MATERNAL CHOLINE SUPPLEMENTATION IN A MOUSE MODEL OF PLACENTAL INSUFFICIENCY

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MATERNAL CHOLINE SUPPLEMENTATION IN A MOUSE MODEL OF PLACENTAL INSUFFICIENCY

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Placental insufficiency is commonly associated with intrauterine growth restriction, preeclampsia, and spontaneous abortion. The essential nutrient choline may mitigate some of these impairments, as suggested by data in humans and trophoblast cell culture. Choline is a precursor for several molecules with crucial roles during pregnancy: the methyl donor betaine, the neurotransmitter acetylcholine, and the phospholipid phosphatidylcholine. Through betaine, it participates in the metabolic network referred to as one-carbon metabolism which is essential for many biochemical processes including nucleotide synthesis, DNA methylation, and amino acid metabolism. Demand for one-carbon (methyl) nutrients, including choline, folate, and vitamin B-12, is high during pregnancy when cells of the placenta and fetus undergo rapid division. This dissertation research sought to examine the effects of choline supplementation during pregnancy in the *Dlx3*+/- mouse, a model of placental insufficiency.

Study 1 investigated the effects of maternal choline supplementation (MCS) on pregnancy outcomes in *Dlx3*+/- mice. This study demonstrated that maternal choline supplementation led to a marked increase in fetal growth by mid-gestation, which resulted in compensatory mechanisms to slow growth by downregulating *Igf1*. Placental betaine concentrations were strongly predictive of fetal weights, suggesting betaine supply may be a determinant of fetal growth.

Study 2 explored the effects of MCS on markers of placental function in the *Dlx3*+/- mouse. The main findings indicate that a higher maternal choline intake can alter placental labyrinth size, modulate expression of angiogenic and inflammatory genes in the placenta, and decrease placental apoptosis; and that these effects occur in a fetal sex-dependent manner.

Study 3 assessed the effects of MCS on vitamin B-12 status in mice with and without placental insufficiency. In addition, data from a human feeding study was used to further explore this relationship. MCS increased the active form of vitamin B-12 in pregnant women; genetic variants that increase choline requirements were also shown to alter vitamin B-12 markers.

Taken together, these studies suggest that higher levels of choline intake during pregnancy may be beneficial for the mother and fetus, and provide support for further research on the use of choline for improving placental function and mitigating placental insufficiency.

BIOGRAPHICAL SKETCH

Julia Heather King was born in Ottawa, Ontario, Canada and graduated with distinction with a Bachelor of Science (Honours) from the University of Guelph, Ontario, Canada in 2012. A keen interest in food and health led her to the Nutritional Science department and she began her research career as an undergraduate research assistant under the guidance of Dr. Marica Bakovic, investigating the link between choline transport and phospholipid metabolism. She subsequently joined the laboratory of Dr. Marie Caudill at Cornell University where she entered the PhD program in Nutrition in Autumn 2012. Concentrating in molecular nutrition with minors in genomics and comparative biomedical sciences, her dissertation project focused on choline supplementation during pregnancy and its effects on maternal and placental outcomes in a mouse model of placental insufficiency. She presented this research at Experimental Biology in 2015 and 2016 and was twice a winner in the Vitamins and Minerals category of the American Society for Nutrition's Emerging Leaders in Nutrition Science Competition. While at Cornell, she enjoyed working as a teaching assistant for a variety of undergraduate courses, including Nutrition and Disease, Food for Contemporary Living, and Anatomy and Physiology.

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LIST OF ABBREVIATIONS

Ahcy	adenosylhomocysteinase
Bhmt	betaine-homocysteine S-methyltransferase
CHDH	choline dehydrogenase
Dlx3	distal-less homeobox 3
Egfr	epidermal growth factor receptor
GPC	glycerophosphocholine
holoTC	holotranscobalamin
IGF	insulin-like growth factor
Igf1	insulin-like growth factor-1
Igf2	insulin-like growth factor-2
Igf1r	insulin-like growth factor receptor-1
Igf2r	insulin-like growth factor receptor-2
Il1b	interleukin 1b
IUGR	intrauterine growth restriction
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LPC	lysophosphatidylcholine
Mat1a	methionine adenosyltransferase 1 alpha
MCS	maternal choline supplementation
MMA	methylmalonic acid
Mmp14	matrix metalloproteinase 14
Mtr	5-methyltetrahydrofolate-homocysteine methyltransferase

