0.24	0.23 ± 0.24	0.99 ± 0.86	0.36 ± 0.27
	OHBUP-free	1.07 ± 0.59	1.54 ± 0.99	0.55 ± 0.35	1.29 ± 1.37	0.80 ± 0.66
	OHBUP-glucuronide	10.6 ± 5.36	12.8 ± 8.61*LATE	11.7 ± 5.86 # LACT	6.67 ± 2.36	7.78 ± 6.34
	TB-free	10.9 ± 10.9	13.1 ± 9.71*LATE	6.01 ± 5.46	8.73 ± 11.7	10.5 ± 13.0
	TB-glucuronide	1.79 ± 1.09	3.87 ± 3.52	3.21 ± 2.30 # LACT	1.85 ± 2.74	1.69 ± 1.27
	EB-free	1.48 ± 1.55	1.45 ± 1.29*LATE	0.47 ± 0.38	1.22 ± 1.70	1.06 ± 1.10
	EB-glucuronide	0.89 ± 0.28	0.87 ± 0.68	0.71 ± 0.50	0.58 ± 0.58	0.80 ± 0.64

Data presented as mean ± standard deviation.
BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; EB, Erythrohydrobupropion; CLR, renal clearance
aThe sample size is the same as indicated in Table 3.
Early pregnancy, 10-14 weeks of gestation; Mid-pregnancy, 22-26 weeks of gestation; Late pregnancy, 34-38 weeks of gestation
*$P < .05$
# $P < .10$ but above .05
LATE, compared to late pregnancy; CR, compared to non-/post-lactating period (control), LACT, compared to lactating period
Modified from Fokina et al., 2016b

2.3.4. Urinary elimination of bupropion and its metabolites

The paired comparisons of estimated parameters for urinary elimination of bupropion and its metabolites are shown in Table 4; the individual comparisons of select parameters are also presented in Figure 5. The mean value of creatinine clearance in mid-pregnancy exceeded that of late pregnancy (185 ± 45 mL/min vs 166 ± 33 mL/min, $P <$ .05, Table 4, Figure 5). In addition, the mean value of creatinine clearance in late pregnancy was higher than that of lactation period (183 ± 54 mL/min vs 128 ± 41 mL/min, $P <$ .05, table 4) and the non/post-lactation period (188 ± 39 mL/min vs 139 ± 24 mL/min, $P <$ .05, Table 4, Figure 5). These results are in agreement with the pregnancy-induced changes in glomerular filtration rate (Hnat and Sibai, 2008). A similar trend was observed in the comparisons across gestation (Table 5), in which the mean creatinine clearance in late pregnancy (n=12) was 180 ± 43 mL/min and was higher than that of lactating period (136 ± 40 mL/min, n=5, $P = .083$) and non-/post-lactating period (145 ± 29 mL/min, n=6, $P <$ .05).

Paired analysis revealed that the renal clearance of bupropion in mid-pregnancy slightly exceeded that of late pregnancy (23.1 ± 12.5 mL/min vs 9.06 ± 5.80 mL/min, n=4, $P =$ .068). Likewise, a comparison across gestation revealed that clearance of bupropion in mid-pregnancy was higher than that of late pregnancy (20.9 ± 11.2 mL/min, n=6, vs 8.67 ± 7.95 mL/min, n=12, $P <$ .05). Neither paired analysis nor comparisons across gestation revealed any difference in the renal clearance of OHBUP, EB and TB.

The molar percentages of bupropion dose eliminated in urine as non-conjugated TB and non-conjugated EB metabolites in mid-pregnancy were slightly higher than the percentages excreted in late gestation as revealed by both pairwise comparisons (for TB-

free, 15.9 ± 11.1% vs 6.37 ± 6.71%, P = .068, and for EB-free 1.71 ± 1.54% vs 0.47 ± 0.48%, P = .068, Table 4) and by analysis across gestation (for TB-free, 13.1 ± 9.71% vs 6.01 ± 5.46%, P < .05 and for EB-free, 1.45 ± 1.29%, n=6, vs 0.47 ± 0.38%, P < .05, Table 5). No differences were observed in the fraction of the drug dose eliminated in a form of an unchanged drug in pregnancy and postpartum.

In pregnancy, 89 ± 9% of the total OHBUP eliminated in the urine was excreted in a form of a conjugate, while the fraction of TB and EB in glucuronidated forms accounted for 28 ± 20% and 46 ± 23% of the total excreted TB and EB, respectively. Pairwise comparisons revealed that the fraction of bupropion dose recovered in the urine in a form of OHBUP-glucuronide in late pregnancy exceeded that of lactating period and non-/post-lactating period (18.2 ± 20.0% vs 6.47 ± 2.61%, P < .05, and 10.0 ± 6.66% vs 5.88 ± 6.64%, P < .05, Table 4, Figure 5). Similarly, the fraction of bupropion dose excreted in the urine as TB-glucuronide in late pregnancy exceeded that of lactating and non/post-lactation period (3.63 ± 2.49% vs 0.71 ± 0.54%, P < .05, and 2.57 ± 1.65% vs 1.19 ± 1.37%, P < .05, Table 4, Figure 5). In addition, analysis across gestation demonstrated that the excretion of both OHBUP- and TB-glucuronide conjugates (as a fraction of bupropion dose) was higher in late pregnancy than in lactating period (TB-glucuronide, 3.21 ± 2.30% vs 1.85 ± 2.74%, P = .083; and OHBUP-glucuronide, 11.7 ± 5.86% vs 6.67 ± 2.36%, P = .083, Table 5). Neither paired analysis, nor comparisons across gestation revealed any difference in the excretion of EB-glucuronide as the percentage of the drug dose.

According to the data from paired analysis, the fraction of bupropion excreted in a form of OHBUP-glucuronide in late pregnancy was slightly higher than that of mid-

pregnancy (11.69 ± 8.10% vs 7.97 ± 4.47%, P = .068, Table 4). However, the opposite was observed comparing mid- and late pregnancy across gestation: 12.8 ± 8.61% vs 11.7 ± 5.86%, P < .05 (Table 5). Inter-individual variability in OHBUP-glucuronide excretion and a small sample size for the groups could have caused the inconsistency in our results in mid- vs late pregnancy comparison.

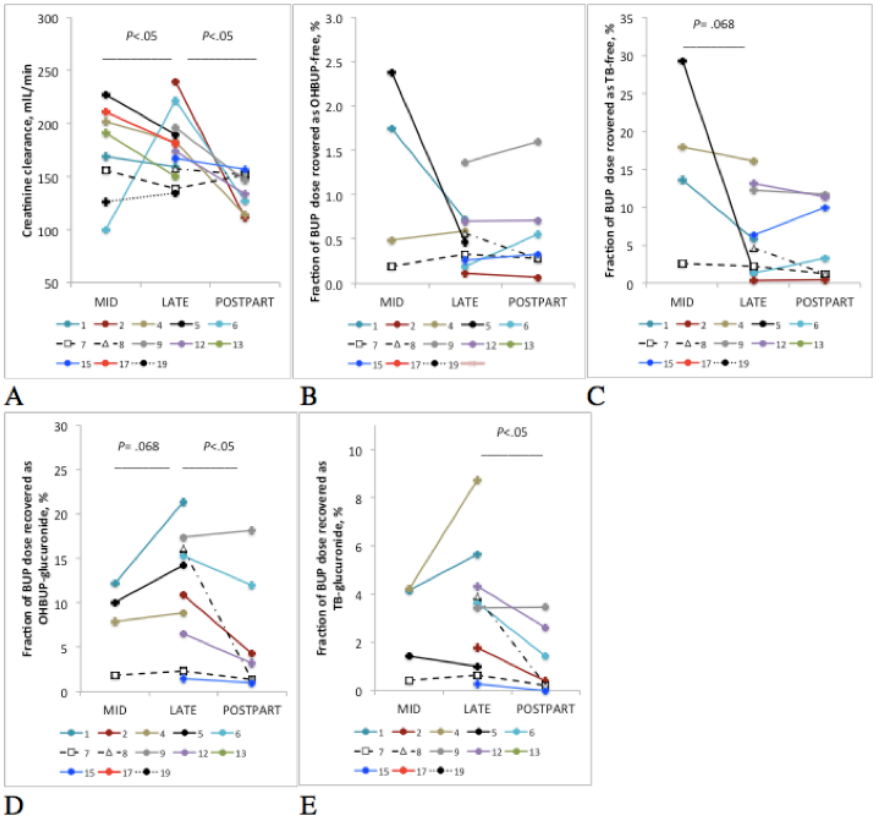

Figure 5. Urinary excretion of bupropion and its metabolites over a dose interval. Individual paired analysis for select pharmacokinetic parameters: mid-pregnancy compared to late pregnancy; and late pregnancy compared to postpartum (non-/post-lactation). BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; postpart, postpartum non-/postlactating. Mid-pregnancy, 22-26 weeks of gestation; Late pregnancy, 34-38 weeks of gestation

2.3.5. *CYP2B6* and *CYP2C19* variant alleles and pharmacokinetics of bupropion in pregnancy

Initially we intended to study the effect of functional genetic variants of *CYP2C19* and *CYP2B6* on pharmacokinetics of bupropion in each of the early, mid- and late pregnancy groups in order to minimize the effect of pregnancy on the pharmacokinetic parameters. However, the insufficient number of subjects enrolled during early pregnancy (n=5) precluded comparative analysis within this group. Thus, the pharmacokinetic parameters of bupropion were compared among the pregnant subjects with and without genetic variant alleles of *CYP2B6* and *CYP2C19* in the mid- and late pregnancy groups. Bupropion CL/F_{ss}, the metabolic ratios and the urine data were compared irrespective of the drug dosing, while the AUC_{ss} data were compared among the subjects taking the same dose of bupropion SR, 150 mg BID.

Thirteen pregnant women participated in the PK study during mid-pregnancy; the *CYP2B6* genotype was determined for ten subjects, of which five were carriers of *1/*1 (wild-type), three were *1/*6, one was *6/*6 and one was *1/*5 (Table 1). Twenty-two subjects participated in the PK study during late pregnancy; the *CYP2B6* genotype was determined for nineteen participants. There were eleven with *1/*1, four with *1/*6, two with *6/*6, one with *1/*9 and one with *4/*4 (Table 1) (Fokina et al., 2016b). Based on the *CYP2B6* allele frequencies in both groups, we compared the PK parameters between the wild type carriers and those who carry *6 allele (Table 6).

In mid-pregnancy, the OHBUP/BUP metabolic ratio was slightly lower in subjects who carry *6 allele (9.46 ± 4.4 vs 32.8 ± 34.0, $P = .086$, Table 6, Figure 6), these data are consistent with the reduced metabolic phenotype of *CYP2B6*6* variant. Although

the comparison of OHBUP AUC_{ss} between the two groups reveled no difference, the mean value of OHBUP AUC_{ss} were slightly higher in *6 carriers as compared to the wild type carriers (742 ± 114 ng*h/ml vs 414 ± 225 ng*h/ml, $P = .077$). Moreover, the mean values for AUC_{ss} of TB and EB in *6 carriers slightly exceeded those of wild type carriers (7263 ± 3116 ng*h/ml vs 2553 ± 2084 ng*h/ml, for TB, $P = .077$, and 1119 ± 393 ng*h/ml vs 477 ± 340 ng*h/ml for EB, $P < .05$). No difference in the PK parameters was observed between those with *6 allele and those with *CYP2B6**1/*1 genotype.

The following *CYP2C19* genotype combinations were determined: mid-pregnancy group, four subjects were with *CYP2C19**1/*1, two with *CYP2C19**1/*17, one with *CYP2C19**17/*17, four with *CYP2C19**1/*2, and one with *CYP2C19**2/*2 (total determined is twelve, Table 1); late pregnancy, ten were with *CYP2C19**1/*1, two with *CYP2C19**1/*17, five with *CYP2C19**1/*2, one with *CYP2C19**2/*17 and one with *CYP2C19**2/*2 (total determined is nineteen, Table 1) (Fokina et al., 2016b). We stratified the subjects in two groups in accordance with their metabolic phenotypes following the description of Scott et al., (2013) (Illustration 6). Thus, for the purpose of our study, the first group included the "extensive" metabolizers (EM) and "ultra-rapid" metabolizers (UM), namely *1/*1, *1/*17, and *17/*17 carriers, and the second group was comprised of "poor" metabolizers (PM) and "intermediate" metabolizers (IM), namely *2/*2 and *1/*2, including *2/*17 (Table 7, Figure 7).

In late pregnancy, the mean values of AUC_{ss} of TB and EB of the "poor/intermediate" metabolizers (PM+IM) group slightly exceeded those of the "extensive/ultra-rapid" (EM+UM) group (5773 ± 2517 ng*h/ml vs 2333 ± 1313 ng*h/ml, $P = .062$; and 782 ± 350 ng*h/ml vs 403 ± 273 ng*h/ml, $P = .088$, respectively, Table 7).

