Title:

Longitudinal Influence of Pregnancy on Nicotine Metabolic Pathways

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Abbreviations:

3HC   3'-hydroxycotinine

CNO   Cotinine N-oxide

COT   Cotinine

COT-GLUC   Cotinine glucuronide

CPD   Cigarettes smoked daily

NCOT   Norcotinine

NIC   Nicotine

NIC-GLUC   Nicotine glucuronide

NNIC   Nornicotine

NNO   Nicotine N-oxide

NRT   Nicotine replacement therapy

TNE   Total nicotine equivalents

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Abstract

Nicotine metabolism increases in pregnancy which may contribute to the difficulties that pregnant women have in quitting smoking. We aimed to determine the extent and timing of changes in nicotine metabolic pathways, including C-oxidation, N-glucuronidation, and the pregnancy-induced influences on the activity of enzymes mediating these pathways (CYP2A6 and UGT2B10, respectively). Current smoking pregnant women (n=47) provided a urine sample during early pregnancy (12.5 weeks), late pregnancy (28.9 weeks), and 6 months postpartum. Concentrations of urinary nicotine and metabolites were analyzed using LCMS/MS and compared using general linear repeated measures analyses. Nicotine C-oxidation was 1.07 (P=0.12) and 1.11 fold (P<0.001) higher at early and late pregnancy, respectively, compared to postpartum. Nicotine N-glucuronidation was 1.33 (P=0.06) and 1.67 fold (P=0.003) higher at early and late pregnancy, respectively, compared to postpartum. The CYP2A6 phenotype ratio (total 3'-hydroxycotinine/cotinine) was significantly higher at early and late pregnancy compared to postpartum (all P<0.05) and correlated with nicotine C-oxidation (all P<0.001), suggesting CYP2A6 activity is induced during pregnancy. The UGT2B10 phenotype ratio (nicotine glucuronide/nicotine) was higher at early and late pregnancy compared to postpartum (P=0.07 and P<0.05, respectively) and correlated with a second UGT2B10 phenotype ratio (cotinine glucuronide/cotinine) (all P<0.001) suggesting UGT2B10 activity is induced during pregnancy. In conclusion, pregnancy-induced increases in nicotine metabolism start by 12 weeks gestation and continue as pregnancy progresses likely due to induction of CYP2A6 and UGT2B10 resulting in potential reductions in the effectiveness of nicotine replacement therapies and an increase in metabolism of other CYP2A6 and UGT2B10 substrates during pregnancy.

Introduction

Maternal cigarette smoking is the leading preventable cause of poor pregnancy outcomes (Ebrahim et al., 2000). Approximately 14% of U.S. women continue to smoke after becoming pregnant (Kurti et al., 2017). Nicotine replacement therapy (NRT) is used in assisting smokers to quit. Despite its effectiveness in non-pregnant populations, NRT is not effective during pregnancy (Coleman et al., 2012; Berlin et al., 2014). One potential reason for this is that nicotine (NIC) metabolic clearance is increased during pregnancy (Dempsey et al., 2002; Bowker et al., 2015) suggesting the dose of NIC in NRT may be insufficient to adequately reduce cigarette cravings. Increased NIC's metabolic clearance

In non-pregnant populations, the major NIC metabolic pathway is the inactivation to cotinine (COT), followed by further metabolism of COT to 3'-hydroxycotinine (3HC); these are C-oxidation processes which are 90% and 100% mediated by the CYP2A6 enzyme, respectively (Benowitz and Jacob, 1994; Nakajima et al., 1996a; Messina et al., 1997). The ratio of 3HC to COT (the nicotine metabolite ratio, NMR), is a phenotypic biomarker of CYP2A6 enzymatic activity and NIC metabolism rate (Dempsey, Tutka et al. 2004). NMR is highly correlated with the rate of total NIC clearance due to the major role of CYP2A6 in NIC's metabolism (Hukkanen, Jacob et al. 2005). Higher NMR is associated with decreased odds of achieving cessation in pregnant (Vaz et al., 2015) and non-pregnant populations (Lerman et al., 2006; Patterson et al., 2008) both in the absence and presence of pharmacotherapy.

Non C-oxidation pathways of NIC metabolism include glucuronide conjugation (Byrd et al., 1992), primarily mediated by UGT2B10, into nicotine glucuronide (NIC-GLUC) (Chen et al., 2007; Kaivosaari et al., 2007; Kato et al., 2013) and N-oxidation, primarily by FMO3, into nicotine N-oxide (NNO) (Byrd et al., 1992; Cashman et al., 1992). Small amounts of nornicotine (NNIC) are also formed as a product of oxidative N-demethylation by CYP2B6 (Yamanaka et al., 2005). COT is further metabolized to cotinine glucuronide (COT-GLUC) primarily by UGT2B10 (Chen et al., 2007). Small amounts of cotinine N-oxide (CNO) and norcotinine (NCOT) are also formed by FMO3 and CYP2B6, respectively (Benowitz

and Jacob, 1994). 3HC is further metabolized into 3HC glucuronide (3HC-GLUC) primarily by UGT2B17 (Byrd et al., 1992; Chen et al., 2012).