Mtrr	5-Methyltetrahydrofolate-homocysteine methyltransferase reductase
Mut	Methylmalonyl-CoA mutase
Nfκb	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NSA	non-swiss albino
PC	phosphatidylcholine
PCho	phosphocholine
PCYT1	phosphocholine cytidylyltransferase
PEMT	phosphatidylethanolamine N-methyltransferase
Pgf	placental growth factor
sFlt-1	soluble fms-like tyrosine kinase-1
SM	sphingomyelin
SMA	smooth muscle actin
Sry	sex-determining region Y
Tbp	TATA box binding protein
Tnfα	tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
Vegf	vascular endothelial growth factor

PREFACE

Choline is an essential nutrient that is required for the synthesis of several molecules with crucial roles during pregnancy: betaine, an osmolyte and methyl donor; acetylcholine, a neurotransmitter and signaling molecule; and phosphatidylcholine (PC), the most prevalent membrane phospholipid. Higher intake of choline during pregnancy has previously been shown to downregulate sFlt-1, a biomarker of preeclampsia; additionally, choline deficiency in a placental cell culture model has been found to impair angiogenesis and increase inflammation and apoptosis.

Given the previous findings suggesting the importance of choline supply during healthy pregnancy, the overarching goal of this dissertation was to extend this work to a model of placental insufficiency, the *Dlx3*+/- mouse. *Dlx3* (distal-less 3 homeobox) is a transcription factor which is required for normal placental development. Mice heterozygous for this gene display inadequate vascularization and development of the placental labyrinth, which is the location of nutrient exchange between mother and fetus. To characterize the effects of maternal choline supplementation (MCS) in the *Dlx3*+/- mouse, we assessed the following specific aims:

Aim 1: To test the hypothesis that maternal choline supplementation (MCS) improves pregnancy outcomes in the *Dlx3*+/- mouse. This aim was accomplished by assessments of litter size and resorption rates, morphometric measurements of fetal

and placental growth, and analysis of potential metabolic and molecular determinants of fetal growth. Results from this study are presented in Chapter 1.

Aim 2: To test the hypothesis that MCS improves placental function in the *Dlx3*+/- mouse. This aim was accomplished by assessing placental morphology and remodeling as well as markers of placental apoptosis, angiogenesis, and inflammation. Results from these experiments are presented in Chapter 2.

Aim 3: To test the hypothesis that choline supply alters markers of vitamin B-12 status in mice with placental insufficiency as well as healthy pregnant women. This aim was accomplished by analyzing vitamin B-12 biomarkers by maternal choline supplementation status as well as choline genetic variant status. These findings are presented in Chapter 3.

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

CHAPTER 1

MATERNAL CHOLINE SUPPLEMENTATION ALTERS FETAL GROWTH PATTERNS IN A MOUSE MODEL OF PLACENTAL INSUFFICIENCY*

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Abstract

Impairments in placental development can adversely affect pregnancy outcomes. The bioactive nutrient choline may mitigate some of these impairments, as suggested by data in humans, animals, and human trophoblasts. Herein, we investigated the effects of maternal choline supplementation (MCS) on parameters of fetal growth in a *Dlx3*+/− (distal-less homeobox 3) mouse model of placental insufficiency. *Dlx3*+/− female mice were assigned to 1X (control), 2X, or 4X choline intake levels during gestation. Dams were sacrificed at embryonic days E10.5, 12.5, 15.5, and 18.5. At E10.5, placental weight, embryo weight, and placental efficiency were higher in 4X versus 1X choline. Higher concentrations of hepatic and placental betaine were detected in 4X versus 1X choline, and placental betaine was positively associated with embryo weight. Placental mRNA expression of *Igf1* was downregulated by 4X (versus 1X) choline at E10.5. No differences in fetal growth parameters were detected at E12.5 and 15.5, whereas a small but significant reduction in fetal weight was detected at E18.5 in 4X versus 1X choline. MCS improved fetal growth during early pregnancy in the *Dlx3*+/− mice with the compensatory downregulation of *Igf1* to slow growth as gestation progressed. Placental betaine may be responsible for the growth-promoting effects of choline.

