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The effect of tobacco use on the expression of placental transporters in Alaska Native women

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Abstract

Prenatal tobacco use among Alaska Native (AN) women has decreased substantially over the past two decades. Previous research suggests that providing AN women with feedback regarding fetal exposure to tobacco may further promote cessation. Transporters in the placenta regulate fetal exposure to nutrients and xenobiotics, including compounds associated with tobacco use. We examined whether prenatal tobacco use impacts transporter expression in the placenta, and whether this is influenced by fetal sex, degree of tobacco exposure, or transporter genotype. At delivery, we obtained placental samples from AN research participants who smoked cigarettes, used commercial chew or iqmik (oral tobacco), or did not use tobacco during pregnancy. Transporter expression was evaluated using qRT-PCR and western blotting and tested for correlations between transcript levels and urinary biomarkers of tobacco use. The impact of *BCRP/ABCG2* and *OATP2B1/SLCO2B1* genotypes on protein expression was also examined.

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AUTHOR CONTRIBUTIONS

E.R.M. and M.P-M. wrote the manuscript; M.P-M. and R.F.T. designed the research; E.R.M., J.K., N.L.B., C.A.P., C.A.H., K.R.K., C.A.F., T.K.T., and V.Y.H. performed the research. E.R.M., J.K., R.F.T., and M.P.M. analyzed the data.

CONFLICT OF INTEREST

Prior to 2020, RFT consulted for Quinn Emanuel and Ethismos Research Inc. MPM sits on the advisory board of Pillcheck but does not receive financial compensation for the role. All other authors declared no competing interests for this work.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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Oral tobacco use was associated with decreased *PGP* and increased *MRP1, MRP3, LAT1*, and *PMAT* mRNA expression. Transcript levels of multiple transporters significantly correlated with tobacco biomarkers in maternal and fetal urine. In women carrying male fetuses, both smoking and oral tobacco were associated with decreased PGP. Oral tobacco was also associated with decreased LAT1 in women carrying females. *BCRP* and *OATP2B1* genotypes did not appear to impact protein expression. In conclusion, prenatal tobacco use is associated with altered expression of multiple placental transporters which differs by fetal sex. As transcript levels of multiple transporters were significantly correlated with tobacco use biomarkers, eliminating prenatal tobacco use should alleviate these changes.

Keywords

Transporters; Drug Transport; Pharmacogenetics; Pregnancy; Addiction; Ethnicity; Gender; Fetus; Single Nucleotide Polymorphism; Women

INTRODUCTION

Given the impact of prenatal tobacco use on fetal outcomes,[1] interventions that promote tobacco cessation during pregnancy are beneficial to neonatal health. In the most recent report from the State of Alaska Pregnancy Risk Assessment Monitoring System (2012–2015), up to 30% of pregnant Alaska Native (AN) women reported use of tobacco during pregnancy.[2] This represents a substantial decrease of 13% from data collected a decade earlier in 2000–2003.[3] A pilot study exploring interventions to increase cessation among pregnant AN women found that participants expressed a desire for objective information about the impact that tobacco-derived chemicals could have on their infants.[4] This inspired a series of studies, entitled "*Biomarker Feedback to Motivate Tobacco Cessation in Pregnant Alaska Native Women (MAW),"* in which AN women have taken an active role in research regarding tobacco use during pregnancy. The studies aimed to use biomarkers in blood and urine to provide feedback on infant exposure to tobacco-associated compounds to promote cessation.[5–7] Two widely used biomarkers of tobacco exposure were examined: cotinine, the primary metabolite of nicotine, and 4-methylnitrosoamino-1-(3-pyridyl)-1-butanol (NNAL), the primary carcinogenic metabolite of a tobacco-derived nitrosamine.[8] Investigators in the MAW study found that maternal cotinine and NNAL urinary levels were highly correlated with those in neonatal urine, thus making them a viable biomarker for infant exposure to tobacco-associated compounds.[9]

Building on the MAW study findings, we sought to investigate the impact of prenatal tobacco use on placental function. The passage of essential nutrients, as well as exogenous substances, across the placenta is regulated by transport proteins expressed in the transporting layer of the placenta, the syncytiotrophoblast.[10] Transporters belonging to the ATP-binding cassette (ABC) superfamily often serve a protective function by preventing harmful compounds from reaching fetal circulation.[11] In contrast, solute carrier (SLC) transporters play a major role in transplacental transport of nutrients and hormones required for fetal development.[12,13] In addition to their endogenous substrates, ABC and SLC transporters transport a wide range of xenobiotics whose impact on fetal development

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is unknown or deleterious.[14] Indeed, multiple chemicals found in tobacco products are transported by various transporters expressed in the placenta.[15–17] Thus, factors that alter placental transporter expression or activity could not only influence the fetal supply of essential nutrients, but also compromise the protective ability of the placenta which could negatively impact fetal development. Importantly, cigarette smoke itself has been shown to alter the expression or activity of a number of transporters in tissues such as the liver and lungs.[18,19] Furthermore, investigators have recently demonstrated that smoking during pregnancy alters transcript levels of a number of SLC nutrient transporters in human placenta.[20] Whether these findings extend to other placental transporters, particularly ABC drug transporters remains unknown.

