# CHOLINE AS A MODULATOR OF PLACENTAL FUNCTION FOR IMPROVING FETAL DEVELOPMENT

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Sze Ting Kwan

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CHOLINE AS A MODULATOR OF PLACENTAL FUNCTION FOR IMPROVING FETAL DEVELOPMENT

Sze Ting Kwan, Ph.D.

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The theory of developmental origins of health and disease proposes that many adult-onset metabolic diseases originate from fetal adaptations to an adverse prenatal environment. These responses often involve altered fetal physiology that affects normal organ functioning, thereby triggering the onset of diseases in later life. Placenta is increasingly recognized to play a central role in this theory. The placenta performs many functions including its major role which is to supply adequate nutrients to the fetus to support its growth and development. When the placenta fails to perform this function, the development of the fetus is adversely impacted. Placental nutrient supply is dependent on placental morphology and vasculature as well as placental nutrient metabolism and transporter abundance, all of which can be affected by the maternal diet during pregnancy. A higher maternal choline intake during pregnancy in animals is known for its beneficial effects on offspring development, in particular, the central nervous system. Nevertheless, its effect on factors that mediate placental nutrient supply remains largely unknown.

Study 1 examined the impact of maternal choline supplementation on biomarkers of placental inflammation, apoptosis and angiogenesis as well as placental morphological and vascular indicators in mice during normal pregnancy. This study demonstrates that maternal choline supplementation modulates the abundance of inflammatory, apoptotic and angiogenic markers in the mouse placenta in a fetal sex- and gestational day-dependent manner. In addition, this study provides evidence of enhanced placental perfusion in response to maternal choline supplementation through increased luminal area of the maternal spiral arteries.

Study 2 investigated the impact of maternal choline supplementation on placental nutrient transporter abundance and placental nutrient metabolism during late gestation of the mouse

pregnancy when fetal growth is maximal. This study indicates that maternal choline supplementation modulates the placental abundance of amino acid, fatty acid, glucose, choline and acetylcholine transporters as well as the placental metabolism of glucose and choline. More importantly, this study provides evidence showing that these choline-induced changes in the placenta alter nutrient availability in the fetal compartment, in particular fetal brain, suggesting that these placental changes may influence the development of the fetus and the normal functioning of its organs.

Study 3 employed an untargeted approach to explore the impact of maternal choline supplementation on placental epigenetic markers during late gestation of mouse pregnancy. This study shows that maternal choline supplementation affects several placental epigenetic markers, including the amount of global DNA methylation, the expression of imprinted genes, as well as the abundance of microRNAs and the expression of their mRNA targets. Although these changes occur in a sexually-dimorphic manner, they all have similar downstream consequences on placental vascular development and nutrient supply system. We hypothesize that the choline-induced changes in these epigenetic markers likely contribute to the improved placental development and functioning observed in Study 1 and Study 2.

Taken together, this dissertation research shows a wide-range of effects of maternal choline supplementation on factors that influence placental nutrient supply and ultimately fetal development and its long term health. Data generated from this dissertation research support a growing body of work suggesting that women of reproductive age should increase their intake of choline-rich foods in order to improve pregnancy outcomes and the lifelong health of their children.

## BIOGRAPHICAL SKETCH

Sze Ting (Cecilia) Kwan was born in Hong Kong. She moved to Sacramento, California with her family when she was 13 years old. After graduating from C.K. McClatchy High School in 2006, she attended UC Davis for her college education. Due to her family influence, she majored in Electrical Engineering. However, after attending the introductory nutrition course during her freshman year, she became fascinated by the powerful impacts diet can have on human health. Because she was eager to learn more about nutrition, she changed her major to Clinical Nutrition. During her undergraduate study, she worked as a nutrition intern in several community centers where she became familiar with different topics related to nutrition during pregnancy and childhood. She also worked as an undergraduate research assistant in the lab of Dr. Bo Lönnerdal where she participated in animal and cell culture studies examining the metabolism of iron and zinc during infancy. All these experiences made her recognize that there are still a lot of unknown questions in the field of nutrition, particularly maternal and child nutrition. After receiving her Bachelor's degree in 2011, she decided to attend Cornell University to receive training in conducting research to address these questions.

Cecilia joined the lab of Dr. Marie Caudill soon after she arrived at Cornell. Her project is an animal feeding study investigating the impact of maternal choline supplementation on the functions of the placenta, a tissue that plays an important role in nourishing the baby but is surprisingly overlooked in the scientific community. She received the Dissertation Fellowship from Egg Nutrition Center, Nell Mondy Research Fellowship from Graduate Women in Science and two scholarships from the Academy of Nutrition and Dietetics to support her research works and professional training. She was also selected as a trainee on the NIH-sponsored Predoctoral Training Program in Maternal and Child Nutrition. As an early career investigator, she received an honorable mention from The International Federation of Placenta Associations in 2016 for her research works. In addition to performing research in the lab, Cecilia completed the Cornell Dietetic Internship in 2016 and is currently certified as a Registered Dietitian.

Dedicated to my family

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I am truly indebted to a lot of people for helping me succeed in my graduate study and become an independent nutrition researcher. Without any of these people, none of what I accomplished in these past 6 years would be a possibility.

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I am extremely thankful to my advisor, Dr. Marie Caudill, and our collaborator, Dr. Mark Roberson for all their mentoring, support in realizing my research and career goals. I truly value each of the challenging questions they have asked me as each stimulates me to think more critically about the data, which makes me a better researcher. I want to thank my committee members, Dr. Andy Clark and Dr. Barbara Strupp for their guidance in completing courses to fulfill my graduation requirements and their support for my research works and academic accomplishments. I also want to thank Dr. Patsy Brannon and Dr. Anna Thalacker-Mercer. I did not like doing oral presentations prior to coming to Cornell, but thanks to Patsy and Anna, I successfully completed the NS 7030 presentations and realize the joy in talking about science. Now, I just cannot wait for every opportunity I can have to share my research with other people!

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## LIST OF ABBREVIATIONS

5-methyl-2'-deoxycytidine, 5mdC

adenosine monophosphate deaminase 3, Ampd3

aquaporin 1, Aqp1

choline transporter-like protein 1, CTL1

decorin, Dcn

dimethylglycine, DMG

docosahexaenoic acid, DHA

endoglin, Eng

false discovery rate, FDR

fatty acid transporter, FATP

gene ontology, GO

glucose transporter, GLUT

glutaminyl cyclase, Qpct

glycine amidinotransferase, Gatm

glycogen branching enzyme 1, GBE1

glycogen phosphorylase, muscle, PYGm

glycogen synthase 1, GYS1

glycogen synthase kinase 3 beta, GSK3β

interleukin 1 beta, Il1b

interleukin 10, Il10

interleukin 6, Il6

long-chain polyunsaturated fatty acids, LCPUFAs

maternal choline supplementation, MCS

matrix metalloproteinase 14, Mmp14

microRNAs, miRNAs

non-Swiss Albino mice, NSA

nuclear factor of kappa light polypeptide gene enhancer in B-cells, Nfkb1

organic cation transporter, OCT

phosphatidylcholine, PC

placental growth factor, Pgf

smooth muscle actin, SMA

soluble endoglin, sENG

soluble fms-like tyrosine kinase-1, sFLT1

system A amino acid transporter, SNAT

TATA box binding protein, Tbp

terminal deoxynucleotidyl transferase dUTP nick end labeling, TUNEL

tissue factor pathway inhibitor 2, Tfpi2

transforming growth factor-β, TGF-β

trimethylamine N-oxide, TMAO

tumor necrosis factor alpha, Tnf

tumor necrosis factor receptor superfamily, member 23, Tnfrsf23

vascular endothelial growth factor, Vegfa

# PREFACE

The placenta is a fetal-derived tissue responsible for providing nutrients to the fetus to support its growth and development. Any abnormal variation in its size, morphology or functional capacity can adversely impact placental nutrient supply, consequently altering the development of the fetus and its risk of disease. As such, the placenta is positioned to play a crucial role in programming offspring health in later life. A growing body of data indicates that suboptimal prenatal conditions (e.g., maternal malnutrition) impair aspects of placental development that affect nutrient supply, and that these placental phenotypes correlate with higher disease susceptibility in the offspring. The overarching goal of this dissertation research is to characterize the impact of maternal choline supplementation (MCS) on factors that determine placental nutrient supply efficiency. To accomplish this research goal, pregnant wild-type non-Swiss Albino (NSA) mice were randomized to receive a diet containing 1X, 2X or 4X the recommended choline level and were sacrificed at one of four gestational days (E10.5, 12.5, 15.5 or 18.5). Maternal liver, serum, placentas and fetuses were collected and used in several experiments to address the following specific aims:

**Aim 1: To test the hypothesis that MCS improves placental morphology and vascularization.** This aim was accomplished by assessing the placental abundance of pro-angiogenic, anti-angiogenic, and pro-inflammatory proteins as well as evaluating placental morphology and vasculature. Results from these experiments are presented in Chapter 1.

**Aim 2: To test the hypothesis that MCS enhances the supply of nutrients to the developing fetus.** In particular, we focused on quantifying the abundance of placental transporters involved in transporting macronutrients and choline metabolites as well as placental enzymes involved in metabolizing glycogen. Concentrations of the glycogen, choline metabolites and DHA were also measured in the placenta and/or fetal brain. Results from these experiments are presented in Chapter 2.

**Aim 3: To test the hypothesis that MCS alters the epigenome of the placenta to regulate processes related to placental nutrient delivery.** This aim was accomplished by employing an untargeted approach to examine placental global DNA methylation, placental expression of imprinted genes and placental abundance of microRNAs. Results from these experiments are presented in Chapter 3.

This dissertation research yielded 1 published primary research article in a peer-reviewed journal (Chapter 1), one original research manuscript that is under peer review for publication (Chapter 2), and one original research manuscript that will be submitted for publication within the next few months (Chapter 3).

# CHAPTER 1

Maternal choline supplementation during murine pregnancy modulates placental markers of inflammation, apoptosis and vascularization in a fetal sex-dependent manner*

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## ABSTRACT

***Introduction:*** Normal placental vascular development is influenced by inflammatory, angiogenic and apoptotic processes, which may be modulated by choline through its role in membrane biosynthesis, cellular signaling and gene expression regulation. The current study examined the effect of maternal choline supplementation (MCS) on placental inflammatory, angiogenic and apoptotic processes during murine pregnancy. ***Method:*** Pregnant dams were randomized to receive 1, 2 or 4 times (X) the normal choline content of rodent diets, and tissues were harvested on embryonic day (E) 10.5, 12.5, 15.5 or 18.5 for gene expression, protein abundance and immunohistochemical analyses. ***Results:*** The choline-induced changes in the inflammatory and angiogenic markers were a function of fetal sex. Specifically, 4X (versus 1X) choline reduced the transcript ($P \leq 0.05$) and protein ($P \leq 0.06$) expression of TNF-a and IL-1β in the male placentas at E10.5 and E18.5, respectively. In the female placentas, 4X (versus 1X) choline modulated the transcript expression of *Il1b* in a biphasic pattern with reduced *Il1b* at E12.5 ($P = 0.045$) and E18.5 ($P = 0.067$) but increased *Il1b* at E15.5 ($P = 0.031$). MCS also induced an upregulation of *Vegfa* expression in the female placentas at E15.5 ($P = 0.034$; 4X versus 2X) and E18.5 ($P = 0.026$; 4X versus 1X). MCS decreased ($P = 0.011$; 4X versus 1X) placental apoptosis at E10.5. Additionally, the luminal area of the maternal spiral arteries was larger ($P \leq 0.05$; 4X versus 1X) in response to extra choline throughout gestation. ***Discussion:*** MCS during murine pregnancy has fetal sex-specific effects on placental inflammation and angiogenesis, with possible consequences on placental vascular development.

## INTRODUCTION

The placenta is the organ of pregnancy that mediates nutrient and oxygen supply to the developing fetus, and is therefore a critical determinant of fetal growth and development. Efficient placental transport requires proper remodeling of the maternal uterine spiral arteries and the development of a vascular network within the chorionic villi (in human placenta) or labyrinth (in mouse placenta) [1, 2]. When placental vascularization is compromised, the placenta is unable to provide sufficient nutrients and oxygen to the developing fetus, which increases the risk of fetal growth restriction and abnormal birth weight [1].

Normal placental vascular development is influenced by the balance of pro- and anti-angiogenic factors. Pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PGF) play a regulatory role in the growth and proliferation of endothelial cells, angiogenesis and vasodilation while anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFLT1) and soluble endoglin (sENG) interfere with normal pro-angiogenic signaling, disrupt endothelial tube formation and damage the placental vasculature [3, 4]. The inflammatory milieu also plays a role in placental vascular development. Heightened levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-a) and interleukin 6 (IL-6) have been shown to cause endothelial cell dysfunction, reduce vascular relaxation, inhibit trophoblast invasion into the maternal decidua and adversely affect placental vascularization [4-6].

Abnormal angiogenesis and inflammation may be causal in pregnancy disorders such as preeclampsia. Aberrant expression of these proteins and others including interleukin 1 beta (IL-1β) and interleukin 10 (IL-10) is detected among women with placental dysfunction [7-11]. Recent work also reveals that placental angiogenesis and inflammation may be a sexual

dimorphic phenomenon, underscoring the importance of considering fetal sex when studying these placental markers [12-14].

Choline is an essential micronutrient required for membrane biosynthesis and cellular signaling, and plays a regulatory role in gene expression via epigenetic processes (e.g., DNA and histone methylation) [15]. Consequently, choline may modulate physiological processes such as inflammation, angiogenesis and apoptosis that are central to placental function and fetal development [15-17]. Notably, we have shown an effect of choline on these processes in a cell culture model of extravillous human trophoblast cells where increasing choline concentrations decreased the abundance of pro-inflammatory, anti-angiogenic and pro-apoptotic markers [18]. Similarly, we found that supplementing the maternal diet of healthy pregnant women with extra choline (930 vs. 480 mg/d) throughout the third trimester of pregnancy decreased placental production and circulating concentrations of sFLT1 [19]. However, apart from the choline-induced reduction in placental sFLT1 expression, it is unknown whether maternal choline supplementation (MCS) can influence inflammatory, angiogenic and apoptotic processes in an *in vivo* model of normal pregnancy. A better understanding of the functional role of choline in placental vascular development is also needed. Accordingly, we conducted a choline supplementation study in pregnant mice and examined biomarkers of placental inflammation, angiogenesis, and apoptosis at four gestational time points. We also conducted a preliminary histological investigation to examine the effect of MCS on vascular indicators within the maternal decidua and the feto-placental unit.

## MATERIALS AND METHODS

### *Mice and diets*

All animal protocols and procedures used in this study were approved by the Institutional Animal Care and Use Committees at Cornell University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Adult male and female non-Swiss Albino (NSA) mice were purchased from Harlan (Indianapolis, IN). The animals were housed in microisolator cages (Ancare) in an environmentally-controlled room (22-25°C and 70% humidity) with a 12-hour light-dark cycle. The mice in the breeding colonies were given *ad libitum* access to a commercially available rodent chow and water. After weaning at 3 weeks of age, both females and males were given *ad libitum* access to the AIN-93G purified rodent diet (Dyets no. 103345; Dyets, Bethlehem, PA) containing 1.4g choline chloride/kg diet (1X choline diet). This dietary regimen was continued until five days prior to mating at which time female mice were randomized to the 1X choline diet, a 2X choline diet containing 2.8g choline chloride/kg diet (Dyets no. 103346; Dyets, Bethlehem, PA), or a 4X choline diet containing 5.6g choline chloride/kg diet (Dyets no. 103347; Dyets, Bethlehem, PA). These dosages were selected based on our studies conducted in third-trimester pregnant women showing a choline lowering effect on sFLT1 with 2X choline supplementation [19] and evidence from rodent studies reporting improvements in brain development in the adult offspring whose mothers were supplemented with 4X choline [20]. Day of conception was determined by the presence of a vaginal plug and was defined as gestational day (E) 0.5. The female mice continued to consume their assigned diet until they were euthanized at one of four gestational time points (i.e., E10.5, E12.5, E15.5 or E18.5; n=6-8 dams/treatment group/time point).

***Tissue collection and processing***

Maternal blood was collected by cardiac puncture into microtainer collection tubes with clot activator and SST gel (Becton Dickinson, Franklin Lakes, NJ), and was allowed to clot at room temperature for one hour. The sample was then centrifuged at 14,000 rpm for 6 minutes, and the serum was collected and stored at -80°C. Maternal liver was removed, immediately frozen in liquid nitrogen and stored at -80°C. The gravid uterus was removed, the fetuses and placentas were then carefully dissected and weighed. One-third of the placental disks were fixed in 10% formalin for histology analysis, while the remaining placental disks were cut in half across the chorionic plate and placed in RNA*later* or immediately frozen in liquid nitrogen and stored at -80°C. The fetuses were imaged to obtain crown rump measurements using the Image J Analysis Software (NIH). Fetal DNA was extracted and subjected to PCR using a commercial kit (Qiagen) for sex determination (Supplemental Table 1).

***Measurement of choline metabolites in maternal liver***

The concentrations of choline and its metabolic derivatives [betaine, dimethylglycine (DMG) and trimethylamine N-oxide (TMAO)] were measured in maternal liver obtained at the last study time point (i.e.: E18.5) by LC/MS according to the method of Holm et al [21] with modifications based on our equipment [22].

***Quantification of placental transcript abundance***

Total RNA was extracted from the placental tissues fixed in RNA*later* by TRIzol reagent (Invitrogen). Reverse transcription was performed using ImProm-II Reverse Transcription System (Promega) with the following reaction conditions: 25°C for 10 minutes, 42°C for 40

minutes and 95°C for 5 minutes. Quantitative PCR was performed using the SYBR Green system in Roche LightCycler480. All primers for the targeted genes (*Tnf, Il1b, Il6, Il10, Nfkb1, Vegfa, Pgf, sFlt1, Eng, Mmp14*) were designed using Primer-BLAST available on the NCBI website (Supplemental Table 1.1). These genes were selected because of their importance in placental development and association with adverse pregnancy outcomes [5, 7-11, 23, 24] and their responsiveness to choline in prior investigations [18, 19]. The reaction conditions were as follows: 95°C for 5 minutes, followed by 40 cycles with 15 sec at 95°C, 30 sec at 63°C, and 30 sec at 72°C. To ensure the specificity of the PCR product, a dissociation stage was included at the end of the amplification cycles. Data are expressed by the $\Delta\Delta C_t$ method, in which the expression level of the gene of interest is normalized by the expression level of the housekeeping gene as fold change before comparison between samples. TATA box binding protein, *Tbp*, was selected as the housekeeping gene because its expression is stable in placental tissue [25] and remains unchanged under different choline intake levels [17].

***Quantification of placental protein abundance***

To evaluate the protein abundance of IL-1β, TNF-a and NF-κB in the placenta, frozen placental samples were homogenized in ten volumes of buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.5% IGEPAL CA-630 (Santa Cruz Biotechnology)] containing protease inhibitor cocktails (Sigma-Aldrich). The homogenates were centrifuged at 13,200 rpm for 25 minutes at 4°C. The total protein concentration in the supernatant was quantified by the Bradford assay (Thermo Scientific Pierce). Protein was loaded onto SDS-PAGE gel, subjected to electrophoresis, and then transferred onto Immobilon FL PVDF membranes (EMD Millipore). Membranes were blocked in blocking buffer (LI-COR). The membranes were then incubated

overnight with primary antibodies for IL-1β (1:200; Santa Cruz Biotechnology), TNF-a, NF-κB or β-actin (1:200, 1:1000 and 1:5000, respectively; Cell Signaling Technology), after which secondary antibodies (IRDye 800CW goat anti-rabbit and IRDye 680RD goat anti-mouse (LI-COR), 1:10,000) were added to the membranes. Protein bands were visualized and quantified by the Odyssey imaging system (LI-COR). Data are expressed as the ratio of the intensity of targeted protein to the intensity of β-actin and compared between samples.

***Measurement of circulating angiogenic factors in maternal serum***

Circulating concentrations of sFLT1 and sENG in the maternal serum were measured using commercial ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

***Assessment of placental apoptosis***

The placental tissues were fixed in 10% formalin, paraffin embedded and sectioned at 10μm. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was conducted using a commercial kit (Millipore, Billerica, MA) to assess placental apoptosis. The total number of cells and the number of TUNEL-positive cells in the placenta were quantified by the Aperio ImageScope software to determine the percentage of TUNEL-positive cells.

***Assessment of maternal spiral artery area and placental labyrinth vasculature***

Some formalin-fixed sections were subjected to immunohistochemistry as described previously [26]. To identify maternal spiral arteries for area evaluation, the placental sections were incubated with a smooth muscle actin (SMA) antibody (1:50, DakoCytomatin, Glostrup,

Denmark), followed by incubation with a secondary antibody. All stained sections were imaged on an Aperio Scanscope (Vista, CA). The maternal spiral arteries were defined manually, and their areas were quantified using the Aperio ImageScope software. Data on the spiral artery area are presented as a ratio of the luminal area to the total vessel area.