Introduction

The placenta mediates the delivery of nutrients to the fetus and is thus a major determinant of fetal growth [1]. Impairments in placental development can lead to placental insufficiency, an inability of the placenta to provide adequate nutrients for the fetus, and contribute to adverse pregnancy outcomes, including intrauterine growth restriction (IUGR), miscarriage, and preeclampsia [2,3]. At present, very few treatment options are available for placental-induced pregnancy complications that adversely affect the health of the mother and child. Nutritional therapies represent a promising area of research for improving maternal and child health [4], especially since some pharmacological treatments may be unsafe during pregnancy. Choline is a water-soluble essential nutrient that is necessary for the synthesis of acetylcholine (a neurotransmitter), phosphatidylcholine (a membrane phospholipid), and betaine (a methyl donor and osmolyte) [5]. During pregnancy, these molecules support rapid cell division, govern genomic methylation patterns, and modulate placental and fetal development [6].

Choline has previously been shown to reduce a risk factor of preeclampsia, sFLT1, in a randomized, controlled feeding trial of maternal choline supplementation (MCS) during the third trimester of pregnancy in healthy women [7]. In addition, choline has been shown to modulate inflammation, oxidative stress, and apoptosis of immortalized human placental trophoblast cells in culture [8] and in normal murine pregnancy [9]. Therefore, we hypothesized that choline supplementation would be beneficial for pregnancies complicated by placental insufficiency.

During pregnancy, the homeodomain-containing transcription factor distal-less homeobox 3 (*Dlx3*) plays a crucial role in the development of the fetal-maternal interface [10]. Mice heterozygous for this gene have been shown to display inadequate vascularization and development of the placental labyrinth, which is the location of nutrient exchange between mother and fetus [11]. Mice that are homozygous null for *Dlx3* die between embryonic days 9.5 and 12.5 due to placental failure and display extremely restricted fetal growth [10]. Heterozygous embryos are viable but have been reported to display placental abnormalities, including a reduced invasion of trophoblasts and impaired remodeling of maternal spiral arteries as well as increased placental oxidative stress and apoptosis within the labyrinth and maternal decidua [11].

A major determinant of early placental and fetal growth in both mice and humans is the insulin-like growth factor (IGF) axis, consisting of IGF1 and IGF2, their receptors IGF1R and IGF2R, and several binding proteins [12]. The placenta accumulates IGFs, IGF receptors, and IGF binding proteins, which help mediate the transfer of nutrients from placenta to fetus [13]. The IGF1 receptor transduces the growth-promoting function of IGFs, while the IGF2 receptor primarily functions to sequester and degrade IGF2 and prevent its binding to IGF1R. Accordingly, the genetic deletion of IGF1, IGF2, and IGF1R severely reduces fetal growth in mice, while the deletion of IGF2R leads to fetal overgrowth [14]. The expression of IGF genes as well as the epidermal growth factor receptor (EGFR), which interacts with IGF1R [15], has been shown to be modulated by choline supply in various tissues and gestational time points of pregnancy [16,17,18].

The present study was undertaken to investigate the effects of MCS on the determinants and parameters of fetal growth in the *Dlx3* murine model of placental insufficiency. We hypothesized that (a) maternal choline supplementation would support fetal growth in placental insufficiency and (b) this effect may occur through the IGF axis.

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 conducted in accordance with the Guide for the Care and Use of Laboratory Animals (protocol number 2001-0034). The *Dlx3*+/− mice were a generous gift from Dr. Maria Morasso (NIH/NIAMS) and were genotyped using a three-primer duplex PCR (polymerase chain reaction) of tail DNA. The primers were designed to amplify wildtype or knockout allele amplicons or both (indicating a heterozygote) (Supplementary Table S1). The mice were housed in microisolator cages (Ancare) in an environmentally-controlled room (22–25 °C and 70% humidity) with a 12-h light-dark cycle. Breeding animals were given ad libitum access to commercial rodent chow (Teklad) and water. Female *Dlx3*+/− mice were mated with *Dlx3*+/− males. Female and male offspring were genotyped at time of weaning (three weeks of age), and *Dlx3*+/− animals were given ad libitum access to an AIN-93G purified rodent diet (Dyets #103345) containing 1.4 g choline chloride/kg diet (1X choline control diet). Five days before mating with *Dlx3*+/− males, *Dlx3*+/− females (n = 116) were randomized to receive either the 1X choline control diet (1.4 g/kg, the standard choline content of the AIN-93G diet providing adequate choline intake), a 2X choline diet containing 2.8 g choline chloride/kg (Dyets #103346), or a 4X choline diet containing 5.6 g choline chloride/kg (Dyets #103347). The 2X treatment was chosen to correspond with the previous human feeding study conducted by our research group [19]. A 4X treatment