In an intravenous pharmacokinetic study of deuterium-labeled NIC-d2 and COT-d4, among ten pregnant smokers, the metabolic clearance of NIC and COT was substantially increased during pregnancy compared to postpartum (Dempsey et al., 2002). More recently, salivary and urinary NMR have been shown to be higher during pregnancy likely due to increased metabolism of COT to 3HC (Bowker, Lewis et al. 2015; Arger, Taghavi et al. 2017 under review). Whether the enzyme is CYP2A6, which is responsible for most COT metabolism in the non-pregnant individual, or whether other C-oxidation enzymes are induced in pregnancy remains to be determined. Potential pregnancy-mediated induction of NIC metabolizing enzymes' activity, including CYP2A6 and UGT2B10, may alter the metabolism of several other clinically relevant substrates.

Using urine samples to provide within-subject measurements of NIC and all nine metabolites at early pregnancy, late pregnancy, and postpartum, our study aimed to describe the timing and magnitude of changes in the profile of all NIC metabolites and metabolic pathways including C-oxidation, N-glucuronidation, O-glucuronidation, and N-oxidation across pregnancy. A second aim was to investigate the impact of pregnancy on activity of NIC metabolizing enzymes using the ratios of a metabolite to the parent compound (metabolite ratios) as biomarkers of enzyme activity.

Methods

Participants

Participants were recruited from a randomized clinical trial examining the effects of financial incentives on smoking abstinence (Higgins et al., 2012). Subjects (n=47) included all current smoking pregnant women who provided a spot urine sample at all three time points, during early pregnancy (estimate gestational age (EGA) 12.5 ± 4.5 weeks), late pregnancy (EGA 28.9 ± 2.0 weeks), and at 6 months

postpartum (24.7 ± 1.2 weeks since birth). All subjects provided written informed consent. This study was approved by the institutional review boards at the University of Vermont and University of Toronto.

Analytical procedures

Urinary concentrations of NIC and its nine metabolites were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (Taghavi, Novalen et al. submitted and under review). Urine samples were diluted and prepared using solid-phase extraction adapted from a previously established method (Miller et al., 2010). The limit of quantification (LOQ) was 1 ng/ml for all compounds.

Creatinine correction

Urinary creatinine concentrations were determined using a colorimetric assay according to protocol provided in the Creatinine Assay Kit (MAK080) purchased from Sigma (St. Louis, MO) with a SynergyMX Analyzer (BioTek, Winooski, VT, USA). There was a significant step-wise increase in urinary creatinine from early to late pregnancy to postpartum (Figure-S1 A), suggesting creatinine levels during pregnancy may no longer accurately represent only urine dilution, but rather reflect a combination of differences in urine dilution and pregnancy-mediated physiological changes in creatinine clearance, as observed previously (Davison and Noble, 1981). Thus, to reduce the effect of pregnancy on creatinine levels in order to provide a better measure of urine dilution, we scaled individual urinary creatinine data at early and late pregnancy to the mean of urinary creatinine data at postpartum taken as baseline (See supplementary material Figure-S1 for details of the scaling procedure). Following this scaling, differences in individual creatinine levels were used as a proxy to correct for urine dilution.

Total nicotine equivalents (TNE), in nmol/mg creatinine, was calculated as the molar sum of NIC and all nine metabolites (COT, 3HC, NIC-GLUC, COT-GLUC, 3HC-GLUC, NNO, CNO, NNIC, and NCOT) and was corrected using scaled creatinine data. Data for TNE without creatinine correction, and corrected

for creatinine using un-scaled data are presented in supplementary Figure-S2. Other variables were expressed as a fraction of TNE, or ratios, and therefore were not creatinine corrected.

Statistical analysis

NIC C-oxidation was measured by the molar sum of all metabolites formed by C-oxidation (i.e. COT, 3HC, COT-GLUC, 3HC-GLUC, CNO, NNIC, and NCOT) as a fraction of TNE. Similarly, NIC non C-oxidation pathway was estimated as the molar sum of metabolites generated via pathways other than by C-oxidation (i.e. NIC-GLUC and NNO) as a fraction of TNE. CYP2A6, UGT2B10, UGT2B17, and FMO3 phenotypes were estimated using the ratio of a metabolite to the parent compound. General linear repeated measures analysis with Bonferroni correction for multiple testing was used to compare patterns of NIC metabolites (individual metabolites as a fraction of TNE), NIC metabolic pathways (C-oxidation, N-glucuronidation, O-glucuronidation, N-oxidation), and enzyme phenotypic metabolite ratios (CYP2A6, UGT2B10, UGT2B17, FMO3) longitudinally across the three time points. Although urinary pH levels increased over the course of pregnancy (Arger, Taghavi et al. 2017 submitted and under review), they did not alter individual metabolite concentrations as a fraction of TNE, metabolic pathways, and enzyme phenotypes; thus, all results presented are unadjusted for pH level. All analyses were conducted with GraphPad Prism (v5.0; La Jolla, CA) and SPSS (v24.0; IBM).