Single nucleotide polymorphisms (SNPs) can also alter transporter expression or activity.[21] Several studies have investigated the impact of SNPs on the ABC transporter P-glycoprotein (PGP) in the placenta.[22] In contrast, research into the impact of SNPs on the expression of other highly expressed transporters in the placenta, such as breast cancer resistance protein (BCRP) and organic anion-transporting polypeptide 2B1 (OATP2B1), has been either limited or inconclusive. The BCRP SNP rs2231142 (421C>A) and the OATP2B1 SNPs rs12422149 (935G>A) and rs2306168 (1457C>T) are either known to or have been proposed to affect protein expression.[23–29] However, studies investigating their impact on BCRP or OATP2B1 expression in the placenta are lacking. Furthermore, the interaction between different genotypes for these SNPs and tobacco-mediated changes in transporter expression has not yet been explored but could have important implications for fetal exposure to nutrients or toxins.

The primary objective of this study was to determine whether prenatal tobacco use impacts placental transporter expression. To do this, we obtained placental samples collected at delivery from AN women who volunteered to participate in the MAW study. We examined the impact of cigarette smoking, as well as the use of smokeless tobacco products, including commercially available chewing tobacco and iqmik, a homemade AN chewing tobacco,[30] on a number of transporters designated as clinically important by the International Transporter Consortium.[14] We also examined other SLC transporters important for endogenous nutrient and neurotransmitter transport. To determine whether changes in transporter expression are correlated with degree of tobacco use, we also tested for possible correlations between placental transporter expression and tobacco biomarker levels in maternal and neonatal urine. Lastly, we investigated the impact of the afore-mentioned genetic variation in the genes coding for BCRP and OATP2B1 on transporter protein expression in the placenta, and whether this is influenced by prenatal tobacco use.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Institutional Review Boards at the University of Toronto and Indian Health Service's Alaska Area. Tribal approval was granted by the Alaska Native Tribal Health Consortium (ANTHC) and Southcentral Foundation (SCF).

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Human placental sample acquisition

Placental samples were originally collected for the MAW study[5–7] and made available to us through a partnership with ANTHC and SCF. These tissues were obtained at delivery from AN eligible and consented women who were 18 years or older with a singleton pregnancy. Tissues were collected between 2012 and 2014 at the Alaska Native Medical Center (ANMC) in Anchorage, Alaska. Women who had not used tobacco for at least six months were eligible for the control group, whereas those reporting any tobacco use in the previous seven days prior to enrollment were assigned to either the smoking, commercial chew, or iqmik group based on the type of tobacco used most frequently. Women were excluded from the study if they had used nicotine replacement therapy or had participated in a behavioral or pharmacological tobacco cessation program within the past 30 days. Alcohol and other drug use or complications including gestational diabetes, obesity, pre-eclampsia, or infection were not exclusionary criteria.

RNA extraction and qRT-PCR

mRNA expression was assessed using qRT-PCR. Briefly, 150–200 mg of placental tissue were homogenized in TRIzol (ThermoFisher Scientific) and RNA was isolated through subsequent addition of chloroform and 2-propanol. The concentration and purity of RNA was determined using a NanoDrop 1000 spectrometer (ThermoFisher). RNA was treated with DNAse prior to reverse transcription with a high-capacity cDNA RT kit (Applied Biosystems). cDNA was quantified using Power SYBR Green (Bio-Rad CFX384 Touch) with primers listed in Table S1. Relative mRNA expression was calculated using the C_t method with the mean of two housekeeping genes, *YWHAZ* and *TOP1*.

Protein extraction and western blotting

Crude membrane fractions were isolated from 400 mg of placental tissue as previously described.[31] Protein concentrations were quantified via Bradford assay. Samples (10–50 μg) were separated using 8 or 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% milk powder in TBST and incubated overnight with the primary antibodies listed in Table S2. Membranes were washed before incubation with HRP-conjugated anti-mouse or anti-rabbit secondary antibody in 2% milk. Bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher) and band intensity was determined using Image Lab (Bio-Rad). Protein expression in each sample was normalized to β-actin. Signal intensity of each sample was normalized to that of a calibration sample loaded on all gels to account for inter-blot variability.

Correlations with biomarker levels in fetal and maternal urine

We performed a secondary analysis on a subset of existing biomarker data previously collected from women whose placentas were used in this study.[9] In the original study that collected this biomarker data, urine was collected from post-partum women and their neonates during the first void after delivery.[9] Cotinine concentrations were measured in maternal urine using gas liquid chromatography and in neonatal urine using liquid chromatography-tandem mass spectrometry (LC-MS-MS). LC-MS-MS was also used to measure NNAL levels in both maternal and fetal urine. Biomarker levels were used

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to perform intraindividual Spearman correlation analyses with mRNA expression of transporters.