is widely used to investigate cognitive effects in rodent models. The presence of a vaginal plug was used to designate embryonic (E) day 0.5. Pregnant mice were euthanized at four different gestational time points (E10.5, E12.5, E15.5, and E18.5) in order to assess the effects of maternal choline supplementation across gestation.

Tissue Collection and Processing and Fetal Anthropometry

At the time of dissection, the maternal liver was removed, flash frozen in liquid nitrogen, and stored at −80 °C. The number of implantations and resorptions in the gravid uterus were recorded. Embryos and placentas were carefully dissected to minimize decidual contamination and weighed. At E12.5, E15.5, and E18.5, placentas were bisected across the chorionic plate; half was placed in RNAlater for mRNA analysis, while the remaining half was immediately frozen in liquid nitrogen and stored at −80 °C for metabolite analysis. Due to the smaller tissue size, E10.5 placentas were alternately designated for mRNA analysis or metabolite analysis. Fetuses were imaged and subsequently frozen in liquid nitrogen and stored at −80 °C. The crown rump measurement of the fetuses was calculated from images using ImageJ software (NIH). Fetal DNA was extracted for *Dlx3* genotyping and sex determination using a commercial kit (Qiagen Inc., Germantown, MD, USA). Sex genotyping was performed by PCR for the Sry gene with a commercial kit (Qiagen Inc., Germantown, MD, USA). The primers are listed in Supplementary Table S1.

Fetal body composition analyses were determined on fetuses acquired from E18.5 using the Leshner method [20]. Fetuses were decapitated at dissection to allow for analyses of the brain in a separate study; all body composition data is reported on

the remaining tissue. Briefly, the fetuses were thawed and weighed, followed by desiccation to determine water weight. Dehydrated fetuses were weighed and pulverized before undergoing ethyl ether/methanol total lipid extraction. Total lipid extractions were successively performed until no change in weight occurred. The protein content was determined colorimetrically, following the digestion of pulverized fetal tissue in radioimmunoprecipitation assay (RIPA) buffer.

Measurements of Hepatic and Placenta Metabolites

Concentrations of choline and the related metabolites betaine, methionine, phosphocholine, glycerophosphocholine, phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin were measured in the maternal livers of dams by LC-MS/MS (liquid chromatography–tandem mass spectrometry) according to the method of Koc et al. [21], with modifications based on our equipment [19]. Choline, betaine, and methionine in the placenta were measured using the method of Holm et al. [22], with modifications based on our equipment [19].

Quantitative Real-Time RT-PCR

RNA was extracted from placentas fixed in RNAlater using TRIzol reagent (Invitrogen, Waltham, MA, USA). Two or three placentas per dam were randomly selected for extraction. RNA concentration and quality were assessed with a NanoDrop ND-1000 instrument (Thermo Fisher Scientific, Waltham, MA, USA), and all samples had an A260/A280 ratio ≥1.8. Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI,

USA). Quantitative PCR was performed using SYBR® Green in a Roche LightCycler480. All primers for the targeted genes (*Igf1, Igf2, Igf1r, Igf2r, Egfr*) were designed using NCBI Primer-BLAST (Supplementary Table S1). The reaction conditions were as follows: 95 °C for 5 min, followed by 40 cycles with 15 s at 95 °C, 30 s at 63 °C, and 30 s at 72 °C. A melting curve analysis was included at the end of the amplification cycles to ensure the specificity of the PCR product. Fold changes were calculated by the ΔCt method [23] normalized to the expression level of housekeeping gene *Tbp* (TATA box binding protein). *Tbp* has been shown to be stable in response to various choline treatments [24].