Results

Baseline characteristics

The baseline demographic and smoking variables for 47 subjects are presented in Table-1. The subjects were predominantly Caucasian and smoked on average 20 cigarettes per day prior to pregnancy.

Influence of pregnancy on the profile of NIC metabolites

Overall, as a fraction of total NIC and metabolites excreted in urine (i.e. TNE), only 3HC-GLUC was not significantly altered during pregnancy and postpartum (Table-2). NIC, NNO, CNO, NNIC, and NCOT as

a fraction of TNE, were lower at early pregnancy compared to postpartum, while COT-GLUC was increased. NIC, COT, NNO, and CNO were lower at late pregnancy compared to postpartum while 3HC, NIC-GLUC, and COT-GLUC were increased. Only NIC and NCOT were different from early to late pregnancy; NIC was significantly lower at late compared to early pregnancy, while NCOT was increased (Table-2).

Influence of pregnancy on NIC's metabolic pathways

Pregnancy resulted in significant changes in the enzymatic pathways of NIC metabolism. NIC C-oxidation, measured as the molar sum of all metabolites generated by C-oxidation as a fraction of TNE, was 1.07 (non-significantly, P=0.12) and 1.11 fold (P<0.001) higher at early and late pregnancy compared to postpartum (Figure-1 & 2B). NIC N-glucuronidation was 1.33 (non-significantly, P=0.06) and 1.67 fold (P=0.003) higher at early and late pregnancy compared to postpartum (Figure-1 & 2C). In contrast, NIC N-oxidation was 1.33 (P=0.005) and 1.60 fold (P<0.001) lower at early and late pregnancy compared to postpartum (Figure-1 & 2D). NIC excreted unchanged, as a fraction of TNE, was 1.83 (P=0.03) and 5.5 fold (P<0.001) lower at early and late pregnancy compared to postpartum (Figure-1 & 2A).

NIC C-oxidation and N-glucuronidation were 1.04 (non-significantly, P=0.11) and 1.25 fold (non-significantly, P=0.24) higher at late compared to early pregnancy, respectively, while NIC N-oxidation was 1.20 fold (non-significantly, P=0.42) lower at late compared to early pregnancy. NIC excreted unchanged, as a fraction of TNE, was 3.00 fold (P=0.001) lower at late compared to early pregnancy (Figure-1 & 2D).

Pregnancy-mediated induction of NIC and COT metabolizing enzymes

To investigate whether the increase in NIC C-oxidation during pregnancy compared to postpartum is related to an induction in the CYP2A6 enzyme, we examined the longitudinal effect of pregnancy on the ratio of 3HC+3HC-GLUC/COT (urinary total 3HC/COT), which is used as a phenotypic measure of

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CYP2A6-mediated C-oxidation activity in non-pregnant smokers (Dempsey, Tutka et al. 2004; Murphy, Park et al. 2014; Arger, Taghavi et al. 2017 submitted and under review). Prior studies have shown that CYP2A6-mediated C-oxidation activity can be measured by various other metabolite ratios (e.g. 3HC/COT and COT/NIC) in plasma or urine depending on smoking status (Bloom et al., 2011; Strasser et al., 2011; Lerman et al., 2015). In urine, the 3HC+3HC-GLUC/COT is the best ratio in regular smokers, both theoretically (i.e. total products over free enzymatic substrate) and because among regular smokers this ratio is independent of time since last cigarette (St Helen et al., 2012) and shows the best correlation to CYP2A6 activity measured in plasma by 3HC/COT. The 3HC+3HC-GLUC/COT ratio was 1.56 (P=0.02) and 2.00 fold (P<0.001) higher at early and late pregnancy compared to postpartum (Table-3, Figure-3). This ratio correlated significantly and similarly with NIC C-oxidation, measured as the molar sum of all metabolites generated by C-oxidation as a fraction of TNE, at each time point (early pregnancy, Spearman rho=0.60, P<0.001; late pregnancy, Spearman rho=0.59, P<0.001; postpartum, Spearman rho=0.55, P<0.001) suggesting CYP2A6 activity, responsible for the first step in NIC C-oxidation, is induced during pregnancy compared to postpartum. When comparing early to late pregnancy, the 3HC+3HC-GLUC/COT ratio was 1.28 fold (non-significantly, P=0.29) higher at late compared to early pregnancy (Table-3, Figure-3), suggesting CYP2A6 activity may increase as pregnancy progresses.