DNA extraction and genotyping

Genomic DNA was extracted from cord blood and maternal blood using a QIAamp DNA Blood Mini Kit (Catalog No. 51106) according to the manufacturer's protocol (Qiagen, Germantown, Maryland, USA). Three genetic variants (*ABCG2* rs2231142, *SLCO2B1* rs12422149, and *SLCO2B1* rs2306168) were genotyped using commercially available TaqMan SNP genotyping assays (Assay IDs C__15854163_70, C___3101331_10, and C__16193013_20, respectively) and a ViiA7 Real-Time PCR system according to the manufacturer's protocol (ThermoFisher).

Statistical analysis

Using GraphPad Prism 6, changes in mRNA and protein expression between treatment groups were determined using one-way ANOVA with Dunnett's multiple comparison test. For analyses comparing both treatment and genotype or treatment and fetal sex, significance was determined using two-way ANOVA with Tukey's multiple comparison test. Clinical characteristics and NNAL/cotinine urine levels were compared using a Kruksal-Wallis test, and Spearman correlations between mRNA and biomarker levels were performed. Lastly, Hardy-Weinberg equilibrium of SNPs was assessed by Chi squared test using the Michael H. Court (2005–2008) calculator.[32] Results are presented as mean ± standard error of the mean (SEM) unless otherwise specified. In all tests, significance was set as $p < 0.05$.

RESULTS

Clinical characteristics

Table 1 shows relevant clinical characteristics of individuals in each observational group. We obtained placental samples from 48 women who did not use tobacco (controls), 40 who smoked (smoking), 7 who used commercial chewing tobacco, and 15 who used iqmik during pregnancy. Compared to women who did not use tobacco, a significantly lower gestational age was associated with women who used iqmik and a significantly lower neonatal birth weight was associated with women who smoked.

Impact of tobacco on placental transporter expression

Use of either commercial or iqmik smokeless chewing tobacco resulted in similar changes in transporter expression. Thus, due to the small sample size of each group, we combined commercial chew and iqmik samples into an "oral" tobacco group for most of our analyses.

Compared to women who did not use tobacco during pregnancy, *MRP1* transcript levels were significantly reduced in placental samples from women who smoked (Figure 1A), whereas protein expression was significantly increased (Figure 2). Oral tobacco use was associated with significantly reduced mRNA levels of *PGP* and significantly increased levels of *MRP1*, *MRP3*, *LAT1*, and *PMAT* (Figure 1B, 1C). However, no significant changes in PGP, PMAT, or LAT1 protein expression were observed in women who either smoked or used oral tobacco (Figure 2).

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There were no significant correlations between transcript and protein levels of PGP, MRP1, or LAT1 within non-tobacco user, cigarette smoker, or oral tobacco use groups (Table S3). While mRNA and protein levels of PMAT were significantly and negatively correlated in non-tobacco user samples, no significant correlation was observed in either the cigarette smoker or oral tobacco use groups.

Impact of fetal sex on tobacco-mediated changes in placental transporter expression

We performed a secondary analysis of tobacco-mediated effects on placental transporter expression stratified by fetal sex. Compared to both women who did not use tobacco and women who smoked, women who used oral tobacco had significantly higher transcript levels of *PMAT* in pregnancies with a female fetus, and higher levels of *OCTN2* in pregnancies carrying either female or male fetuses (Figure S1). Oral tobacco use was also associated with significantly induced mRNA levels of *MRP1* and *OAT4* in pregnancies with female fetuses when compared to women who smoked, but not when compared to women who did not use tobacco. Smoking was associated with significantly induced *OCT3* in pregnancies with male fetuses compared to women who used oral tobacco, and reduced *OATP2B1* mRNA expression in women carrying female fetuses who either used oral tobacco or did not use tobacco during pregnancy.

When protein expression was analyzed for male and female fetuses separately, PGP expression was significantly reduced in women carrying a male fetus who either smoked or used oral tobacco as compared to non-users (Figure S3). Compared to non-users, LAT1 protein expression was significantly decreased in women who used oral tobacco carrying female fetuses. MRP1 protein expression trended towards an increase in women carrying male fetuses who used oral tobacco compared to controls (multiplicity-adjusted p-value = 0.0581); however, this did not reach significance. PMAT protein expression was not significantly altered when analyzed by fetal sex.

Correlations between transporter mRNA expression and nicotine biomarkers

Average levels of cotinine and NNAL in maternal and neonatal urine for each observational group are listed in Table 2. Maternal and neonatal cotinine levels did not differ significantly between women who either smoked, used commercial chew, or used iqmik. In contrast, levels of NNAL in both maternal and neonatal urine differed significantly between women who used commercial chew versus iqmik. As a result, commercial chew and iqmik groups were not combined for analyses using biomarker data.

MRP3 transcript levels were significantly and positively correlated with both maternal and neonatal cotinine as well as maternal NNAL levels in the smoking group (Tables S4, S5, Figures S3, S4). *LAT1* and *LAT2* mRNA levels were significantly and positively correlated with neonatal cotinine levels in women who used commercial chew. *LAT1* was also significantly correlated with maternal cotinine in women who used iqmik. Furthermore, *PMAT* was positively correlated with neonatal NNAL, and *OATP2B1* was positively correlated with maternal NNAL in women who smoked. Lastly, *OCTN2* was positively correlated with neonatal cotinine levels in women who used commercial chew.