Statistical Analysis

For maternal measurements, data were analyzed using general linear models with treatment and litter size as independent variables. Comparisons between embryonic genotype distributions were made using Fisher's exact test with two-tailed P values. For placental and fetal measurements, data were analyzed using a linear mixed model. The model included choline treatment, fetal sex, and litter size as fixed effects and maternal identifier as a random effect. Data was either stratified by fetal *Dlx3* genotype or, when all genotypes were combined, was adjusted for fetal genotype as a fixed effect. Data were assessed for normality and log-transformed if the residuals were not normally distributed. Corrections for multiple analyses were not performed due to the hypothesis-driven nature of the study and the relatively limited sample size. Data are presented as mean ± SEM. $p \leq 0.05$ was considered statistically significant,

and $0.05 < p < 0.10$ was considered to indicate trends. All analyses were performed using SPSS software, Version 23 (IBM).

Results

Effects of Maternal Choline Supplementation

Pregnancy Outcomes

To determine whether choline treatment during pregnancy could improve viability, we examined indicators of embryo survival. MCS did not significantly impact the number of implantations or the percentage of resorptions at any gestational time point in *Dlx3*+/− litters (Supplementary Table S2). We also assessed whether MCS could increase or prolong the survival of homozygous null fetuses at E10.5 and E12.5 (Supplementary Table S3). A small number of *Dlx3*−/− fetuses (n = 10) survived to E12.5, with the majority of these found in the 4X group (n = 7). Choline treatment at 2X or 4X did not significantly alter embryo genotype distributions at any gestational time point.

Fetal and Placental Growth

Since *Dlx3*+/− has been reported to be a model of placental insufficiency, we sought to determine whether MCS could improve placental efficiency as well as fetal growth. At E10.5, embryo weight was ~70% higher in 4X choline pups of all three genotypes compared to 1X controls ($p < 0.001$, 0.001, 0.033 for wildtypes, heterozygotes, and homozygous nulls, respectively). 2X (versus 1X) choline also yielded significantly higher embryo weights in wildtype pups ($p = 0.038$) (Figure 1A). At E18.5, the embryo weight of the 4X pups was slightly (but significantly) lower in both wildtype (~12%, $p = 0.003$) and heterozygote (~10%, $p = 0.005$) pups compared to 1X pups (Figure 1B).