To evaluate the vascular structure in the placental labyrinth, the placental sections were incubated with isolectin (1:100, Vector Laboratories, Burlingame, CA), which is a marker of the endothelial cells and has been used to stain the vasculature in other mouse tissues [27, 28], and then counterstained with hematoxylin. The placental labyrinth compartment was defined manually, and the intensity of the isolectin staining was determined using the Aperio ImageScope software. Data are expressed as the staining intensity per unit area of placental labyrinth.

***Statistical analysis***

Fetal measurements and the placental transcript and protein data were analyzed separately for each gestational day and fetal sex using a mixed linear model. Because some fetuses were fixed in formalin together with their placentas, fetal DNA was degraded and was not available for sex genotyping. Therefore, histology data were analyzed without stratifying by fetal sex. All mixed linear models included choline treatment as an independent fixed effect and maternal identification as an independent random effect. Litter size was included in the model as a covariate when it achieved $P \leq 0.05$. For the maternal measurements, data were analyzed separately for each gestational day using one-way ANOVA. The model included choline treatment as an independent fixed effect, and litter size as a covariate when it had a $P \leq 0.05$. Correlations between the choline metabolites in maternal liver and placental inflammatory or

angiogenic markers at E18.5 were assessed using Pearson's correlation analysis (with log-transformed variables as needed). Bonferroni correction was used to adjust for multiple comparisons. Data are presented as means ± SEM. SPSS software, Version 23 (SPSS Inc, Chicago, IL) was used to perform the statistical analysis and differences were considered statistically significant when $P_{adjusted} \leq 0.05$. Given that we hypothesized (*a priori*) that supplementing the maternal diet with extra choline would influence the outcome variables, unadjusted *P*-values ($P_{unadjusted}$) are also presented for variables whose significance was lost after adjusting for multiple testing.

## RESULTS

### *Concentrations of choline (and its metabolites) in the maternal liver*

Maternal liver concentration of choline was higher in response to MCS, but only the difference between 1X and 4X choline groups remained significant after adjusting for multiple testing (4X vs 1X choline: $P_{adjusted} \leq 0.001$; 4X vs 2X choline: $P_{unadjusted} = 0.02$, $P_{adjusted} = 0.06$; 2X vs 1X choline: $P_{unadjusted} = 0.032$, $P_{adjusted} = 0.09$; Figure 1.1A). Maternal liver concentrations of betaine, DMG and TMAO were higher in response to 2X and 4X choline ($P_{adjusted} < 0.05$ vs 1X choline; Figure 1.1B-1.1D).

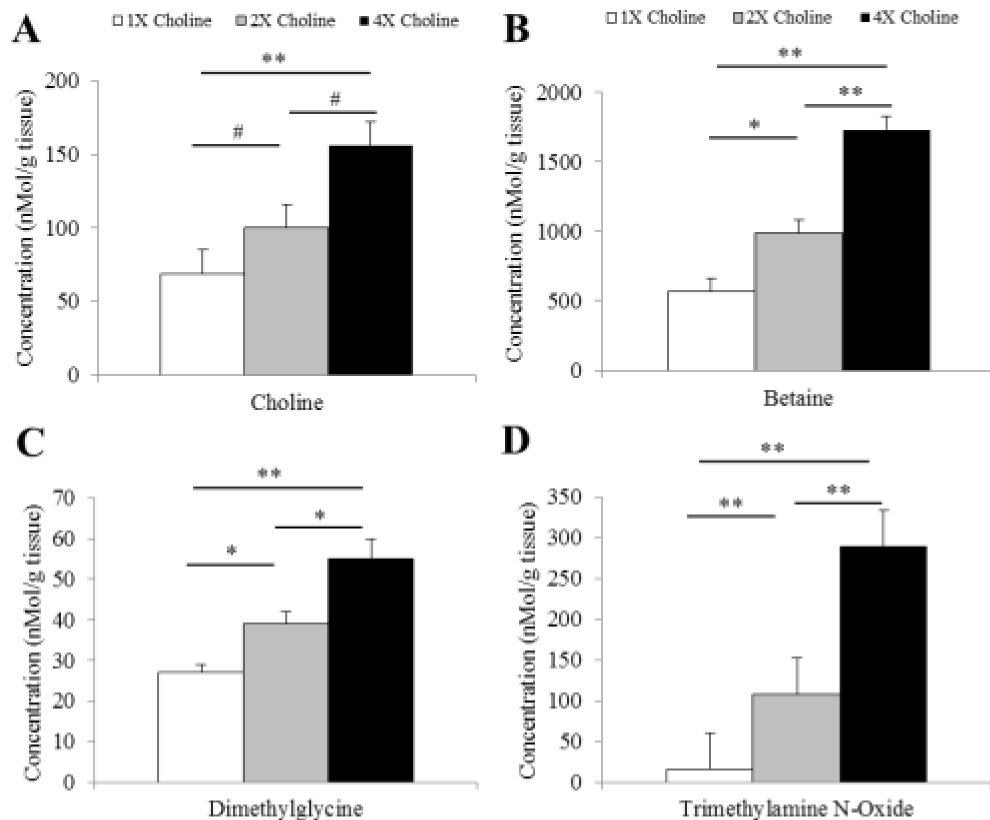


**Figure 1.1.** Maternal hepatic concentrations of **A)** choline, **B)** betaine, **C)** dimethylglycine and **D)** trimethylamine *N*-oxide at E18.5 in response to three different choline treatments (1X, 2X and 4X). Data were analyzed using ANOVA followed by post-hoc Bonferroni corrections. Values are presented as mean ± SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$. $^{\#}$ $P_{unadjusted} \leq 0.05$, $P_{adjusted} > 0.05$.

### *Placental inflammation*

In the female placentas, MCS influenced the transcript abundance of *Il1b* with the 4X choline group having lower abundance at E12.5 ($P_{adjusted}$ = 0.045 vs 1X choline) and higher abundance at E15.5 ($P_{adjusted}$ = 0.031 vs 1X choline; $P_{adjusted}$ = 0.006 vs 2X choline). A lower *Il1b* transcript abundance in response to 4X choline was also detected at E18.5 ($P_{unadjusted}$ = 0.022 vs 1X choline), but this difference was lost after adjusting for multiple testing ($P_{adjusted}$ = 0.067 vs 1X choline) (Figure 1.2A). Protein concentrations of IL-1β exhibited expression patterns that mirrored those of mRNA abundance at E12.5 (4X vs 1X choline: $P_{unadjusted}$ = 0.039, $P_{adjusted}$ = 0.11), E15.5 (4X vs 2X choline: $P_{unadjusted}$ = 0.041, $P_{adjusted}$ = 0.12), and E18.5 (4X vs 1X choline: $P_{unadjusted}$ = 0.022, $P_{adjusted}$ = 0.065) (Figure 1.2B-C).

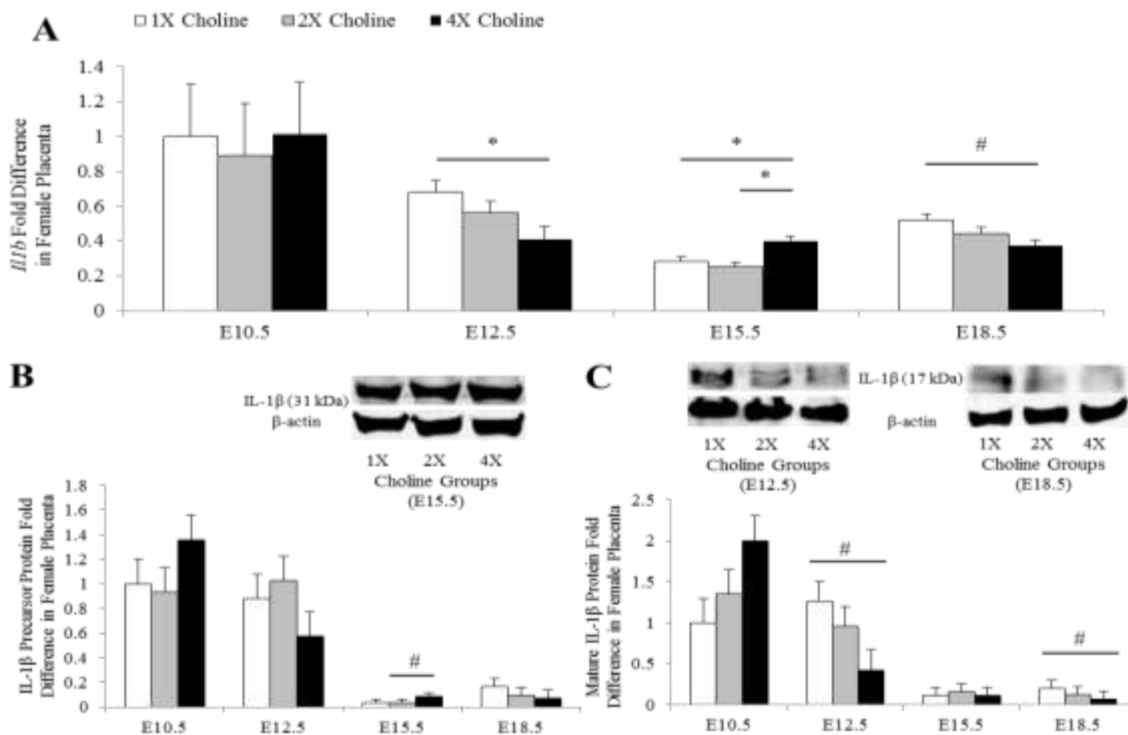


**Figure 1.2. A)** Transcript and **B-C)** protein abundance of IL-1β in the female placentas obtained from dams receiving 1X, 2X or 4X choline treatments at E10.5, E12.5, E15.5 and E18.5. The

transcript data are expressed as fold-change relative to the housekeeping gene *Tbp* and the protein data are expressed relative to β-actin. After normalization, the mean value of the control group at E10.5 was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Statistical analysis was done using the mixed linear model followed by post-hoc Bonferroni corrections. Values are given as mean ± SEM. *$P \leq 0.05$. $^{\#}P_{unadjusted} \leq 0.05$, $P_{adjusted} > 0.05$.

*Nfkb1* transcript abundance in the female placentas was higher at E18.5 in the 4X choline group ($P_{adjusted}$ = 0.014 vs 1X choline). Protein concentration of NF-κB exhibited an expression pattern similar to mRNA abundance but did not achieve statistical significance (4X vs 1X choline: $P_{unadjusted}$ = 0.059, $P_{adjusted}$ = 0.177) (Figure 1.3A-B). MCS had no detectable effects on the transcript abundance of *Tnf, Il6* and *Il10* ($P \geq 0.12$) in the female placentas at any time points. Correlation analyses indicated a modest but significant negative correlation ($r$ = -0.54, $P$ = 0.02) of *Il1b* abundance in the E18.5 placentas with TMAO concentration in the maternal liver. The placental *Nfkb1* transcript abundance at E18.5 was also positively associated with the concentrations of choline ($r$ = 0.7, $P$ = 0.001), betaine ($r$ = 0.65, $P$ = 0.004) and DMG ($r$ = 0.48, $P$ = 0.044) in the maternal liver.

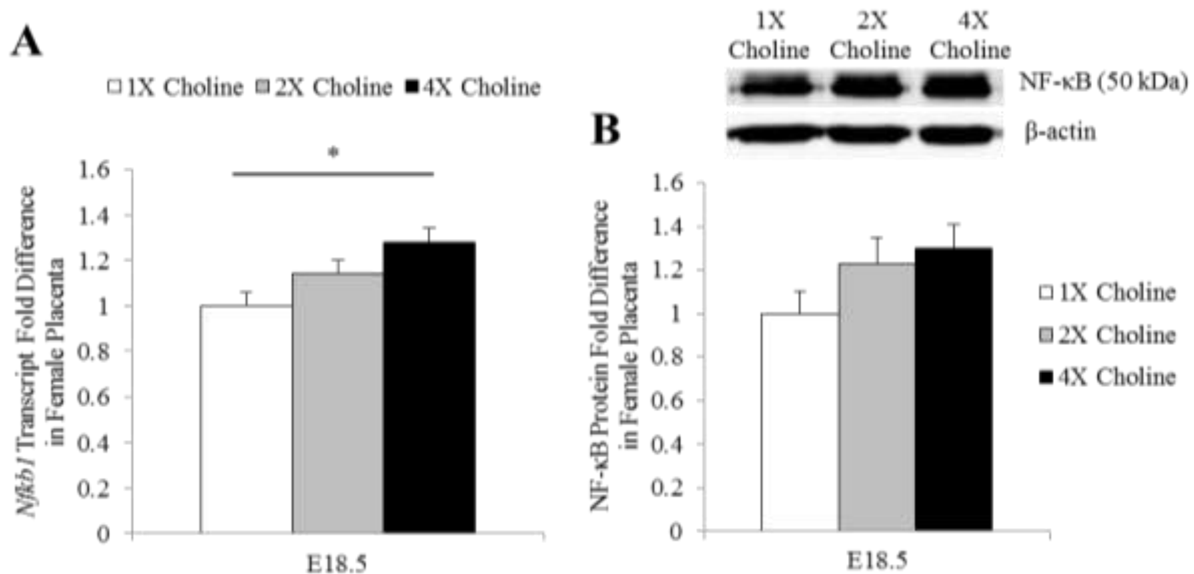


**Figure 1.3. A)** Transcript and **B)** protein expression of NF-κB in the E18.5 female placentas obtained from dams receiving 1X, 2X or 4X choline treatments. The transcript data are expressed as fold-change relative to the housekeeping gene *Tbp* and the protein data are expressed relative to β-actin. After normalization, the mean value of the control group was assigned a value of 1 and the mean values of the treatment groups were presented as a fraction of this value. Statistical analysis was done using the mixed linear model followed by post-hoc Bonferroni corrections. Values are given as mean ± SEM. *$P \leq 0.05$.

In the male placentas, 4X choline decreased the transcript abundance of *Il1b* at E18.5 ($P_{adjusted}$ = 0.035 vs 1X choline) (Figure 1.4A). The protein abundance of the precursor form of IL-1β was also reduced in the 4X choline group ($P_{adjusted}$ = 0.01 vs 1X choline). Similarly, a reduction in the mature form of IL-1β was detected in the 4X choline group ($P_{unadjusted}$ = 0.035 vs 1X choline) but statistical significance was lost after adjusting for multiple testing ($P_{adjusted}$ = 0.1 vs 1X choline; Figure 1.4B).

The male placentas in the 2X and 4X choline groups also had lower ($P_{adjusted}$ = 0.008 and 0.033 vs 1X choline, respectively) transcript abundance of *Tnf* at E10.5. Similarly, the protein concentration of TNF-a was lower in the 2X and 4X choline groups at E10.5 ($P_{unadjusted}$ = 0.05 and 0.02 vs 1X choline, respectively) but statistical significance was lost after adjusting for multiple testing ($P_{adjusted}$ = 0.15 and 0.06 vs 1X choline, respectively) (Figure 1.4C-D). MCS had no effects ($P \geq 0.1$) on the transcript abundance of *Il6, Il10* and *Nfkb1* or the protein concentration of NF-κB in the male placentas.

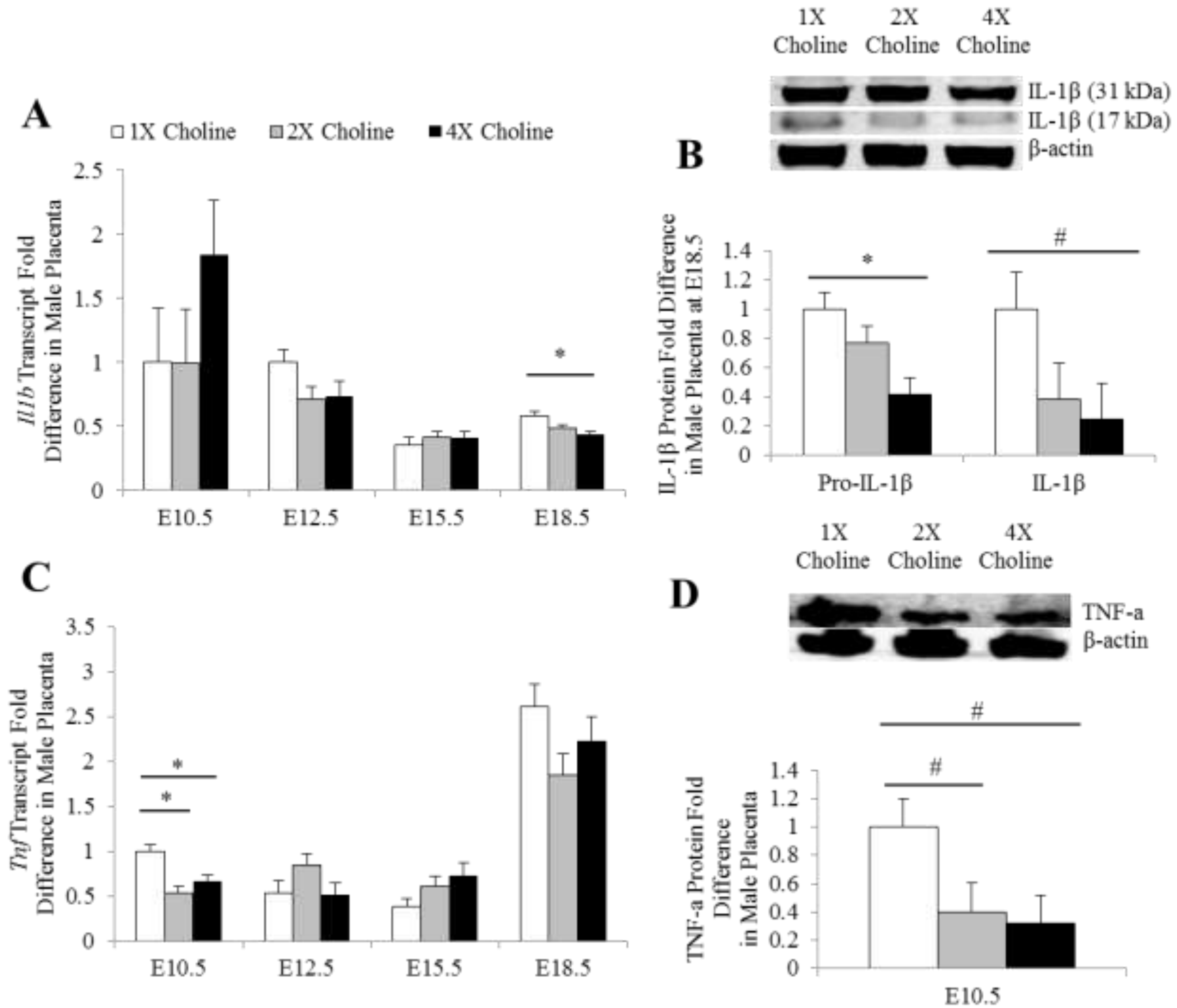


**Figure 1.4.** mRNA and protein abundance of **A-B)** IL-1β and **C-D)** TNF-a in the male placentas obtained from dams receiving 1X, 2X or 4X choline treatments. The transcript data are expressed as fold-change relative to the housekeeping gene *Tbp* and the protein data are expressed relative to β-actin. After normalization, the mean value of the control group at E10.5 (for mRNA data) or the mean value of the control group (for protein data) was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Statistical analysis was done using the mixed linear model followed by post-hoc Bonferroni corrections. Values are given as mean ± SEM. $*P \leq 0.05$. $^{\#}P_{unadjusted} \leq 0.05$, $P_{adjusted} > 0.05$.

***Placental angiogenic markers***

In the female placentas, a higher expression of *Vegfa* was observed at E15.5 ($P_{adjusted}$ = 0.034 vs 2X choline) and E18.5 ($P_{adjusted}$ = 0.026 vs 1X choline) in response to 4X choline (Figure 1.5). Correlation analyses showed significant modest correlations between *Vegfa* abundance in the E18.5 placentas and all four choline metabolites in the maternal liver (choline: $r$ = 0.57, $P$ = 0.014; betaine: $r$ = 0.48, $P$ = 0.045; DMG: $r$ = 0.51, $P$ = 0.032; TMAO: $r$ = 0.58, $P$ = 0.011). MCS had no detectable effects on the transcript abundance of *Pgf, sFlt1*, *Mmp14* and *Eng* ($P \geq$ 0.1).