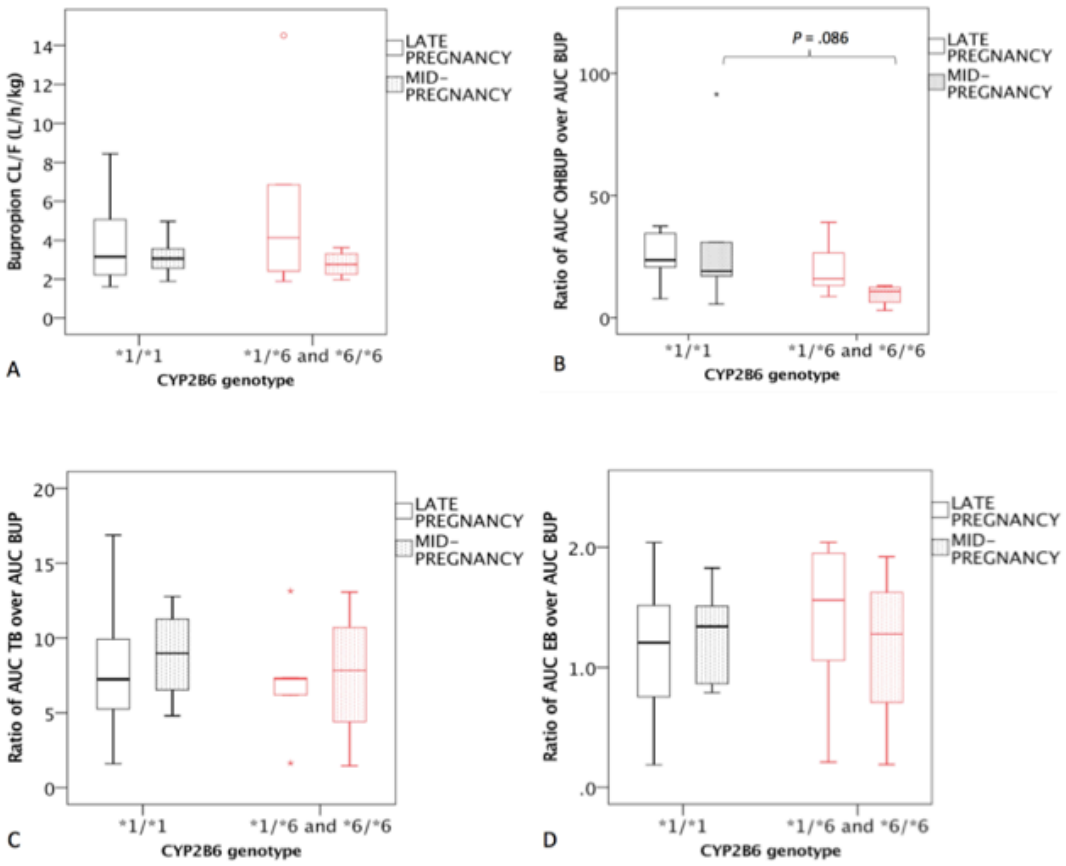

Figure 6. The effect of CYP2B6*6 variant allele on the pharmacokinetic parameters of bupropion. Late pregnancy, 34-38 weeks of gestation; mid-pregnancy, 22-26 weeks of gestation; AUC, area under the curve; BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; EB, erythrohydrobupropion; CL/F, apparent oral clearance. Modified from Fokina et al., 2016b.

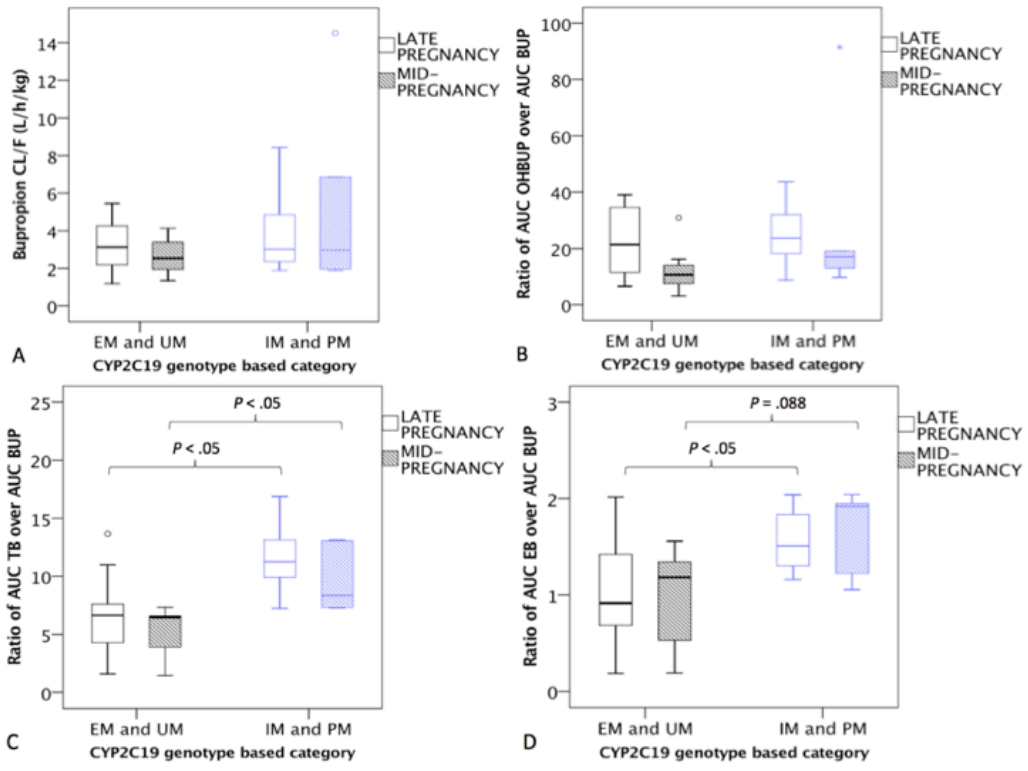

Figure 7. The effect of CYP2C19 genotype based metabolizer status on the pharmacokinetic parameters of bupropion. Late pregnancy, 34-38 weeks of gestation; mid-pregnancy, 22-26 weeks of gestation; AUC, area under the curve; BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; EB, erythrohydrobupropion; CL/F, apparent oral clearance; EM, extensive metabolizer; UM, ultrarapid metabolizer; IM, intermediate metabolizer; PM, poor metabolizer. Modified from Fokina et al., 2016b.

Table 6. Effect of CYP2B6 genetic variability on the pharmacokinetic parameters and urinary excretion of bupropion and its metabolites in mid- and late pregnancy.

	Mid-pregnancy (22-26 weeks)			Late pregnancy (34-38 weeks)		
***CYP2B6* genotypes**	**All[a]**	***1/*1**	***1/*6 and *6/*6**	**All**	***1/*1**	***1/*6 and *6/*6**
BUP all doses	**(n=10)**	**(n=5)**	**(n=4)**	**(n=19)**	**(n=11)**	**(n=6)**
CL/F_{ss} (L/h)	340 ± 344	458 ± 481	212 ± 31	300 ± 128	323 ± 121	275 ± 138
CL/F_{ss} (L/h/kg)	4.40 ± 3.83	5.96 ± 5.15	2.77 ± 0.69	3.50 ± 1.75	3.78 ± 2.04	3.18 ± 1.04
OHBUP/BUP M.R.	21.8 ± 25.6	32.8 ± 34.0	9.46 ± 4.44 #	23.3 ± 11.6	24.8 ± 10.2	20.0 ± 11.0
TB/BUP M.R.	7.22 ± 3.89	7.11 ± 4.10	7.55 ± 4.77	8.44 ± 4.05	8.00 ± 4.55	8.89 ± 3.19
EB/BUP M.R.	1.28 ± 0.66	1.36 ± 0.75	1.17 ± 0.72	1.23 ± 0.52	1.18 ± 0.60	1.28 ± 0.39
% of BUP dose recovered as	**(n=8)**	**(n=4)**	**(n=3)**	**(n=16)**	**(n=9)**	**(n=5)**
BUP-free	0.40 ± 0.20	0.43 ± 0.16	0.42 ± 0.28	0.31 ± 0.45	0.22 ± 024	0.51 ± 0.76
OHBUP-free	1.23 ± 1.01	1.06 ± 0.85	1.01 ± 1.20	1.03 ± 1.39	0.80 ± 0.80	1.52 ± 2.31
OHBUP-glucuronide	10.86 ± 8.33	12.3 ± 10.4	6.44 ± 4.49	14.1 ± 13.0	17.6 ± 16.2	9.68 ± 3.47
TB-free	10.9 ± 9.24	6.79 ± 4.78	16.6 ± 13.5	8.40 ± 8.65	5.55 ± 5.73	13.2 ± 12.8
TB-glucuronide	3.48 ± 3.25	2.90 ± 1.82	1.96 ± 2.04	3.32 ± 2.51	3.62 ± 2.26	3.36 ± 3.28
EB-free	1.21 ± 1.29	0.64 ± 0.57	1.90 ± 1.99	0.67 ± 0.65	0.47 ± 0.48	0.97 ± 0.93
EB-glucuronide	0.73 ± 0.64	0.69 ± 0.54	0.98 ± 0.84	0.67 ± 0.51	0.69 ± 0.46	0.71 ± 0.73
BUP dose 150 mg BID	**(n=8)**	**(n=4)**	**(n=3)**	**(n=12)**	**(n=6)**	**(n=5)**
AUC_{ss} BUP (ng*h/ml)	556 ± 228	414 ± 225	742 ± 114 #	545 ± 211	510 ± 207	617 ± 234
AUC_{ss} OHBUP (ng*h/ml)	8952 ± 3224	9188 ± 4672	8572 ± 1815	10873 ± 4144	10656 ± 4361	10118 ± 4031
AUC_{ss} TB (ng*h/ml)	4458 ± 3189	2553 ± 2084	7263 ± 3116 #	4053 ± 2625	2799 ± 1501	5435 ± 3361
AUC_{ss} EB (ng*h/ml)	754 ± 441	477 ± 340	1119 ± 393 *	592 ± 359	432 ± 276	776 ± 421

BID, twice a day; AUCss, area under the curve at steady state; BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; EB, erythrohydrobupropion; M.R., metabolic ratio, defined as the ratio of AUCs, corrected for molecular weight

Data presented as mean ± standard deviation

[a] Subjects with undetermined CYP2B6 genotype were not included

*$P < .05$; # $P < .10$ but above .05

Modified from Fokina et al., 2016b

Table 7. Effect of CYP2C19 genetic variability on the pharmacokinetic parameters and urinary excretion of bupropion and its metabolites in mid- and late pregnancy.

	Mid-pregnancy (22-26 weeks)			Late pregnancy (34-38 weeks)		
***CYP2C19* genotypes**	**All[a]**	**EM and UM**	**PM and IM**	**All**	**EM and UM**	**PM and IM**
BUP all doses	**(n=12)**	**(n=7)**	**(n=5)**	**(n=19)**	**(n=12)**	**(n=7)**
CL/F_{ss} (L/h)	308 ± 321	210 ± 50	445 ± 489	300 ± 128	296 ± 127	305 ± 138
CL/F_{ss} (L/h/kg)	3.90 ± 3.66	2.66 ± 1.05	5.63 ± 5.35	3.50 ± 1.75	3.23 ± 1.36	3.96 ± 2.32
OHBUP/BUP M.R.	19.9 ± 23.6	12.6 ± 9.12	30.1 ± 34.5	23.3 ± 11.6	22.2 ± 11.7	22.2 ± 11.7
TB/BUP M.R.	7.12 ± 3.53	5.18 ± 2.50	9.83 ± 3.02 *	8.44 ± 4.05	6.58 ± 3.33	11.6 ± 3.16 *
EB/BUP M.R.	1.24 ± 0.61	0.95 ± 0.56	1.64 ± 0.46 #	1.23 ± 0.52	1.03 ± 0.52	1.57 ± 0.34 *
% of BUP dose recovered as	**(n=9)**	**(n=6)**	**(n=3)**	**(n=16)**	**(n=11)**	**(n=5)**
BUP-free	0.38 ± 0.19	0.40 ± 0.21	0.34 ± 0.20	0.31 ± 0.45	0.38 ± 0.54	0.18 ± 0.13
OHBUP-free	1.14 ± 0.99	1.26 ± 1.14	0.90 ± 0.73	1.03 ± 1.39	1.17 ± 1.66	0.71 ± 0.42
OHBUP-glucuronide	9.97 ± 8.24	10.2 ± 10.3	9.60 ± 2.27	14.1 ± 13.0	14.2 ± 15.4	13.9 ± 6.11
TB-free	10.7 ± 8.67	9.71 ± 10.1	12.51 ± 6.02	8.40 ± 8.65	7.82 ± 9.83	9.69 ± 6.01
TB-glucuronide	3.23 ± 3.13	2.72 ± 3.84	4.25 ± 0.11	3.32 ± 2.51	2.49 ± 2.24	5.15 ± 2.17 *
EB-free	1.16 ± 1.22	1.18 ± 1.49	1.11 ± 0.62	0.67 ± 0.65	0.64 ± 0.74	0.72 ± 0.45
EB-glucuronide	0.69 ± 0.61	0.43 ± 0.39	1.22 ± 0.68 #	0.67 ± 0.51	0.74 ± 0.44	0.91 ± 0.63
BUP dose 150 mg BID	**(n=8)**	**(n=4)**	**(n=4)**	**(n=12)**	**(n=6)**	**(n=6)**
AUC_{ss} BUP (ng*h/ml)	586 ± 232	628 ± 130	534 ± 338	545 ± 211	536 ± 211	554 ± 246
AUC_{ss} OHBUP (ng*h/ml)	8826 ± 3039	8546 ± 4041	9177 ± 1603	10873 ± 4144	10424 ± 4546	11322 ± 4101
AUC_{ss} TB (ng*h/ml)	4574 ± 3003	3537± 1668	5870 ± 4039	4053 ± 2625	2333 ± 1313	5773 ± 2517#
AUC_{ss} EB (ng*h/ml)	749 ± 413	634 ± 281	893 ± 548	592 ± 359	403 ± 273	782 ± 350 #

BID, twice a day; AUCss, area under the curve at steady state; BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; EB, erythrohydrobupropion; EM, extensive metabolizer phenotype; UM, ultra-rapid metabolizer phenotype; PM, poor metabolizer phenotype, IM, intermediate metabolizer phenotype; M.R., metabolic ratio, defined as the ratio of AUCs, corrected for molecular weight.

Data presented as mean ± standard deviation.

[a] Subjects with undetermined CYP2C19 genotype were not included.

*$P < .05$; # $P < .10$ but above .05

Modified from Fokina et al., 2016b

Consequently, the TB/BUP and EB/BUP metabolic ratios in PM+IM group were higher than those in the EM+UM group (11.6 ± 3.16 vs 6.58 ± 3.33, P <.05; and 1.57 ± 0.34 vs 1.03 ± 0.52, P < .05, respectively, Figure 7); no difference in the AUC_{ss} of bupropion was observed. Likewise, the TB/BUP and EB/BUP ratios in the PM+IM group in mid-pregnancy were slightly higher than those in the EM+UM group (9.83 ± 3.02 vs 5.18 ± 2.50, P < .05, and 1.64 ± 0.46 vs 0.95 ± 0.56, P = .088, respectively, Figure 7); although we observed no differences in the AUC_{ss} of bupropion, TB and EB (Table 7). Further, in late pregnancy, the fraction of bupropion excreted with the urine in a form of TB-glucuronide in the PM+IM group of subjects was 5.15 ± 2.17 and was higher than that of the EM+UM group (2.49 ± 2.24, P < .05). Likewise, the fraction of bupropion excreted in a form of EB-glucuronide was slightly higher in the PM+IM group than in the EM+UM group (1.22 ± 0.68, vs 0.43 ± 0.39, P = .071, Table 7).