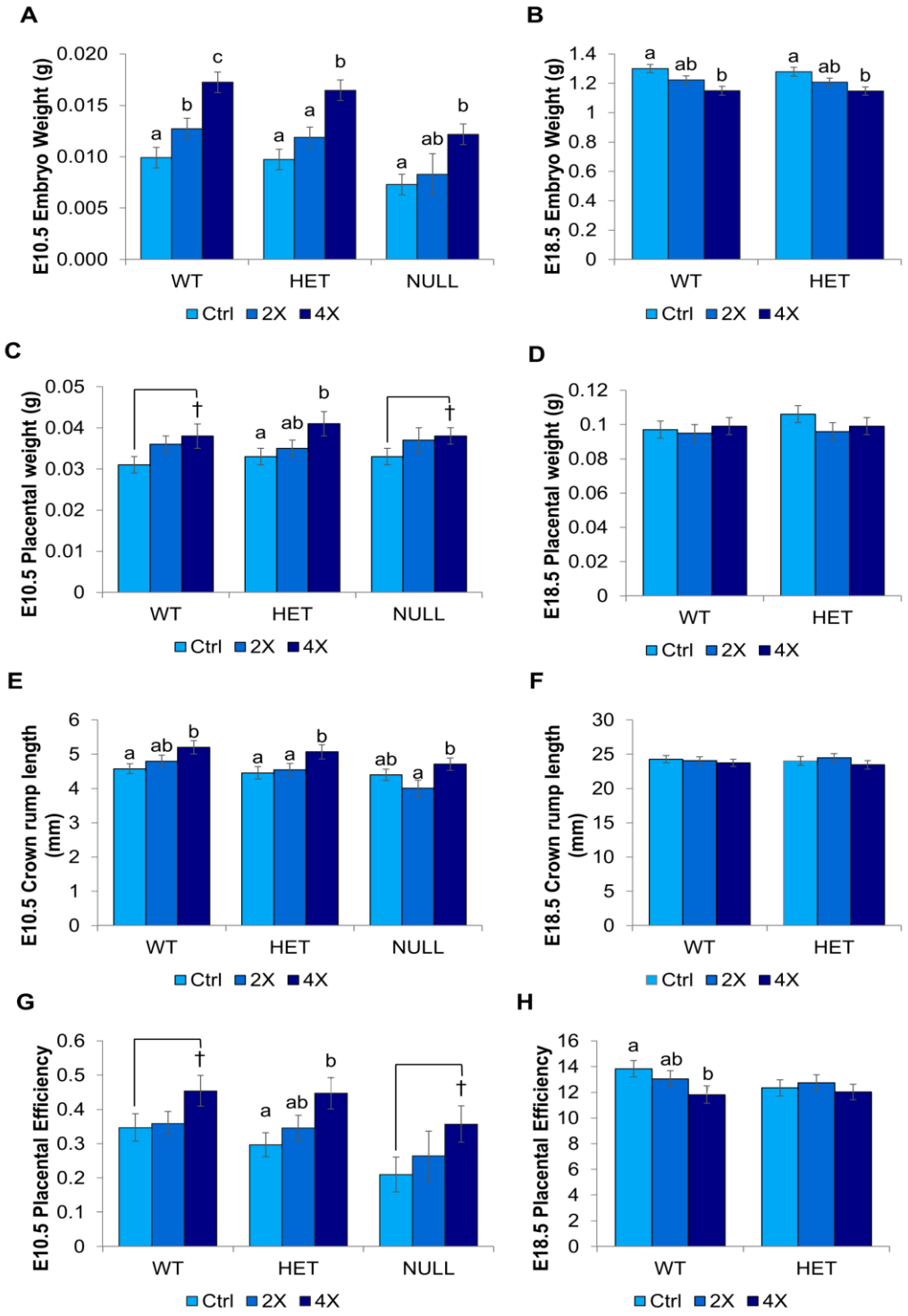


Figure 1. Fetal and placental growth characteristics by choline treatment (1X control, 2X, and 4X) and *Dlx3* genotype. Embryo weight at (A) E10.5, and (B) E18.5. Placental weight at (C) E10.5, and (D) E18.5. Crown-rump length at (E) E10.5 and (F) E18.5. Placental efficiency (defined as embryo weight/placental weight) at (G) E10.5 and (H) E18.5. Data were analyzed using mixed linear models controlling for maternal identifier, fetal sex, and litter size. Values are presented as mean ± SEM. Differing letters denote $p \leq 0.05$. † denotes $p < 0.10$. n = seven to 10 dams per treatment per time point.

Placental weight was significantly higher at E10.5 in 4X heterozygotes (~24%, $p = 0.024$ versus 1X) and tended to be higher in 4X wildtypes (~23%, $p = 0.053$ versus 1X) (Figure 1C). Placental weight was not influenced by choline treatment at E18.5 (Figure 1D). Fetal crown-rump length was ~14% higher at E10.5 in 4X choline wildtypes ($p = 0.022$) and heterozygotes ($p = 0.033$) compared to 1X controls but did not differ at E18.5 (Figure 1E,F). Placental efficiency, calculated as the ratio of embryo weight to placental weight, was ~51% higher at E10.5 in 4X choline heterozygotes ($p = 0.016$, vs. 1X) and tended to be higher in wildtypes (~31%, $p = 0.088$ vs. 1X) and homozygous nulls (~70%, $p = 0.076$ versus 1X) (Figure 1G). At E18.5, placental efficiency was ~15% lower in 4X wildtypes ($p = 0.045$ vs. 1X) but did not differ in heterozygotes (Figure 1H).

At E12.5 and E15.5, the 2X and 4X choline groups of all genotypes did not significantly differ from 1X controls in embryo weight, placenta weight, crown-rump length, or placental efficiency (Supplementary Table S4). However, when comparing 2X to 4X, heterozygotes in the 4X group had slightly lower embryo weights at E12.5 (~19%, $p = 0.011$).