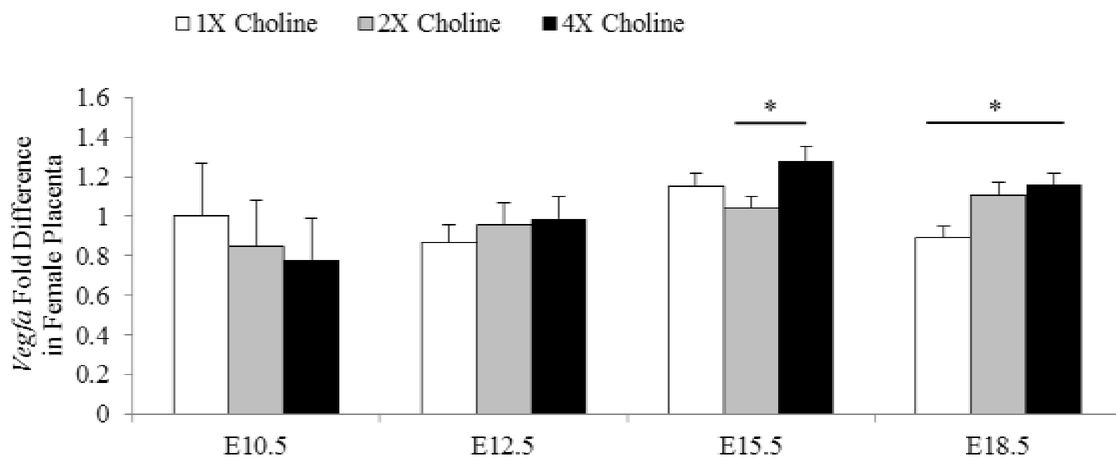


**Figure 1.5.** mRNA abundance of *Vegfa* in the female placentas obtained from dams receiving 1X, 2X or 4X choline treatments at E10.5, E12.5, E15.5 and E18.5. Data are expressed as fold-change relative to the housekeeping gene *Tbp*. After normalization, the mean value of the control group at E10.5 was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Statistical analysis was done using the mixed linear model followed by post-hoc Bonferroni corrections. Values are given as mean ± SEM. *$P \leq 0.05$.

In the male placentas, *sFlt1* transcript abundance tended to be lower in response to 2X and 4X choline ($P_{unadjusted}$ = 0.07 vs 1X choline) at E18.5; however, this tendency was not detected after adjusting for multiple testing ($P_{adjusted}$ = 0.22 vs 1X choline). Other angiogenic factors in the male placentas remained unchanged ($P \geq 0.1$) in response to MCS.

***Maternal circulating concentration of sFLT1 and sENG***

4X choline decreased sFLT1 concentration in the maternal serum at E18.5 ($P_{unadjusted}$ = 0.05 vs 1X choline), but this difference was lost after adjusting for multiple testing ($P_{adjusted}$ = 0.15 vs 1X choline). MCS did not affect the concentration of sENG ($P \geq 0.5$) in the maternal serum.

***Placental apoptosis***

Fewer TUNEL-positive cells were detected in the placentas of the 2X ($P_{adjusted}$ = 0.04 vs 1X choline) and 4X choline ($P_{adjusted}$ = 0.011 vs 1X choline) groups at E10.5 (Figure 1.6A-B). No effects of MCS ($P \geq 0.18$) were detected on the apoptotic index in the placentas at any other time points (Figure 1.6A).

***Placental vasculature***

Placentas from the 2X and 4X choline groups exhibited a larger ($P_{adjusted} \leq 0.05$) maternal spiral artery luminal area across all four gestational time points as compared to the 1X choline group (Figure 1.6C-D). The isolectin staining intensity in the placental labyrinth did not differ in response to MCS at any of the gestational time points ($P \geq 0.13$).

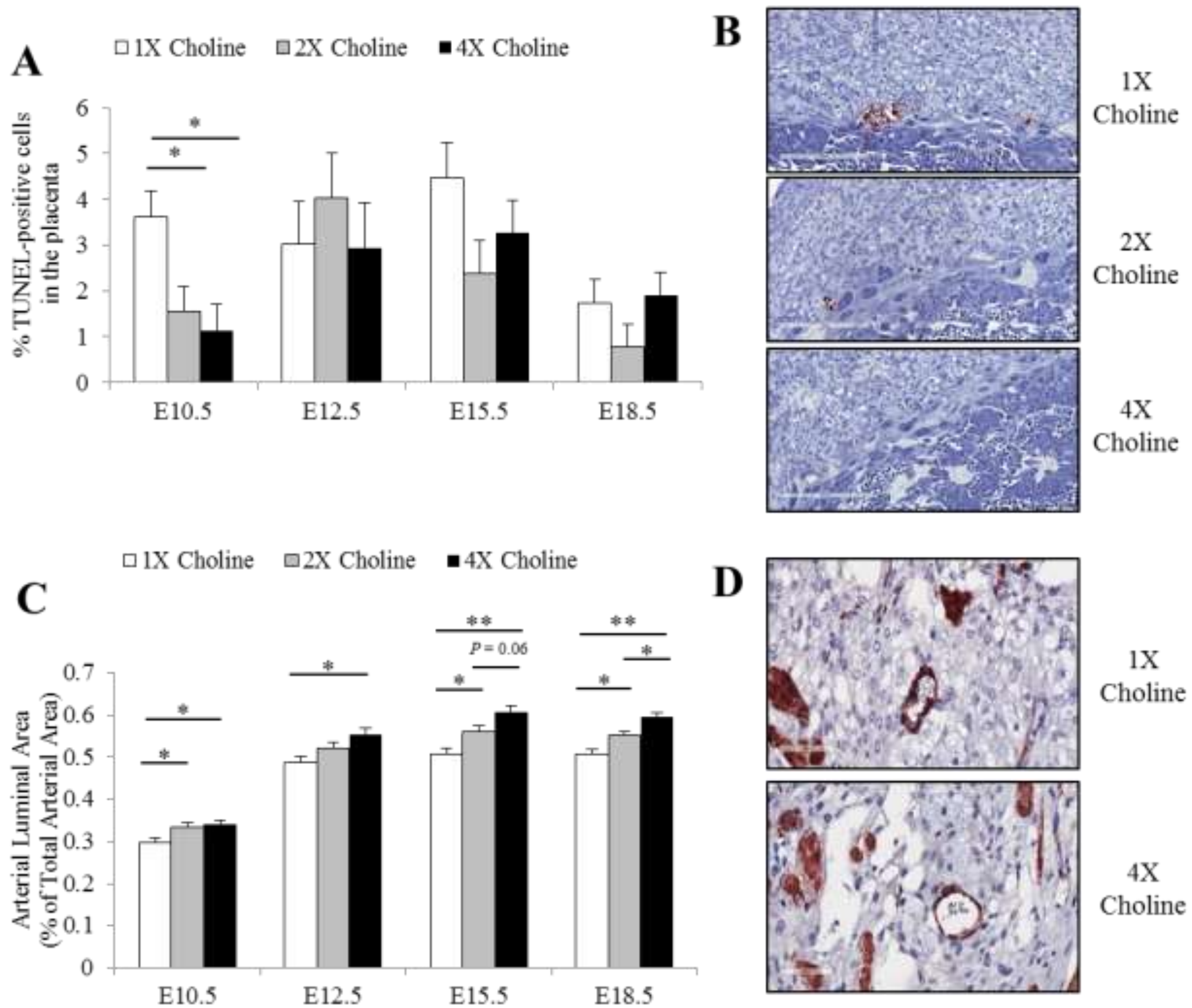


**Figure 1.6. A)** The percentage of TUNEL-positive cells in the placenta from dams receiving 1X, 2X or 4X choline treatments at E10.5, E12.5, E15.5 and E18.5. **B)** Representative images of the apoptotic nuclei within the E10.5 placentas are shown. **C)** Arterial luminal area in the maternal decidua from dams receiving 1X, 2X or 4X choline treatments at E10.5, E12.5, E15.5 and E18.5. **D)** Representative images of the smooth muscle actin staining within the maternal decidua are shown. Data were analyzed using the mixed linear model followed by post-hoc Bonferroni corrections. Values are presented as mean ± SEM. *$P \leq 0.05$, **$P \leq 0.001$.

***Phenotypic measurements of the fetus and the placenta***

Fetal weight and crown rump measurements were not affected ($P \geq 0.28$ and $P \geq 0.6$, respectively) by MCS. Maternal choline treatment also had no effects on placental weight ($P \geq 0.23$) or placental efficiency (the ratio of fetal weight to placental weight; $P \geq 0.19$) (Supplemental table 1.2).

## DISCUSSION

Previous investigations from our group have shown that extra dietary choline during the third trimester of human pregnancy suppresses placental production of an anti-angiogenic factor sFLT1 [19] while choline inadequacy in a cell culture model leads to a molecular profile that impairs trophoblast function and *in vitro* angiogenesis [18]. In the current study, we show effects of MCS on placental markers of inflammation, angiogenesis and apoptosis, all of which can influence placental vascular development. We also demonstrate that most of these choline-induced effects manifest in a fetal sex- and gestational day-dependent manner. Finally, we present preliminary *in vivo* evidence suggesting that a higher maternal choline intake during murine pregnancy improves remodeling of the maternal spiral arteries, a finding that merits additional investigation in the future.

### *MCS alters the placental abundance of inflammatory and angiogenic markers in a fetal sex- and gestational day-dependent manner*

Choline is an essential nutrient known to have an important role in fetal development [15]. In rodent studies, offspring from dams who received 4X choline (as compared to 1X choline) during pregnancy have improved cognitive function and attenuated age-related memory decline [20]. These neuroprotective consequences of extra maternal choline have been associated with inflammatory and angiogenic processes in the nervous system [17, 29]. We extend these findings to the mouse placenta whereby maternal choline supply modulated these same biological processes but in a manner that was dependent on fetal sex and gestational time point. Because aberrant expression of the inflammatory and angiogenic markers is associated with placental dysfunction, the choline-induced changes of these markers shown in the present study may have

important clinical implications on pregnancy outcomes.

One striking difference between male and female placentas in response to MCS was the expression of the pro-inflammatory cytokine *Il1b*. In the female placentas, 4X (versus 1X) choline induced a 40% reduction at E12.5, a 43% increase at E15.5, and a 30% reduction at E18.5. Although statistical significance was not achieved after adjusting for multiple testing, IL-1β protein abundance exhibited an expression pattern that paralleled those of the transcript. In contrast, IL-1β expression remained largely unchanged in the male placentas until E18.5, when 4X choline yielded a 26% reduction in *Il1b* transcript abundance and a 55% reduction in IL-1β protein abundance as compared to 1X choline. As some immune responses are shown to be more active and stronger in females compared to males [30], we speculate that the less pronounced effects of MCS on the expression of IL-1β in the male placentas may relate to the sex-specific differences in immune regulation. Notably, however, the sex-specific immune response to maternal choline may also be cytokine dependent, as suggested by the downregulation of *Tnf* expression in the male placentas at E10.5, but not in the female placentas, in response to 4X choline supplementation.

The observed choline-induced downregulation of placental pro-inflammatory cytokines at several gestational time points may be beneficial to placental development. Excessive production of placental TNF-a and IL-1β have been shown to impair vascular remodeling [31] and increase the risk of adverse pregnancy outcomes in both animals [5] and humans [9, 32, 33]. Consistent with these data, pharmacological targeting of these pro-inflammatory cytokines in animal models reverses some of the placental vascular abnormalities and improves pregnancy outcomes [34, 35]. Therefore, supplementing the maternal diet with extra choline may be a nutritional strategy for lowering the risk of developing pregnancy disorders characterized by an intensified placental

pro-inflammatory response.

As our prior investigation in extravillous human trophoblasts [18] found that cells cultured in a medium deficient in choline have an increased abundance of NF-κB, we expected that placental *Nfkb1* expression would be downregulated in response to MCS in the present study. Contrary to our hypothesis, the transcript abundance of *Nfkb1* at E18.5 was 28% higher in the 4X choline group, and this change was detected only in the female placentas. Although statistical significance was not achieved, the protein abundance in these placentas also showed a 30% increase, which was comparable to the change detected at the transcript level. The reason for this sex-specific difference and choline-induced upregulation of placental *Nfκb1* is unclear. However, consistent with the greater investment of female placentas in the maintenance of pregnancy [36], we hypothesize this induction of a pro-inflammatory state during late gestation may facilitate nutrient transport to the rapidly growing fetus [37].

MCS also affected the transcript abundance of angiogenic proteins in the female placentas, as evidenced by an approximately 30% upregulation of the pro-angiogenic factor *Vegfa* in late gestation. VEGF promotes endothelial cell proliferation and new blood vessel formation, and stimulates relaxation of the vascular system by increasing the production of nitric oxide [4]. Notably, reduced expression of VEGF is observed in placentas from preeclamptic women as compared to placentas from normotensive women [38], and adenoviral-mediated delivery of Vegf in a mouse model of preeclampsia resolves the maternal preeclamptic phenotype [23]. Taken together, these data suggest that the choline-induced increase of *Vegfa* may beneficially influence placental angiogenic balance, vascular development and pregnancy outcome.

Although the objectives of the present study did not focus on exploring the mechanisms

by which extra choline affects placental inflammatory and angiogenic processes, we suggest that some of these choline-induced effects are mediated by signaling pathways related to acetylcholine and protein kinase C (PKC). Choline is a precursor of acetylcholine and we have shown that MCS during pregnancy increased the placental concentration of acetylcholine and upregulated placental cholinergic receptor muscarinic 4 (*CHRM4)* expression [19, 22]. Others have shown that acetylcholine can signal through the alpha-7 nicotinic acetylcholine receptor, resulting in the recruitment of VEGF and blood vessel formation [39] as well as the reduction of pro-inflammatory cytokines [40]. Furthermore, biosynthesis of phosphatidylcholine from choline can prevent the accumulation of diacylglycerol and subsequent activation of PKC [15] which induces TNF-a production [41] and attenuates the actions of VEGF [42, 43]. In previous work, we demonstrated that the addition of a PKC inhibitor partially rescues aberrant *IL1B* expression induced by choline inadequacy in a cell culture model of extravillous human trophoblasts [18]. Because choline can be oxidized to generate the methyl donor betaine, it may be possible that an epigenetic mechanism is also involved in mediating these choline-induced effects.

The reasons for the sexual dimorphic placental response to MCS are also unclear, but it may relate to different rates of fetal development and different strategies to meet nutrient demands [44]. Regardless, these observations are consistent with the theory of fetal programming suggesting that female fetuses tend to generate a more adaptive response to environmental triggers (such as maternal diet) and invest more resources in developing their placentas [44, 45].

***MCS decreases placental apoptosis in early gestation***

Consistent with our prior investigation in extravillous human trophoblasts [18], we found that

supplementing the maternal diet with 2X and 4X choline decreased placental apoptosis at E10.5 by 57% and 68%, respectively. This reduction may be beneficial because it could increase the survival of trophoblasts and endothelial cells thereby enhancing the development of the placental vasculature.

***MCS increases the luminal area of the maternal spiral arteries***

In the present study, we found that placentas from the 4X choline supplemented groups exhibited larger maternal spiral artery luminal areas than the 1X choline group. To the best of our knowledge, these data are the first *in vivo* evidence indicating extra maternal choline may improve remodeling of the maternal spiral arteries. Nonetheless, blood flow measurements are needed to determine if the choline-induced increase in luminal area leads to enhanced uteroplacental perfusion.

***Conclusion***

Supplementing the maternal diet of mice with extra choline influences placental inflammatory, angiogenic and apoptotic processes, with possible consequences on placental vascular development. Of note, most of these choline-induced effects occur in a fetal sex- and gestational day-dependent manner, highlighting the importance of these variables in studies that examine the effects of dietary manipulation on placental development. A higher maternal choline intake also increased the luminal area of the maternal spiral arteries, which may influence placental perfusion. Overall, our data provide additional support for increasing maternal choline intake during normal pregnancy as a nutritional strategy to improve placenta-related pregnancy outcomes.

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**Supplemental Table 1.1.** Primers for PCR and quantitative PCR.

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| *Sry* | 5' TGGGACTGGTGACAATTGTC 3' | 5' GAGTACAGGTGTGCAGCTCT 3' |
| *Tnf* | 5’ AAGTTCCCAAATGGCCTCCC 3’ | 5’ TGGTGGTTTGCTACGACGTG 3’ |
| *Il1b* | 5’ TGCCACCTTTTGACAGTGATG 3’ | 5’ GCTCTTGTTGATGTGCTGCT 3’ |
| *Il6* | 5’ GGATACCACTCCCAACAGACC 3’ | 5’ GCCATTGCACAACTCTTTTCT 3’ |
| *Il10* | 5’ TAATAAGCTCCAAGACCAAGGTG 3’ | 5’ TCCAGCAGACTCAATACACACT 3’ |
| *Nfkb1* | 5’ AGCAACCAAAACAGAGGGGA 3’ | 5’ TTTGCAGGCCCCACATAGTT 3’ |
| *Vegfa* | 5’ CACTGGACCCTGGCTTTACT 3’ | 5’ ACTTGATCACTTCATGGGACTTCT 3’ |
| *Pgf* | 5’ TGTGCCGATAAAGACAGCCA 3’ | 5’ TCGTCTCCAGAATAGGTCTGC 3’ |
| *sFlt1* | 5’ GTCACAGATGTGCCGAATGG 3’ | 5’ TGGAGATCCGAGAGAAAATGGC 3’ |
| *Eng* | 5’ ATCAGTTTCCCGTCAGGCTC 3’ | 5’ GTTCGATGGTGTTGGATGCC 3’ |
| *Mmp14* | 5’ GCCCTCTGTCCCAGATAAGC 3’ | 5’ TTGGTTATTCCTCACCCGCC 3’ |
| *Tbp* | 5’ AGGAGCCAAGAGTGAAGAACAA 3’ | 5’ AACTTCACATCACAGCTCCCC 3’ |

**Supplemental Table 1.2.** Fetal and placental phenotypic measurements in response to three different maternal choline treatments (1X, 2X and 4X) at E10.5, E12.5, E15.5 and E18.5. Data were analyzed using a mixed linear model. Values are presented as mean ± SEM.

| **Fetal Weight (g)** | E10.5 | E12.5 | E15.5 | E18.5 |
|---|---|---|---|---|
| 1X Choline | 0.01 ± 0.001 | 0.08 ± 0.006 | 0.39 ± 0.01 | 1.16 ± 0.03 |
| 2X Choline | 0.02 ± 0.002 | 0.09 ± 0.007 | 0.39 ± 0.01 | 1.21 ± 0.02 |
| 4X Choline | 0.01 ± 0.001 | 0.09 ± 0.007 | 0.41 ± 0.01 | 1.21 ± 0.03 |
| *P* Value | *P* = 0.550 | *P* = 0.971 | *P* = 0.482 | *P* = 0.280 |
| **Crown Rump (mm)** | E10.5 | E12.5 | E15.5 | E18.5 |
| 1X Choline | 4.73 ± 0.13 | 9.07 ± 0.17 | 14.45 ± 0.25 | 23.98 ± 0.51 |
| 2X Choline | 4.67 ± 0.16 | 8.98 ± 0.20 | 14.17 ± 0.24 | 24.06 ± 0.50 |
| 4X Choline | 4.84 ± 0.13 | 8.92 ± 0.18 | 14.49 ± 0.26 | 23.74 ± 0.51 |
| *P* Value | *P* = 0.689 | *P* = 0.821 | *P* = 0.619 | *P* = 0.893 |
| **Placental Weight (g)** | E10.5 | E12.5 | E15.5 | E18.5 |
| 1X Choline | 0.03 ± 0.002 | 0.06 ± 0.002 | 0.08 ± 0.004 | 0.09 ± 0.004 |
| 2X Choline | 0.04 ± 0.002 | 0.06 ± 0.003 | 0.08 ± 0.004 | 0.09 ± 0.004 |
| 4X Choline | 0.03 ± 0.002 | 0.05 ± 0.003 | 0.08 ± 0.004 | 0.09 ± 0.004 |
| *P* Value | *P* = 0.225 | *P* = 0.278 | *P* = 0.974 | *P* = 0.661 |
| **Placental Efficiency** | E10.5 | E12.5 | E15.5 | E18.5 |
| 1X Choline | 0.39 ± 0.03 | 1.48 ± 0.07 | 5.04 ± 0.33 | 13.75 ± 0.56 |
| 2X Choline | 0.40 ± 0.04 | 1.45 ± 0.08 | 4.87 ± 0.32 | 13.77 ± 0.53 |
| 4X Choline | 0.39 ± 0.03 | 1.63 ± 0.07 | 5.33 ± 0.35 | 13.90 ± 0.56 |
| *P* Value | *P* = 0.989 | *P* = 0.191 | *P* = 0.632 | *P* = 0.977 |

# CHAPTER 2

Maternal choline supplementation modulates placental nutrient transport and metabolism in late gestation of mouse pregnancy*

*Kwan STC, King JH, Yan J, Wang Z, Jiang X, Hutzler JS, Klein HR, Brenna JT, Roberson MS, Caudill MA. Maternal choline supplementation modulates placental nutrient transport and metabolism in late gestation of mouse pregnancy. *Under Review.*

**ABSTRACT**

**Background:** Fetal growth is dependent upon placental nutrient supply, which is influenced by placental perfusion and transporter abundance. Previous research indicates that adequate choline nutrition during pregnancy improves placental vascular development, supporting the hypothesis that choline may affect placental nutrient transport efficiency.