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Impact of SNPs on placental BCRP and OATP2B1 protein expression

The impact of genotype and smoking were examined for the *ABCG2/BCRP* SNP rs2231142 (C→A) and the *SLCO2B1/OATP2B1* SNPs rs12422149 (G→A) and rs2306168 (C→T). The number of placental samples per genotype and minor allele frequency for each SNP are listed in Table 3. All three SNPs were in Hardy-Weinberg equilibrium.

BCRP protein expression was not significantly altered for minor allele carriers (the CA genotype) compared to major allele homozygotes (CC) in women who did not use tobacco (Figure 3A). As we only had three AA non-user samples, we did not proceed with statistical analysis for comparison to CC protein expression. While smoking was not associated with significantly altered BCRP expression in either the CC or CA genotype, BCRP expression trended towards higher levels in the CA genotype in women who smoked.

In women who did not use tobacco, placental tissue with GG/CT genotype for rs12422149/rs2306168, respectively, had significantly lower OATP2B1 protein expression than those with the GA/CC genotype (Figure 3B). In contrast, basal expression of OATP2B1 in placentas carrying minor alleles for either SNP was not significantly altered compared to placentas that were homozygous for major alleles in both SNPs. Smoking was not associated with any significant effect on OATP2B1 protein expression for any of the three genotypes examined.

In general, mRNA expression was not significantly correlated with protein expression for BCRP or OATP2B1 in placental samples from women who either smoked or did not use tobacco (Table S6). The correlation between protein and mRNA was only significant for the *BCRP* CA genotype in women who did not use tobacco.

DISCUSSION

Placental transporters play integral roles in supplying the fetus with nutrients required for proper development and protecting the fetus from xenobiotics in maternal circulation. However, research into whether tobacco use during pregnancy alters the expression of placental transporters has been limited. Here, we demonstrate that smoking and oral tobacco use during pregnancy are associated with altered expression of multiple ABC and SLC transporters in the human placenta, and that fetal sex influences these changes. In addition to investigating the absolute effect of tobacco use on transporter expression, we also demonstrate that changes in transcript levels of some transporters are correlated with levels of tobacco biomarkers. In contrast, genotype does not appear to significantly impact changes in BCRP or OATP2B1 expression in placental samples from women who smoke.

PGP is one of the most abundant ABC efflux transporters in the placenta[33] and plays a critical role in preventing xenobiotics from entering the fetal compartment.[34] Oral tobacco use during pregnancy was associated with reduced placental mRNA expression of *PGP*. Similarly, women who smoked or used oral tobacco had significantly reduced placental PGP protein expression in pregnancies carrying a male fetus. These changes are consistent with a previous report that nicotine exposure reduced the mRNA and protein expression of PGP in pregnant rats.[35] PGP is located on the apical surface of the syncytiotrophoblast

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where it largely plays a protective role by effluxing substrates that enter the placenta into maternal circulation.[34] Substrates of PGP include a wide variety of clinically relevant drugs, including those often used during pregnancy such as cardiac medications and HIV antiretrovirals.[36] Furthermore, PGP has been shown to transport benzo(a)pyrene, a mutagen present in tobacco smoke.[37,38] Thus, reduced placental PGP expression could increase fetal exposure to maternally-administered drugs or tobacco-associated toxins, which could have deleterious effects on fetal development.

The multidrug resistance-associated protein (MRP) 1 and MRP3 are efflux transporters with broad substrate selectivity which includes anionic conjugated metabolites, HIV protease inhibitors, folates, and certain anticancer drugs.[34] Expression of these transporters is highest in the fetal endothelium, thereby functioning to export substrates from the fetal circulation.[34] Transcript levels of both *MRP1* and *MRP3* were significantly induced in women who used oral tobacco, and *MRP3* was positively correlated with tobacco biomarkers in women who smoked. In contrast, smoking was associated with significantly decreased *MRP1* mRNA levels, but significantly increased MRP1 protein expression. To our knowledge, this is the first study to investigate the impact of tobacco use on placental MRP expression. The induction of placental *MRP1* and *3* mRNA in women who used oral tobacco, and MRP1 protein in women who smoked appears to be consistent with reports in lung tissue.[19] In addition to transporting drugs, glutathione conjugates of arsenic (found in cigarette smoke),[15] and NNAL,[16] MRP1 transports endogenous substrates including glutathione disulfide which is thought to constitute a defensive mechanism against oxidative stress.[34] Increased markers of oxidative stress are evident in placentas of women who use tobacco;[39] thus, the induction of MRP1 in our study could potentially be a defensive measure against this.