We also assessed the impact of altered growth patterns on fetal body composition at E18.5. Choline treatment at 2X or 4X did not alter the percentage of body water, fat, or protein compared to 1X choline (Supplementary Table S2).

Hepatic and Placental Choline Metabolites

Since the maternal liver is the source of choline metabolites, which are used by the placenta to support fetal growth and development, we measured the concentration of maternal hepatic choline metabolites (Table 1). At all four gestational time points, 4X (versus 1X) choline yielded significantly higher betaine concentrations by ~112%, ~114%, ~92%, and ~348% respectively ($p < 0.001$ for E10.5, 12.5 and 18.5; $p = 0.001$ for E15.5). 2X choline yielded higher betaine (~153% of 1X choline) at E18.5 ($p = 0.022$ vs. 1X). In the 1X control group, hepatic betaine concentration declined by ~35% from E10.5 to E18.5 ($p = 0.042$), whereas the 2X and 4X choline groups showed no significant change in hepatic betaine concentrations across gestation. At E12.5, glycerophosphocholine (GPC) was lower in both the 2X (~21%, $p = 0.031$) and 4X choline groups (~22%, $p = 0.025$) compared to the 1X choline group. No significant differences in GPC at E10.5, 15.5, or 18.5 were detected. At E15.5, phosphocholine was 133% higher in the 4X versus 1X choline group ($p = 0.014$). No significant differences in hepatic choline, methionine, phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin were detected at any gestational time point (Table 1).

Placental concentrations of betaine were also higher in the 4X group at E10.5 (~39%, $p = 0.007$ vs. 1X) and tended to be 19% higher at E12.5 ($p = 0.088$ vs. 1X) (Table 2). However, betaine concentration did not differ by treatment in late pregnancy (E15.5 and E18.5). Placental concentrations of free choline and methionine were not significantly affected by choline treatment at any time point; however, methionine tended to be lower at E18.5 in the 4X group (~22%, $p = 0.088$, versus 1X).

Table 1. Maternal liver concentrations of choline metabolites (per gram tissue) at E10.5, E12.5, E15.5, and E18.5 by choline treatment. Data were analyzed using general linear models with treatment and litter size as fixed factors. Values are presented as mean ± SEM. Log-transformed variables are represented by back-transformed means and 95% confidence intervals (in parentheses). * Significantly different versus control diet, $p \leq 0.05$. ** Significantly different versus 1X and 2X diets, $p \leq 0.05$. GPC, glycerophosphocholine; GPC, phosphatidylcholine; PCho, phosphocholine; LPC, lysophosphatidylcholine; SM, sphingomyelin. n = seven to 10 dams per treatment, per time point.

Time point	Diet	Choline (nmol/g)	Methionine (nmol/g)	GPC (nmol/g)	LPC (nmol/g)	Betaine (µmol/g)	PCho (µmol/g)	PC (µmol/g)	SM (µmol/g)
10.5	**1X**	214 (173, 265)	124 (95, 161)	948 ± 105	332 (269, 411)	0.81 ± 0.1	1.03 ± 0.2	22.2 ± 0.9	3.94 ± 0.2
	2X	242 (186, 313)	92.7 (67, 127)	1021 ± 126	326 (252, 421)	1.49 ± 0.2*	1.41 ± 0.2	21.6 ± 1.1	3.70 ± 0.2
	4X	247 (197, 310)	99.1 (75, 131)	895 ± 110	347 (277, 435)	1.71 ± 0.1*	1.07 ± 0.2	22.2 ± 0.9	4.16 ± 0.2
12.5	**1X**	206 (173, 245)	85.5 (70, 104)	865 ± 54	363 (302, 437)	0.99 ± 0.1	1.08 (0.7, 1.6)	22.6 ± 0.7	3.97 ± 0.2
	2X	188 (156, 226)	108 (88, 133)	687 ± 57*	353 (292, 429)	1.77 ± 0.2*	1.20 (0.8, 1.8)	22.5 ± 0.7	4.03 ± 0.2
	4X	197 (166, 233)	97.5 (81, 118)	687 ± 52*	371 (308, 446)	2.13 ± 0.1*	1.38 (1.0, 2.0)	22.5 ± 0.7	3.75 ± 0.2
15.5	**1X**	138 (116, 163)	88.7 ± 5.9	294 (225, 382)	339 (259, 443)	1.21 ± 0.2	0.70 ± 0.2	22.4 ± 0.8	3.44 ± 0.2
	2X	133 (111, 160)	88.7 ± 6.4	337 (252, 449)	322 (245, 424)	1.60 ± 0.2*	0.73 ± 0.3	21.6 ± 0.8	3.43 ± 0.2
	4X	144 (121, 171)	75.6 ± 6.0	262 (200, 344)	318 (243, 414)	2.32 ± 0.2*	1.63 ± 0.3*	22.3 ± 0.8	3.20 ± 0.2
18.5	**1X**	117 (97, 141)	104 ± 7.1	301 ± 34	286 (231, 355)	0.53 ± 0.2	0.58 ± 0.2	22.3 ± 1.0	3.10 ± 0.1
	2X	141 (116, 170)	83.3 ± 7.2	302 ± 34	262 (211, 325)	1.34 ± 0.2*	1.04 ± 0.2	21.3 ± 1.0	3.23 ± 0.1
	4X	137 (113, 168)	97.4 ± 7.6	257 ± 36	252 (200, 316)	2.35 ± 0.3**	0.91 ± 0.2	20.8 ±1.0	2.94 ± 0.1