**Objective:** The present study sought to determine the impact of maternal choline supplementation (MCS) on placental nutrient transporter abundance and placental nutrient metabolism during late gestation when fetal growth is maximal.

**Methods:** Pregnant mice were randomized to receive 1, 2 or 4 times (X) the normal choline content of rodent diets. The placentas and fetuses were harvested on embryonic day (E) 15.5 and 18.5. The placental abundance of amino acid (*Snat*), fatty acid (*Fatp)*, glucose (*Glut*), choline (*Ctl1*) and acetylcholine (*Oct3*) transporters, glycogen metabolic enzymes (*Gys1, Gbe1, Pygm* and *Gsk3β*) as well as placental concentration of glycogen were quantified. Concentrations of choline metabolites were also measured in the placentas and fetal brains.

**Results:** In the female placentas, MCS downregulated *Snat4*, *Glut1*, *Gys1* and *Gbe1* but upregulated *Fatp4* and *Pygm* at E15.5. At E18.5, MCS upregulated *Snat1*, *Glut3*, *Gys1* and *Gbe1*, and increased glycogen concentration in the female placentas. In the male placentas, MCS decreased the abundance of *Glut1* and *Gys1* at E15.5, and increased the abundance of *Snat1* and *Fatp4* at E18.5. Higher placental *Ctl1* and *Oct3* expressions were also detected in response to MCS in both sexes, with subsequent effects on the concentration of choline metabolites in the placentas and fetal brains.

**Conclusions:** These data suggest that MCS affected placental nutrient transporter abundance and nutrient metabolism in a fetal-sex specific manner during late gestation of mouse pregnancy.

## INTRODUCTION

The theory of fetal programming posits that the intrauterine environment plays a key role in determining offspring health later in life. By functioning as a nutrient sensor, the placenta is positioned to play an integral role in fetal programming because it actively modifies processes involved in uteroplacental perfusion, placental nutrient transport and metabolism to modulate the efficiency by which nutrients are transported to the fetus (1). Numerous studies (2-9) have reported aberrant expression and activity of placental macronutrient transporters and metabolic enzymes in preeclampsia, intrauterine growth restriction, maternal obesity and gestational diabetes, all of which have adverse consequences on fetal development.

Amino acids are essential for normal fetal growth because they are used in protein synthesis, energy production and signaling pathways (10). The placental system A amino acid transporters are responsible for the uptake of nonessential amino acids such as glycine, which can subsequently be used in exchange for the uptake of essential amino acids such as leucine via other transporters (1). Therefore, system A amino acid transporters are important for transporting both nonessential and essential amino acids. The major placental system A amino acid transporters are SLC38A1 (SNAT1), SLC38A2 (SNAT2) and SLC38A4 (SNAT4) (1).

Long-chain polyunsaturated fatty acids (LCPUFAs) are also needed for fetal development. Not only do they provide energy, but they are also components of cell membranes and precursors to signaling molecules such as eicosanoids (11). Sufficient supply of LCPUFAs, particularly docosahexaenoic acid (DHA), is critical for normal development of the fetal nervous system, and has been correlated with better cognitive outcomes in postnatal life (11, 12). However, both the placenta and fetus have minimal enzymatic activity to generate LCPUFAs. Therefore, placental transfer of LCPUFAs from the maternal circulation is the major source of

fetal LCPUFAs (12). Placentas have several fatty acid transporters, but a study conducted in healthy pregnant women indicates that only SLC27A1 (FATP1) and SLC27A4 (FATP4) are correlated with placental and fetal DHA concentrations (13).

Similar to fatty acids, the fetus has limited ability to synthesize glucose, which is the major energy substrate used in fetal metabolism. Consequently, the fetus relies on the placenta to transport glucose from the maternal circulation (1). Two major glucose transporters are found in the placenta: SLC2A1 (GLUT1) and SLC2A3 (GLUT3). Placental trophoblasts also store a large amount of glycogen (14) and express major enzymes in the glycogen metabolic pathway (8, 15, 16). As such, placental glycogen can be mobilized to provide glucose to the developing fetus at times when fetal metabolic demand exceeds maternal supply (14).

Many studies (15, 17-23) have demonstrated that different diet manipulations modulate the expression and activity of these nutrient transporters and enzymes. A higher maternal intake of choline, an essential micronutrient, during pregnancy has been shown to influence placental vascular development and offspring cognition (24-27), but its impact on placental nutrient transporters and metabolic enzymes remains largely unknown. Therefore, the current study was conducted to provide insights into the effect of maternal choline supplementation (MCS) on factors that mediate placental nutrient delivery and metabolism during late gestation of mouse pregnancy.

## METHODS

### Animals and Diets

All animal protocols and procedures used in this study were approved by the Institutional Animal Care and Use Committees at Cornell University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Adult male and female Non-Swiss Albino (NSA) mice were obtained from Harlan (Indianapolis, IN) and used for breeding during which time they were allowed *ad libitum* access to rodent chow and water. The offspring of the breeding pairs were weaned at 3 weeks old and provided the 1X choline diet (Dyets, Bethlehem, PA; Table 2.1). Five days prior to mating, female mice were randomized to receive the 1X choline diet, 2X choline diet or 4X choline diet (Dyets, Bethlehem, PA; Table 2.1). These dosages were based on previous studies indicating improved vascular development in the human placenta with 2X choline supplementation (26) and improved brain development in the mouse offspring with 4X choline supplementation (27). Presence of a vaginal plug indicated conception and was designated as gestational day (E) 0.5. The female mice continued to consume their assigned diet until they were euthanized at E15.5 or E18.5. These late gestational time-points represent periods of rapid growth and increased fetal nutrient demand.

**Table 2.1.** Composition of the experimental diets.

| Dietary Components | 1X Choline Diet (Dyet #103345) | 2X Choline Diet (Dyet #103346) | 4X Choline Diet (Dyet #103347) |
|---|---|---|---|
| Protein, % kcal | 19 | 19 | 19 |
| Carbohydrate, % kcal | 64 | 64 | 64 |
| Fat, % kcal | 17 | 17 | 17 |
| Total, % kcal | 100 | 100 | 100 |
| **Ingredients, g/kg** | | | |
| Casein | 200 | 200 | 200 |
| L-Cystine | 3 | 3 | 3 |
| Sucrose | 100 | 100 | 100 |
| Cornstarch | 398.586 | 397.186 | 394.386 |
| Dyetrose | 132 | 132 | 132 |
| Soybean Oil | 70 | 70 | 70 |
| t-Butylhydroquinone | 0.014 | 0.014 | 0.014 |
| Cellulose | 50 | 50 | 50 |
| Mineral Mix #210025 | 35 | 35 | 35 |
| Vitamin Mix #310025 | 10 | 10 | 10 |
| Choline Chloride | 1.4 | 2.8 | 5.6 |

## Tissue Collection and Processing

Both the placentas and fetuses were collected. The placentas were weighed, cut in half and placed in RNA*later* or flash frozen in liquid nitrogen. The fetuses were weighed and flash frozen in liquid nitrogen. All tissues were stored at -80°C until analytical measurements were performed. Placentas and fetuses from 6-8 dams per each treatment group at each gestational time point were used for all the measurements.

## Analytical Measurements

### *Sex genotyping*

DNA was extracted from the fetuses, and PCR was performed using a commercial kit (Qiagen) to determine fetal sex (Supplemental Table 2.1).

### *mRNA abundance of placental transporters and enzymes*

Placental RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed using ImProm-II Reverse Transcription System (Promega) with the following conditions: 25°C for 10 minutes, 42°C for 40 minutes and 95°C for 5 minutes. Quantitative PCR was performed using the SYBR® Green system in Roche LightCycler480 with the following conditions: 95°C for 5 minutes, followed by 40 cycles with 15 sec at 95°C, 30 sec at 63°C, and 30 sec at 72°C. A dissociation stage was added at the end of the amplification cycles to evaluate the specificity of the final PCR products. Data are expressed by the ΔΔCt method where the expression of the targeted gene is normalized by the expression of the housekeeping gene as fold change before comparison between samples. Genes of interest include *Snat1, Snat2, Snat4, Fatp1, Fatp4, Glut1, Glut3, Gys1, Gbe1, Pygm, Gsk3β, Ctl1* and *Oct3*. TATA box binding

protein, *Tbp*, was used as the housekeeping gene because its expression is stable in placental tissue (28) and under different choline intake levels (29). All primers were designed using Primer-BLAST available on the NCBI website (Supplemental Table 2.1).

*Transporter proteins abundance in the placental membrane*

Placental tissues were homogenized in ten volumes of Buffer A [50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktails (Sigma-Aldrich)] and centrifuged at 800g for 5 minutes at 4°C. The supernatant was then centrifuged again at 17,000g for 15 minutes at 4°C. After centrifugation, the pellet, which contained the membrane-bound proteins, was resuspended in ten volumes of Buffer B [50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 2% IPEGAL CA-630 (Santa Cruz Biotechnology) and protease inhibitor cocktails (Sigma-Aldrich)]. Total membrane protein concentration was determined by the Bradford assay (Thermo Scientific Pierce). 40μg of the extracts was subjected to SDS-PAGE electrophoresis and transferred onto Immobilon FL PVDF membranes (EMD Millipore). Membranes were blocked in LI-COR blocking buffer and then incubated overnight with primary antibodies for GLUT1 (ProteinTech; 1:200), GLUT3 (EMD Millipore; 1:1000) and β-actin (Cell Signaling Technology; 1:5000). Secondary antibodies (IRDye 800CW goat anti-rabbit and IRDye 680RD goat anti-mouse (LI-COR); 1:10,000) were added to the membranes and incubated for one hour. Protein bands were visualized and quantified by the Odyssey imaging system (LI-COR). Data are expressed as the ratio of the intensity of targeted protein to the intensity of β-actin before comparison between samples.

*Glycogen concentration in the placenta*

Placental glycogen concentration was quantified using a commercial glycogen assay kit (Abnova) following the manufacturer's protocol. Data are expressed as milligram of glycogen per gram of tissue.

*Placental concentration of choline metabolites*

Concentrations of choline, betaine, phosphocholine, phosphatidylcholine, glycerophosphocholine and sphingomyelin in the placentas were determined using LC/MS-MS according to Koc et al (30). Placental acetylcholine and trimethylamine N-oxide (TMAO) concentrations were determined using LC/MS-MS according to Holm et al (31) with modifications based on our equipment (32).

*Concentration of choline metabolites in fetal brain*

Choline, betaine, acetylcholine, phosphocholine, phosphatidylcholine and glycerophosphocholine concentrations in the fetal brain were determined using LC/MS-MS according to Koc et al (30).

*Concentration of DHA in fetal brain*

Fetal brain DHA analysis was accomplished as described previously (33). Briefly, fatty acids were extracted and methylated to generate fatty acid methyl esters (FAME) following a modified one-step hydrolysis and methylation protocol. Heptadecanoic acid (17:0) was added as an internal standard. FAME quantification was performed by a gas chromatograph with a flame ionization detector (GC-FID), and FAME identification was done using GC-covalent adduct chemical ionization mass spectrometry (GC-CACI-MS). DHA concentration was directly

calibrated to an internal standard and expressed as milligram of DHA per milligram of tissue.

## Statistical Analysis

All data were stratified by gestational day and fetal sex, and then analyzed using a mixed linear model followed by post-hoc Fisher's Least Significant Difference test. Choline treatment was included as an independent fixed effect, maternal identification as an independent random effect, and litter size as a covariate if it had $P \leq 0.05$. All statistical analyses were performed using SPSS software, Version 23 (SPSS Inc, Chicago, IL). Data are presented as means ± SEM, and differences were considered to be statistically significant at $P \leq 0.05$.

## RESULTS

### Placental Amino Acid Transporters

The expression of *Snat4* in the female placentas at E15.5 was reduced in response to 2X choline ($P$ = 0.005 vs 1X choline) and 4X choline ($P$ = 0.025 vs 1X choline), but these effects disappeared by E18.5 and were not observed in the male placentas. MCS did not affect placental *Snat1* expression at E15.5; however, 4X choline upregulated *Snat1* expression in the male placentas ($P$ = 0.023 vs 1X choline, $P$ = 0.002 vs 2X choline) and in the female placentas ($P$ = 0.028 vs 1X choline) at E18.5. Placental *Snat2* expression was unaffected by MCS (Figure 2.1).

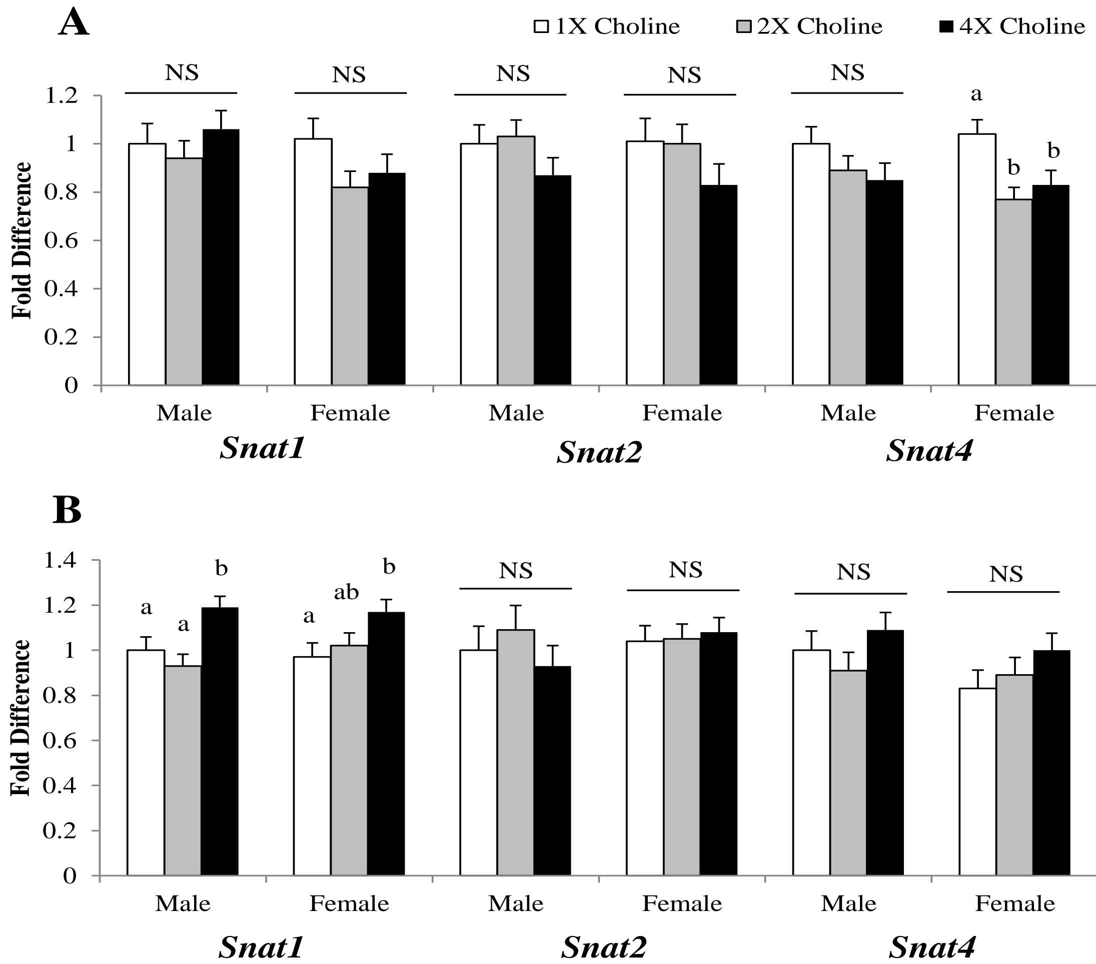


**Figure 2.1.** Transcript abundance of the amino acid transporters (*Snat1, Snat2, Snat4*) in the placentas from dams in the 1X, 2X and 4X choline group at **A)** E15.5 and **B)** E18.5 (n=6-8 dams per group, per gestational day). Data are expressed as fold-change relative to the housekeeping gene *Tbp*. After normalization, the mean value of the male in 1X choline group for each gene was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Values are given as mean ± SEM. Means without a common letter are significantly different ($P \leq 0.05$). NS = not significant.

**Placental Fatty Acid Transporters**

Both 2X choline and 4X choline increased the abundance of *Fatp4* in the female placentas at E15.5 (*P* = 0.017 and 0.011 vs 1X choline, respectively), but not at E18.5. The expression of *Fatp4* in the male placentas was higher at E18.5 in response to 4X choline (*P* = 0.05 vs 1X choline, *P* = 0.035 vs 2X choline) (Figure 2.2). Placental *Fatp1* expression was unaffected by MCS (data not shown).

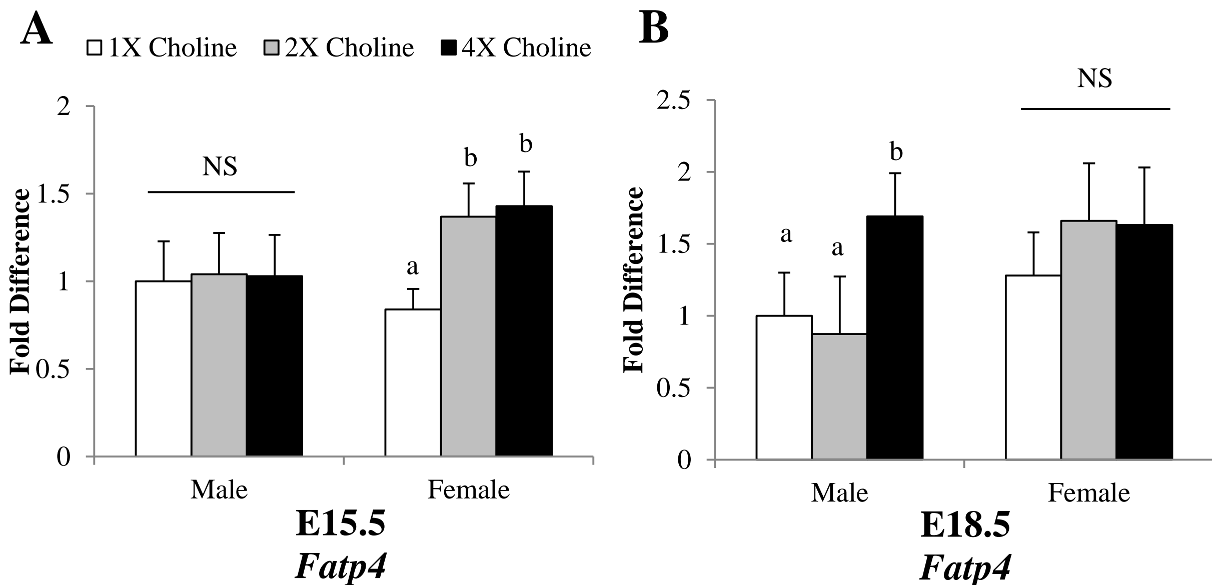


**Figure 2.2.** Transcript abundance of the fatty acid transporter, *Fatp4*, in the placentas from dams in the 1X, 2X and 4X choline group at **A)** E15.5 and **B)** E18.5 (n=6 dams per group, per gestational day). Data are expressed as fold-change relative to the housekeeping gene *Tbp*. After normalization, the mean value of the male in 1X choline group was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Values are given as mean ± SEM. Means without a common letter are significantly different ($P \leq 0.05$). NS = not significant.

**Placental Glucose Transporters**

In response to 4X choline, lower *Glut1* transcript abundance was detected in both the male ($P$ = 0.028 vs 1X choline, $P$ = 0.008 vs 2X choline) and female ($P$ = 0.038 vs 1X choline, $P$ = 0.024 vs 2X choline) placentas at E15.5. MCS also decreased the number of GLUT1 transporter proteins in the membrane of both male ($P$ = 0.041 for 4X choline vs 1X choline, $P$ = 0.043 for 2X choline vs 1X choline) and female ($P$ = 0.023 for 4X choline vs 1X choline, $P$ = 0.039 for 4X choline vs 2X choline) placentas (Figure 2.3A). No detectable effects of choline on the transcript or protein abundance of GLUT1 were found at E18.5 (data not shown).

Placental GLUT3 transcript and protein expression did not differ among the choline groups at E15.5 (data not shown). Although no changes in *Glut3* transcript abundance were detected, more GLUT3 transporter proteins were found in the membrane of the female placentas from 4X choline group ($P$ = 0.016 vs 1X choline) at E18.5 (Figure 2.3B). The effects of MCS were sex–specific since similar changes were not observed in the male placentas at this time.