We also found that the protein expression of the large amino acid transporter 1 (LAT1) was significantly lower in placentas of women who used oral tobacco, but only in those carrying a female fetus. LAT1 is highly expressed at the maternal-facing microvillous membrane of the placenta where it facilitates the uptake of leucine, isoleucine, and valine from maternal circulation.[40] As these amino acids are essential, placental transport to fetal circulation is required for proper fetal growth. Indeed, reduced placental LAT1 and leucine uptake have been associated with intrauterine growth restriction.[41,42] Thus, a reduction of placental LAT1 could contribute to the fetal growth restriction that is associated with tobacco use during pregnancy.

On the other hand, we observed significantly higher mRNA of *LAT1* in placentas from women who used oral tobacco. Furthermore, *LAT1* transcript levels were significantly and positively correlated with neonatal or maternal cotinine in women who used oral, smokeless tobacco. Likewise, *LAT2* mRNA was significantly correlated with neonatal cotinine levels in women who used commercial chew. Interestingly, the oral tobacco groups had the highest levels of neonatal and maternal cotinine. This implies that nicotine-based harm reduction interventions focused on reducing tobacco intake during pregnancy could potentially be beneficial for alleviating changes in transporter expression. On the other hand, we did not find any association between transcript levels of *LAT1* or *LAT2* and smoking. This is

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consistent with previous reports for *LAT1*, but studies examining *LAT2* report conflicting results.[20,43]

BCRP is the most highly expressed efflux transporter in the placenta[33] and plays a predominant role in placental efflux of xenobiotics, thereby limiting fetal drug exposure.[34] BCRP exhibits a wide substrate specificity and transports a number of chemotherapeutics, glucuronide and glutathione conjugates, and hormones. Given the integral protective role that BCRP plays in the placenta, SNPs in the gene encoding BCRP have been proposed to be clinically relevant to fetal drug exposure.[44] As such, we also wanted to investigate the frequency and impact of BCRP SNPs. Reduced BCRP protein stability and expression have been reported for the SNP rs2231142 which results in a non-synonymous amino acid substitution (Q141K).[23] This SNP has also been shown to alter drug disposition in non-placental tissues.[44] We observed a minor allele frequency of approximately 17% for this SNP in this population of AN women. However, analysis of CC and CA genotypes showed only a trend towards 21% reduction in BCRP membrane protein expression in the CA genotype. Our results are in line with a previous report in a Japanese population in which placental BCRP protein expression was significantly decreased in AA genotypes, but not CA genotypes.[29] However, a more recent study conducted in an ethnically diverse population reported significantly reduced placental BCRP protein expression in both CA and AA genotypes as compared to CC.[45] We also found that smoking during pregnancy was not associated with changes in BCRP protein expression in placentas with CC genotype. Similarly, while BCRP expression in the smoking group was increased over 2-fold compared to the non-tobacco use group in placentas with the CA genotype, this did not reach statistical significance. Thus, while we were potentially underpowered to look at the interaction between genotype and tobacco use, BCRP genotype for rs2231142 does not appear to significantly influence the impact of smoking on placental BCRP protein expression.

In contrast to the previously described efflux transporters, OATP2B1 is an SLC uptake transporter that facilitates placental uptake of fetal-derived precursors used for placental steroid synthesis.[44] We also examined two SNPs in the gene encoding OATP2B1: rs12422149 (935G>A) and rs2306168 (1457C>T), both of which are non-synonymous and had minor allele frequencies of approximately 32% and 15% in our study population. These SNPs are associated with reduced OATP2B1 function.[24–29] Having one minor allele for either rs12422149 or rs2306168 did not significantly impact placental OATP2B1 expression as compared to major allele homozygotes for both SNPs. However, the membrane protein expression of OATP2B1 was significantly lower in minor allele carriers for rs2306168 as compared to minor allele carriers for rs12422149. While this has not been previously examined in the placenta, significant associations between these SNPs and transporter expression were not seen in liver tissues.[27] Lastly, it does not appear that smoking significantly impacts OATP2B1 protein expression in the placenta for any of the three genotypes examined.

There are a few important limitations of our study. Firstly, some women within each treatment group had complications which could affect transporter expression including pre-eclampsia, gestational diabetes, obesity, and infection.[46] These complications could potentially contribute to or negate changes associated with tobacco use and require further

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investigation. For example, there was a high incidence of gestational diabetes in the chewing tobacco group, which likely increased the fetal birth weight of this sample group[47] and may have negated the impact of tobacco use on this outcome. Secondly, while the sample sizes for women who did not use tobacco or smoked were relatively large, commercial chew and iqmik sample sizes were substantially smaller, an unavoidable consequence of working with pre-collected biobank samples. We tried to mitigate the impact of this by combining the iqmik and commercial chew groups into the oral tobacco group when results did not significantly differ between the two groups. Sample size similarly limited our ability to investigate the impact of SNPs on basal expression of BCRP or OATP2B1, in addition to the impact of tobacco use within genotypes, especially in the case of placentas that were homozygous for minor alleles.