2.4. Discussion

Bupropion has a potential to be a safe and effective medication for smoking cessation in pregnancy. Bupropion is extensively metabolized; its main plasma metabolite, OHBUP, is pharmacologically active and is thought to contribute to antismoking capacities of the drug (Zhu et al., 2012). The pregnancy-induced changes in maternal physiology may affect the disposition of the drug and thus its efficacy in promoting cessation from smoking in pregnant women. Thus, in the first part of this study we investigated the effect of pregnancy on the pharmacokinetics of bupropion.

Pairwise comparisons of the PK parameters of bupropion were conducted in mid- against late pregnancy, and late pregnancy against postpartum periods, namely, lactating

and non-/post-lactating. Paired comparisons allowed accounting for inter-individual differences, however the same dose and formulation of bupropion was an essential criterion for this type of analysis. To supplement paired analysis, the PK parameters were compared across gestation and postpartum for the subjects who were treated with bupropion SR 150 mg BID.

The C_{max} of bupropion in postpartum lactating period was higher than that of late pregnancy. This suggests a higher rate of bupropion absorption during lactation, which can be explained in part by the functional and possibly structural changes in the gastrointestinal tract and walls of the stomach during lactating period (Hammond, 1997; Butte and Hopkinson, 1998).

Bupropion has been historically used as a probe substrate marker for CYP2B6 enzyme *in vitro* (Nirogi et al., 2015). The activity of CYP2B6 in bupropion hydroxylation *in vivo* is assessed by the ratio of OHBUP AUC over that of bupropion. It has been suggested that alterations in the activity of CYP2B6 affects primarily the levels of OHBUP but not those of bupropion (Zhu et al., 2012; Kirchheiner et al., 2003). In our study we observed that the OHBUP/BUP metabolic ratio in late pregnancy exceeded that of postpartum, which was consistent with the pregnancy-associated upregulation of *CYP2B6*. However, no changes in the OHBUP AUC in pregnancy were evident.

Further, by analogy with the OHBUP/BUP metabolic ratio, the TB/BUP and EB/BUP metabolic ratios would reflect, to a certain extent, the activity of enzymes icatalyzing the formation of TB and EB from bupropion. We observed that the TB/BUP metabolic ratio in late pregnancy exceeded that of postpartum, while the EB/BUP ratio in mid-pregnancy was higher than that of late pregnancy. Therefore, it suggests pregnancy-

induced increase in the reductive metabolism of bupropion. However, the contribution of CYP2C19 to the hydroxylation of bupropion, TB and EB was reported (Zhu et al., 2014; Chen et al., 2010), and *CYP2C19* is downregulated due to pregnancy (McGready et al., 2003). Therefore, the changes in TB/BUP and EB/BUP metabolic ratios in pregnancy should be interpreted with caution. We observed that the TB AUC_{ss} and EB AUC_{ss} were higher in mid-pregnancy as compared to late pregnancy. These changes in the TB and EB levels could be caused by pregnancy-induced downregulation of *CYP2C19*. Hence, the increased levels of TB and EB could contribute towards the higher values of TB/BUP and EB/BUP metabolic ratios in pregnancy as compared to the non-pregnant state. The donwregulation of *CYP2C19* due to pregnancy could also decrease the CYP2C19-mediated metabolism of bupropion. However, it was not evident from our data, probably due to the opposite effect of pregnancy on the expression of *CYP2B6,* which leads to increase in the CYP2B6-mediated hydroxylation of bupropion. Thus, the cumulative effect of pregnancy on *CYP2C19* and *CYP2B6* resulted in a slight decrease in the AUC_{ss} of bupropion and a consequent slight increase in CL/F of the drug.

Changes occurring in the renal system during pregnancy could also affect the pharmacokinetics of bupropion and its metabolites. The renal clearance of OHBUP was the lowest in pregnant women, followed by those of bupropion, EB and TB, which was in line with the report on men and non-pregnant females (Benowitz et al., 2013). In men and non-pregnant women, the renal clearance of bupropion and its main plasma metabolites is much lower than the glomerular filtration rate (GFR) (Benowitz et al., 2013). Data obtained from pregnant and non-pregnant females in our study also indicated that the renal clearance of the drug and its metabolites is lower than the GFR. Thus, the

pregnancy-associated increase in renal blood flow and GFR should not result in drastic changes in the elimination rates of bupropion and its main metabolites. Moreover, the potential role of tubular absorption in renal elimination of bupropion and its derivatives cannot be discounted, and pregnancy-associated changes in tubular reabsorption could counterbalance those of the GFR (Giacoia and Mattison, 2009).

The enhanced renal clearance of bupropion in mid-pregnancy as compared to late pregnancy is most likely caused by the pregnancy-associated increase in GFR, which is the highest in mid-pregnancy period (Hnat and Sibai, 2008). It appears that while we detected the increase in AUC_{ss} for TB and EB in mid-pregnancy and attributed it to the downregulation of *CYP2C19*, the increased renal elimination of bupropion in mid-pregnancy could counterbalance the increase in plasma levels (and consequently AUC_{ss}) of bupropion.

The fractions of bupropion dose recovered in the urine as metabolites, namely, OHBUP, TB and EB, were determined in a free form and in a form of conjugates. In pregnancy, OHBUP was the most appreciably conjugated, followed by TB and EB. These results were in agreement with the data obtained previously by Benowitz et al (2013) on men and non-pregnant women (Benowitz et al., 2013). The fractions of bupropion recovered in the urine as unconjugated (free) TB and EB in mid-pregnancy were higher than those of late pregnancy (Table 4 & 5). This could be cause by the increased GFR in mid-pregnancy (Hnat and Sibai, 2008), however it could also reflect the observed increase in plasma levels (and thus AUC_{ss}) of both TB and EB in mid-pregnancy (Table 2). Further, the fractions of bupropion recovered in the urine in a form of OHBUP- and TB-glucuronides were higher in late pregnancy than in postpartum.

Several studied suggested that a number of hepatic UGT enzymes are upregulated in pregnancy, during late trimester in particular (Jeong et al., 2008; Abernethy et al., 1982; Ohman et al., 2008). This results in the enhanced renal clearance of certain medications during pregnancy (Abernethy et al., 1982; Ohman et al., 2008). The particular enzymes catalyzing conjugation of OHBUP and TB have not been investigated thoroughly, although a recent study identified UGT2B7 as the primary UGT isoform responsible for OHBUP glucuronidation (Gufford et al., 2016). Moreover, we did not quantify the OHBUP- and TB-glucuronide in plasma, thus we cannot estimate the renal clearance of conjugated OHBUP and TB. Nevertheless, our data suggest that the increase in the formation of OHBUP due to pregnancy-induced upregulation of *CYP2B6* could not be observed because of the enhanced glucurondation of OHBUP and its subsequent renal excretion. Likewise, it appears that the increased AUC_{ss} of TB in late pregnancy due to downregulation of *CYP2C19* could have been observed if not counterbalanced by the higher rate of TB glucuronidation.

In the second part of this study, the effect of functional polymorphisms of *CYP2B6* and *CYP2C19* on bupropion biodisposition in pregnancy was investigated. Bupropion and its major pharmacologically active metabolites exhibit linear pharmacokinetics at steady state (Findlay et al., 1982), therefore all the estimated pharmacokinetic parameters, with the exception of AUC_{ss}, were deemed dose-independent.

The higher AUC_{ss} of TB and EB, higher TB/BUP and EB/BUP metabolic ratios and higher fraction of the drug dose excreted in a form of TB-and EB-conjugates were observed in pregnant women with the *CYP2C19* *1/*2, *2/*2 and *2/*17 (therefore,

classified as PM and IM). A similar effect of the *CYP2C19**2 variant allele on the levels of TB and EB in non-pregnant subjects was reported by Zhu et al., (2014). In addition, Zhu and colleagues demonstrated that the *CYP2C19**2 variant allele resulted in a higher AUC of bupropion and consequently lower CL/F in non-pregnant subjects, however we did not observe similar results in pregnant subjects in our study. We can speculate that the biotransformation of bupropion in pregnant subjects in our study could have been shunted from reduction pathways towards hydroxylation, however no increase in the OHBUP AUC_{ss} was observed in the PM+IM group as compared to the EM+UM group in pregnancy. It is possible that our small sample size precluded detecting the difference in the AUC_{ss} of OHBUP between the studied groups.

In mid-pregnancy, the OHBUP/BUP metabolic ratio in subjects who carry the *CYP2B6**6 allele was lower than in those who were homozygous for the wild type *CYP2B6*. These data suggest that the *CYP2B6**6 allele, which confers reduced CYP2B6 function, is associated with lower rate of bupropion hydroxylation. Thus, the effect of the *CYP2B6**6 variant allele on bupropion hydroxylation in pregnancy is similar to that of the non-pregnant state (Benowitz et al., 2013). However, it appears that the levels of OHBUP were not affected by the *CYP2B6**6 variant allele, although the difference might not have been evident due to our small sample size in both mid- and late pregnancy groups. Interestingly, in mid-pregnancy group, the AUC_{ss} of bupropion, TB and EB were higher in the carriers of *CYP2B6**6 allele than in subjects homozygous for the wild type *CYP2B6* variant. Based on these data we can speculate that the metabolism of bupropion was rerouted towards reductive pathways in those subjects with the *CYP2B6**6 variant allele. It has to be noted that the imbalance of *CYP2B6* and *CYP2C19* genotypes in these

subjects could also be the source of the observed differences in bupropion, TB and EB AUCs. However, those eligible for AUC_{ss} comparison in the mid-pregnancy group had either CYP2C19 IM or CYP2C19 PM status.

Nevertheless, it appears that the PM and/or IM CYP2C19 status coupled with the pregnancy-induced downregulation of *CYP2C19* could lead to a higher exposure to TB and EB in pregnancy during bupropion therapy. *In vivo* studies suggested that bupropion is a potent inhibitor of CYP2D6 enzyme, thus drug-drug interaction is plausible in instances when bupropion is co-administered with medication(s) metabolized by CYP2D6 (Kotlyar et al., 2005). Moreover, *in vitro* studies revealed that suppression of CYP2D6 activity during bupropion therapy occurs due to the inhibitory effects of TB and EB rather than that of the parent drug (Parkinson et al., 2010). Due to pregnancy-induced upregulation of *CYP2D6* (Ke et al., 2013), a dose adjustment of CYP2D6-metabolized medications might be considered in pregnancy (Ke et al., 2013; Ryu et al., 2015). In such instances, the drug-drug interaction of bupropion and CYP2C6 substrates cannot be ruled out, particularly in those patients with the loss-of-function *CYP2C19* variant allele(s).

2.5. Conclusion

The isoform-specific effect of pregnancy on bupropion-metabolizing enzymes, namely, CYP2B6, CYP2C19 and UGT, in combination with the pregnancy-associated changes in renal elimination of the drug and its metabolites could lead to a slight increase in bupropion CL/F in pregnancy. It appears that the increased rate of OHBUP formation in pregnancy is not evident due to the accelerated glucuronidation of OHBUP and its subsequent renal excretion. Therefore, while systemic exposure to the parent drug,

bupropion, could be slightly lower in pregnancy, the exposure to the main pharmacologically active metabolite, OHBUP, should be similar to that of the non-pregnant state. The pharmacokinetic profile of bupropion in pregnancy is not drastically different to that of postpartum; therefore it should not directly influence the efficacy of the drug in promoting smoking cessation during pregnancy. In addition, due to the multifactorial effect of pregnancy on bupropion biodisposition, the OHBUP/BUP metabolic ratio could not serve as an adequate measure for the activity of CYP2B6 in pregnancy.

The effects of *CYP2B6**6, the reduced-function allele of *CYP2B6*, and *CYP2C19**2, the loss-of-function allele of *CYP2C19*, on bupropion biodisposition in pregnancy are similar to those in the non-pregnant state. Further studies are needed to confirm whether pregnant smokers with the *CYP2B6**6 variant have a greater chance to achieve cessation with bupropion than *CYP2B6* wild type carriers.

2.6. Study limitations and future directions

Initially, only those pregnant subjects treated for depression with bupropion SR 150 mg BID were eligible to participate in this opportunistic study. This would have allowed studying the effect of pregnancy on the disposition of the drug at the same formulation and dose regimen as indicated for promoting smoking cessation in non-pregnant smokers. However, later in the study, the dose restriction was removed to overcome slow recruitment. Further, we aimed to conduct paired comparisons of the PK parameters derived in each of the study window in pregnancy, namely early, middle and late, with those derived in postpartum period(s) (lactating or non-/post-lactating,

depending on availability). However, several subjects completed only one or two PK studies during pregnancy, and/or were unavailable to complete postpartum PK studies. Therefore, paired analysis of early pregnancy against postpartum and mid-pregnancy against postpartum were not conducted due to insufficient sample size. Furthermore, bupropion dose was adjusted for several subjects based on the therapeutic needs irrespective of the study. As a result, these subjects could not be included in the further PK study visits because same dose of the drug was a required criterion for an adequate paired analysis.

Taken together, we acknowledge that a small sample size was the main limitation of the current study. On average, each subject completed two PK studies. Thus, although we were able to conduct paired analysis between select treatment windows, this analysis could not fully reflect the continuum of changes in the pharmacokinetics of bupropion as pregnancy progresses. Therefore, in order to supplement the paired analysis, we conducted the comparisons across gestation for those subjects who were treated with bupropion SR 150 mg BID.

Insufficient number of subjects also precluded evaluating the potential role of *CYP2B6* and *CYP2C19* genetic polymorphisms in the magnitude of pregnancy-induced changes on the pharmacokinetics of bupropion and its major metabolites. Moreover, differentiating each CYP2C19 metabolic phenotype, namely PM, IM, EM, and UM, in the data analysis was not possible. Likewise, homozygotes and heterozygotes for the *CYP2B6**6 variant alleles were grouped together.

Several variant alleles (such as *CYP2B6**4) were underrepresented in our studied population, and others (such as *CYP2C19**3) were not presented due to low frequency of

these alleles. Another potential outcome of the small number of subjects was the imbalance of *CYP2B6* and *CYP2C19* genotypes that could have influenced our results or interpretation of the data.

We did not include testing for the *CYP2B6**18 reduced variant allele in our study. *CYP2B6**18 variant is exclusive for individuals of African descent and has a relatively low frequency (Zanger and Klein, 2013). There were two self-declared African American pregnant participants in our cohort of subjects, however neither were included in the *CYP2B6* variant alleles comparisons due to insufficient DNA sample. Nevertheless, the majority of our subjects were white/non-Hispanic, suggesting the lack of diversity, which might affect the generalizability of the results.