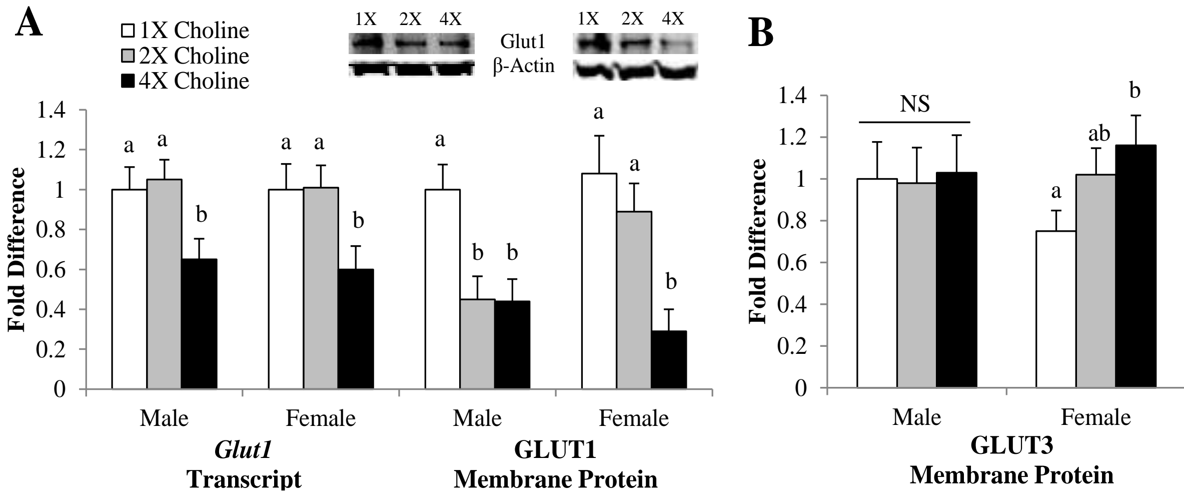


**Figure 2.3. A)** Transcript and membrane protein abundance of the glucose transporter 1 (GLUT1) in the placentas from dams in the 1X, 2X and 4X choline group at E15.5 (n=6-7 dams per group). **B)** Membrane protein abundance of the glucose transporter 3 (GLUT3) in the placentas from dams in the 1X, 2X and 4X choline group at E18.5 (n=7 dams per group). Gene data are expressed as fold-change relative to the housekeeping gene *Tbp*. Protein data are expressed as fold-change relative to β-actin. After normalization, the mean value of the male in 1X choline group was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Values are given as mean ± SEM. Means without a common letter are significantly different ($P \leq 0.05$). NS = not significant.

**Placental Glycogen Metabolic Enzymes**

MCS reduced the expression of the glycogen synthesizing enzyme *Gys1* ($P$ = 0.046 for 2X choline vs 1X choline, $P$ = 0.05 for 4X choline vs 1X choline) in the male placentas at E15.5. In the female placentas, the expression of *Gys1* ($P$ = 0.024 for 2X choline vs 1X choline, $P$ = 0.04 for 4X choline vs 1X choline) and *Gbe1* ($P$ = 0.047 for 2X choline vs 1X choline, $P$ = 0.04 for 4X choline vs 1X choline) were downregulated in response to MCS. 4X choline also upregulated the expression of *Pygm*, an enzyme involved in glycogen breakdown ($P$ = 0.027 vs 2X choline) at this time point. No difference in the placental expression of *Gsk3β* was detected in response to MCS (Figure 2.4A).

By E18.5, MCS upregulated the expression of these glycogen metabolic enzymes in the female placentas. Specifically, both 2X choline ($P$ = 0.025 vs 1X choline) and 4X choline ($P$ = 0.004 vs 1X choline) increased placental *Gys1* abundance. 4X choline also increased the placental abundance of *Gbe1* ($P$ = 0.024 vs 1X choline). The effects of choline were sex-specific since MCS had no comparable impacts on the expression of these enzymes in the male placentas. Placental *Pygm* and *Gsk3β* were unaffected by extra choline at this time point (Figure 2.4B).

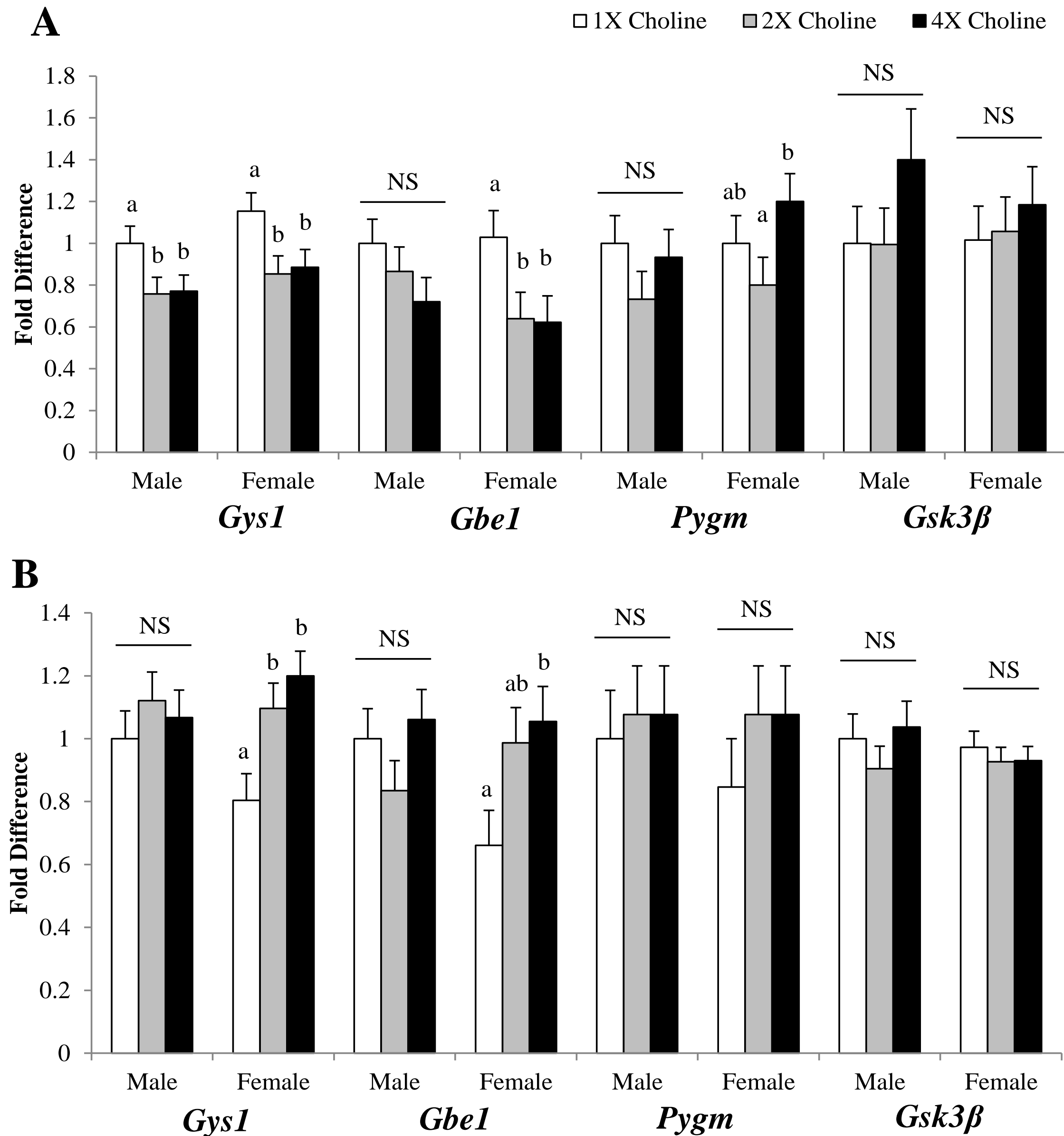


**Figure 2.4.** Transcript abundance of the enzymes involved in glycogen metabolism (*Gys1, Gbe1, Pygm* and *Gsk3β*) in the placentas from dams in the 1X, 2X and 4X choline group at **A)** E15.5 and **B)** E18.5 (n=6-8 dams per group, per gestational day). Data are expressed as fold-change relative to the housekeeping gene *Tbp*. After normalization, the mean value of the male in 1X choline group for each gene was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Values are given as mean ± SEM. Means without a common letter are significantly different ($P \leq 0.05$). NS = not significant.

**Placental Glycogen Concentration**

A generalized trend for a reduced glycogen concentration in the female placentas at E15.5 was detected in response to MCS, but it did not reach statistical significance ($P \geq 0.06$). In contrast, glycogen concentration doubled in the female placentas from 4X choline group at E18.5 ($P = 0.01$ vs 1X choline). Borderline higher concentrations of glycogen were also detected in the female placentas from the 2X choline group at E18.5 ($P = 0.051$ vs 1X choline). The glycogen concentration in the male placentas remained largely unaffected by MCS at either time point (Table 2.2).

**Table 2.2.** Effect of maternal choline supplementation on placental glycogen concentration at E15.5 and E18.5.[1]

| | Male | | | Female | | |
|---|---|---|---|---|---|---|
| **E15.5** | 1X Choline | 2X Choline | 4X Choline | 1X Choline | 2X Choline | 4X Choline |
| Glycogen Concentration (mg/g tissue) | 19.2 ± 2.6 | 17.1 ± 2.2 | 15.8 ± 2.6 | 19.1 ± 2.0 | 13.9 ± 1.9 | 14.4 ± 2.0 |
| **E18.5** | 1X Choline | 2X Choline | 4X Choline | 1X Choline | 2X Choline | 4X Choline |
| Glycogen Concentration (mg/g tissue) | 9.8 ± 2.8 | 8.9 ± 2.8 | 10.4 ± 2.8 | 6.0 ± 2.0 | 10.9 ± 2.0 # | 12.7 ± 2.0 * |

[1] Values are given as mean ± SEM. *$P = 0.01$ vs 1X choline. #$P = 0.051$ vs 1X choline.

**Placental Choline Transporters and Choline Metabolites Concentration**

Placental choline transporter *Ctl1* was upregulated in the male placentas in response to 4X choline at E15.5 ($P = 0.036$ vs 1X choline, $P = 0.05$ vs 2X choline) and at E18.5 ($P = 0.002$ vs 1X choline, $P < 0.001$ vs 2X choline). In the female placentas, 2X choline ($P = 0.055$ vs 1X choline) and 4X choline ($P = 0.015$ vs 1X choline) increased *Ctl1* expression at E15.5. Similar upregulation was observed at E18.5 ($P = 0.049$ for 2X choline vs 1X choline, $P = 0.007$ for 4X choline vs 1X choline) (Figure 2.5B). The expression of *Oct3*, which transports acetylcholine to the fetus, was higher in response to 4X choline in both the male and female placentas at E15.5 ($P = 0.038$ and 0.023 vs 1X choline, respectively) (Figure 2.5C).

In the male placentas, 4X choline reduced the acetylcholine concentration ($P < 0.001$ vs 1X choline and vs 2X choline; Figure 2.5E) at E15.5. The TMAO concentration in these placentas increased in response to both 2X choline ($P = 0.007$ vs 1X choline) and 4X choline ($P < 0.001$ vs 1X choline and vs 2X choline; Figure 2.5F). At E18.5, male placentas from 4X choline group had a lower concentration of choline ($P = 0.032$ vs 1X choline; Figure 5D) and phosphocholine ($P = 0.028$ vs 1X choline; Figure 2.5G), but a higher concentration for both acetylcholine ($P < 0.001$ vs 1X choline, $P = 0.029$ vs 2X choline; Figure 2.5E) and TMAO ($P < 0.001$ vs 1X choline and vs 2X choline; Figure 2.5F). Male placentas from 2X choline group at E18.5 also had a lower phosphocholine concentration ($P = 0.01$ vs 1X choline; Figure 2.5G) as well as a higher acetylcholine ($P = 0.034$ vs 1X choline; Figure 2.5E) and TMAO ($P = 0.025$ vs 1X choline; Figure 2.5F) concentration.

The concentration of acetylcholine in the female placentas at E15.5 decreased in response to 2X choline ($P = 0.034$ vs 1X choline) and 4X choline ($P < 0.001$ vs 1X choline; Figure 2.5E), whereas a higher concentration of TMAO ($P = 0.003$ for 2X choline vs 1X choline, $P < 0.001$ for

4X choline vs 1X choline and vs 2X choline; Figure 2.5F) and glycerophosphocholine (*P* = 0.02 for 4X choline vs 1X choline; Figure 2.5I) were found in these placentas. At E18.5, MCS upregulated the concentration of acetylcholine (*P* = 0.032 for 2X choline vs 1X choline, *P* < 0.001 for 4X choline vs 1X choline and vs 2X choline; Figure 2.5E), TMAO (*P* = 0.009 for 2X choline vs 1X choline, *P* < 0.001 for 4X choline vs 1X choline and vs 2X choline; Figure 2.5F), phosphocholine (*P* = 0.015 for 2X choline vs 1X choline, *P* = 0.008 for 4X choline vs 1X choline; Figure 2.5G), and phosphatidylcholine (*P* = 0.006 for 2X choline vs 1X choline, *P* < 0.001 for 4X choline vs 1X choline; Figure 2.5H) in the female placentas.

MCS did not affect the concentrations of betaine and sphingomyelin in the male and female placentas at either study time point (data not shown).

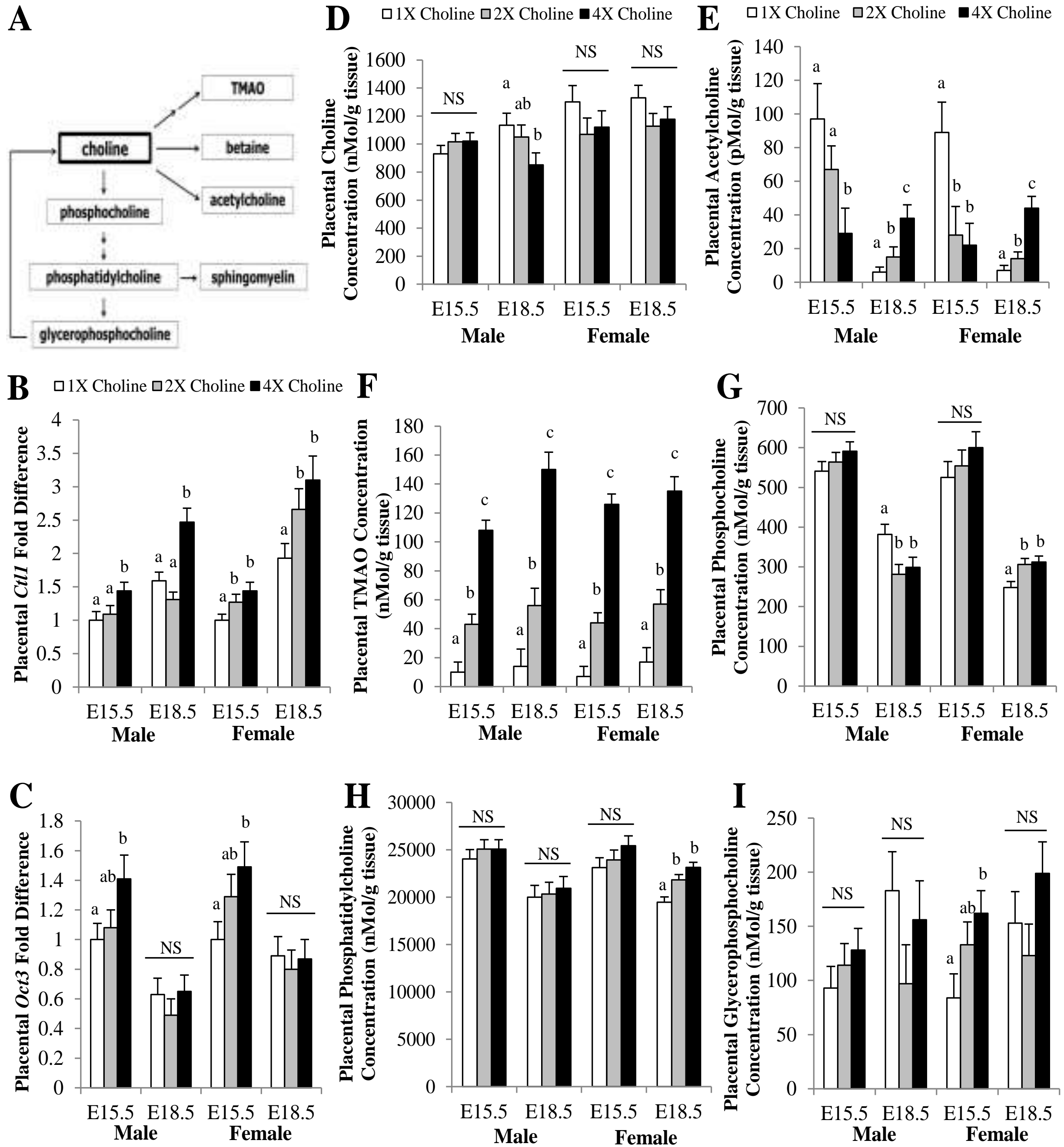


**Figure 2.5. A)** Choline metabolic pathways. Transcript abundance of **B)** *Ctl1* and **C)** *Oct3* in the placentas from dams in the 1X, 2X and 4X choline group at E15.5 and E18.5 (n=6 dams per group, per gestational day). Data are expressed as fold-change relative to the housekeeping gene *Tbp*. After normalization, the mean value of the 1X choline group at E15.5 for each sex was assigned a value of 1 and the mean values of the other groups were presented as a fraction of this value. Concentration of **D)** choline, **E)** acetylcholine, **F)** TMAO, **G)** phosphocholine, **H)** phosphatidylcholine, and **I)** glycerophosphocholine in the placentas from dams in the 1X, 2X and 4X choline group at E15.5 and E18.5 (n=7 dams per group, per gestational day). Values are given as mean ± SEM. Means without a common letter are significantly different ($P \leq 0.05$). NS = not significant.

**Placental and Fetal Weight**

No differences in the placental weight ($P \geq 0.66$) or fetal weight ($P \geq 0.28$) were found among the choline groups when the data were analyzed without stratifying by fetal sex (Table 2.3; (24)). When the data were analyzed separately for each sex, a trend ($P = 0.07$) for a higher weight was detected in the female fetuses from 4X choline group at E18.5 compared to those from the 1X choline group. No difference was detected in the weight of their placentas. The weights of the male fetuses and their placentas were also unaffected by MCS (Table 2.3).

**Table 2.3.** Effect of maternal choline supplementation on fetal and placental weight at E15.5 and E18.5.[1]

| | Overall effects | | | Male | | | Female | | |
|---|---|---|---|---|---|---|---|---|---|
| **E15.5** | 1X Choline | 2X Choline | 4X Choline | 1X Choline | 2X Choline | 4X Choline | 1X Choline | 2X Choline | 4X Choline |
| Fetal Weight (g) | 0.39 ± 0.01 | 0.39 ± 0.01 | 0.41 ± 0.01 | 0.390 ± 0.015 | 0.387 ± 0.014 | 0.411 ± 0.015 | 0.391 ± 0.013 | 0.387 ± 0.014 | 0.405 ± 0.015 |
| Placental Weight (g) | 0.08 ± 0.004 | 0.08 ± 0.004 | 0.08 ± 0.004 | 0.086 ± 0.004 | 0.081 ± 0.004 | 0.083 ± 0.004 | 0.078 ± 0.004 | 0.084 ± 0.004 | 0.079 ± 0.005 |
| **E18.5** | 1X Choline | 2X Choline | 4X Choline | 1X Choline | 2X Choline | 4X Choline | 1X Choline | 2X Choline | 4X Choline |
| Fetal Weight (g) | 1.16 ± 0.03 | 1.21 ± 0.02 | 1.21 ± 0.03 | 1.183 ± 0.027 | 1.248 ± 0.027 | 1.205 ± 0.027 | 1.133 ± 0.027 | 1.173 ± 0.025 | 1.204 ± 0.026$^{\#}$ |
| Placental Weight (g) | 0.09 ± 0.004 | 0.09 ± 0.004 | 0.09 ± 0.004 | 0.090 ± 0.005 | 0.091 ± 0.005 | 0.091 ± 0.005 | 0.081 ± 0.004 | 0.090 ± 0.004 | 0.085 ± 0.004 |

[1] Values are given as mean ± SEM. $^{\#}P = 0.07$ vs 1X choline.

## Choline Metabolites in Fetal Brain

There was more choline in the brain of the male fetuses from 4X choline group at E15.5 (*P* = 0.007 vs 1X choline; Figure 2.6A). More acetylcholine was also detected in their brain in response to 4X choline (*P* = 0.021 vs 1X choline, *P* = 0.08 vs 2X choline; Figure 2.6B). At E18.5, 4X choline increased the concentration of phosphocholine in the brain of the male fetuses (*P* = 0.026 vs 1X choline; Figure 2.6C).