Our results could form the basis of multiple avenues for further research. Firstly, the mechanism underlying tobacco-mediated changes in transporter expression remains to be elucidated. Many compounds found in cigarette smoke are ligands for the aryl hydrocarbon receptor (AHR),[48] a transcription factor that has been shown to regulate transporter expression.[49] We observed a significant induction of transcript levels of the AHR target gene CYP1A1 in placentas from women who smoked, and a trend towards increased expression in women who used oral tobacco, implying activation of AHR in the placenta (data not shown). Whether this activation of AHR is involved in downstream changes in transporter expression will need to be further investigated. Moreover, how different forms of tobacco differentially impact placental transporter expression is unclear. We observed more extensive dysregulation of transporter expression in the oral tobacco group than in women who smoked, implying that these methods of tobacco use impact transporter expression via different mechanisms. One critical difference between smoking cigarettes and smokeless tobacco products is that smoking is associated with higher levels of inflammation and oxidative stress.[50] Another differentiating factor is the pharmacokinetics of nicotine absorption, which differs between smoking and oral tobacco use. The higher pH of smokeless tobacco products such as iqmik results in increased nicotine absorption,[51,52] which has been proposed to result in nicotine toxicity.[51] Moreover, whereas smoking results in rapid increases in blood concentrations of nicotine which peak upon completion of smoking, blood concentrations of nicotine during oral tobacco use rise gradually and then persist and decline slowly over a few hours.[53] The levels of nicotine biomarkers did not differ significantly between women who smoked and women who used oral chewing tobacco in the current study, so whether this could explain differing impacts on the placenta will require further research.

Another unanswered question is how fetal sex influences the regulation of placental transporter expression. The placenta is largely fetal-derived and reflects the biological sex of the fetus. As such, the placenta exhibits sexually dimorphic basal gene expression and responses to stressors including environmental chemicals, stress hormones, and inflammation.[54] A number of studies have also documented sex-specific changes in placental transporter expression in result to different stimuli.[55–57] The underlying reasons for this differential regulation are not clear, but could involve sex chromosomes or sex steroids.[54,58] This will be an important area for future research.

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In conclusion, we demonstrated that tobacco use during pregnancy in AN women is associated with differences in the expression of multiple placental transporters; the data suggest these differences in expression may be fetal sex-specific. Several of the transporters affected play significant roles in fetal supply of essential nutrients or protection from xenobiotics, so tobacco-mediated changes in their expression could have negative implications for fetal development. As we observed significant correlations between transcript levels of some transporters and biomarkers of tobacco use in maternal and fetal urine, it is possible that harm reduction strategies focused on eliminating tobacco use during pregnancy could prevent or lessen these effects. Secondly, we report the minor allele frequencies of three common SNPs affecting BCRP or OATP2B1 in AN women. While limited by small sample sizes for genotype analysis, there does not appear to be an association between placental transporter expression and genotype, nor a genotype-by-tobacco use interaction with expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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STUDY HIGHLIGHTS

What is the current knowledge on the topic?

Alaska Native (AN) women have requested information on how prenatal tobacco use may impact fetal health. *In vitro* studies have demonstrated an impact of tobacco on transporter expression in liver and lung cell lines. However, research regarding its impact on transporters in the placenta, which regulates fetal exposure to nutrients and xenobiotics, is limited.

What question did this study address?

Whether smoking cigarettes or using chewing tobacco during pregnancy is associated with changes in the expression of ABC and SLC transporters in placentas obtained from Alaska Native women.

What does this study add to our knowledge?

Prenatal tobacco use is associated with altered expression of drug and nutrient transporters in the placenta. These changes differ by fetal sex, and in some cases correlate with biomarkers of tobacco use.

How might this change clinical pharmacology or translational science?

This study serves as justification to further investigate the impact of prenatal tobacco use on transport of nutrients and xenobiotics across the placenta. These findings may also be used as incentive to avoid tobacco use during pregnancy.