Similarly, we used the ratio of NIC-GLUC/NIC and COT-GLUC/COT as phenotypic measures of UGT2B10-mediated N-glucuronidation activity, as UGT2B10 is the main enzyme involved in NIC and COT N-glucuronidation in non-pregnant smokers (Byrd et al., 1992; Chen et al., 2007). NIC-GLUC/NIC was 2.13 (non-significantly, P=0.08) and 4.22 fold (P=0.02) higher at early and late pregnancy compared to postpartum (Table-3, Figure-3). COT-GLUC/COT was 1.89 (P=0.006) and 2.50 fold (P<0.001) higher at early and late pregnancy compared to postpartum (Table-3, Figure-3). Both ratios were significantly and similarly correlated with each other at each time point (early pregnancy, Spearman rho=0.47, P=0.001; late pregnancy, Spearman rho=0.46, P=0.001; postpartum, Spearman rho=0.50, P<0.001). Higher NIC and COT N-glucuronidation ratios at early and late pregnancy compared to postpartum

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suggests UGT2B10 activity is induced during pregnancy compared to postpartum. When comparing early to late pregnancy, NIC-GLUC/NIC and COT-GLUC/COT ratios were 1.98 (non-significantly, P=0.27) and 1.32 fold (non-significantly, P=0.40) higher at late compared to early pregnancy, respectively (Table-3, Figure-3), suggesting UGT2B10 activity may increase as pregnancy progresses.

Lastly, we used the ratio of 3HC-GLUC/3HC as a phenotypic measure for UGT2B17-mediated O-glucuronidation activity, as UGT2B17 is the main enzyme involved in 3HC O-glucuronidation in non-pregnant smokers (Kuehl and Murphy, 2003). The ratio of 3HC-GLUC/3HC was not altered at early (P=1.00) and late pregnancy (P=0.41) compared to postpartum consistent with no change in the proportion of TNE excreted as 3HC-GLUC (Table-2 & 3), suggesting UGT2B17 activity is not changed during pregnancy compared to postpartum. Similarly, when comparing early to late pregnancy, the 3HC-GLUC/3HC ratio remained more or less constant (P=0.79) (Table-3), suggesting UGT2B17 activity is not altered as pregnancy progresses. The FMO3-mediated N-oxidation (NNO/NIC and CNO/COT) and CYP2B6-mediated N-demethylation (NNIC/NIC and NCOT/COT) phenotype ratios were higher at early and late pregnancy compared to postpartum, but this difference did not reach statistical significance.

Discussion

Our study describes the urinary profile of NIC and all nine metabolites from 12 weeks gestation to 6 months postpartum. First, we found the excretion of NIC was substantially decreased, with a small increase in the excretion of NIC-GLUC, and a substantial increase in the excretion of COT-GLUC and 3HC, and no change in the excretion of 3HC-GLUC. Second, we found a substantial increase in NIC C-oxidation and N-glucuronidation with a small decrease in N-oxidation and no change in O-glucuronidation pathways. Third, we found evidence for CYP2A6 and UGT2B10 induction including (1) a substantial increase in and (2) a high correlation between two metabolite phenotype ratios commonly used to phenotype each of CYP2A6 and UGT2B10 in non-pregnant populations during pregnancy. Most

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changes were present as early as 12 weeks gestation in early pregnancy and were even greater at 29 weeks gestation, during late pregnancy, before decreasing following delivery.

Our data suggests NIC metabolic clearance is accelerated during pregnancy primarily through faster C-oxidation. CYP2A6 is the major enzyme involved in NIC C-oxidation in non-pregnant populations (Messina et al., 1997), but other enzymes such as CYP2B6 are also involved (Nakajima et al., 1996b; Al Koudsi and Tyndale, 2010). Unlike for NIC, CYP2B6 does not contribute to C-oxidation of COT to 3HC. As such, we used the ratio of total 3HC to COT (NMR) and the sum of all metabolites produced by C-oxidation as a fraction of TNE to estimate CYP2A6 activity. We found both indices increased as early as 12 weeks gestation and continued to increase at 29 weeks gestation compared to postpartum. We also found the two indices to be similarly (i.e. with a similar correlation coefficient) and significantly correlated with each other at each time point providing indirect evidence that CYP2A6 (and likely not CYP2B6) is induced during pregnancy. This finding has implications for other clinically relevant substrates of CYP2A6; pregnant women who are being treated with CYP2A6 substrate drugs may require dose adjustments to achieve therapeutic effects.

Our data suggests higher NIC N-glucuronidation also contributes to the increased NIC metabolic clearance during pregnancy. UGT2B10 is the primary enzyme involved in NIC N-glucuronidation in non-pregnant populations (Chen et al., 2007; Kaivosaari et al., 2007; Kato et al., 2013), but UGT1A3, 1A4, 1A9, and 2B7 are also involved (Nakajima et al., 2002; Kuehl and Murphy, 2003; Kaivosaari et al., 2007). UGT2B10 is responsible for N-glucuronidation of both NIC and COT (Chen et al., 2007). COT N-glucuronidation has not been detected by UGT1A3 and 1A9, or has been detected at negligibly low levels by UGT1A4 and 2B7 (Kuehl and Murphy, 2003; Al Koudsi et al., 2006; Kaivosaari et al., 2007). As such, we used the ratio of NIC-GLUC to NIC and COT-GLUC to COT to estimate UGT2B10 activity. Consistent with higher NIC-GLUC and COT-GLUC excreted as a fraction of TNE, we found both indices increased as early as 12 weeks gestation and continued to increase at 29 weeks gestation compared to postpartum. We also found the two indices to be similarly and significantly correlated with each other at

each stage; thus, providing indirect evidence that UGT2B10 is induced during pregnancy. Therefore, pregnant women who are being treated with UGT2B10 substrates may also require dose adjustments to achieve therapeutic effects.