It appears that the activity of UGT enzymes catalyzing conjugation of bupropion metabolites is increased in pregnancy, thus the elimination rate of OHBUP, the main and pharmacologically active derivative of bupropion, is accelerated. UGT2B7 was recently identified as the main enzyme catalyzing the formation of OHBUP-glucuronides (Gufford et al., 2016). Although clinical pharmacokinetic studies reported stimulatory effect of pregnancy on select UGT enzymes (Jeong et al., 2008), the effect is isoform-specific (Anderson, 2005). In addition, the clinical relevance of UGT functional polymorphism in the disposition of a variety of xenobiotics has been recognized (Stingl et al., 2013). Studying the effect of pregnancy on OHBUP conjugation along with the effect of genetic polymorphism on UGT2B7 activity would help to determine the alterations in phase II metabolism of bupropion and their impact on the drug biodisposition.

In the current investigation we did not measure the enantiomers of OHBUP, which could be recognized as another limitation of this study. As was mentioned in the Section 1.4, the pharmacological activity of OHBUP is stereo-specific: 2S,3S-OHBUP is more potent than 2R,3R-OHBUP (Damaj et al., 2004), however it is less abundant than 2R,3R-OHBUP (Kharash et al., 2008). It was also reported that glucuronidation of OHBUP is stereoselective (Gufford et al., 2016). Therefore, although we did not observe alterations in the plasma levels of OHBUP in pregnancy, it is possible that the levels of the most pharmacologically active enantiomer of OHBUP, 2S,3S-OHBUP, have been altered due to stereoselectivity in the effects of pregnancy-associated changes on the drug biodisposition. Further studies are needed to investigate the potential effect of pregnancy on the pharmacokinetics of each of OHBUP enantiomer, and whether the levels of OHBUP, and 2S,3S-OHBUP in particular, play role in the efficacy of bupropion as a smoking cessation aid for pregnant women.

CHAPTER 3: CARBONYL REDUCTION OF BUPROPION AND 4-METHY;NITROZAMINO-1-(3-PYRIDYL)-1-BUTANONE BY HUMAN PLACENTA

3.1. Introduction

As mentioned in the Section 1.9, human placenta biotransforms bupropion predominantly via reductive pathway leading to the formation of TB and EB, with the latter being the major metabolite of bupropion. *In vitro* studies also suggest that the formation of TB and EB in placental tissue is catalyzed primarely by membrane-bound carbonyl reductases, specifically 11βHSD and aldo-ketoreductases (Wang et al., 2010).

NNK, a component of cigarette smoke, is a potent pulmonary and transplacental carcinogen (Correa et al., 1990). Exposure to NNK *in utero* via maternal smoking can lead to increased risks of developing lung cancers in adulthood (Ter-Minassian et al., 2011). The reductive metabolism of NNK results in the formation of NNAL and constitutes a crucial step in the detoxification of NNK. Soluble and membrane-bound placental carbonyl reducing enzymes are involved in the formation of NNAL from NNK (Atalla and Maser, 2001). Thus, effective detoxification of NNK can decrease the susceptibility to cancer associated with NNK exposure (Ter-Minassian et al., 2011; Finckh et al., 2001).

The higher rate of TB and EB formation from bupropion in placentas obtained from smokers suggests the inductive effect of exposure to cigarette smoke on the membrane-bound carbonyl reductases responsible for the formation of TB and EB (Wang et al., 2010). Similarly, the potential effect of exposure to bupropion on the activity of placental carbonyl reducing enzymes cannot be ruled out. Given that both NNK and bupropion undergo reductive metabolism by placental enzymes, the placenta can become

a site for a "drug-drug" interaction. Any impact on the NNK-reducing enzymes as well as NNK-activating enzymes could affect the risks of the NNK-associated carcinogenesis.

Therefore, the objective of this study was to investigate the effect of bupropion on the activity of the placental carbonyl reductases. In addition, we reexamined the *in vitro* placental metabolism of NNK and studied the effect of smoking on the formation of NNAL.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals

Chemicals were purchased from the following companies: bupropion, phenacetin, Ncotinamide adenine dinucleatide phosphate (NADP+), glucose 6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, and ammonium acetate, from Sigma-Aldrich (St. Louis, MO); hydroxybupropion (OHBUP), threohydrobupropion (TB), erythrohydrobupropion (EB), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), 4-methylnitrosamino-1-(3-pyridyl)-1-butanol (NNAL), from Toronto Research Chemicals Inc. (North York, Canada); LC/MS-grade methanol, LC/MS-grade acetonitrile, ethyl acetate, formic acid, acetic acid, trichloroacetic acid (TCA), potassium phosphate mono- and dibasic from Fisher Scientific (Fair Lawn, NJ).

3.2.2. Preparation of subcellular fraction from placental trophoblast tissue

Human placentas were obtained from the women exposed to bupropion (n=15) and non-exposed (n=37) immediately after delivery in accordance to the protocol approved by the Institutional Review Board of the University of Texas Medical Branch at Galveston (UTMB). The placentas were subdivided further into the following groups: bupropion exposed/smokers (n=4), bupropion exposed non-smokers (n=11), non-

exposed/non-smokers (n=19), non-exposed smokers who smoked ≤ 10 cigarettes per day (CPD), n=9; and non-exposed smokers who smoked ≥ 20 CPD (n=9).

The subcellular fractions were prepared by differential centrifugation according to the protocol established in our laboratory (Zharikova et al., 2006). Briefly, villous tissue were excised, rinsed with ice-cold saline, and homogenized in the 0.1M potassium phosphate buffer. Crude microsomal and cytosolic subcellular fractions were prepared from the homogenate by differential centrifugation (Zharikova et al., 2006). The protein content was determined by commercially available kit (Bio-Rad Laboratories kit, Hercules, CA) using bovine serum albumin as a standard. The microsomal and cytosolic fractions were used individually in the experiments; pooled 8 microsomal or cytosolic fractions derived from placentas of non-smokers were used as noted below.

3.2.3. Biotransformation of NNK by microsomal and cytosolic placental subcellular fractions

The activities of individual placental subcellular fraction in metabolizing NNK were determined. The total reaction volume was 250 μL of 0.1M of potassium phosphate buffer at pH=7.4. The protein amount and incubation time were selected so the formation of NNAL was linear. Each reaction contained 0.25 mg of microsomal or cytosolic protein and 8mM final concentration of NNK for microsomes (4-5 times apparent K_m) and 12mM for cytosolic fraction (3 times apparent K_m). The reaction mixture was pre-incubated for 5 min at 37 C. The reaction was initiated by addition of 25 μL of NADPH-regenerating system (0.4 mM NADP+, 4 mM glucose 6-phosphate, 1 U/mL glucose-6-phosphate dehyrogenase, and 2 mM $MgCl_2$). The reaction was held for 10 min at 37°C and terminated by adding 100 μL of 1M Na_2CO_3, mixed and chilled on ice. The samples were processed as described below. The effect of protein amount (0.125-0.75 mg of pooled microsomal or cytosolic subcellular fractions) and incubation time (0-60 min) on the NNAL formation was investigated at pH=7.4. The effect of pH on the activity of

microsomal carbonyl reductases was determined using pooled preparations; the pH ranging from pH=5 to pH=7.4 was tested. In addition, the effect of NNK concentration (0-12mM) on the reaction velocity in individual placental fractions was studied to construct saturation curves and determine apparent K_m values.

3.2.4. Inhibitory effect of bupropion and its metabolites, OHBUP, TB and EB on the formation of NNAL from NNK

The effect of bupropion and its major plasma metabolites on the formation of NNAL from NNK by placental subcellular fractions was studied *in vitro*. Stock solution for bupropion was prepared in potassium phosphate buffer (pH=7.4). Stock solutions for OHBUP, TB and EB were prepared in 15% methanol; the final concentration of methanol in the reaction mixture did not exceed 3%. The inhibitors were, namely, bupropion, OHBUP, TB and EB, and each inhibitor was used at the concentration equimolar to that of the substrate (NNK) in respective subcellular fractions. Each reaction solutions contained the following components in the 0.1M potassium phosphate buffer (pH=7.4): inhibitor, 0.25 mg of pooled placental microsomal or cytosolic protein, NNK at the final concentrations of 1000 μM for placental microsomes and 2000 μM for cytosolic fractions (both concentrations were equal approximately one respective apparent K_m as determined for respective pooled placental subcellular fractions). The reaction solutions were pre-incubated for 10 min at 37°C, the reactions were initiated by addition of NADPH, incubated for 10 min at 37°C and terminated as described above. The control reaction contained the same above-mentioned components, with the exception of the inhibitors.

3.2.5. Extraction and recovery of NNAL

The extraction method was modified from Skarydova et al., (2012). Each reaction solution was extracted with 1 mL of ethyl acetate by vigorous shaking for 15 min, centrifuged for 10 min at 12000 x g, organic layers was transferred to another tube. The

extraction procedure was repeated, the organic layers combined and dried at 40°C under a stream of nitrogen. The residue was reconstituted in 200 µl of the initial mobile phase, and 75 µl was injected into the HPLC system for analysis. Alternatively, the residue was reconstituted in 1000 µl of the initial mobile phase, and 10 µl was injected into the LC-MS system for analysis.

3.2.6. Instrumental and analytical conditions

NNAL was determined either by HPLC/UV analysis or by LC-MS analysis as described below.

HPLC/UV analysis

The HPLC method was adapted from Atalla and Maser (2001). The HPLC system consisted of Waters 1525 binary pump and 717 plus autosampler controlled by Empower data software (Waters, Milford, MI). A 2489 dual λ absorbance detector (Waters, Milford, MI) was used for detection of NNAL. The separation of the analytes was achieved by a Phenomenex Gemini-NX C18 column (150mm X 4.6mm, 5µm) connected to a Phenomenex C18 guard column (40mm X 3.0mm). The mobile phase consisted of 92% of 1mM potassium phosphate buffer, pH=7.4, and 8% acetonitrile; the elution was isocratic at a rate of 1.5 ml/min. The NNAL was monitored at λ = 230 nm. After NNAL elution at 14 min, the column was flushed with 90% of acetonitrile, and equilibrated with the initial mobile phase prior to the next injection.

LC-MS analysis

The analysis of NNAL was conducted by an Agilent HPLC 1260 Infinity system coupled with an Agilent 6130 single quadrupole mass spectrometer (Santa Clara, CA, USA). The HPLC system consisted of a degasser (G4225A), apump delivery system (G1212B), an autosampler (G1329B) and a column compartment (G1316A) controlled

by Agilent OpenLAB CDS ChemStation software (Santa Clara, CA, USA). The mass spectrometer was equipped with an ion source (ESI) operated in a positive mode. The fragmentor voltage was 130V, the gas temperature was 350°C. Separation of NNAL was achieved by a Phenomenex Gemini-NX C18 column (150 X 4.6 mm, 5 μm) using isocratic elution at a rate of 1 ml/min; a splitter was used to direct 20% of the eluent towards mass spectrometer. The mobile phase consisted of 10% acetonitrile and 90% 2mM ammonium formate. NNAL was monitored at SIM m/z=210. After NNAL elution at 10 min, the column was flushed with 90% acetonitrile for 7 min and equilibrated with the initial mobile phase prior to the next injection.

3.2.7. Biotransformation of bupropion by placental microsomes

The specific activity of individual placental microsomal fractions in metabolizing bupropion into TB and EB were determined. The final concentration of bupropion was 80 μM and was approximately 3-folds of the apparent K_m based on the data published previously (Wang et al., 2010). The experiment was conducted as described above for NNK metabolism by placental microsomes; the reaction was terminated by addition of 40 μL of 40% TCA (8% final, w/v) and extracted with 1.5 mL of acetonitrile as described in Chapter 1 for plasma samples.

The effect of protein amount (0.125-0.75 mg), incubation time (0-60 min), and pH (5.5-7.4) on the formation of TB and EB from bupropion was confirmed using pooled microsomal fractions.

3.2.8. Inhibitory effect of NNK on bupropion metabolism by placental microsomes

We studied the inhibitory effect of NNK on the formation of OHBUP, TB and EB from bupropion by placental microsomal fractions *in vitro*. Stock solution of NNK was prepared in the 0.1M potassium phosphate buffer (pH=7.4). Each reaction solutions contained the following components in 0.1M potassium phosphate buffer (pH=7.4): 0.25

mg of placental microsomal protein, bupropion at the final concentration of 32 μM (equals to an apparent K_m based on the specific activity reported previously by Wang et al., 2010), and NNK at the final concentrations of either 200 μM or 2 mM. The reaction solutions were pre-incubated for 10 min at 37°C, the reactions were initiated by addition of NADPH, incubated for 10 min at 37°C and terminated as described above (Section 3.2.3.). The control reaction contained the same above-mentioned components with the exception of NNK.

3.2.9. Quantitative determination of OHBUP, TB and EB by LC-MS

Bupropion metabolites were quantified using the LC-MS method described in details in Section 2.2.5.3. For the analysis of OHBUP, TB and EB formed from bupropion by placental microsomes *in vitro*, the LOD for each of the metabolite was 0.5 ng/mL, the LLOQ was 2 ng/mL, and linearity was achieved within 2 ng/ml -300 ng/mL.

3.2.10. Data analysis

The values of K_m for NNK and V_{max} for NNAL formation were determined using non-linear regression analysis of the Michaelis-Menten equation (GraphPad Prizm 5, Vision 5.01, Graph Pad Software, Inc.); the data presented as mean ± STDEV. The rates of formation of each of the products, namely, NNAL, TB and EB, by placental preparations obtained from bupropion-exposed and non-exposed, smokers and non-smokers were compared using Mann-Whitney U Test (SPSS 20 for Windows, SPSS Inc, Chicago, IL). *P* values < .05 were considered statistically significant. *P* values above .05 but below .10 were also reported. The effect of respective inhibitors on the formation of NNAL, TB and EB was evaluated as follows: the metabolites formation in the control reaction was set as 100%, and their formation in the presence of respective inhibitors were calculated as % of control.

3.3. RESULTS

3.3.1. Biotransformation of NNK and bupropion by placental subcellular fractions

The optimal pH for the formation of NNAL from NNK by placental microsomes was ~ 6 (Figure 8) and was consistent with the data on the activity placental 11βHSD enzymes *in vitro* reported previously (Lakshmi et al., 1993). The formation of TB and EB from bupropion was the highest at pH=6.8 - 7 (Figure 9). All further experiment in this study were conducted at the physiological pH=7.4.