At E15.5, borderline higher acetylcholine concentration was detected in the brain of female fetuses from the 4X choline group (*P* = 0.052 vs 1X choline). The female fetuses from the 2X choline group also tended to have more acetylcholine in their brain (*P* = 0.08 vs 1X choline; Figure 2.6B). In response to 4X choline, higher concentrations of phosphocholine (*P* = 0.05 vs 1X choline and *P* = 0.003 vs 2X choline) and betaine (*P* = 0.018 vs 1X choline) were found in the brain of the female fetuses at E18.5 (Figure 2.6C-D).

MCS did not affect the brain concentrations of phosphatidylcholine and glycerophosphocholine in the female and male fetuses at either study time point (data not shown).

## DHA Concentration in Fetal Brain

Because the placental *Fatp4* expression was altered by maternal choline treatments, we also measured the concentration of DHA in fetal brain. DHA concentration in male fetal brains tended to be lower at E15.5 in the 4X choline group compared to 1X choline (*P* = 0.054) and 2X choline (*P* = 0.056), but no differences were detected at E18.5 among the choline groups. In female fetal brains, DHA concentration was significantly higher in response to 2X choline (*P* = 0.013 vs 1X choline) and 4X choline (*P* = 0.028 vs 1X choline) at E18.5 (Figure 2.6E). Similar

results were obtained with the inclusion of fetal brain weight as a covariate in the statistical model (data not shown).

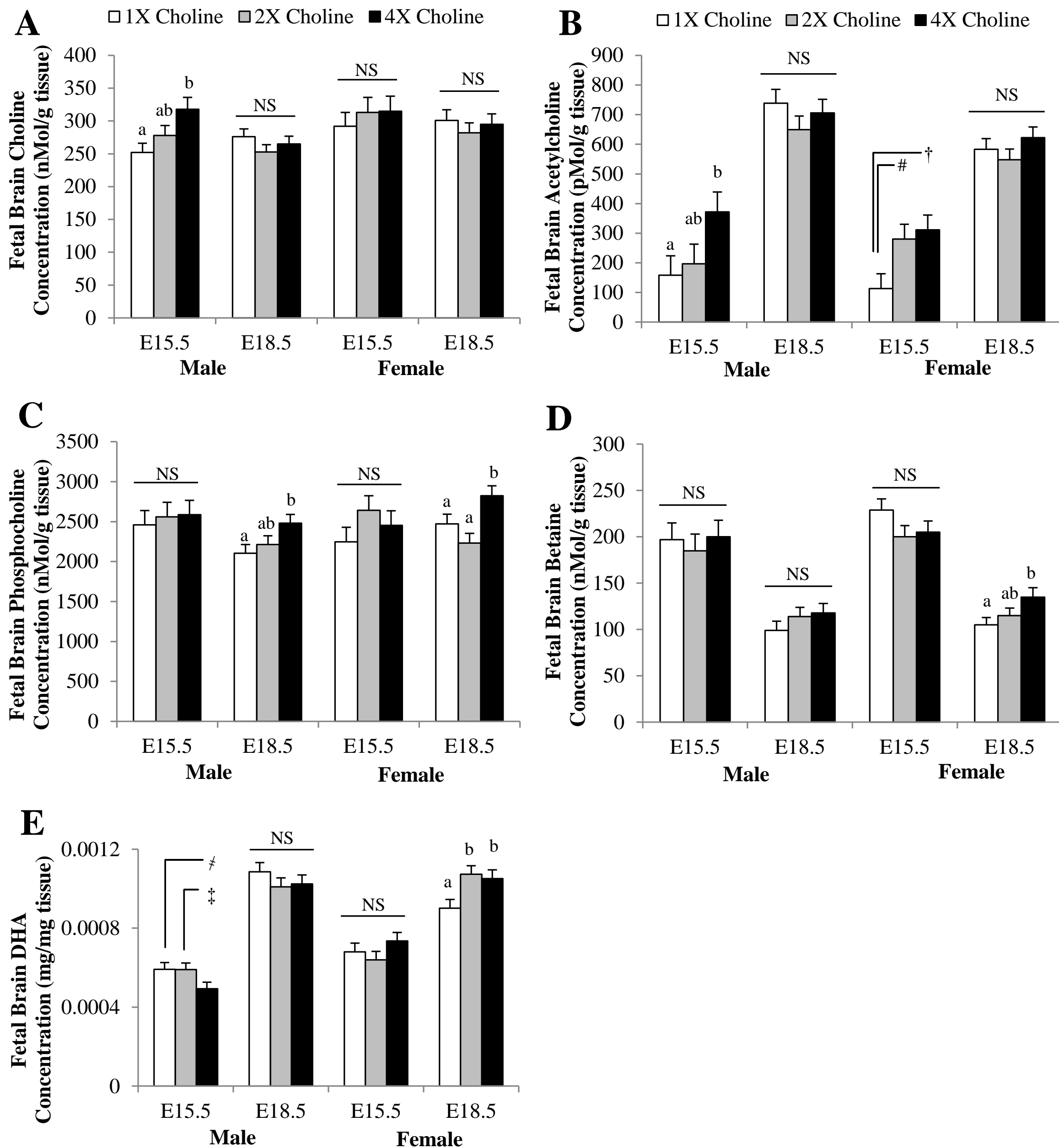


**Figure 2.6.** Concentration of **A)** choline, **B)** acetylcholine, **C)** phosphocholine, **D)** betaine and **E)** DHA in the brain of fetuses whose mothers were in the 1X, 2X and 4X choline group at E15.5 and E18.5 (n=7 dams per group, per gestational day). Values are given as mean ± SEM. Means without a common letter are significantly different ($P \leq 0.05$). $^{\#}P = 0.08$ vs 1X choline. $^{\dagger}P = 0.052$ vs 1X choline. $^{\nmid}P = 0.054$ vs 1X choline. $^{\ddagger}P = 0.056$ vs 2X choline. NS = not significant.

**DISCUSSION**

In the present study, we demonstrate effects of maternal choline supplementation during late gestation of mouse pregnancy on placental abundance of nutrient transporters and placental nutrient metabolism (i.e., glucose and choline). Notably, these choline-induced effects were a function of gestational day and were more pronounced in the female placentas than the male placentas.

**Maternal choline supplementation alters the placental abundance of macronutrient transporters and the placental metabolism of glucose**

Supplementing the maternal diet with extra choline decreased the number of *Glut1* transporters at E15.5 in both male and female placentas. A significant reduction in *Snat4*, an amino acid transporter, was also detected in female placentas at E15.5. Importantly, however, no adverse effects of extra choline were detected on placental or fetal weights at this time point, suggesting that another source of glucose, such as placental glycogen, was used to meet fetal glucose demands. Indeed, we found that MCS downregulated the expression of glycogen synthesizing enzymes *Gys1* in both male and female placentas and *Gbe1* in female placentas, while upregulated expression of the glycogen degrading enzyme *Pygm* was detected in female placentas. While speculative based upon statistical significance ($P \geq 0.06$), we also detected a numerically lower glycogen concentration in the female placentas, indicating that placental glycogen was being broken down to maintain a constant glucose supply to the fetus. The mechanism by which choline reduced *Glut1* and *Snat4* abundance is unclear but may be a secondary response to enhanced placental perfusion (which would enhance nutrient delivery) in the choline-supplemented dams. In this regard, we previously reported that MCS significantly

increased the luminal area of the maternal spiral arteries in mice fed a diet containing the same choline concentration (24). The subsequent changes in glycogen metabolism are consistent with the tendency for regulatory systems to temporarily overshoot or overcompensate to maintain homeostasis (34) and to increase body capability to deal with more difficult metabolic challenges in the future (35).

At E18.5, MCS upregulated *Snat1* transporter in both male and female placentas, increasing the availability of substrates such as glycine, which can be used to generate glucose via gluconeogenesis (36, 37). In response to MCS, the number of GLUT3 transporters in the female placentas also increased, which would be expected to increase the amount of glucose supplied to the fetus. However, an upregulation in the glycogen synthesizing genes, *Gys1* and *Gbe1*, was detected along with more glycogen in these placentas. The greater glycogen storage in MCS mice may be due to a glucose-sparing effect of glycine (36, 37). Alternatively, transport of glucose via the high-affinity GLUT3 transporter may be reversible when the fetus is hyperglycemic (38). Although speculative based upon statistical significant ($P = 0.07$), female fetuses in the 4X choline group tended to weigh more at E18.5 compared to those from the 1X choline group which is consistent with a hyperglycemic environment in the fetal compartment. Collectively, these data suggest that the transport of glucose from fetal circulation back into the placenta at E18.5 was favored in an attempt to avoid glucose surplus in the fetal compartment and fetal overgrowth. Additional studies employing labeled glucose methodology to measure glucose flux between the maternal, placental and fetal compartments will be needed to verify this hypothesis.

In addition to altered expression of amino acid and glucose transporters, maternal choline supplementation increased the placental abundance of *Fatp4* transporters, which mediates the

placental transfer of DHA to fetal circulation (1). The metabolism of choline and DHA intersects at the PEMT pathway which utilizes a phosphatidylethanolamine (PE) molecule enriched in DHA to make a phosphatidylcholine (PC) molecule enriched in DHA (i.e., PC-DHA). Notably, the PEMT pathway can be enhanced by choline supplementation (39, 40), subsequently increasing the production of PC-DHA to generate a supply of DHA for placental uptake and transport to the fetus by FATP4. Indeed, others have reported a strong correlation between DHA in maternal plasma phospholipids, placental phospholipids, cord blood phospholipids and placental *FATP4* transcript abundance (13). Our finding of the choline-induced upregulation of *Fatp4* transporter prompted us to measure DHA concentration in the fetal brain, which was significantly higher among female fetuses from the choline-supplemented groups at E18.5. Taken together, these data suggest that supplementing the maternal diet with extra choline upregulates placental *Fatp4* in response to the choline-induced increase in PC-DHA production, ultimately increasing DHA supply to the developing fetus.

**Maternal choline supplementation impacts placental transport of choline and its metabolic derivatives**

Supplementing the maternal diet with extra choline increased the placental abundance of the choline transporter *Ctl1* at E15.5 and E18.5, a finding indicative of enhanced placental choline uptake. Paradoxically, however, the placental concentrations of several choline metabolites were lower in the choline supplemented groups. For example, diminished concentrations of acetylcholine were detected at E15.5 in placentas obtained from the choline-supplemented groups. Nonetheless, this lower placental concentration of acetylcholine coincided with a higher placental *Oct3* abundance which transports acetylcholine from the placenta to the

fetus in mouse pregnancy (41, 42) and a higher acetylcholine concentration in the fetal brain. As such, these data collectively suggest that maternal choline supplementation increased acetylcholine delivery to the developing fetal brain during the early stages of late gestation, which would be expected to enhance neuron functioning (43) and may contribute to the neuroprotective effects of prenatal choline supplementation (27).

Choline and its metabolic derivatives (Figure 2.5A) have important regulatory roles in placental development and function. As an example, acetylcholine modulates amino acid transport (44), which may explain the concurrent choline-induced changes of placental acetylcholine concentration and placental *Snat* abundance. Notably, the placental TMAO concentration was significantly higher in response to MCS. TMAO can be synthesized in the maternal liver, which was significantly higher in response to MCS in these mice (24), and then transported into the placenta. Alternatively, it may be produced by microbiota residing within the placenta tissue (45). Because TMAO is an osmolyte that maintains cell volume and stabilizes proteins and nucleic acids (46), a higher concentration of this metabolite in the placenta may be beneficial, rather than harmful, for normal placental development and function. Additional studies are needed to fully elucidate the functions of TMAO in the placenta.

## Conclusion

In sum, maternal choline supplementation modulates placental nutrient transporter abundance as well as glucose and choline metabolism in late gestation of mouse pregnancy in a manner that is dependent on fetal sex and gestational day. Many of the choline-induced effects appear to be secondary responses that could be related to choline’s beneficial effects on placental perfusion and vascularization (24). We also provide evidence of altered nutrient availability in

the fetal brain in response to these choline-induced placental changes. As uteroplacental perfusion, placental nutrient transporters and metabolism affect nutrient supply to the fetus, these choline-induced placental responses may have lasting impacts on fetal organ development and functioning, with potential long-term health implications.

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**Supplemental Table 2.1.** Primers for PCR and qPCR.

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| *Sry* | 5'-TGGGACTGGTGACAATTGTC-3' | 5'-GAGTACAGGTGTGCAGCTCT-3' |
| *Slc38a1/Snat1* | 5'-TGGAAGAGAGGAAAGGACCCA-3' | 5'-TCCGCAGTCAAAAGTCAACC-3' |
| *Slc38a2/Snat2* | 5'-GGCATTCAATAGCACCGCAG-3' | 5'-CGGGATGGCAGACAAAGGAA-3' |
| *Slc38a4/Snat4* | 5'-GCTCGTCATGTACCTTCTTGC-3' | 5'-CCATGAGAAGAGCCGTATCAAA-3' |
| *Fatp1* | 5'-CTTCTGTGAGAACCTGCGAG-3' | 5'-TTGTCCTAGATCCAAGCCCC-3' |
| *Fatp4* | 5'-CATCAAGACGGTCAGGAGAGAT-3' | 5'-TACCATTGAAGCAAACAGCAGG-3' |
| *Slc2a1/Glut1* | 5'-TCTGTCGGGGGCATGATTG-3' | 5'-TGGAGAAGCCCATAAGCACAG-3' |
| *Slc2a3/Glut3* | 5'-TGCCCTGAGAGTCCAAGATTC-3' | 5'-CACTCTCATCCTTCATCTCCTGG-3' |
| *Gys1* | 5'-GTGTGAGGACGCAGGTAGAG-3' | 5'-CCAACGCCCAAAATACACCTTA-3' |
| *Gbe1* | 5'-ATCACCACGGAATGGGTCAA-3' | 5'-ATCGAGTCTGGGTACAACGTG-3' |
| *Pygm* | 5'-AAGGACCCCAAGAGGATCTACTA-3' | 5'-TCATCACAGGCGTTCTCCAAG-3' |
| *Gsk3β* | 5'-TCCTTATCCCTCCACATGCT-3' | 5'-GGCGTTATTGGTCTGTCCAC-3' |
| *Slc44a1/Ctl1* | 5'-TCAAGAGTGCTAGTGTGGATTCT-3' | 5'-TGCTTCTTTGGGGGACCTTC-3' |
| *Slc22a3/Oct3* | 5'-TGCTTCGTGATCGTGACAGA-3' | 5'-TGGGCGTGAAGTAGGCAATC-3' |
| *Tbp* | 5'-AGGAGCCAAGAGTGAAGAACAA-3' | 5'-AACTTCACATCACAGCTCCCC-3' |

# CHAPTER 3

Maternal choline supplementation triggers fetal sex-specific changes in the epigenome and transcriptome of the mouse placenta*

*Kwan STC, King JH, Yan J, Jiang X, Roberson MS, Caudill MA. Maternal choline supplementation triggers fetal sex-specific changes in the epigenome and transcriptome of the mouse placenta. *In Prep.*

**ABSTRACT**

The placental epigenome regulates processes that affect placental and fetal development, and could be mediating some of the previously reported effects of maternal choline supplementation (MCS) on placental vascular development and nutrient delivery. As such, the present study employed an untargeted approach to examine placental global DNA methylation, placental expression of imprinted genes and placental abundance of microRNAs. DNA, mRNA and miRNA were extracted from placentas collected on gestational day 15.5 from mice fed the 1X or 4X choline diet. Placental global DNA methylation was examined using LC-MS/MS. Placental transcriptome, imprintome and miRNA profiles were examined using genome-wide sequencing procedures. MCS increased placental global DNA methylation ($P = 0.015$), and led to sex-specific changes in the placental transcriptome and imprintome. In female placentas, MCS altered the expression of 187 genes, four of which were imprinted genes (*Ampd3*, *Tfpi2*, *Gatm* and *Aqp1*; FDR $\leq 0.05$). In male placentas, MCS affected 141 genes, including three imprinted genes (*Dcn*, *Qpct* and *Tnfrsf23*; FDR $\leq 0.01$). MCS also reduced (FDR = 0.13) miR-2137 abundance in the placenta, resulting in the upregulation ($P < 0.05$) of its target genes. Gene ontology analyses on the differentially-expressed genes revealed that many processes implicated in placental vascular development, placental nutrient delivery and fetal organ development were affected. Overall, these data indicate that the placental epigenome is responsive to maternal choline intake during pregnancy, which may be one mechanism mediating the beneficial effects of maternal choline supplementation on various placental and fetal outcomes.

## INTRODUCTION

Epidemiological evidence has shown that an adverse *in utero* environment is associated with a higher risk of developing obesity, metabolic syndrome, cardiovascular diseases and other chronic diseases later in life.[1-3] These data have led to the fetal programming hypothesis, which suggests that the developing fetus will generate an adaptive response to the suboptimal prenatal environment in order to increase its survival. This adaptive response involves changing the development of the placenta,[4] as well as the fetus and its organs, with permanent effects on their normal functioning.[3] Although there is a growing body of evidence in support of this hypothesis, the molecular mechanisms mediating the programming phenomenon are less clear. One proposed mechanism linking prenatal exposure to later health outcomes is the modulation of gene expression via epigenetic processes.

The best characterized epigenetic mechanism is DNA methylation, which adds methyl groups to the cytosine residues in CpG dinucleotides to modulate gene expression. One group of genes known to be regulated by DNA methylation is the imprinted genes, which are expressed according to parental origin.[5, 6] Many of the imprinted genes are expressed in the placenta where they synthesize proteins to control the cell cycle, cell signaling, vascularization, nutrient uptake, utilization and storage,[5, 6] ultimately affecting placental nutrient supply efficiency. As expected, aberrant expression of placental imprinted genes alters fetal growth and adversely affects birth weight.[5, 7] The expression of placental imprinted genes may also serve as a biomarker for future health outcomes, such as infant neurodevelopment and bone health at the age of four.[8, 9]

Another epigenetic mechanism that has received increased attention in recent years involves the microRNAs (miRNAs). These are small noncoding RNA molecules with 19-24 nucleotides that post-transcriptionally regulate gene expression.[10] Specifically, miRNA base-

pairs with its mRNA targets in a sequence-specific manner to trigger mRNA transcript degradation and translational repression.[10, 11] As such, a higher abundance of miRNAs will reduce the expression of its mRNA targets. The placenta produces many miRNAs, and their target genes are involved in cell proliferation, apoptosis, invasion and angiogenesis,[11, 12] which are essential to normal placental morphological and vascular development. Some miRNAs also regulate immune cell development at the maternal-fetal interface and mediate immune response and maternal tolerance to the fetus.[12] Given their roles in many aspects of placental development and function, it is not surprising that miRNA deregulation is associated with pregnancy disorders that impair fetal growth.[10, 13-15]

Choline is an essential nutrient involved in one-carbon metabolism where its methyl groups are used in cellular methylation reactions such as DNA methylation.[16] Our previous investigations indicate that maternal choline supplementation (MCS) improves placental vascularization and perfusion,[17, 18] enhances placental nutrient supply efficiency[19] and alters fetal growth.[20] Given the roles of imprinted genes and miRNAs in these placental and fetal outcomes, we sought to test the hypothesis that these epigenetic processes could be mediating some of the aforementioned choline-induced effects on placental and fetal development.

## METHODS

### Animals and Diets

This study was an extension of an animal feeding study[17, 19] where we examined the impact of MCS on placental vascularization and nutrient transport system. Briefly, non-Swiss Albino (NSA) mice (Harlan, Indianapolis, IN) that would be mated in subsequent experiments were fed a 1X choline diet (1.4g choline chloride/kg diet; Dyets #103345; Dyets, Bethlehem, PA) upon weaning at 3 weeks old. Five days prior to mating, female mice were randomized to one of three treatment groups: 1X choline group, 2X choline group (2.8g choline chloride/kg diet; Dyets #103346) or 4X choline group (5.6g choline chloride/kg diet; Dyets #103347). Presence of a vaginal plug was defined as gestational day (E) 0.5. Pregnant female mice continued on their diet until they were sacrificed at one of four gestational time points: E10.5, E12.5, E15.5 or E18.5. The present study used the tissues collected from dams in the 1X choline and 4X choline groups at E15.5 (n=3 dams per group, per fetal sex). The 4X choline group was chosen because our previous findings[17, 19] indicate a pronounced effect of this dosage on placental development and function, while E15.5 was chosen because it represents the time when the placenta reaches its maximal size[21] and the fetus is rapidly growing.[22] To minimize decidual contamination, the maternal decidua was removed during the dissection. The remaining placental disks were cut in half across the chorionic plate. The fetuses were also collected during the dissection. Both the placental and fetal tissues were flash frozen in liquid nitrogen before storage at -80°C for further analysis. All animal protocols were approved by the Institutional Animal Care and Use Committees at Cornell University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

## Fetal Sex Genotyping

Fetal DNA was extracted using the DNeasy blood and tissue kit (Qiagen), and fetal sex was determined by PCR for the *Sry* gene, using forward 5'-TGGGACTGGTGACAATTGTC-3' and reverse 5'-GAGTACAGGTGTGCAGCTCT-3' primers.