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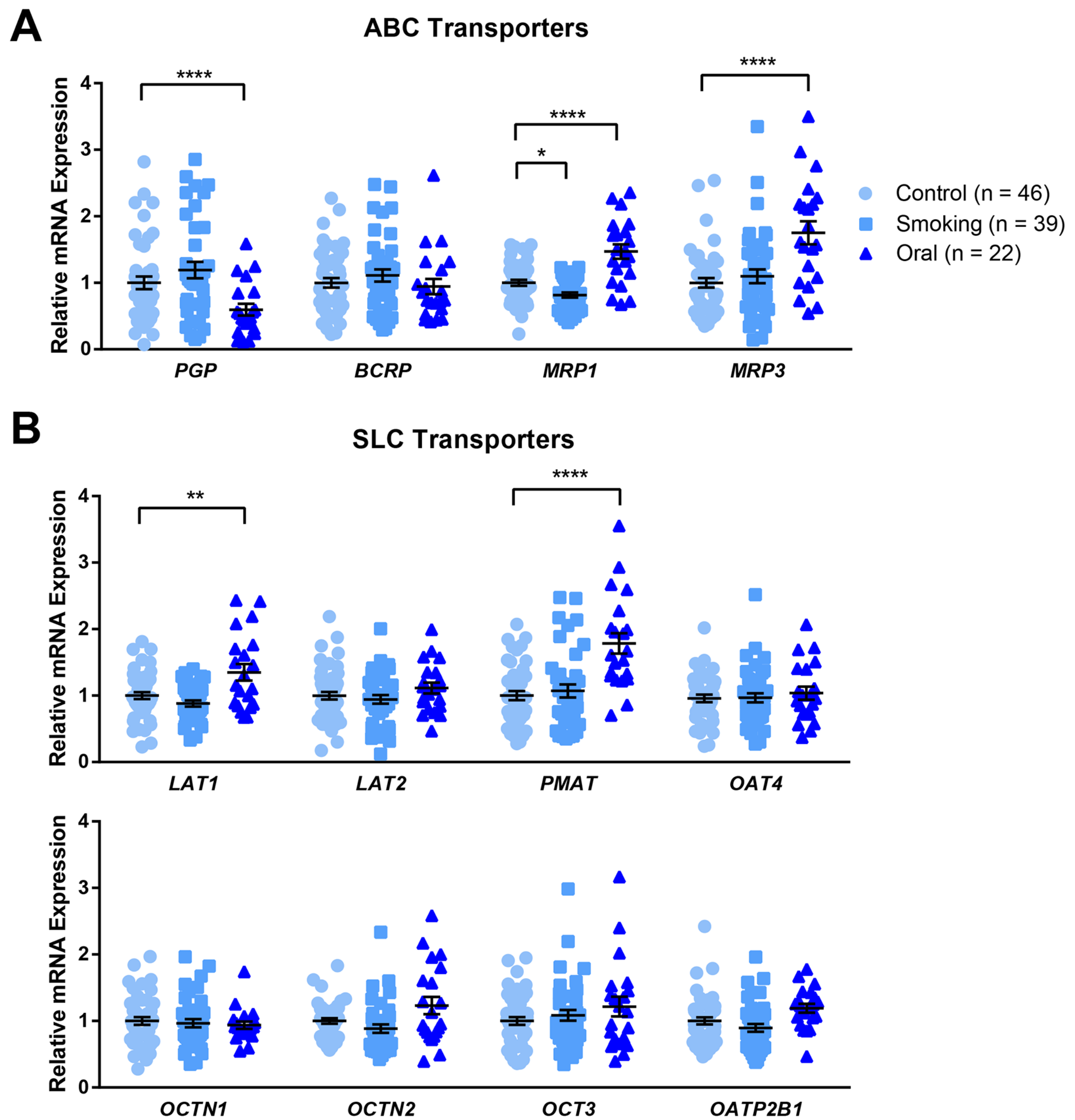

Figure 1: mRNA expression of transporters in term human placentas.

Relative expression of (A) ABC and (B) SLC transporters was determined using qRT-PCR and normalized to the geometric mean of YWHAZ and TOP1. Expression is shown as average mRNA expression ± SEM relative to control. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, **** $p \ll 0.001$). Control: n = 46; smoking: n = 39; oral: n = 22.

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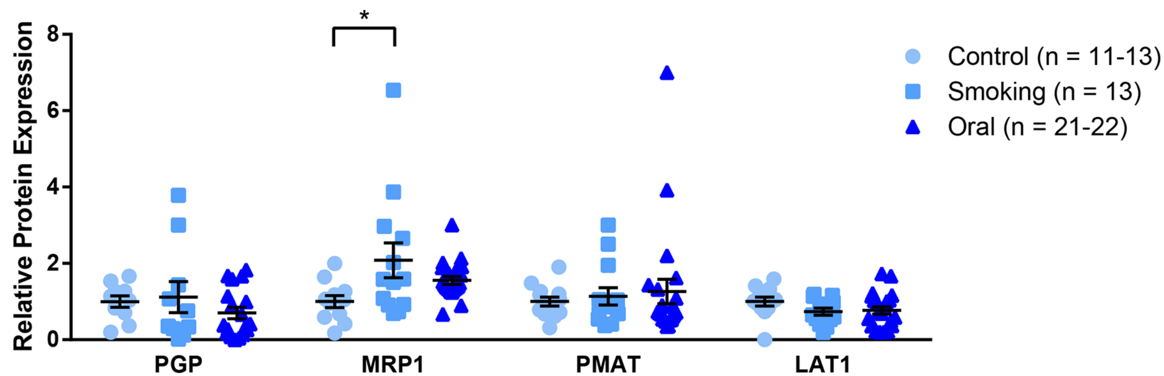

Figure 2: Protein expression of transporters in term human placentas.

Protein expression was examined in crude membrane protein fractions of placentas from mothers with no tobacco exposure (control, n = 11–13), cigarette smokers (n = 13), or oral users (n = 21–22). Relative expression was determined using Western blotting, normalized to β-actin, and is expressed as average expression ± SEM relative to control. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test.