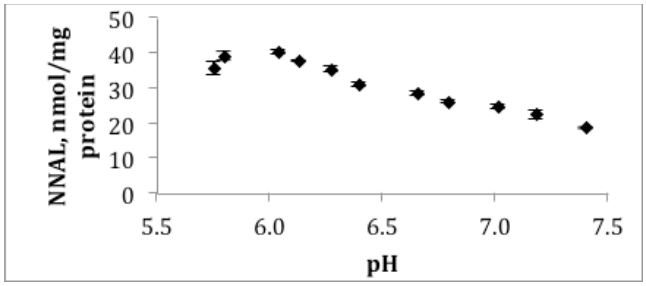

Figure 8. The effect of pH on the biotransformation of NNK into NNAL by human placental microsomes. NNK, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone; NNAL, 4-methylnitrosamino-1-(3-pyridyl)-1-butanol

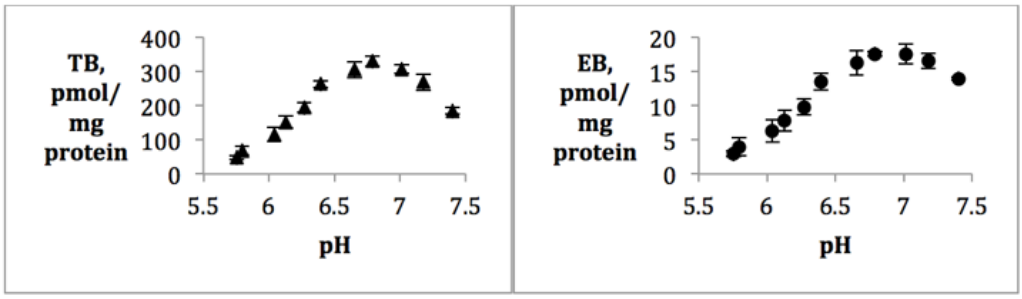

Figure 9. The effect of pH on the formation of TB and EB from bupropion by human placental microsomes. TB, threohydrobupropion; EB, erythrohydrobupropion

Under the experimental condition, the amount of NNAL formed by placental microsomes was linear with protein concentrations up to 0.75 mg per reaction volume

and time up to 20 min. The amount of NNAL formed by placental cytosolic fraction was linear with protein concentration up to 0.75 mg and time up to 60 min. No endogenous NNK and/or NNAL were detected in the subcellular preparations of placentas obtained from smoking mothers.

The amounts of OHBUP, TB and EB formed from bupropion by placental microsomes were linear with protein concentration up to 0.75 mg and time up to 20 min. The endogenous bupropion and its metabolites were present in the microsomal preparations from placentas obtained from mothers treated with bupropion during pregnancy. The preparations from bupropion-exposed placentas were not included in the incubations with bupropion as a substrate.

3.3.2. Validation of HPLC/UV and LC-MS methods for quantitative determination of NNAL

The HPLC/UV and LC-MS methods for quantitative determination of NNAL were validated for specificity, selectivity, precision and accuracy.

Figure 10 A-D shows the representative UV chromatograms for the blank placental microsomes, NNAL standard spiked with blank microsmes resulting in a final concentration of 2 μg/mL, and NNAL formed from the 10 min incubation of placental microsomes with 2mM NNK and cofactors. The two peaks of NNAL represent the enantiomers of the product formed from the respective enantiomers of NNK, Figure 10 D, which was consistent with the previous reports (Skarydova et al., 2012). The peaks were chromatographically separated but their areas were summarized for the quantitative analysis of NNAL. A ratio of peak areas to one another in each sample was the same irrespective of the subcellular placental preparation and the effect of inhibitors.

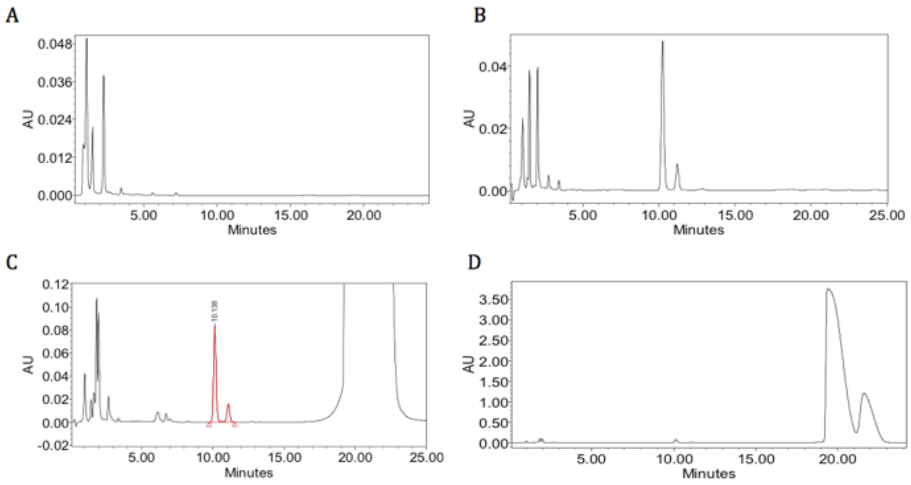

Figure 10. Representative HPLC/UV chromatograms (A) blank placental mcirosomes; (B) blank placental microsomes spiked with NNAL standard, 2 μg/mL final concentrations; (C) NNAL formed from NNK by placental microsomes; (D) same as (C) lower resolution to show chromatographic separation of NNK stereoisomers. NNK, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone; NNAL, 4-methylnitrosamino-1-(3-pyridyl)-1-butanol

For the HPLC/UV method, the extraction recovery ranged from 95.9% to 99.9% with RSD ≤ 6.5%. The LOD and LLOQ were 0.025 μg/mL and 0.1 μg/mL, respectively, and the linearity was achieved within 0.1-20 μg/mL, which was sufficient for the present analysis. The intra-day accuracy ranged between 95.7% and 103.5% with RSD ≤ 6.5%.

For the LC-MS, the LOD and LLOQ were 0.5 ng/mL and 1.25 ng/mL, respectively, and the linearity was achieved within 1.25 ng/ml - 5000 ng/mL, which was sufficient for the present analysis. The inter-day accuracy was between 88.2% and 110.6% with RSD ≤ 3.1%, while the inter-day accuracy ranged from 103.0% and 108.2% with RSD ≤ 5.7%.

3.3.3. Metabolism of NNK by human placental microsomal and cytosolic fractions

The microsomal and cytosolic placental subcellular fractions had similar activity in the reductive metabolism of NNK to NNAL. The rate of NNAL formation by

microsomes and cytosolic fractions of human placenta was dependent on NNK concentration and exhibited saturation kinetics (Figure 11 A-B). The average values for apparent K_m and V_{max} for placental microsomes (n=5) were 1.7 ± 0.5 mM and 2.8 ± 0.5 nmol.mgP^{-1}.min^{-1}, respectively. The average values for apparent K_m and V_{max} for placental cytosolic fractions (n=3) were 4.2 ± 0.3 mM and 2.2 ± 0.8 nmol.mgP^{-1}.min^{-1}, respectively.

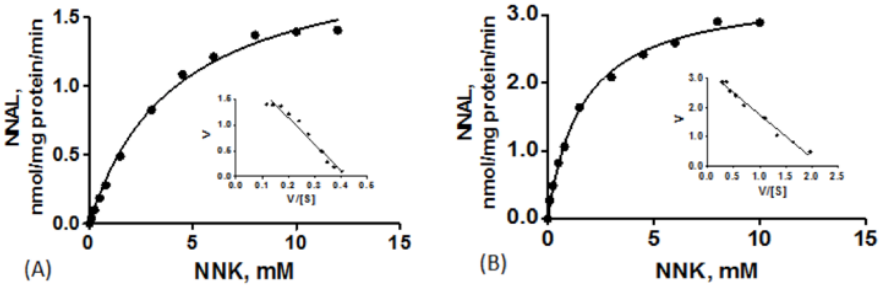

Figure 11. Representative saturation curves of NNAL formation by placental subcellular fractions: (A) cytosolic; (B) microsomal. Eadie-Hofstee plot (insert) of reaction velocity (V) against V/[S] confirmed monophasic kinetics of the reactions. NNK, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone; NNAL, 4-methylnitrosamino-1-(3-pyridyl)-1-butanol

3.3.4. Effect of cigarette smoking and exposure to bupropion on reductive metabolism of NNK by placental subcellular fractions

The effect of cigarette smoking and exposure to bupropion on the reductive metabolism of NNK was determined in five groups of placental subcellular fractions, namely, bupropion-exposed smokers, bupropion-exposed non-smokers, non-exposed non-smokers (control), non-exposed "light" smokers (10CPD), and non-exposed "heavy" smokers (≥20CPD).

The formation of NNAL from NNK by placental microsomes did not differ across the groups (Figure 12). However, the formation of NNAL from NNK by placental cytosolic fractions from non-exposed "heavy" smokers was 12.1 ± 3.5 nmol.mgP^{-1} and was lower than that of "light" smokers (17.4 ± 6.2 nmol.mgP^{-1}, *P*= .085) and control

(16.5 ± 6.0 nmol.mgP^{-1}, $P < .05$). Further, the formation NNAL in cytosolic fractions of bupropion-exposed smokers group was 23.2 ± 2.7 nmol.mgP^{-1} and was higher than that of control (16.5 ± 6.0 nmol.mgP^{-1}, $P < .05$) and "heavy" smokers (12.1 ± 3.5 nmol.mgP^{-1}, $P < .05$).

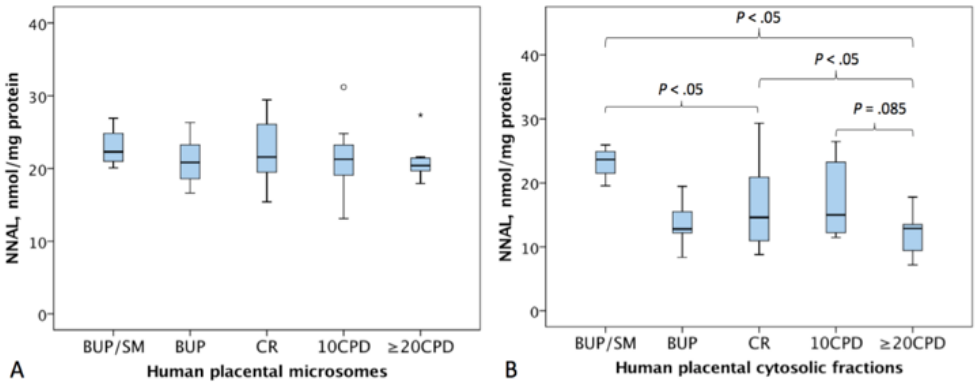

Figure 12. The formation of NNAL from NNK by placental microsomes (A) and cytosolic fraction (B). BUP/SM, bupropion exposed/smokers; BUP, bupropion exposed/non-smokers; CR, non-exposed/non-smokers; 10CPD, non-exposde/smokers ≤10 cigarettes per day; ≥20CPD, non-exposed/smokers ≥20 cigarettes per day; NNAL, 4-methylnitrosamino-1-(3-pyridyl)-1-butanol; NNK, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone

3.3.5. Effect of cigarette smoking on the reductive metabolism of bupropion

As reported previously, the formation of TB and EB was lower in microsomes from placentas obtained from non-smokers as compared to those who smoked ≥20CPD (Wang et al., 2010). We confirmed those results in our investigation by comparing the formation of TB and EB from bupropion in 3 groups of placental microsomes, namely, control (n=16), "light" smokers (n=9), and "heavy" smokers (n=9) (all non-exposed to bupropion).

Our results indicated that the formation of TB from bupropion in "light" smokers, 465 ± 297 pmol.mgP^{-1}, and in "heavy" smokers group, 431 ± 235 pmol.mgP^{-1}, was higher than that of control, 291 ± 120 pmol.mgP^{-1}, with $P = .062$ and $P = .10$, respectively

(Figure 13 A). Moreover, the formation of TB by the combined group of smokers (n=18) was 448 ± 260 pmol.mgP^{-1} and was higher then that of control ($P < .05$) (Figure 14 A). No difference in the formation of EB was observed between smokers and non-smokers (Figure 13 B and 14 B).

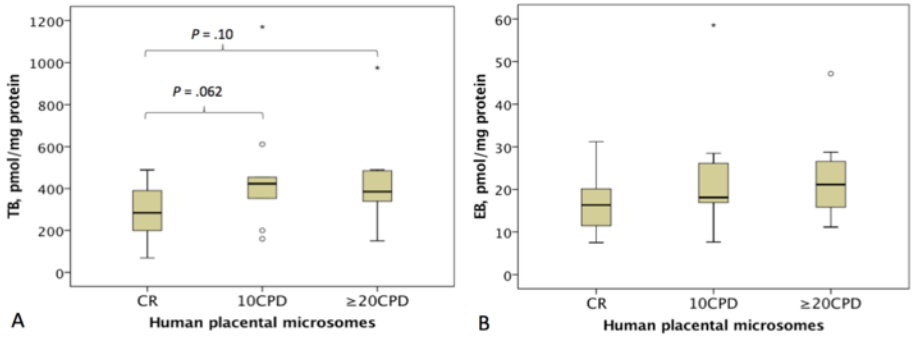

Figure 13. The formation of TB (A) and EB (B) from bupropion by placental microsomes from smokers and non-smokers (both groups non-exposed to bupropion). CR, non-exposed/non-smokers; 10CPD, non-exposde/smokers ≤10 cigarettes per day; ≥20CPD, non-exposed/smokers ≥20 cigarettes per day; TB, threohydrobupropion; EB, erythrohydrobupropion.

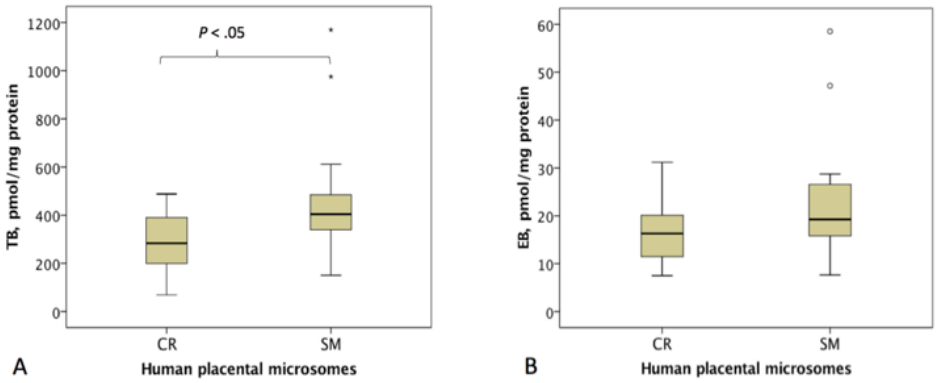

Figure 14. The formation of TB (A) and EB (B) from bupropion by placental microsomes from smokers and non-smokers (both groups non-exposed to bupropion). CR, non-exposed/non-smokers; SM, non-exposed/smokers; TB, threohydrobupropion; EB, erythrohydrobupropion.