## Placental DNA Extraction

Genomic DNA was extracted from the placenta using the DNeasy blood and tissue kit (Qiagen), with the addition of RNase A (Qiagen) to remove any co-purified RNA. DNA concentration and purity were evaluated using a NanoDrop spectrophotometer.

## Global DNA Methylation Assay and Data Analysis

Global DNA methylation was measured using LC-MS/MS, as described previously[23] with modifications based on our instrument.[24] Briefly, 300ng genomic DNA was digested with nuclease P1, followed by digestion with phosphodiesterase 1, and digestion with alkaline phosphatase (Sigma-Aldrich). Samples were diluted with 0.1% formic acid in water and injected into the instrument for analysis. Global methylation is presented as a percentage of the amount of 5-methyl-2'-deoxycytidine (5mdC) relative to the total amount of cytosine [i.e.: 5mdC/(dC + 5mdC)]. One-way ANOVA was used to analyze the data, and statistical significance was defined at $P \leq 0.05$. Data are presented as means with 95% confidence intervals. All the statistical analyses were done in the SPSS software, Version 23 (SPSS Inc, Chicago, IL).

## Placental RNA Extraction

Total RNA was extracted from the placentas using Trizol (Thermo Fisher) according to

the manufacturer's instructions with the following modifications: (i) an extra chloroform extraction step of the aqueous layer after the first phase separation; (ii) addition of 1uL Glyco-blue (Thermo Fisher) before the isopropanol precipitation; and (iii) two washes of the RNA pellet with 75% ethanol. RNA concentration and purity were determined using a NanoDrop spectrophotometer. RNA integrity and presence of small RNAs (<<200 nucleotides) were determined with a Fragment Analyzer (Advanced Analytical).

**Placental mRNA Sequencing and Data Analysis**

NEBNext Ultra Directional RNA Library Prep Kit (New England Biolabs) was used to make libraries, which were sequenced on Illumina NextSeq500. After processing, reads were mapped to the reference mouse transcriptome (UCSC mm10) with Tophat, Version 2.0. FPKM values were generated and statistical analysis was performed using Cufflinks, Version 2.2. Genes were considered to have significantly different expression when the false discovery rate (FDR) was less than 0.2. Gene ontology was performed on the differentially-expressed genes using PANTHER, Version 11.1.[25, 26] Differentially-expressed imprinted genes were identified as listed on the MRC Harwell Imprinting Webpages (http://www.har.mrc.ac.uk/research/genomic_imprinting/).[27]

**Placental miRNA Sequencing and Data Analysis**

Libraries were made using the NEBNext Small RNA Library Prep Kit (New England Biolabs), and were sequenced on Illumina HiSeq2500. After processing and mapping the reads to the reference mouse genome, reads were mapped to mature miRNAs (miRBase, Version 21) using MirDeep2, and differential expression was determined using EdgeR. Statistical

significance was defined at FDR < 0.2. Predicted mRNA targets of the differentially-expressed miRNA were identified using TargetScan, Version 7.1.[28] Strong targets were defined similarly as in other investigations[29, 30] and have a context++ score ≤ -0.2, where a more negative score will indicate a greater repression. Gene ontology was conducted for the mRNA targets using PANTHER, Version 11.1.[25, 26] The expression of each of the mRNA targets identified from TargetScan was obtained from the mRNA sequencing dataset.

## RESULTS

### Placental DNA Methylation

Regardless of fetal sex, global DNA methylation was 21% higher ($P = 0.015$) in the 4X choline placentas compared to the 1X choline placentas (Figure 3.1). In the female placentas, 4X choline supplementation yielded 15% higher ($P = 0.035$) global DNA methylation than 1X choline. A nonsignificant ($P = 0.086$) higher abundance of global DNA methylation was also detected in the male placentas in response to 4X choline (vs 1X choline; Figure 3.1).

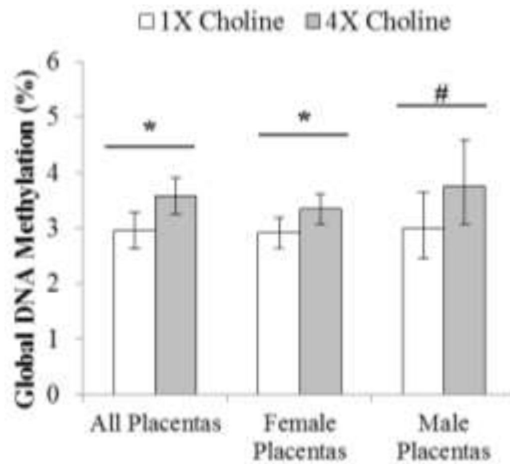


**Figure 3.1.** Percentage of global DNA methylation in the placentas from dams in the 1X and 4X choline group. Data are presented as means with 95% confidence intervals. $^{*}P \leq 0.05$. $^{\#}P = 0.086$.

**Placental Transcriptome and Imprinting Profile**

4X maternal choline supplementation during pregnancy altered (FDR < 0.2) the expression of 131 genes in the placentas (data not shown). When the analysis was done separately for each sex, 44 genes were downregulated (FDR < 0.2) and 143 genes were upregulated (FDR < 0.2) in the female placentas in response to 4X versus 1X choline supplementation (Supplemental Figure 3.1). Among these 187 genes exhibiting differential expression, 4 were suggested to be imprinted genes: *Aqp1* (FDR = 0.009), *Tfpi2* (FDR = 0.009), *Ampd3* (FDR = 0.009) and *Gatm* (FDR = 0.054). Their expression in the female placentas was all increased by additional maternal choline (Table 3.1). Overall, 26 biological processes in the female placentas were affected ($P < 0.05$; Table 3.2).

**Table 3.1.** Differentially-expressed imprinted genes in the female placentas in response to 4X versus 1X maternal choline supplementation.

| Gene Symbol | Gene Name | Fold Difference | FDR Value |
|---|---|---|---|
| *Aqp1* | aquaporin 1 | 2.23 | 0.009 |
| *Tfpi2* | tissue factor pathway inhibitor 2 | 2.16 | 0.009 |
| *Ampd3* | adenosine monophosphate deaminase 3 | 1.62 | 0.009 |
| *Gatm* | glycine amidinotransferase | 1.65 | 0.054 |

**Table 3.2.** Selected biological processes in the female placentas affected by 4X versus 1X maternal choline supplementation.

| Biological Processes | *P* Value | List of Changed Genes |
|---|---|---|
| response to external stimulus (GO:0009605) | 0.0005 | *Ambp, Ccl2, Cubn, Cuzd1, Fgb, F2, G6b, Kng1, Mgl2, Tfpi2* |
| vitamin transport (GO:0051180) | 0.0009 | *Cubn, Cuzd1, Rbp4, Ttr* |
| protein metabolic process (GO:0019538) | 0.0037 | *Adamts5, Ambp, Apoa1, Apoa2, Apoa4, Cd109, Cd5l, Clgn, Cpn1, Cryab, Ctsk, Ctsm, Cubn, Cuzd1, C4b, F2, Galnt6, Galnt15, Hspb7, Htra1, Itih1, Itih2, Itih3, Itih4, Plg, Rimklb, Slpi, St6galnac1, Tfpi2, Ube2ql1,* |
| lipid metabolic process (GO:0006629) | 0.0043 | *Acsf2, Anxa1, Apoa1, Apoa2, Apoa4, Gdpd3, Hpgd, Hsd11b1, Hsd11b2, Lcn2, Slc27a2, St6galnac1* |
| cell adhesion (GO:0007155) | 0.0078 | *Cd5l, Col5a2, Cthrc1, Cubn, C9, Fga, Fgb, Fbln2, Mgl2, Msr1* |
| immune system process (GO:0002376) | 0.0091 | *Bai1, Ccl2, Cd109, Cd5l, Clec2h, Col5a2, Cryab, Cubn, C4b, C9, Fbln2, Fcgrt, G6b, Hspb7, Lphn1, Mgl2, Msr1, Pla2g4f, Slc27a2, Slfn4* |
| fatty acid metabolic process (GO:0006631) | 0.0108 | *Acsf2, Anxa1, Apoa1, Apoa4, Lcn2, Slc27a2* |
| transport (GO:0006810) | 0.0144 | *Afp, Alb, Apoa1, Apoa4, Aqp1, Arl4d, Bai1, Cd5l, Clgn, Cubn, Cuzd1, Lcn2, Lphn1, Mgl2, Mlph, Rbp4, Slc5a1, Slc7a8, Slc7a9, Slc13a3, Slc27a2, Slc43a2, Steap4, Tbc1d9, Ttc30b, Ttr* |
| amino acid transport (GO:0006865) | 0.0195 | *Slc7a8, Slc7a9, Slc43a2* |
| carbohydrate transport (GO:0008643) | 0.0480 | *Aqp1, Slc5a1* |

In the male placentas, 4X versus 1X choline decreased (FDR < 0.2) the expression of 79 genes and increased (FDR < 0.2) the expression of 62 genes (Supplemental Figure 3.2). Among these 141 genes affected by 4X choline, 3 were suggested to be imprinted genes: *Qpct* (FDR = 0.012), *Dcn* (FDR = 0.012) and *Tnfrsf23* (FDR = 0.012). All of these imprinted genes were downregulated in the male placentas in response to additional maternal choline intake (Table 3.3). Overall, 4X choline altered ($P < 0.05$) 12 biological processes in the male placentas (Table 3.4).

**Table 3.3.** Differentially-expressed imprinted genes in the male placentas in response to 4X versus 1X maternal choline supplementation.

| Gene Symbol | Gene Name | Fold Difference | FDR Value |
|---|---|---|---|
| *Qpct* | glutaminyl cyclase | 0.46 | 0.012 |
| *Dcn* | decorin | 0.58 | 0.012 |
| *Tnfrsf23* | tumor necrosis factor receptor superfamily, member 23 | 0.64 | 0.012 |

**Table 3.4.** All the biological processes in the male placentas affected by 4X versus 1X maternal choline supplementation.

| Biological Processes | *P* Value | List of Changed Genes |
|---|---|---|
| immune response (GO:0006955) | 0.0001 | *Bai1, Ctsg, Eva1c, Gpr125, Gzmb, Gzmc, Gzmd, Gzme, Gzmf, Gzmg, Mcpt8, Nptx1, Prf1, Tnfrsf1b, Tnfrsf23* |
| immune system process (GO:0002376) | 0.0002 | *Bai1, Cd93, Cryaa, Ctsg, Eva1c, Gpr125,Gzmb, Gzmc, Gzmd, Gzme, Gzmf, Gzmg, Hspb7, Lbp, Mcpt8, Nptx1, Pla2g7, Prf1, Tnfrsf1b, Tnfrsf23* |
| protein folding (GO:0006457) | 0.0063 | *Clip4, Cryaa, Hspb7, Htra1* |
| visual perception (GO:0007601) | 0.0064 | *Ccbe1, Cryaa, Hsd11b2, Hspb7, Pde4b* |
| heart development (GO:0007507) | 0.0132 | *Bai1, Eva1c, Gpr125, Hand2* |
| cholesterol metabolic process (GO:0008203) | 0.0160 | *Apold1, Hsd3b6, Lbp* |
| protein metabolic process (GO:0019538) | 0.0225 | *Adamts5, Clip4, Cryaa, Ctsg, Ctsk, Ggt6, Gzmb, Gzmc, Gzmd, Gzme, Gzmf, Gzmg, Hspb7, Htra1, Ipp, Lce1g, Mcpt8, Ptprn2, Rimklb, St6galnac1, 4930486L24Rik* |
| cytokine-mediated signaling pathway (GO:0019221) | 0.0245 | *Cxcl12, Dcn, Tnfrsf1b, Tnfrsf23* |
| cell-cell signaling (GO:0007267) | 0.0259 | *Bai1, Cd93, Cxcl14, Doc2b, Eva1c, Nov, Raasl11b* |
| steroid metabolic process (GO:0008202) | 0.0326 | *Apold1, Hsd3b6, Hsd11b2, Lbp* |
| cellular component movement (GO:0006928) | 0.0376 | *Calm4, Cd93, Cxcl12, Cxcl14, Kif21b, Tnfrsf23* |
| cellular amino acid catabolic process (GO:0009063) | 0.0463 | *Aspg, Tdo2* |

**Placental miRNAs Expression Profile**

Prior to controlling for false discovery rate, 10 placental miRNAs displayed differential expression ($P \leq 0.05$) in response to 4X versus 1X maternal choline supplementation. When data were stratified by fetal sex, altered abundance of 30 miRNAs in the female placentas and seven miRNAs in the male placentas was detected ($P \leq 0.05$). After adjustment for the false discovery rate, 11 miRNAs in the female placentas remained significantly different among the choline treatment groups (FDR < 0.2). However, this differential finding may have been driven by one female placental sample in the 1X choline group (Supplemental Table 3.1), which had a lower miRNA-mapped read frequency than the other samples possibly due to sample degradation and increased background signaling. Upon excluding this sample, the new analyses showed that miR-2137 was significantly downregulated (FDR = 0.125, $P_{unadjusted} = 2.05 \times 10^{-4}$) in response to 4X versus 1X maternal choline supplementation. Although significance was not achieved upon stratification by fetal sex, both female and male placentas exhibited a 61% and 65% downregulation in the abundance of miR-2137, respectively.

Based on TargetScan prediction, miR-2137 has 170 mRNA targets. These mRNA targets are related to 27 biological processes (Table 3.5). In response to 4X versus 1X choline, five of the predicted mRNA targets were upregulated ($P \leq 0.05$) in the female placentas (Table 3.6). These included *Cd109* ($P < 0.01$), *Mt3* ($P < 0.01$), *Plg* ($P < 0.01$), *Gja4* ($P = 0.01$), and *Psrc1* ($P = 0.05$). In the male placentas, 4X versus 1X choline also upregulated ($P \leq 0.05$) four of the predicted mRNA targets (Table 3.6), which were *Pmaip1* ($P = 0.02$), *Pcdh1* ($P$=0.04), *Mt3* ($P = 0.04$), and *Cd28* ($P = 0.05$).

**Table 3.5.** All the biological processes affected by the predicted mRNA targets of miR-2137.

| | **Processes** | ***P* Values** |
|---|---|---|
| **Biological Processes Overrepresented by The Predicted Targets of miR-2137** | regulation of transcription from RNA polymerase II promoter (GO:0006357) | <0.0001 |
| | transcription from RNA polymerase II promoter (GO:0006366) | <0.0001 |
| | developmental process (GO:0032502) | <0.0001 |
| | muscle organ development (GO:0007517) | <0.0001 |
| | transcription, DNA-dependent (GO:0006351) | <0.0001 |
| | segment specification (GO:0007379) | <0.0001 |
| | nervous system development (GO:0007399) | 0.0001 |
| | system development (GO:0048731) | 0.0001 |
| | RNA metabolic process (GO:0016070) | 0.0001 |
| | mesoderm development (GO:0007498) | 0.0002 |
| | ectoderm development (GO:0007398) | 0.0002 |
| | pattern specification process (GO:0007389) | 0.0003 |
| | synaptic transmission (GO:0007268) | 0.0033 |
| | skeletal system development (GO:0001501) | 0.0052 |
| | heart development (GO:0007507) | 0.0057 |
| | nucleobase-containing compound metabolic process (GO:0006139) | 0.0073 |
| | cell-cell signaling (GO:0007267) | 0.0102 |
| | dorsal/ventral axis specification (GO:0009950) | 0.0169 |
| | embryo development (GO:0009790) | 0.0268 |
| | female gamete generation (GO:0007292) | 0.0290 |
| | transmembrane receptor protein serine/threonine kinase signaling pathway (GO:0007178) | 0.0307 |
| | response to endogenous stimulus (GO:0009719) | 0.0401 |
| | apoptotic process (GO:0006915) | 0.0418 |
| | death (GO:0016265) | 0.0478 |
| | cell death (GO:0008219) | 0.0478 |
| | behavior (GO:0007610) | 0.0481 |
| | tRNA metabolic process (GO:0006399) | 0.0496 |

**Table 3.6.** Predicted mRNA targets of miR-2137 that displayed significant differential expression in the female and male placentas in response to 4X choline supplementation.

| | Gene Symbol | Gene Name | Fold Difference |
|---|---|---|---|
| **Female Placentas** | *Gja4* | gap junction protein, alpha 4 | 1.33 |
| | *Psrc1* | proline/serine-rich coiled-coil 1 | 1.49 |
| | *Cd109* | CD109 antigen | 1.55 |
| | *Mt3* | metallothionein 3 | 3.85 |
| | *Plg* | plasminogen | 3.91 |
| **Male Placentas** | *Pcdh1* | protocadherin 1 | 1.25 |
| | *Pmaip1* | phorbol-12-myristate-13-acetate-induced protein 1 | 1.39 |
| | *Cd28* | CD28 antigen | 1.64 |
| | *Mt3* | metallothionein 3 | 1.69 |

## DISCUSSION

To the best of our knowledge, this is the first study to survey the effects of maternal choline supplementation on placental epigenetic markers in an untargeted, genome-wide manner. We demonstrate choline-induced effects on global DNA methylation, imprinted gene expression and miRNAs abundance in mouse placenta, all of which could be mediating some of the previously observed beneficial effects of maternal choline supplementation on placental outcomes.

### Maternal choline supplementation alters the expression of numerous imprinted genes in the placenta

Placental expression of several imprinted genes was altered in a sex-dependent manner in response to a higher maternal choline intake. In the female placentas, maternal choline supplementation upregulated the expression levels of *Gatm*, *Tfpi2*, *Aqp1* and *Ampd3*. Because a lower placental *Gatm* abundance is associated with IUGR,[31] the choline-induced upregulation in *Gatm* abundance in the present study suggests a possible benefit of MCS on fetal growth. The other choline-altered imprinted genes in the female placentas are implicated in processes essential to normal placental vascular development and thereby also affect fetal growth. Specifically, *Tfpi2* inhibits the activity of matrix metalloproteinases and regulates placental perfusion.[32, 33] Deficiency of *Aqp1*, a recently identified placenta-specific imprinted gene, causes aberrant placental vascularization and fetal overgrowth.[34] Although the consequences of altered *Ampd3* expression in placenta remain to be examined, *Ampd3* deficiency in cancer cells has been shown to inhibit cell proliferation and invasion.[35] Collectively, the choline-induced upregulation of all these placental imprinted genes would be expected to improve placental vascularization

and perfusion, likely contributing to the choline-induced beneficial effects reported previously.[17]

The downregulation of the imprinted genes, *Dcn* and *Tnfrsf23*, in the male placentas in response to MCS may also result in an improved placental vascular network. Reduced placental *Dcn* expression enhances endothelial cell migration and remodeling of the placental vasculature[36], whereas a lower expression of *Tnfrsf23* reduces apoptosis[37] and modulates the inflammatory responses during trophoblast invasion.[38] Additionally, in the male placentas, 4X choline downregulated the abundance of *Qpct*, which controls placental nutrient delivery.[39] An upregulation of *Qpct* is frequently detected in preeclamptic placentas,[40] possibly as a compensatory response to poor placental perfusion. Therefore, the choline-induced downregulation of *Qpct* in the present study may indicate a sufficiently perfused placenta which is consistent with the previously reported choline-induced enlargement of the maternal spiral arteries. In sum, altered abundance of these placental imprinted genes in response to MCS may be one mechanism by which a higher maternal choline intake improves placental vascular development.[17]

**Maternal choline supplementation changes many processes important for fetal development**

Gene ontology analyses on all the differentially-expressed genes revealed that seven placental processes were similarly influenced in both female and male placentas. These processes related mostly to the metabolism of lipids (GO: 0008203, 0008202) and proteins (GO: 0019538, 0009063, 0006457). In the female placentas, many processes impacted by MCS were also related to the transport and metabolism of macronutrients (GO: 0006865, 0008643, 0006810, 0008652 and 0006633), which is consistent with our previous findings that MCS

altered the abundance of several macronutrient transporters and glycogen metabolic enzymes in these placentas.[19] Furthermore, the analyses in the present study indicated that processes related to the transport of micronutrients such as vitamins A, B-12 and minerals (GO: 0051180, 0006820 and 0006811) were affected by MCS. Interestingly, processes related to homeostasis (GO: 0042592) and responding to stimulus (GO: 0009605, 0007596) were affected. Based on our prior work, we have proposed that the female placentas initiate changes in the placental vascular development as well as placental nutrient metabolism and transport in response to changes in maternal nutrient intake (e.g.: MCS) to maintain homeostasis.[19] The finding on these three gene ontology in the current study supports our hypothesis.