Non-pregnant women who use estrogen-containing contraceptives or hormonal replacement therapies metabolize NIC faster compared to women using non-estrogen versions (or not taking any hormones) consistent with the estrogen induction of CYP2A6 (Higashi et al., 2007) and faster NIC metabolism by women than men (Benowitz et al., 2006). During pregnancy, estrogen levels begin to rise after conception and are approximately 5-fold and 20-fold higher at early and late pregnancy, respectively, compared to pre-pregnancy levels. Consistent with the increase in estrogen between early and late pregnancy, we found an increase in CYP2A6-mediated C-oxidation in early pregnancy which increased further at late pregnancy before decreasing following delivery. This suggests that the elevated estrogen during pregnancy may induce CYP2A6 leading to faster NIC metabolism.

Although NIC and COT are both metabolized by CYP2A6 and UGT2B10, NIC's metabolism appears to be more affected than COT's metabolism as evident by a larger decrease in the proportion of NIC compared to the proportion of COT that is excreted unchanged (as a fraction of TNE) at early and late pregnancy compared to postpartum. The pregnancy-mediated increase in NIC and COT metabolism may be multifactorial. NIC is a high extraction ratio drug and its rate of clearance is primarily controlled by the liver blood flow (Lee et al., 1989; Nakajima et al., 1996a). COT is a low extraction ratio drug and its rate of clearance is primarily determined by the level of metabolizing enzymes in the liver (Nakajima et al., 1996a). There is a substantial increase in cardiac output during pregnancy, which would be expected to be associated with increased liver blood flow. Thus, the increase in liver blood flow may disproportionately increase NIC clearance. Of note, pregnancy-mediated increases in liver blood flow may have implications for clearance of other drugs with high extraction ratio, such as morphine and lidocaine.

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During pregnancy, it is possible that placenta may be involved in the increased clearance of NIC and COT. NIC crosses the placental barrier but there is no evidence of the nicotine metabolite COT in placental tissue or microsomal fractions (Pastrakuljic et al., 1998). This is consistent with *in vitro* findings of very little CYP2A6 activity in the placenta. Low glucuronidation activity has been detected in the placenta, but it is not clear whether the UGT2B10 isoform is present (Collier et al., 2002; Tutka et al., 2008). The observation that NIC crosses the placenta with little evidence of metabolism to COT suggests that placenta is unlikely to be a major contributor to higher NIC metabolism in pregnancy. Similarly, during pregnancy, it is possible that fetal metabolism may account for a percentage of NIC total clearance. The pharmacokinetics of NIC differ in the fetus compared to the mother. The primary mechanism of clearance of NIC from the fetus is via transfer back to the maternal circulation (Suzuki et al., 1974). Thus, fetal metabolism accounts for only a small percentage of total NIC clearance and is unlikely to contribute substantially to higher NIC metabolism observed in pregnancy.

The increase in the metabolic clearance of NIC would be expected to result in a compensatory increase in NIC intake among pregnant smokers. However, a recent study found no evidence of compensatory smoking when comparing the smoking topography of pregnant and non-pregnant female smokers (Bergeria et al., 2017). Consistent with this, the daily intake of NIC (when measured by the TNE) was reduced during pregnancy compared to postpartum in the present study. The majority of pregnant smokers who quit or make reductions in their smoking report doing so soon after learning they are pregnant (Heil et al., 2014); therefore, the desire to protect the fetus and sensitivity to social expectations around smoking during pregnancy likely outweighs the influence of increased NIC metabolism on tobacco consumption during this time.

A strength of our study is the within-subject design where all subjects provided urine samples at all three time points allowing us to demonstrate the consistency of our metabolic and enzyme pathway findings. Our study expands on previous *in vivo* pharmacokinetics and metabolite data (Dempsey et al., 2002) by examining a much larger sample set and having additional time points allowing for comparison of the

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early and late stages of pregnancy while maintaining the same within-subject design. We also expand on previous findings of overall higher salivary NMR during pregnancy (Bowker et al., 2015) by providing a detailed analysis of individual NIC metabolic pathways including C-oxidation, N-glucuronidation, O-glucuronidation, and N-oxidation.

A limitation of our study is the assumption that the 6 months postpartum sample provides metabolism measurements that are comparable to that of baseline prior to pregnancy. Ideally, the baseline sample would have been collected before conception and participants would have been followed into pregnancy, however, this was not possible in the current study design. A second limitation is that we did not assess contraceptive/hormonal use at 6 months postpartum. If subjects were taking estrogen-containing contraceptives at 6 months postpartum, the relative changes during pregnancy may have been underestimated. Moreover, information on the timing of sample collection, or the time since smoking, for each participant before sample collection was not collected, but should not substantially alter this urinary metabolic ratio among current regular smokers due to cotinine's long half-life, and 3HC's formation dependence (Benowitz et al., 1983; Benowitz and Jacob, 2001; Lea et al., 2006; St Helen et al., 2012).