3.3.6. Effect of bupropion and its major metabolites on the formation of NNAL from NNK by placental subcellular fractions

In the presence of bupropion, OHBUP, TB and EB the formation of NNAL from NNK in human placental microsomes was 5.3 ± 0.5 nmol.mgP^{-1}, 4.3 ± 0.6 nmol.mgP^{-1}, 3.9 ± 0.1 nmol.mgP^{-1}, and 4.4 ± 0.1 nmol.mgP^{-1}, respectively, and was not different from that of the control reaction (5.0 ± 0.5 nmol.mgP^{-1}) (Figure 15A). The formation of NNAL by placental cytosolic fraction was 4.2 ± 0.6 nmol.mgP^{-1} in the absence of inhibitors, while in the presence of inhibitors, namely, bupropion, OHBUP, TB and EB, the formation of NNAL was as follows: 4.0 ± 0.6 nmol.mgP^{-1}, 2.1 ± 0.3 nmol.mgP^{-1}, 3.7 ± 0.8 nmol.mgP^{-1}, and 3.3 ± 0.1 nmol.mgP^{-1}, respectively (Figure 15B). The results indicate that in the presence of OHBUP and EB, the remaining activity of carbonyl reductases in NNK metabolism was 45 ± 4.3% and 71 ± 1.8%, respectively.

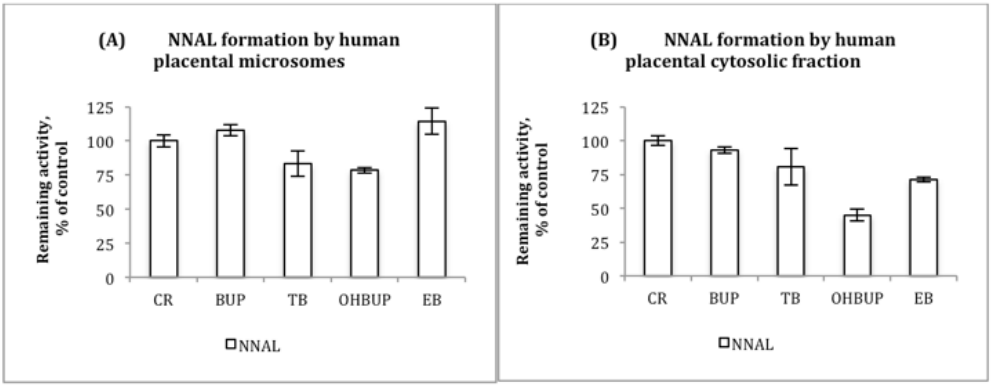

Figure 15. The formation of NNAL in the presence of bupropion, TB, OHBUP and EB by placental microsomes (A) and cytosolic fraction (B). BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; EB, erythrohydrobupropion; CR, control (absence of inhibitors); NNAL, 4-methylnitrosamino-1-(3-pyridyl)-1-butanol

3.3.7. Effect of NNK on the formation of OHBUP, TB and EB from bupropion by placental microsomes

In the absence of NNK, the formation of OHBUP, TB and EB from bupropion in placental microsomes was as follows: OHBUP, 6.6 ± 0.5 pmol.mgP^{-1}; TB, 100 ± 1.4 pmol.mgP^{-1}; and EB, 7.9 ± 1.0 pmol.mgP^{-1}. In the presence of NNK at the concentration of 200 μM, the formation of OHBUP was 4.8 ± 0.4 pmol.mgP^{-1} and constituted 75 ± 6% of the control reaction. In the presence of NNK at the concentration of 2 mM, the formation of OHBUP was 4.82 ± 0.9 pmol.mgP^{-1} and was 72.8 ± 13.6% of the control reaction (Figure 16). The presence of NNK did not affect the formation of TB and EB (Figure 16).

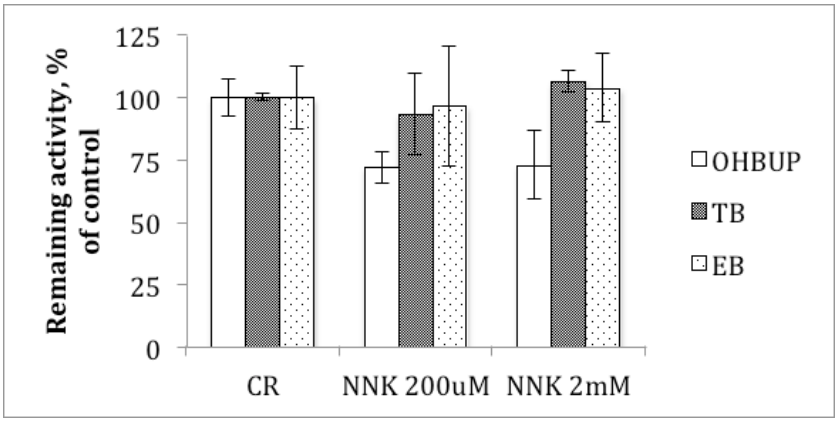

Figure 16. The effect of NNK on formation of OHBUP, TB and EB from bupropion by placental microsomes. OHBUP, hydroxybupropion; TB, threohydrobupropion; EB, erythrohydrobupropion; CR, control (absence of inhibitors); NNK, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone

3.4. DISCUSSION

It appears that both NNK, a component of cigarette smoke, and bupropion, a medication used to promote cessation from smoking, are the substrates for the placental carbonyl reductases. The reductive metabolism of bupropion was catalyzed primarily by the membrane-bound enzyme(s), while both microsomal and cytosolic placental carbonyl reductases were involved in the formation of NNAL from NNK. For the latter, the similar values for apparent K_m and V_{max} in placental microsomes and cytosolic fractions

suggest similar specific activities of placental soluble and membrane-bound carbonyl reducing enzymes in NNK metabolism. These results are consistent with the data reported previously (Atalla and Maser, 2001).

The placental 11βHSD and, to a lesser extent, aldo-keto reductases were identified to catalyze the formation of TB and EB from bupropion (Wang et al., 2010). Placental 11βHSDs were also reported to be involved in the NNK metabolism (Atalla and Maser, 2001). There are two isoforms of 11βHSD enzymes in humans, 11βHSD1 and 11βHSD2, and both are expressed in placental tissue (Stewart et al., 1994; Albiston et al., 1994; Tannin et al, 1991). Both enzymes are the members of a short-chain dehydrogenase-reductase family of carbonyl reductases, and the lack of specific inhibitor precluded identification of the particular isoform responsible for bupropion reduction or confirming the contribution of both types of 11βHSDs in the reaction (Wang et al, 2010). Similarly, the particular type of11βHSD responsible for the formation of NNAL from NNK was not reported (Atalla and Maser, 2001).

The presence of NNK did not affect the rate of TB and EB formation from bupropion by placental microsomes, and neither bupropion, nor its metabolites had any effect on the reductive metabolism of NNK by the microsomes. In addition, while the optimal pH for NNAL formation was pH=6, the rate of TB and EB formation was maximum at the pH level between 6.5 and 7. Moreover, it appears that smoking during pregnancy has opposite effect on the membrane bound enzymes catalyzing the reductive metabolism of bupropion and NNK: while the formation of TB and EB from bupropion is increased in placentas of smokers, the NNAL formation from NNK is slightly decreased. Taken together these data suggest that different carbonyl reductases are responsible for the reductive metabolism of bupropion and NNK in human placenta. It is also possible that the same multiple enzymes are involved in the reaction, but their contribution in reductive metabolism of NNK and bupropion is not equal.

We observed the decreased formation of NNAL from NNK in both microsomal and cytosolic placental fractions. Nicotine and polycyclic hydrocarbons of cigarette smoke stimulate the expression and, subsequently, activity of CYP2A1 and 1A2 in fetal, newborn and adult rat organs and human placenta (Czekaj et al., 2005). The CYP enzymes mentioned above are involved in metabolizing of NNK into carcinogenic derivatives (Mori et al., 2001; Mori et al., 2003). We can speculate that an increased activity of these CYPs in the microsomes obtained from the placentas of smoking mothers caused switching of NNK metabolism from reduction (detoxification) towards hydroxylation (activation). However, the concentrations of NNK were few folds over the apparent K_m for microsomal carbonyl reductases and were in a mM range (based on the formation of NNAL). Although we did not determine the apparent K_m for NNK based on the formation of its oxidative metabolite(s) by placental microsomes, the reported apparent K_m for NNK in this reaction in other tissues were in a μM range (Hong et al., 1992; Smith et al, 1992). Therefore, the concentration of NNK used in our experimental condition was saturated for both oxidative and reductive NNK biotransformation. Further, glycyrrhizic acid is a non-selective inhibitor of both isoforms of 11βHSD enzyme (Hult et al., 1998; Maser et al., 2003); and it was shown to be a potent inhibitor in a range of nM concentrations (Monder et al., 1989). Glycyrrhizic acid is a component of licorice supplements used in tobacco processing and its final concentrations in tobacco can reach 0.11% (1.1 mg per 1 g of tobacco) (Carmines, 2002; Maser, 2004;). Therefore, the inhibitory effect of glycyrrhizic acid on carbonyl reductases in heavy smokers cannot be ruled out and could explain the decreased formation of NNAL from NNK in placental microsomes obtained from women who were heavy smokers during pregnancy.

The presence of bupropion did not affect the reductive metabolism of NNK by placental microsomes. However, the activity of soluble carbonyl reductases from placentas of bupropion-exposed smokers was higher than those of non-exposed smokers and non-exposed non-smokers. We can speculate that bupropion, in combination with

smoking, can produce a stimulatory effect on the carbonyl reductases in a synergistic manner, thus resulting in a higher detoxifying capacity of these enzymes. However, we have to acknowledge the small sample size (n=4) in the bupropion-exposed smokers group, which could skew the results.

The purpose of our inhibition studies was to evaluate the potential "drug-drug" interaction in placenta, namely, the effect of the presence of bupropion and its metabolites on NNK reductive biotransformation, and the presence of NNK on the TB and EB formation from bupropion. In addition, we measured the potential inhibitory effect of NNK on the formation of OHBUP from bupropion. The OHBUP, TB and EB were used as inhibitors because the plasma levels of bupropion main metabolites *in vivo* are few-fold higher than those of the parent drug (Findlay et al., 1981). The presence of NNK did not affect bupropion reductive metabolism by placental microsomes, however OHBUP and EB inhibit the reductive capacity of placental membrane-bound enzymes, thus the formation of NNAL from NNK was decreased. The estimated plasma Cmax of bupropion and its metabolites were: bupropion, 0.67 μM; OHBUP, 4.01 μM; TB, 2.34 μM and EB, 0.43 μM (Findlay et al., 1981; Reese et al, 2008); and the concentrations of NNK in blood plasma of a smoker ranged from 1 to 15 pM (Schrader et al, 2000). Thus, the concentrations used in the inhibition experiments *in vitro* were 1000-folds higher then those *in vivo*, therefore the impairment of NNK reductive metabolism caused by OHBUP and TB in placenta is unlikely to take place *in vivo*.

3.5. Conclusion

Taken together, our results suggest that heavy smoking during pregnancy can negatively influence the detoxifying capacity of carbonyl reductases and thus promote NNK-induced damage in the placenta and the fetus. Moreover, the placental enzymes responsible for reductive metabolism of bupropion are not the same as those catalyzing

NNAL formation from NNK. Exposure to bupropion during pregnancy should not inhibit the detoxifying capacity of soluble and membrane-bound carbonyl reductases.

3.6. Study limitations and future directions

The relatively small sample size for the placental tissue from bupropion-exposed smokers was a limitation of our study. We observed higher activity of cytosolyc carbonyl-reducing enzymes in the formation of NNAL from NNK in bupropion-exposed smokers placentas. Neither exposure to bupropion only, nor exposure to the cigarette smoke only produced similar effect on NNK reductive metabolism. Larger sample size is needed to confirm our results. In addition, the residual presence of bupropion and its metabolites in the preparations of placentas obtained from the drug-exposed females precluded studying bupropion metabolism by these placental subcellular fractions. Moreover, the individual placental preparations could differ in their CYP enzyme(s) activity in NNK hydroxylation due to the effect of bupropion exposure with or without exposure to cigarette smoke. Studying NNK activation pathway in individual placental preparations along with the NNK reduction would allow better understanding of the factors influencing the detoxification capacity of the placenta in NNK elimination.

The activated NNK can bind to DNA and form DNA adducts which can lead to mutations (Akopyan and Bonavyda, 2006). In the placentas of smokers, the number of DNA adducts and double-strand breaks are higher than in the placentas of non-smokers (Everson et al., 1989; Slatter et al., 2014). The quantitative assessment of DNA adducts and double strand breaks in the placentas of bupropion-exposed (smokers and non-smokers), and non-exposed (smokers and non-smokers) would demonstrate whether exposure to the drug with or without the exposure to cigarette smoke affects the occurrence of DNA damage.

CHAPTER 4: FETAL EXPOSURE TO MATERNALLY ADMINISTERED BUPROPION: THE CONCENTRATIONS OF THE DRUG, HYDROXYBUPROPION, AND THREOHYDROBUPROPION IN UMBILICAL CORD PLASMA AND AMNIOTIC FLUID

4.1. Introduction

Ex vivo dual perfusion of a placental lobule is the only experimental method that allows studying human placental passage of substances across organized placental tissue (Kay et al., 2011). Placental perfusion can be performed in two configurations, namely, open configuration, with the constant supply of the perfusate containing the same concentration of a studied drug, and the closed configuration, with the recirculation of the media. The former is useful to estimate the drug clearance, while the latter is suitable to estimate the drug distribution (Kay et al., 2011).

The placental transfer of bupropion and its pharmacologically active metabolite, OHBUP, were studied *ex vivo* utilizing closed placental perfusion configuration (Earhart et al., 2010). Both drugs cross human placenta and appear in the fetal side of the perfusion system. Bupropion is efficiently retained by the tissue and is metabolized into TB, while OHBUP is not metabolized by placental tissue and is transferred to the fetal side at the greater extent than the parent drug (Earhart et al., 2010; Hemauer et al., 2010). The *ex vivo* placental perfusion technique is a valuable tool to study the transfer of therapeutic agents across placenta, however it cannot integrate maternal or fetal pharmacokinetic factors, therefore it does not always accurately approximate the *in vivo* conditions (Hutson, 2011). For examples, *in vivo* placental passages of tricyclic

antidepressant nortriptyline and its active metabolite, cis-10-hydroxynortriptyline, estimated as a ratio of umbilical cord to maternal concentrations, were significantly greater than those predicted *ex vivo* using the perfusion of the placental lobule (Loughhead et al., 2006b).