MCS also affected processes regulating nutrient delivery in the male placentas. However, rather than altering processes directly involved in nutrient transport and metabolism, MCS modulated processes in the male placentas that are largely implicated in normal placental morphological and vascular development, such as signal transduction (GO: 0007267, 0019221) and immune response (GO: 0002376, 0006955). Interestingly, one process altered by MCS was related to heart development (GO: 0007507). The programming effect of maternal undernutrition on the risk of cardiovascular diseases is well-illustrated by the Dutch Famine Cohort.[41-44] Furthermore, MCS is shown to normalize the blood pressure of adult offspring from dams fed a low-protein diet throughout gestation.[45] According to our analyses, we hypothesize that the programming effects of maternal diets on future cardiovascular health may be mediated in part by a placental-cardiovascular axis.[46, 47] Taken together, these data support a role of MCS in altering various placental processes in both female and male placentas that have subsequent impact on placental nutrient supply efficiency as well as the growth and development of the fetus.

**Maternal choline supplementation increases global DNA methylation in the placenta**

Similar to our previous findings in humans,[24] a higher global DNA methylation in both female and male placentas was found in response to additional maternal choline intake during pregnancy. Because DNA hypomethylation often leads to genomic instability that increases mutation frequency and disease susceptibility,[48] the choline-induced hypermethylation in these placentas is expected to stabilize the placental genome, which minimizes any adverse effects on normal placental development and function.

**Maternal choline supplementation reduces placental miR-2137 abundance, with downstream effects on the expression of its target genes**

We identified that placental miR-2137 was downregulated by MCS. The bioinformatics analyses indicated that miR-2137 targets genes important to several developmental processes. For example, it affects processes related to cell death (GO: 0006915, 0016265, 0008219), which is crucial for normal placental morphological development. The processes related to cell signaling (e.g.: GO: 0007267, 0007178) are affected as well, including the transmembrane receptor protein serine/threonine kinase signaling pathway. This pathway includes proteins in the transforming growth factor-β (TGF-β) superfamily,[49, 50] which are known to regulate placental vascularization.[51] By modulating placental miRNA abundance, MCS indirectly affects these biological processes, possibly leading to improved placental vascularization as observed in our prior study.[17]

miR-2137 also impacts processes related to the development and function of different organs (e.g.: GO: 0007517, 0007399, 0001501, 0007507), including the cardiovascular and

nervous system. These data support not only the existence of a placental-cardiovascular axis, but also a placental-brain axis[46] that may explain the interactive effects of prenatal choline supply[52] and placenta[53] on programming offspring neurodevelopment.

Although miR-2137 has not been experimentally studied in the placenta, it has been examined in other tissues.[54-57] Consistent with our bioinformatics analyses, these studies show that an altered miR-2137 abundance changes processes related to apoptosis as well as heart and brain functioning. Interestingly, micronutrient supplementation in a paternal undernutrition mouse model also changes miR-2137 expression in the offspring pancreas,[58] indicating that this miRNA may be particularly sensitive to nutritional manipulation. In addition to the bioinformatics analyses, we found that the choline-induced miR-2137 downregulation led to higher expression of several predicted mRNAs. While different between the female and male placentas, these genes all play some roles in apoptotic, vascular, and TGF-β signaling processes. Taken together, changes induced by miR-2137 in response to MCS may benefit placental development and offspring health.

**Conclusion and Future Directions**

Findings from the present study add to the growing body of research that illustrates the responsiveness of the placental epigenome to maternal choline intake during pregnancy. Moreover, this study identifies several epigenetic markers that could be mediating some of the previously reported choline-induced effects on placental and fetal development. Additional studies are needed to explore the clinical relevance of these placental markers in predicting pregnancy outcomes and future offspring health.

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**Supplemental Figure 3.1**. Differentially-expressed genes in the female placentas in response to 4X choline supplementation.

| Gene Symbol | Fold Difference | Gene Symbol | Fold Difference | Gene Symbol | Fold Difference | Gene Symbol | Fold Difference |
|---|---|---|---|---|---|---|---|
| *Ccr1* | 0.12 | *Ephb4* | 1.40 | *Mfi2* | 2.04 | *Slc39a5* | 3.19 |
| *H2-Q7,H2-Q9* | 0.16 | *Cyp11a1* | 1.41 | *Krt16* | 2.07 | *Cubn* | 3.22 |
| *Cd5l* | 0.26 | *Hist1h1c* | 1.42 | *Prl8a2* | 2.08 | *Cpn1* | 3.26 |
| *Sycp1* | 0.27 | *Cdo1* | 1.46 | *Nrgn* | 2.10 | *Sfrp4* | 3.35 |
| *Kap* | 0.27 | *Prl3a1* | 1.49 | *Doxl2* | 2.11 | *Ly6g6c* | 3.55 |
| *Cuzd1* | 0.30 | *Vgf* | 1.49 | *Steap4* | 2.11 | *Igfals* | 3.83 |
| *Fxyd1* | 0.30 | *F11* | 1.50 | *Crabp1* | 2.12 | *Mt3* | 3.85 |
| *Lcn2* | 0.31 | *Fabp4* | 1.51 | *BC049730* | 2.12 | *Pi15* | 3.85 |
| *Prap1* | 0.34 | *Tbc1d9* | 1.51 | *Htra1* | 2.13 | *Spink3* | 3.89 |
| *Ltf* | 0.35 | *Adm* | 1.52 | *Spon1* | 2.13 | *Plg* | 3.92 |
| *Tex13* | 0.40 | *Anxa1* | 1.54 | *Map7d2* | 2.15 | *Apom* | 4.01 |
| *Ccl2* | 0.44 | *Fbln2* | 1.55 | *Cyp21a1* | 2.17 | *Fga* | 4.04 |
| *Prl3d1* | 0.46 | *Antxr1* | 1.55 | *Tfpi2* | 2.17 | *Amn* | 4.08 |
| *Arg1* | 0.47 | *Cd109* | 1.55 | *Tacstd2* | 2.17 | *Itih1* | 4.11 |
| *Ccrn4l* | 0.52 | *Ctsk* | 1.56 | *Tmem98* | 2.20 | *Fgb* | 4.19 |
| *Pi16* | 0.53 | *Rnase4* | 1.56 | *Chac1* | 2.20 | *Fgg* | 4.25 |
| *Elk4* | 0.54 | *Fcgrt* | 1.56 | *Kng1* | 2.22 | *Pklr* | 4.43 |
| *Gm8883* | 0.55 | *Cryab* | 1.58 | *Hpgd* | 2.24 | *Gjb1* | 4.52 |
| *Trim68* | 0.56 | *Mfap5* | 1.59 | *Erv3* | 2.25 | *Mgl2* | 4.65 |
| *Cbl* | 0.56 | *Hoxa10* | 1.60 | *Apoa4* | 2.25 | *Spp2* | 4.69 |
| *Prl4a1* | 0.58 | *Ampd3* | 1.63 | *Aqp1* | 2.25 | *Serpinf2* | 4.84 |
| *Gjb4* | 0.59 | *Ramp1* | 1.63 | *Ube2ql1* | 2.28 | *Clgn* | 4.88 |
| *Msr1* | 0.59 | *Acsf2* | 1.64 | *Galnt15* | 2.29 | *Slc7a9* | 4.95 |
| *Cthrc1* | 0.60 | *Slit3* | 1.64 | *Aspg* | 2.31 | *Fxyd2* | 4.96 |
| *Crym* | 0.61 | *Col5a2* | 1.65 | *Prl5a1* | 2.35 | *Aass* | 5.05 |
| *Trim12c* | 0.61 | *Gatm* | 1.65 | *Endou* | 2.36 | *Maob* | 5.15 |
| *Zfp949* | 0.61 | *Hsd11b1* | 1.68 | *AU023871* | 2.40 | *Apob* | 5.35 |
| *Evpl* | 0.62 | *Arl4d* | 1.68 | *C4b* | 2.41 | *Hgd* | 5.40 |
| *C1qc* | 0.63 | *Trf* | 1.69 | *Epdr1* | 2.43 | *Serpina1a* | 5.44 |
| *Hsd11b2* | 0.64 | *Fetub* | 1.71 | *Serpina1b* | 2.48 | *Apoa2* | 5.52 |
| *9430008C03Rik* | 0.65 | *Slpi* | 1.72 | *Mamdc2* | 2.50 | *Clec2h* | 5.68 |
| *Slfn4* | 0.65 | *Adamts5* | 1.73 | *Col6a5* | 2.56 | *Aldob* | 5.93 |
| *Ctsm* | 0.66 | *Sphk1* | 1.73 | *Slc27a2* | 2.59 | *Ttr* | 6.09 |
| *Gna14* | 0.66 | *Hspb7* | 1.73 | *Rbp4* | 2.60 | *C9* | 6.25 |
| *Col6a3* | 0.67 | *Igfbp6* | 1.74 | *Lrp2* | 2.63 | *Olfr224* | 6.40 |
| *Lphn1* | 0.67 | *Nccrp1* | 1.74 | *Tdo2* | 2.69 | *Afp* | 6.86 |
| *Pla2g4f* | 0.67 | *Jph2* | 1.74 | *Bai1* | 2.70 | *Agt* | 7.46 |
| *Mpzl2* | 0.67 | *Snta1* | 1.74 | *Slc13a3* | 2.75 | *St6galnac1* | 7.96 |
| *Stox2* | 0.68 | *Sfrp5* | 1.75 | *Cfi* | 2.80 | *Psca* | 8.01 |
| *Aldh1a3* | 0.68 | *1600015I10Rik* | 1.79 | *Slc5a1* | 2.80 | *2610035D17Rik* | 8.19 |
| *Tnks* | 0.70 | *Mlph* | 1.82 | *Pdzk1ip1* | 2.83 | *Apoa1* | 9.23 |
| *Eif2c2* | 0.71 | *F2* | 1.86 | *Itih4* | 2.87 | *Gc* | 9.86 |
| *Galnt6* | 0.71 | *Rrm2* | 1.87 | *Trpm2* | 2.88 | *Gdpd3* | 12.80 |
| *Herpud1* | 0.72 | *Rimklb* | 1.91 | *Bex2* | 2.88 | *Cps1* | 13.91 |
| *Ccng1* | 1.38 | *Cldn1* | 1.94 | *Itih3* | 2.94 | *Itih2* | 14.41 |
| *Slc7a8* | 1.38 | *Cldn10* | 2.01 | *Cdhr2* | 2.95 | *Alb* | 29.20 |
| *Slc43a2* | 1.38 | *Ttc30b* | 2.02 | *Ambp* | 3.06 | | |

**Supplemental Figure 3.2**. Differentially-expressed genes in the male placentas in response to 4X choline supplementation.

| Gene Symbol | Fold Difference | Gene Symbol | Fold Difference | Gene Symbol | Fold Difference |
|---|---|---|---|---|---|
| *Ggt6* | 0.10 | *Cysltr2* | 0.53 | *Ccrn4l* | 1.56 |
| *Ceacam18* | 0.20 | *Il1r2* | 0.53 | *Tspan2* | 1.56 |
| *Mcpt8* | 0.21 | *Lum* | 0.55 | *Cd93* | 1.57 |
| *Psca* | 0.23 | *Syngr1* | 0.55 | *Adamts5* | 1.59 |
| *Npb* | 0.28 | *Gm9199* | 0.55 | *Lyve1* | 1.61 |
| *Lect1* | 0.28 | *Eva1c* | 0.56 | *Ccbe1* | 1.61 |
| *BC002163* | 0.29 | *Rbm38* | 0.57 | *Doxl2* | 1.63 |
| *Nptx1* | 0.30 | *Ipp* | 0.57 | *Fst* | 1.63 |
| *Klk15* | 0.32 | *Dcn* | 0.58 | *Prl2a1* | 1.64 |
| *Scgb1a1* | 0.33 | *Tox2* | 0.58 | *Srek1ip1* | 1.67 |
| *Ctsk* | 0.35 | *Serping1* | 0.58 | *Serpine1* | 1.68 |
| *Alb* | 0.36 | *Gja4* | 0.58 | *Ramp3* | 1.69 |
| *Zfp738* | 0.36 | *Kcnq4* | 0.59 | *Gm8883* | 1.71 |
| *Ctsg* | 0.37 | *Eno2* | 0.59 | *Sfrp5* | 1.75 |
| *Mtus2* | 0.37 | *Pla1a* | 0.59 | *Sphk1* | 1.80 |
| *Ntrk2* | 0.39 | *Serpina3n* | 0.59 | *Sfrp4* | 1.82 |
| *Bai1* | 0.40 | *Gda* | 0.60 | *Prl8a2* | 1.83 |
| *Cryaa* | 0.40 | *Havcr2* | 0.61 | *Pde4b* | 1.84 |
| *Klk4* | 0.40 | *Tnfrsf23* | 0.62 | *Hspb7* | 1.85 |
| *Tmem45a* | 0.40 | *Hsd11b2* | 0.63 | *AW011738* | 1.87 |
| *Egln3* | 0.41 | *Cxcl12* | 0.63 | *Htra1* | 1.89 |
| *Gzmc* | 0.42 | *Plxdc2* | 0.63 | *Hand2* | 1.92 |
| *Car12* | 0.42 | *Srgn* | 0.64 | *Igsf11* | 1.93 |
| *Doc2b* | 0.43 | *Kif21b* | 0.64 | *Hhipl1* | 1.94 |
| *Tdo2* | 0.43 | *Unc5a* | 0.66 | *Cldn10* | 2.00 |
| *Cxcl14* | 0.43 | *Rasl11b* | 0.67 | *Golga7b* | 2.01 |
| *Lbp* | 0.44 | *Gpr125* | 0.68 | *Krt16* | 2.05 |
| *Hsd3b6* | 0.44 | *Cdo1* | 0.69 | *Apold1* | 2.15 |
| *Cldn11* | 0.44 | *Col12a1* | 0.70 | *Fggy* | 2.22 |
| *Gjb3* | 0.45 | *H2-D1* | 0.70 | *Ttc18* | 2.37 |
| *Prf1* | 0.45 | *Tuft1* | 0.70 | *Clca5* | 2.38 |
| *Nov* | 0.45 | *Lpcat1* | 0.71 | *Col6a5* | 2.41 |
| *Gzmd* | 0.46 | *Sparcl1* | 1.38 | *Rimklb* | 2.44 |
| *2610528A11Rik* | 0.46 | *Aqp8* | 1.38 | *Kcnj10* | 2.47 |
| *Qpct* | 0.46 | *Prl7a1* | 1.42 | *Slc15a2* | 2.72 |
| *Dio2* | 0.46 | *Tnfrsf1b* | 1.44 | *Clip4* | 2.75 |
| *Gzmg* | 0.48 | *Add3* | 1.44 | *Wnt10a* | 2.79 |
| *Gzmf* | 0.48 | *Gm14403* | 1.46 | *Trpm2* | 2.80 |
| *Ceacam10* | 0.49 | *Prl7b1* | 1.47 | *Calm4* | 2.83 |
| *Gzmb* | 0.50 | *Aspg* | 1.49 | *Ear11* | 2.83 |
| *Selenbp1* | 0.50 | *Pla2g7* | 1.50 | *Crabp1* | 3.45 |
| *Fgl2* | 0.50 | *Acpp* | 1.50 | *St6galnac1* | 3.46 |
| *Gpr133* | 0.51 | *Gm14295* | 1.50 | *Gm3558* | 3.76 |
| *4930486L24Rik* | 0.52 | *Igfbp3* | 1.53 | *Ifi202b* | 4.19 |
| *Ptprn2* | 0.52 | *Tacstd2* | 1.55 | *Lce1g* | 8.00 |
| *Gzme* | 0.52 | *Jam2* | 1.55 | *Gjb4* | 9.12 |
| *Mal* | 0.52 | *Nccrp1* | 1.56 | *Ang2* | 11.00 |

**Supplemental Table 3.1.** Results from the miRNA-sequencing experiment when statistical analyses were performed including all the placental samples.[1]

| miRNA | 1X Choline Group | | | 4X Choline Group | | | FDR |
|---|---|---|---|---|---|---|---|
| | Sample 1* | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | |
| miR-712-5p | 187.53 | 1.06 | 1.12 | <0.01 | <0.01 | 0.41 | 0.019 |
| miR-6538 | 118.98 | 1.86 | 7.87 | 0.37 | 0.62 | 2.89 | 0.031 |
| miR-3470a | 902.06 | 14.22 | 10.11 | 8.73 | 9.68 | 8.06 | 0.031 |
| miR-6240 | 1141.97 | 12.76 | 39.33 | 13.38 | 9.99 | 25.22 | 0.031 |
| miR-5126 | 31.04 | 1.33 | 3.37 | 0.37 | 0.31 | 0.83 | 0.033 |
| miR-3470b | 1077.95 | 24.32 | 22.19 | 16.17 | 19.35 | 31.21 | 0.056 |
| miR-6380 | 18.11 | 1.33 | 0.56 | 0.37 | 0.31 | <0.01 | 0.155 |
| miR-96-3p | 9.05 | 9.57 | 11.80 | 11.89 | 23.73 | 19.02 | 0.160 |
| miR-3535 | 870.38 | 71.76 | 85.40 | 53.15 | 76.17 | 58.29 | 0.160 |
| miR-3471 | 15.52 | 0.53 | 0.70 | 0.19 | 0.31 | <0.01 | 0.160 |
| miR-690 | 494.03 | 31.63 | 33.15 | 21.93 | 36.21 | 28.52 | 0.160 |

[1]Data are shown as normalized counts (per million miRNA reads). *Sample 1 is the sample with lower miRNA-mapped read frequency.

# AFTERWORD

The overarching goal of this dissertation research is to determine the effects of supplementing the maternal diet with additional choline on factors that influence placental nutrient supply, which determines fetal growth and development. Employing a multi-disciplinary research approach integrating nutrition, metabolism, biochemistry, genomics, and reproductive biology, a variety of biomarkers was analyzed in the placental and fetal tissues collected on four different gestational days from a healthy pregnant mouse model consuming three different levels of choline intake. The major findings and potential implications are discussed below.

***Maternal choline supplementation improves placental vascularization and perfusion***

This study sought to determine the impact of additional maternal choline intake during pregnancy on indicators related to placental vascularization and perfusion. The results show that maternal choline supplementation modulates placental inflammation, apoptosis and angiogenesis. The remodeling process of maternal spiral arteries is also more efficient in response to extra choline intake. As a result, placental perfusion is enhanced, which is one important factor for an adequate placental nutrient supply. Interestingly, the effects of choline on these processes are strongly dependent on fetal sex and gestational stage, highlighting the need for considering these variables when studying the effects of maternal diets on placental development. Because abnormal placental vascularization and insufficient placental perfusion are characteristics of many pregnancy disorders that impair fetal development, additional works are needed to explore the impact of maternal choline supplementation in preventing and/or ameliorating these pregnancy problems.

***Maternal choline supplementation alters placental nutrient transport and metabolism***

This study aimed to examine the impact of additional maternal choline intake during pregnancy on placental nutrient transporter abundance and placental nutrient metabolism. The findings

indicate that the metabolism and transporter abundance of the macronutrients as well as choline and its metabolites are all altered by maternal choline supplementation, again in a manner dependent on fetal sex and gestational stage. Most importantly, these choline-induced changes in the placental nutrient supply system affect the amount of nutrients available to the fetus for its growth and development. Because the abundance of these placental nutrient transporters and metabolic enzymes in response to a higher maternal choline intake is largely unknown in human pregnancy, more research is needed to determine the effect of choline on these markers in the human placentas. As the hypothesis of fetal programming proposes that the development of the placenta and fetus plays an important role in determining the offspring postnatal health and risk for different diseases, further studies are also warranted to examine the long-term impacts of these placental responses to a higher maternal choline consumption during pregnancy.

***Maternal choline supplementation modulates several epigenetic processes in the placenta with important consequences on placental and fetal development***

The objective of this study was to characterize changes in placental epigenetic processes in response to maternal choline supplementation with the long-term goal of better understanding mechanisms involved in choline's beneficial effects on placental vascular development, placental nutrient delivery and fetal development. The data illustrate that maternal choline supplementation changes the global DNA methylation, imprinted and non-imprinted gene expression, and microRNA abundance in the mouse placenta. All these changes have subsequent impacts on many downstream processes important for normal development. Consistent with prior findings, these effects differ greatly between the females and males. As an untargeted experimental approach was employed, several potentially important epigenetic markers are identified, allowing future investigations to further elucidate their roles in mediating the effect of choline on different placental and fetal developmental outcomes.

In sum, findings from this dissertation research further advance our fundamental understanding regarding the role of choline in reproductive health. Not only do these data support our previous findings from a human feeding trial indicating that maternal choline supplementation improves placental vascular development, they also provide additional insights regarding the impact of maternal choline supplementation on other aspects of placental development and functions. Given the importance of these new insights in optimizing pregnancy outcomes and offspring health, future students in our research group can use these results as a justification for designing additional experiments in normal human pregnancy and in human pregnancy disorders. Altogether, results from this animal feeding study, along with our previous works conducted in healthy pregnant women, support the recommendation that women of reproductive age should increase their intake of choline-rich foods in order to improve pregnancy outcomes and the lifelong health of their children.