In conclusion, we showed that the metabolic clearance of NIC is increased during pregnancy via faster C-oxidation and N-glucuronidation and provided indirect evidence suggesting the activity of CYP2A6 and UGT2B10 enzymes are responsible for this increase. Our findings have implications for the use of clinically relevant substrates of these enzymes in pregnancy, in particular NRT, and provide insights into smoking behavior and cessation in pregnancy.

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Authorship Contributions:

Participated in research design: *Heil, Higgins, and Tyndale*

Conducted experiments: *Taghavi and Arger,*

Contributed new reagents or analytic tools: *Taghavi, Arger, Heil, Higgins, and Tyndale*

Performed data analysis: *Taghavi*

Wrote or contributed to the writing of the manuscript: *Taghavi, Arger, Heil, Higgins, and Tyndale*

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Footnotes:

A preliminary account of this work will be presented at the following meeting: Taghavi T, Arger CA,

Heil SH, Higgins ST, and Tyndale RF (2017) Changes in nicotine metabolic pathways across pregnancy:

A longitudinal study, presented at the 21st North American ISSX Meeting; 2017 September 24-28;

Providence, RI.

Figure legends:

Figure-1: Change in nicotine metabolite profile during pregnancy and postpartum

The metabolic profile of nicotine is depicted by pie charts representing the molar amount of each metabolite found in urine, expressed as a percentage of TNE. Also shown are the molar sum of all nicotine metabolites formed by C-oxidation (i.e. cotinine (COT), 3HC, cotinine glucuronide (COT GLUC), 3HC glucuronide (3HC GLUC), nornicotine (NNIC), norcotinine (NCOT), and cotinine N-oxide (CNO)), and metabolites generated via pathways other than by C-oxidation (nicotine glucuronide (NIC GLUC), nicotine N-oxide (NNO)), and nicotine (NIC) excreted unchanged as a percentage of TNE. The size of each pie reflects the 41% and 48% lower level of TNE at early and late pregnancy, respectively, compared to postpartum. Statically significant differences between the proportions of metabolic pathways (C-oxidation, non C-oxidation, nicotine (unchanged)) are indicated by an asterisk (*) on the early and late pregnancy pie charts and are for comparisons of early and late pregnancy stages to postpartum. Statistically significant differences indicated by a pound (#) on the early pregnancy pie chart are for comparison of early pregnancy to late pregnancy.

* Indicates significant differences from postpartum as $P<0.05$.

*** Indicates significant differences from postpartum as $P<0.001$.

indicates significant differences from early to late pregnancy as $P<0.01$.

Figure-2: Influence of pregnancy on nicotine metabolic pathways during pregnancy and postpartum

Data are shown for individual subjects with lines connecting each subject's pregnancy and postpartum data. Bars indicate mean ± SEM. *P*-values are for the main effect of time derived from general linear repeated measures analysis. Statistics for post-hoc comparisons between each stage of pregnancy are presented in the results section. One outlier was removed from illustration in (D), but included in the statistical analysis.

Figure-3: Influence of pregnancy on CYP2A6 (A), UGT2B10 (B-C), UGT2B17 (D), and FMO3 (E)

phenotypic metabolic ratios during pregnancy and postpartum

Data are shown for individual subjects with lines connecting each subject's pregnancy and postpartum

data. Bars indicate mean ± SEM. *P*-values are for the main effect of time derived from general linear

repeated measures analysis. Statistics for post-hoc comparisons between each stage of pregnancy are

presented in the results section. One outlier was removed from illustration in (D), but included in the

statistical analysis.

JPET #245126

Table-1: Baseline smoking and demographic characteristics

% or mean ± SEM	Total sample (n=47)
Age of smoking initiation	15.2 ± 0.5
Age at intake	24.5 ± 0.8
% Caucasian	89%
Education years	12.3 ± 0.2
MNWS Score	1.5 ± 0.1
CPD prior to pregnancy	19.6 ± 1.3
CPD at early pregnancy	10.4 ± 1.0
CPD at late pregnancy	7.2 ± 0.9
CPD at 6 months post-partum	8.6 ± 0.8
% with ≥1 quit attempts after learning of pregnancy	23%

Abbreviations: SEM, standard error of the mean; MNWS, Minnesota Nicotine

Withdrawal Scale; CPD, cigarettes smoked per day.