Although the use of bupropion for treatment of depression during pregnancy is consistent with several practice guidelines, the fetal exposure to the drug has not been studied extensively. Quantifying the extent of placental passage of the drug and its active metabolites *in vivo* would be informative for estimation of potential fetal exposure to maternally administered bupropion.

Therefore, the primary objective of this investigation was to examine the extent of *in vivo* placental passage of bupropion and its major pharmacologically active metabolites. We also determined the concentrations of bupropion and its metabolites in the amniotic fluid to evaluate potential fetal exposure via pathway beyond placental transfer.

4.2. MATERIALS AND METHODS

4.2.1. Study overview

The subjects of this opportunistic study were pregnant women enrolled in the study to determine the pharmacokinetics of bupropion during pregnancy (Chapter 2). The subject were prescribed bupropion by their regular care provider for treatment of depression, the dose and regimen was determined based on the clinical need of each patient and did not depend on the current study. The exclusion criteria listed in the study overview, Section 2.2.2, were applicable in the current investigation. The eligible

participants in this investigation were pregnant women taking bupropion within the last 7 days prior to delivery. The subjects were recruited at UTMB, Galveston, TX and the affiliated clinics in Pasadena and Pearland, TX. The study was carried out following the protocol approved by the UTMB Institutional Review Board; each subject provided written informed consent prior to data collection.

Maternal venous blood samples were obtained less than 4 hours prior to delivery or immediately after delivery. The samples were placed in BD Vacutainer heparinized tubes, plasma was separated by centrifugation and transferred to cryovials (Corning, NY). Umbilical cord venous and arterial blood samples were obtained after delivery of the placenta; the plasma was separated as described above. When feasible, amniotic fluid samples were collected from the planned Cesarean section deliveries, using a sterile 30cc needleless syringe after rupture of membranes. All samples were stored at -80°C until analysis.

4.2.2. Quantitative determination of bupropion, OHBUP and TB

The concentrations of bupropion, OHBUP and TB in the maternal plasma, umbilical cord venous and arterial plasma, and amniotic fluid were determined using the LC-MS/MS method as described in Section 2.2.5.1. The LOD and LLOQ concentrations for the bupropion and its metabolites in a 200 µl of a plasma and amniotic fluid sample were as follows: bupropion, 0.0625 and 0.125 ng/ml, OHBUP, 0.2 and 0.5 ng/ml, and TB, 0.12 and 0.3 ng/ml.

4.2.3. Data analysis

The levels of bupropion, OHBUP and TB in the blood plasma and amniotic fluid samples below their respective LLOQ were considered undetectable and were excluded from the subsequent analysis. The extent of placental transfer of bupropion, OHBUP and TB was estimated by a ratio of the analyte concentration in the umbilical cord venous plasma to that of the corresponding maternal plasma. Data are presented as median and mean ± STDEV when specified.

4.3. RESULTS

Twenty-two pregnant subjects participated in the study; they were receiving the following bupropion therapy: 150 mg twice a day (n=8), 300 mg daily (n=3), 75 mg twice a day (n=3), 150 mg daily (n=6), 100 mg daily (n=1) and 75 mg daily (n=1) (Table 8). The time of the last dose before delivery was available for 14 subjects only (Table 8). The average age for the participating subjects was 29.5 ± 6.6 years, and average gestational age at delivery was 384 ± 16 weeks. For the samples of maternal blood (n=22), the corresponding umbilical cord venous (n=22) and arterial (n=17) blood samples were obtained. Amniotic fluid samples were available from nine deliveries.

The concentrations of bupropion, OHBUP and TB were below LOD and LLOQ in one maternal plasma sample (Subject F), suggesting that the subject did not take the drug within a few days prior to delivery. Neither analyte was detected in the corresponding umbilical cord venous and arterial plasma samples. The median concentrations in the umbilical cord venous plasma were: bupropion, 5.3 ng/ml, OHBUP, 103.6 ng/ml, and TB, 59.6 ng/ml (Table 8). The concentrations of bupropion, OHBUP

and TB in the umbilical cord arterial plasma samples were 113 ± 26% of those in the cord venous plasma.

Bupropion and OHBUP were undetectable in four samples of the umbilical cord venous plasma (subjects B, C, F, and U). The individual placental transfer ratios for the drug and its metabolites are shown in Figure 17, and the mean values of their ratios are presented in Table 8. In all examined pairs of corresponding maternal plasma and umbilical cord venous plasma, the concentrations of OHBUP and TB in maternal samples exceeded those in the cord sample, and the fetal-to-maternal ratios were: OHBUP, 0.22 ± 0.09; TB, 0.60 ± 0.11 (Table 8). Same pattern was observed for bupropion in all but four maternal-cord plasma pairs: in subjects K, M, N, and V, the concentrations of the parent drug in the umbilical cord venous plasma were higher than those it the corresponding maternal plasma (Table 8).

There was a high correlation between the concentrations of TB and OHBUP in the matched maternal and umbilical cord venous plasma; the corresponding Pearson's correlation coefficients *r* were 0.99 and 0.86 (Figure 18 B&C). High correlation between the levels of bupropion in the maternal-fetal pairs was observed as well (r=0.87). However, the majority of maternal plasma samples had bupropion concentrations below 10 ng/ml, and for those matching maternal-cord plasma samples the correlation for bupropion concentrations was weak (Figure 18 A).

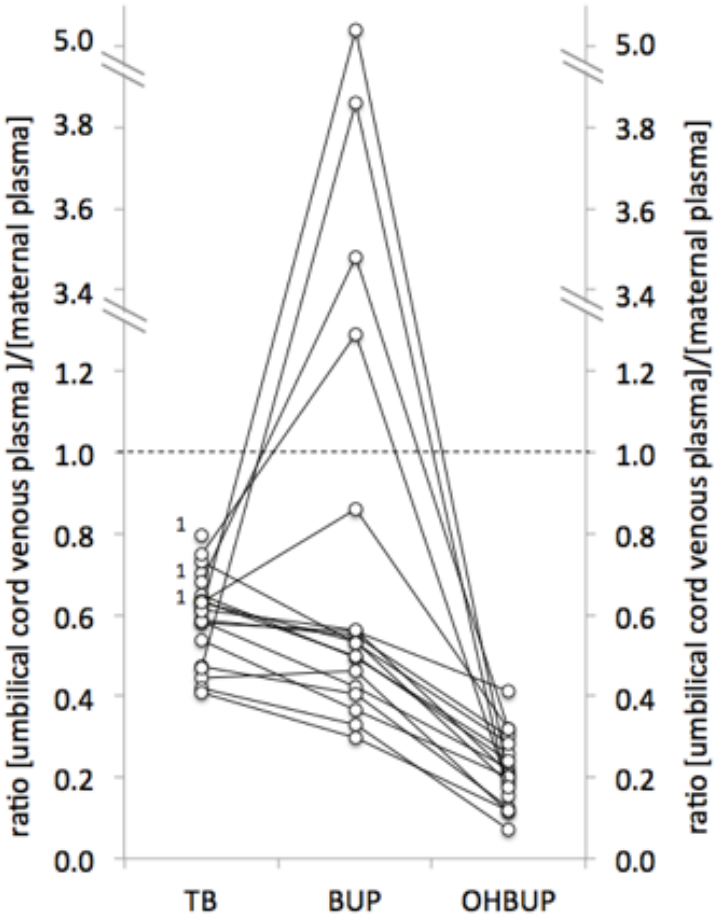

Figure 17. The individual umbilical cord venous plasma/maternal plasma ratios for bupropion and its metabolites. The lines connect the ratios obtained from a single subject. The dotted line at the ratio of 1.0 is provided for reference. BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion.1 BUP and OHBUP not available. Modified from Fokina et al., 2016a.

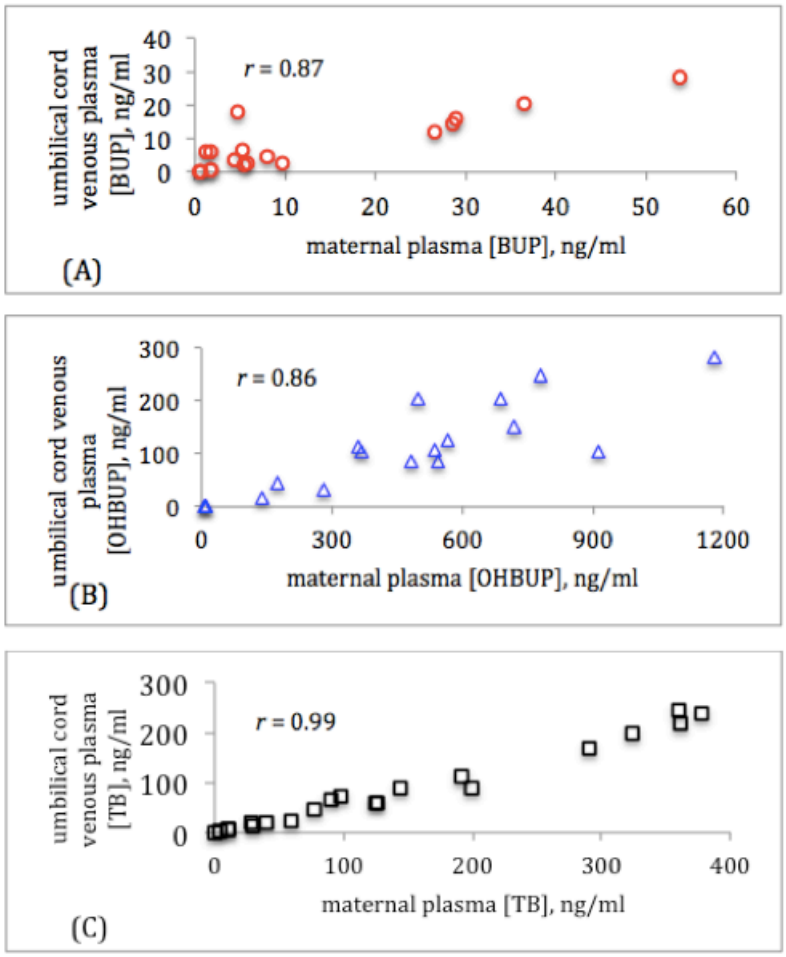

Figure 18. The concentrations of bupropion (A), OHBUP (B) and TB (C) in the umbilical cord venous plasma against those in the maternal plasma. BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; *r*, Pearson's correlation coefficient. Modified from Fokina et al., 2016a.

Table 8. Ratio of bupropion, TB and OHBUP in the umbilical cord venous plasma to the maternal plasma.

Subject ID	Age, years	Race/ethnicity	GA, Weeks	Bupropion dose,		Time between the last dose and delivery, hrs	Umbilical cord venous plasma (ng/ml)			Maternal venous plasma (ng/ml)			Umbilical cord venous plasma to maternal plasma ratio		
				mg	Frequency		BUP	OHBUP	TB	BUP	OHBUP	TB	BUP	OHBUP	TB
A	32	White/non-Hispanic	37[4]	150	BID	12	4.5	204.6	168.3	8.0	497.2	289.9	0.56	0.41	0.58
B	23	White/non-Hispanic	37[1]	150	BID	16	<LOD	<LLOQ	3.1	0.13	1.4	4.4	ND	ND	0.70
C	33	White/Hispanic	39	150	BID	NA	<LOD	<LLOQ	6.4	0.22	1.5	9.8	ND	ND	0.65
D	38	White/non-Hispanic	36[6]	150	BID	10	15.9	205.8	111.9	29.0	687.4	190.9	0.55	0.30	0.59
E	38	White/non-Hispanic	39[0]	150	BID	29	2.5	125.5	45.0	5.9	565.6	77.0	0.42	0.22	0.59
F	25	White/non-Hispanic	39[1]	150	BID	NA	<LOD	<LOD	<LOD	<LOD	<LOD	<LLOQ	ND	ND	ND
G	30	White/Hispanic	38[2]	150	BID	NA	0.24	0.83	6.6	0.4	4.4	10.4	0.53	0.19	0.63
H	30	Black/non-Hispanic	34[5]	150	BID	2	2.2	17.4	59.6	5.4	138.1	125.8	0.41	0.13	0.47
I	42	White/non-Hispanic	37[5]	300	QD	21	12.2	103.1	88.6	26.5	912.4	198.8	0.46	0.11	0.45
J	24	White/non-Hispanic	37[3]	300	QD	NA	20.4	150.5	198.0	36.4	716.6	323.8	0.56	0.21	0.61
K	25	White/non-Hispanic	40[1]	300	QD	NA	6.2	249.1	244.5	1.8	780.7	359.5	3.48	0.32	0.68
L	42	White/Hispanic	38[2]	75	BID	25	0.91	44.7	18.6	1.8	172.5	28.7	0.50	0.26	0.65
M	31	White/Hispanic	36[5]	75	BID	45	6.1	108.9	219.5	1.2	536.7	360.6	4.94	0.20	0.61
N	29	White/non-Hispanic	37[4]	75	BID	NA	18.1	84.3	58.7	4.7	542.5	124.9	3.86	0.16	0.47
O	24	White/non-Hispanic	39[2]	150	QD	20	2.9	32.9	24.0	9.7	279.6	58.9	0.30	0.12	0.41
P	21	White/non-Hispanic	39[0]	150	QD	48	0.7	2.3	21.1	1.9	11.8	39.4	0.36	0.20	0.54
Q	25	White/non-Hispanic	39[0]	150	QD	48	14.3	282.6	239.7	28.6	1,180.9	378.2	0.50	0.24	0.63
R	28	White/non-Hispanic	39[0]	150	QD	4	28.5	104.2	66.2	53.7	366.7	90.4	0.53	0.28	0.73
S	39	White/non-Hispanic	39[4]	150	QD	30	3.8	115.2	90.7	4.4	362.3	143.9	0.86	0.32	0.63
T	22	White/Hispanic	37[0]	150	QD	NA	0.22	0.70	12.4	0.68	9.7	29.6	0.33	0.07	0.42
U	25	White/Hispanic	41[2]	100	QD	NA	<LOD	<LOD	0.4	<LOD	<LLOQ	0.5	ND	ND	0.80
V	23	White/non-Hispanic	38[2]	75	QD	43	6.8	84.3	72.8	5.3	482.7	97.3	1.29	0.17	0.75
Median	28.5		38[4]				5.3	103.6	59.6	5.0	424.7	97.3	0.53	0.21	0.61
Mean	29.5		38[4]										1.14	0.22	0.60
STDEV	6.6		1[6]										1.40	0.09	0.11

QD, once daily, BID, twice per day; GA, gestational age; NA, not available; ND, not determined; BUP, bupropion; OHBUP, hydroxybupropion; TB, threohydrobupropion; LLOQ, lower limit of quantification; LOD, lower limit of detection.
Modified from Fokina et al., 2016a.