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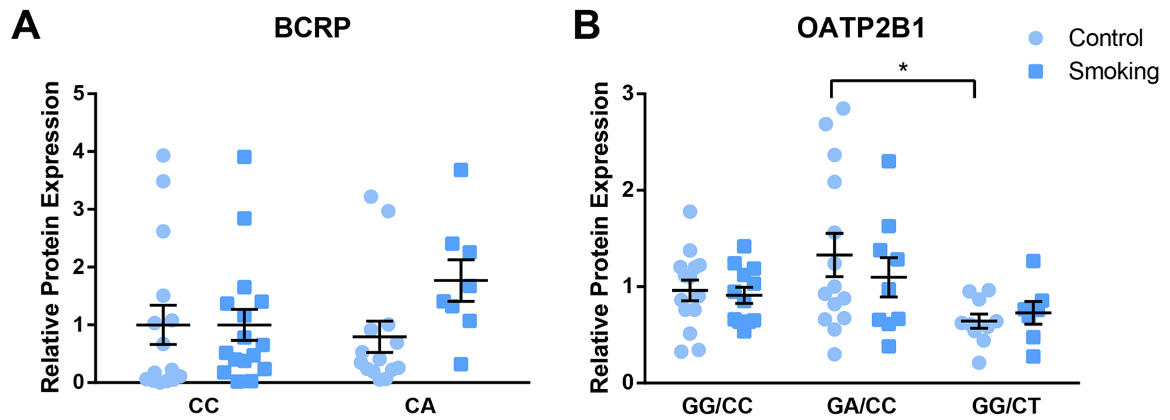

Figure 3: Data analysis by placental genotype of (A) BCRP or (B) OATP2B1.

For OATP2B1, genotype is expressed as rs12422149 genotype (G→A)/rs2306168 genotype (C→T). Relative protein expression was determined using Western blotting, normalized to β-actin, and is expressed as average expression ± SEM relative to control and major allele homozygous genotype. Significance was determined using a two-way ANOVA with Tukey's multiple comparisons test (* $p < 0.05$). Control: n = 10–15; smoking: 7–16.

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Table 1:

Clinical characteristics of placentas from each sample group.[†]

Characteristic	Control (Non-user) (n = 48)	Smoking (n = 40)	Commercial Chewing Tobacco (n = 7)	Iqmik (n = 15)
Gestational Age (weeks)	39 ± 3	39 ± 1	39 ± 1	38 ± 1 **
Complications				
GDM	7 (15%)	8 (20%)	4 (57%)	3 (20%)
Obesity	6 (13%)	5 (13%)	4 (57%)	1 (7%)
Pre-eclampsia	6 (13%)	4 (10%)	1 (14%)	2 (13%)
Infection[††]	5 (10%)	3 (8%)	1 (14%)	0
Neonatal Birth Weight (g)	3544 ± 535	3284 ± 563 *	3697 ± 533	3407 ± 310
Neonatal Sex				
Male	24 (50%)	18 (45%)	6 (86%)	10 (67%)
Female	24 (50%)	22 (55%)	1 (14%)	5 (33%)

[†]Demographics are given as numbers followed by percent of total per treatment group in brackets. Significance was determined using a Kruksal-Wallis test with Dunn's multiple comparison test.

[*]$p < 0.05$,

[**]$p < 0.01$.

[††]Infections included urinary tract infections, chorioamnionitis, rubella, and chlamydia.

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Table 2:

Levels of cotinine and NNAL in maternal and fetal urine at birth.

	Smoking (n = 25–26)		Commercial Chewing Tobacco (n = 7)		Iqmik (n = 12–13)	
Biomarker	**GM**	**95% CI**	**GM**	**95% CI**	**GM**	**95% CI**
Maternal Cotinine (ng/mg creatinine)	402	223–725	347	60–2012	702	287–1719
Maternal NNAL (pg/mg creatinine)	316	201–497	657	97–4463	138 *	70–273
Neonatal Cotinine (ng/mL)	61	35–105	114.5	30–439	76	26–218
Neonatal NNAL (pg/mL)	16	10–25	63.9	21–199	6 **	3–11

† Data is depicted as geometric mean (GM) with 95% confidence interval. Significance was determined using a Kruskal-Wallis test with Dunn's multiple comparison test.

* denotes significance between commercial chew and iqmik groups with * $p < 0.05$ and ** $p < 0.01$. Data represents a subset of data previously collected by Benowitz *et al.*[9]

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Table 3:

Number of samples per genotype and minor allele frequencies for each SNP.

Gene (Protein)	SNP	Genotype	Number of Samples	MAF (n minor alleles/n total alleles)	Hardy-Weinberg Equilibrium
ABCG2 **(BCRP)**	rs2231142	CC CA AA	73 28 4	0.171429	$\chi^2 = 0.395$ p = 0.5299
SLCO2B1 **(OATP2B1)**	rs12422149	GG GA AA	47 49 9	0.319048	$\chi^2 = 0.575$ p = 0.4483
SLCO2B1 **(OATP2B1)**	rs2306168	CC CT TT	73 30 0	0.145631	$\chi^2 = 2.993$ p = 0.0836

[†] Hardy-Weinberg Equilibrium was assessed by Chi squared test using the Michael H. Court (2005–2008) calculator.[32]