JPET #245126

Table-2: Nicotine and metabolites, as a fraction of TNE, during pregnancy and postpartum

Fraction of compounds	Early Pregnancy	Late Pregnancy	Postpartum	Main effect of time
NIC	6% (7%)*##	2%(3%)***	11% (11%)	**P<0.001**
COT	10% (6%)	8% (5%)***	12% (5%)	**P=0.001**
3HC	40% (12%)	43% (14%)*	37% (13%)	**P=0.026**
NIC-GLUC	8% (7%)	10% (8%)**	6% (3%)	**P=0.004**
COT-GLUC	20% (9%)**	24% (10%)***	17% (7%)	**P<0.001**
3HC-GLUC	5% (2%)	5% (2%)	4% (2%)	P=0.068
NNO	6% (4%)**	5% (4%)***	8% (4%)	**P<0.001**
CNO	3% (1%)***	3% (1%)***	4% (1%)	**P<0.001**
NNIC	0.6% (0.4%)*	0.7% (0.8%)	1% (1%)	**P=0.035**
NCOT	0.2% (1%)***#	1% (2%)	1% (0.6%)	**P<0.001**

Mean and standard deviation (in parentheses, illustrating the group variation) of each metabolite as a fraction (i.e. percentage) of creatinine-corrected TNE and results from general linear repeated measures models with post-hoc analyses correcting for multiple testing (Bonferroni) across the three pregnancy stages.

* Indicates significant differences from postpartum as *P*<0.05.

** Indicates significant differences from postpartum as *P*<0.01.

*** Indicates significant differences from postpartum as *P*<0.001.

\# indicates significant differences from early to late pregnancy as *P*<0.05.

\#\# indicates significant differences from early to late pregnancy as *P*<0.01.

Table-3: Phenotype ratios of nicotine metabolism enzymes during pregnancy and postpartum

Enzyme phenotypes	Early Pregnancy	Late Pregnancy	Postpartum	Main effect of time
3HC+3HC-GLUC/COT	8.1 (8.0)*	10.4 (8.0)***	5.2 (3.5)	***P*<0.001**
NIC-GLUC/NIC	4.9 (7.0)	9.7 (17.5) *	2.3 (4.0)	***P*=0.006**
COT-GLUC/COT	3.4 (3.8)**	4.5 (3.6) ***	1.8 (1.3)	***P*<0.001**
3HC-GLUC/3HC	0.12 (0.04)	0.12 (0.04)	0.11 (0.06)	*P*=0.19
NNO/NIC	4.0 (6.8)	4.1 (3.9)	2.8 (4.7)	*P*=0.33
CNO/COT	0.44 (0.33)	0.53 (0.34)	0.40 (0.22)	*P*=0.08
NNIC/NIC	0.45 (1.14)	0.57 (0.73)	0.32 (0.68)	*P*=0.18
NCOT/COT	0.12 (0.07)	0.16 (0.24)	0.10 (0.08)	*P*=0.19

Mean (SD) of each phenotype ratio and results from general linear repeated measures models with post-hoc analyses correcting for multiple testing (Bonferroni) across the three pregnancy stages. No statistically significant differences were found between early and late pregnancy stages.
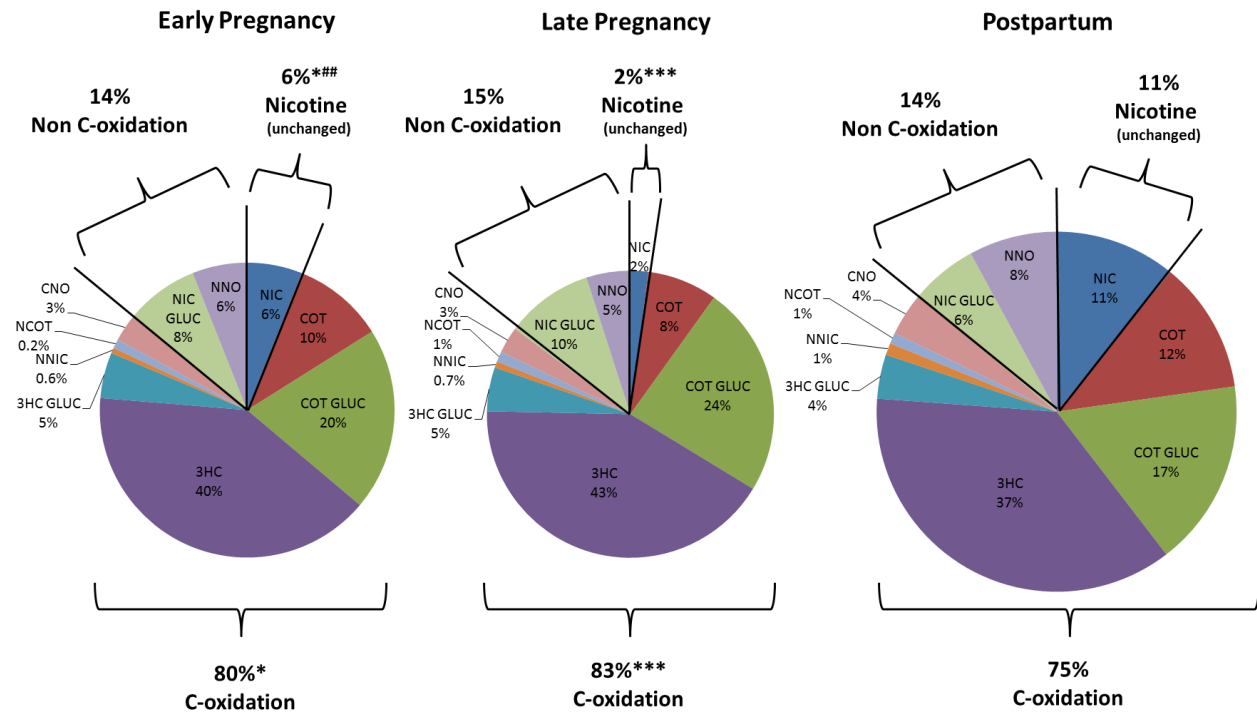
* Indicates significant differences from postpartum as *P*<0.05.