Table 9. The concentrations of bupropion, TB and OHBUP in the amniotic fluid and corresponding umbilical cord venous plasma and maternal plasma

Subject ID	Bupropion dose,		Bupropion, ng/ml			OHBUP, ng/ml			TB, ng/ml			
	mg	Frequency	Umbilical cord venous plasma	Amniotic fluid	Maternal plasma	Umbilical cord venous plasma	Amniotic fluid	Maternal plasma	Umbilical cord venous plasma	Amniotic fluid	Maternal plasma	Amniotic fluid: umbilical cord venous plasma ratio
C	150	BID	<LOD	<LOD	0.2	<LLOQ	<LLOQ	1.5	6.4	13.9	9.8	2.2
D	150	BID	15.9	48.0	29.0	205.8	185.2	687.4	111.9	399.4	190.9	3.6
E	150	BID	2.5	3.0	5.9	125.5	72.0	565.6	45.0	101.2	77.0	2.2
I	300	QD	12.2	13.3	26.5	103.1	123.2	912.4	88.6	157.1	198.8	1.8
J	300	QD	20.4	27.6	36.4	150.5	104.6	716.6	198.0	339.5	323.8	1.7
L	75	BID	0.9	1.9	1.8	44.7	38.8	172.5	18.6	63.6	28.7	3.4
O	150	QD	2.9	8.6	9.7	32.9	40.0	279.6	24.0	112.5	58.9	4.7
P	150	QD	0.7	0.7	1.9	2.3	1.2	11.8	21.1	52.7	39.4	2.5
Q	150	QD	14.3	14.1	28.6	282.6	77.3	1,180.9	239.7	291.0	378.2	1.2
Median			7.6	10.9	9.7	114.3	74.6	565.6	45.0	112.5	77.0	2.2
Mean												2.6
STDEV												1.1

QD, once daily, BID, twice per day; OHBUP, hydroxybupropion, TB, threohydrobupropion; LLOQ, lower limit of quantification; LOD, lower limit of detection
Modified from Fokina et al., 2016a.

The concentrations of bupropion and its metabolites in the amniotic fluid and the corresponding maternal plasma and umbilical cord plasma are presented in Table 9. The levels of bupropion and OHBUP were below their respective LOD and LLOQ in one sample of the amniotic fluid (subject C). The concentrations of OHBUP in the amniotic fluid were lower than in the maternal plasma and lower or similar to those in the venous cord plasma. The concentrations of bupropion in the amniotic fluid were lower than in the maternal plasma (with one exception, subject D). No patter was observed in the concentrations of the drug in the amniotic fluid relative to those in the umbilical venous plasma. On the other hand, the concentrations of TB in the amniotic fluid samples were unanimously higher than those in the corresponding cord venous plasma samples. Moreover, in seven out of nine subjects, the concentrations of TB in the amniotic fluid exceeded those in the maternal plasma (Table 9).

4.4. DISCUSSION

In treatment of depression with bupropion the dose of the drug ranges from 75mg to 450 mg per day and titrated depending on the patient's response to the therapy. The pregnant subjects enrolled in this study were receiving bupropion in the indicated doses irrespective of the current study, therefore variability in the plasma levels of the drug and its metabolites was expected. However, we observed that the concentrations of the drug and its metabolites in the maternal plasma at delivery varied widely among the individuals who were taking the same dose and formulation of the drug (Table 8). This could be explained in part by the differences in a time of the last dose prior to delivery (Table 8), however inter-individual variability in bupropion metabolism could be the

main factor contributing to the substantial differences in the plasma levels of the drug and its metabolites (Benowitz et al., 2013; Jefferson et al., 2005). As was mentioned in Section 1.4, bupropion undergoes extensive hepatic biotransformation via oxidative and reductive pathways (Bondarev et al., 2003). The formation of the primary metabolite, OHBUP, is predominantly catalyzed by CYP2B6 (Jefferson et al., 2005), while the formation of reductive metabolites, TB and EB, catalyzed by hepatic 11beta HSD1 and carbonyl reductases (Molnari and Myers, 2012). CYP2C19 also contributes to the metabolism of bupropion and its reductive metabolites, TB and EB (Zhu et al., 2012). Both CYP2C19 and CYP2B6 are highly polymorphic and the genetic variability of *CYP2C19* and *CYP2B6* has been associated with the altered plasma concentrations of bupropion and its metabolites in pregnancy (Sections 2.3 and 2.4) similar to the non-pregnant state (Benowitz et al., 2013; Zhu et al., 2014). In addition, data from *in vitro* and *ex vivo* experiments suggest that human placenta biotransformes bupropion (Earhart et al., 2010; Wang et al., 2010). TB, the major placental metabolite, was formed in the course of a 4-hour placental perfusion and was released into the maternal and fetal circulation of the model system (Earhart et al., 2010). We can speculate whether placental metabolism of bupropion *in vivo* is substantial enough to affect the levels of the drug and its metabolites in the maternal circulation. Nevertheless, placental active role in the disposition of bupropion is crucial in regulating fetal exposure to maternally administered drug and its metabolites.

In the current study, the concentrations of OHBUP and TB in the maternal plasma were invariably higher than in the corresponding umbilical cord venous plasma (Table 8). However, in 22% of maternal-fetal pairs (4 out of 18 available for this analysis), the

levels of bupropion in the umbilical cord venous plasma were higher than in the corresponding maternal plasma. These data suggest that maternally administered bupropion might accumulate in the fetal circulation, however these results could also be attributed in part to the retention of the drug by the placental tissue and the its following release in the fetal circulation. In addition, earlier report suggested that if sufficient time lapsed from the last dose the concentration of maternally administered medication in the fetal circulation can be higher than in the maternal, this occurs due to higher maternal clearance of the drug as compared to the fetal and placental clearance (Anderson et al., 1980).

We observed high linear correlation of OHBUP and TB concentrations in the umbilical cord venous plasma with the corresponding concentrations in the maternal plasma. This suggests that the levels of OHBUP and TB in the fetal circulation can be predicted form those in the maternal circulation. High correlation between the maternal and fetal levels was observed for bupropion, however the majority of the maternal plasma samples contained less than 10 ng/ml of the drug, and for those samples the correlation with the umbilical cord venous plasma was weak. Therefore, it appears that the fetal levels of bupropion can be predicted from the maternal levels in instances when bupropion levels in maternal plasma exceed 10 ng/ml. However, it should be noted that these conclusions are valid for term pregnancy and could not be directly extrapolated for earlier stages of gestation. Nevertheless, the concentrations of OHBUP and TB in the umbilical cord plasma were higher than those of bupropion, suggesting that fetus would be exposed *in utero* primarily to bupropion metabolites, which are less pharmacologically active as compared to the parent drug.

The presence of bupropion and its metabolites in the umbilical cord arterial plasma and their concentrations relative to those in the umbilical cord venous plasma would indicate whether umbilical artery is the main route for the elimination of the drug and its metabolites by the fetus. Further, higher levels of the metabolites in the umbilical cord arterial plasma than in the cord venous plasma would suggest that fetus is capable of metabolizing bupropion. In the majority of the available umbilical cord arterial and venous plasma pairs the concentrations of the drug and its metabolites were similar. Therefore, even if the fetus is capable to appreciably metabolize bupropion, it was not evident from the concentrations of OHBUP and TB in the umbilical cord arterial plasma relative to those in the umbilical cord venous plasma.

The concentrations of OHBUP in the amniotic fluid were similar to the umbilical cord venous plasma and lower than those of the maternal plasma. There was no such pattern for bupropion concentrations in the amniotic fluid, umbilical cord venous plasma and maternal plasma. However, the levels of TB were higher in the amniotic fluid samples than in all the corresponding umbilical cord venous plasma and, in several instances, higher than those in the maternal plasma.

During late gestation, fetal urine is the main constituent of the amniotic fluid and is produced at the rate up to 1000 ml/day. The rate of fetal swallowing of the amniotic fluid can reach up to 760 ml/day; fetal swallowing constitutes the main route of the amniotic fluid removal. Several reports demonstrated that CYP2B6 and 11βHSD are expressed in the fetal liver however their activity is low (Croom et al., 2009; Gupta et al., 2003; Ring et al., 1999). In addition, the expression and activity of fetal phase II enzymes was reported (Ekstrom et al, 2013; Divakaran et al., 2014). Specifically, UGT2B7, which

is responsible for OHBUP glucuronidation (Gufford et al., 2016), is expressed in fetal liver, lungs and kidneys (Ekstrom et al, 2013). Bupropion, along with its metabolites, is carried from the placenta by the umbilical cord vein towards the fetus, enters fetal liver; thus, it possibly undergoes hepatic metabolism. The waste products are then excreted into the amniotic fluid and recycled via swallowing, entering the fetal hepatic circulation. The observed higher concentration of TB in the amniotic fluid as compared to the cord venous plasma could indicate that reductive metabolism of bupropion does take place in the fetal liver. In addition, we can speculate that due to recirculation of the drug and its metabolites with the amniotic fluid, the fetal exposure to bupropion and its pharmacologically active derivatives could still occur even after pregnant patient stops taking the drug.

4.5. CONCLUSION

We confirmed *in vivo* that maternally administered bupropion and its metabolites cross the human placenta. The concentrations of OHBUP and TB in the fetal circulation can be predicted from the corresponding concentrations in the maternal plasma. The levels of OHBUP, TB and, with a few exceptions, bupropion in the fetal circulation were lower than those in the maternal, suggesting limited fetal exposure to the drug and its pharmacologically active metabolites. However, the higher blood brain barrier permeability in the fetus and the lower affinity of the fetal plasma proteins to xenobiotics can promote fetal exposure to bupropion and its metabolites that reached fetal circulation (Tong et al., 2009). Furthermore, the continuous fetal exposure to the drug and its metabolites via recirculation of the amniotic fluid is likely to occur.

4.6. Study limitations and future directions

We quantified bupropion, OHBUP and TB in the umbilical cord plasma at delivery in order to estimate the placental passage of the drug and its pharmacologically active metabolites in pregnant women taking the drug for treatment of depression. In addition, the levels of bupropion and its metabolites in the amniotic fluid were determined at delivery. The time of the last dose before delivery was not gathered for a number of subjects. However, when available, the time of the last dose indicated that a number of subjects skipped one or more recent dose(s) most likely due to hospitalization to the labor and deliver ward. Thus, the concentrations of the drug and its metabolites in maternal plasma did not correspond to the steady state, which could affect the estimated values obtained for the transplacental passage of the drug and its metabolites. Nevertheless, the data allowed estimating the extent of potential fetal exposure to maternally administered bupropion, although the results are valid for term pregnancy only. It is very likely that the extent of placental passage of the drug and its metabolites changes with the progression of pregnancy, similar to the gestational age-dependent changes in the enzymatic capacity of fetal liver. In addition, the samples of placental tissue were not collected in our study; therefore, we could not estimate the drug and metabolites retention by the tissue *in vivo*, which was a limitation of our investigation.

This was an opportunistic study; therefore, gathering the obstetrical and neonatal outcomes was not feasible. Bupropion and its pharmacologically active metabolites that are present in the fetal circulation can interfere with the cholinergic signaling in the fetal central nervous system affecting neurodevelopment. To date, little is known about the

possible impact of the maternally administered bupropion on the long-term neuropsychological development of the offspring. A recent study determined the association between the exposure to bupropion *in utero* and the risk of developing attention deficit hyperactivity disorder (ADHD), however the causal effect of the drug on this disorder has not been confirmed (Figueroa, 2010). Additional studies are needed to investigate whether the extent of fetal exposure to the drug is correlated with the neonatal outcomes and behavioral problems.

Further, similar to the pharmacokinetic study of bupropion in pregnancy (Section 2.6), we did not measure the enantiomers of bupropion and its metabolites, which was a limitation of our study. As was mention in Section 1.4, bupropion is a racemic mixture; 2S,2S-OHBUP enantiomer has higher potency than the parent drug and 2R,2R-OHBUP enantiomer, however the levels of 2S,2S-OHBUP in the blood are lower than those of 2R,2R-OHBUP. Further investigations are needed to determine whether the placental passage of OHBUP is stereospecific. Higher rate of placental transfer of 2S,2S-OHBUP than 2R,2R-OHBUP could lead to undesirable fetal exposure to the derivative of bupropion which has higher pharmacological activity than the parent drug. In addition, quantification of conjugated TB and OHBUP in the umbilical cord plasma and amniotic fluid would help to determine the extent of glucuronidation in the fetal elimination of maternally administered bupropion and its pharmacologically active metabolites.

CHAPTER 5: SUMMARY

Bupropion is used in treatment of depression during pregnancy, however the drug is not routinely used as a smoking cessation aid for pregnant smokers. It appears that the effect of pregnancy on biodisposition of bupropion and its pharmacologically active metabolites is multifactorial. As a result, the systemic exposure to the parent drug could slightly decrease in pregnancy, while the exposure to its pharmacologically active metabolite, OHBUP, appears similar to that of the non-pregnant state. Our data suggest that the effects of *CYP2B6* and *CYP2C19* functional polymorphisms on the phenotypic variations in bupropion pharmacokinetics in pregnancy are similar to those observed in men and non-pregnant females. Additional studies are needed to determine whether there is a correlation between the *CYP2B6*6* variant allele and the quit rate in pregnant smokers treated with bupropion for smoking cessation. Data from *in vitro* studies indicated that bupropion therapy during pregnancy should not impact the detoxifying capacity of placental carbonyl reducing enzymes towards NNK, the potent pro-carcinogen of cigarette smoke. Data from *in vivo* investigation revealed that fetal exposure to maternally administered bupropion could occur via transplacental transfer and amniotic fluid recirculation. Further studies are needed to investigate whether the exposure to bupropion and its metabolites *in utero* can have long-term behavioral and neurodevelopmental consequences.

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