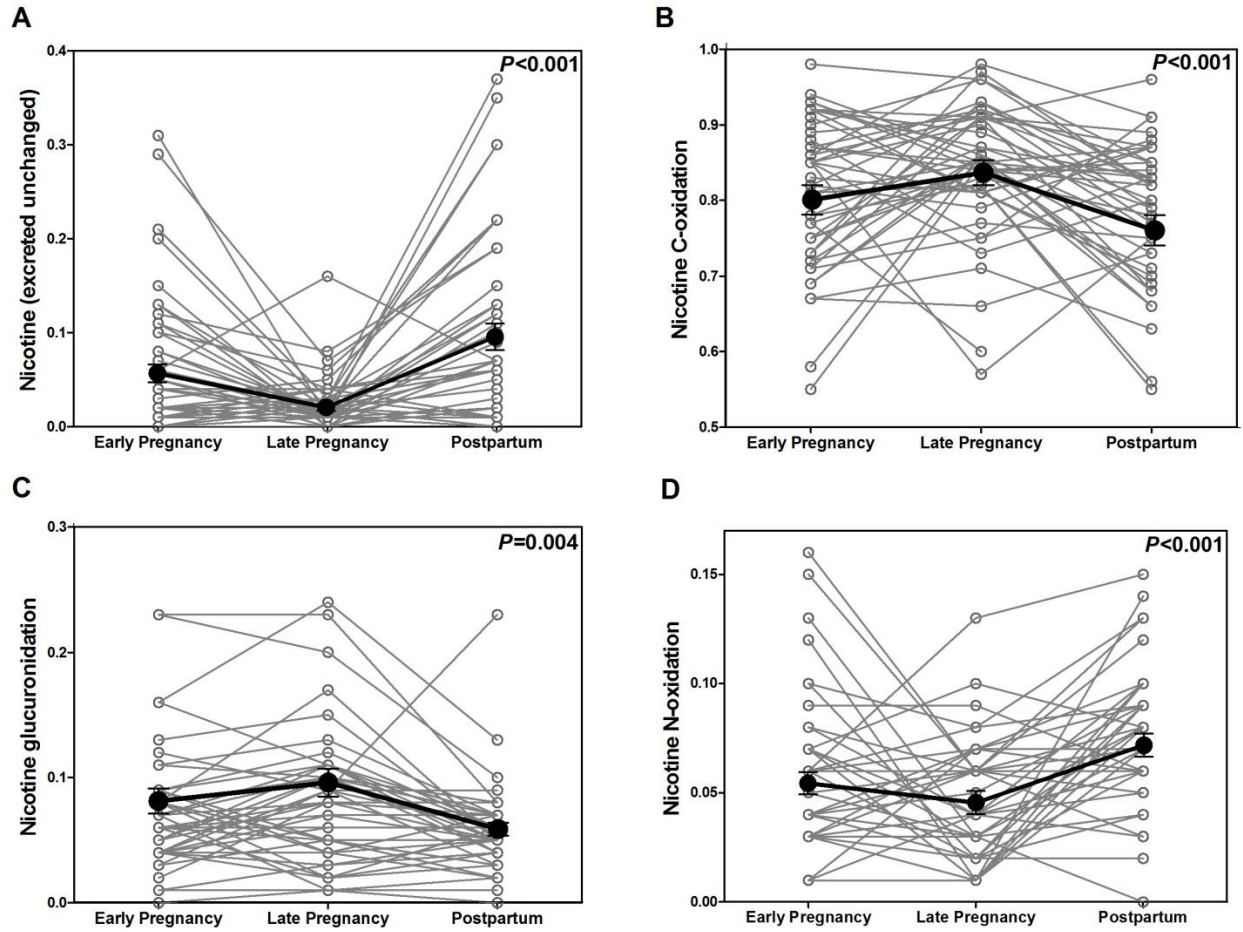****** Indicates significant differences from postpartum as *P*<0.01.

*** Indicates significant differences from postpartum as *P*<0.001.

Figure-1: Change in nicotine metabolite profile during pregnancy and postpartum

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Figure-2: Influence of pregnancy on nicotine metabolic pathways during pregnancy and postpartum illustrating the inter-individual variation, and consistency of pregnancy effect, among the subjects (n=47)

Figure-3: Influence of pregnancy on CYP2A6 (A), UGT2B10 (B-C), UGT2B17 (D), and FMO3 (E) phenotypic metabolic ratios during pregnancy and postpartum, illustrating the inter-individual variation, and consistency of pregnancy effect, among the subjects (n=47)