Table 2. Placental concentrations of choline metabolites (µmol metabolite/g tissue) at E10.5, E12.5, E15.5, and E18.5 by choline treatment. Data were analyzed using mixed linear models with treatment, genotype, sex, and litter size as fixed effects and maternal ID as random effect. Values are presented as mean ± SEM. * Significantly different versus control diet, $p \leq 0.05$. ** Significantly different versus 1X and 2X diets, $p \leq 0.05$. # $p < 0.1$ vs. 1X diet. n = 20 placentas per treatment per time point (two to three per dam).

Time point	Diet	Choline	Methionine	Betaine
E10.5	**1X**	0.50 ± 0.04	0.27 ± 0.02	3.09 ± 0.24
	2X	0.56 ± 0.04	0.30 ± 0.02	3.31 ± 0.27*
	4X	0.52 ± 0.05	0.29 ± 0.03	4.28 ± 0.31**
E12.5	**1X**	0.65 ± 0.06	0.25 ± 0.03	5.34 ± 0.50
	2X	0.62 ± 0.06	0.23 ± 0.03	5.59 ± 0.51
	4X	0.57 ± 0.05	0.25 ± 0.02	6.33 ± 0.42#
E15.5	**1X**	0.90 ± 0.06	0.19 ± 0.02	7.99 ± 0.40
	2X	0.94 ± 0.06	0.21 ± 0.02	8.74 ± 0.43
	4X	0.87 ± 0.07	0.19 ± 0.02	8.27 ± 0.48
E18.5	**1X**	1.08 ± 0.08	0.200 ± 0.02	7.66 ± 0.46
	2X	1.14 ± 0.07	0.182 ± 0.02	7.42 ± 0.41
	4X	1.03 ± 0.08	0.156 ± 0.02#	7.80 ± 0.42

Placental Growth Factor Gene Expression

Since changes in fetal and placental growth were seen with choline treatment at E10.5 and E18.5, we measured the mRNA expression of several key growth-related genes in *Dlx3*+/− placentas at these time points. The placental expression of *Igf1* in the 4X (versus 1X) choline group was ~52% lower at E10.5 ($p = 0.005$) and, although not significant, was also lower (~34%) at E18.5 (Figure 2A). The placental expression of *Igf2* did not significantly differ at either time point (Figure 2B). Igf1r tended to have slightly higher expression (~35%) at E10.5 in the 4X choline group when compared to the 1X ($p = 0.087$) and 2X groups ($p = 0.059$) (Figure 2C). Similarly, the expression of *Igf2r* was higher (~22%) at E10.5 in the 4X groups compared to 1X ($p = 0.035$) (Figure 2D). Neither *Igf1r* nor *Igf2r* was significantly affected by choline treatment at E18.5. The expression of *Egfr* tended to be ~27% lower in the 4X choline treatment group at E18.5 versus the 1X controls (Figure 2E).

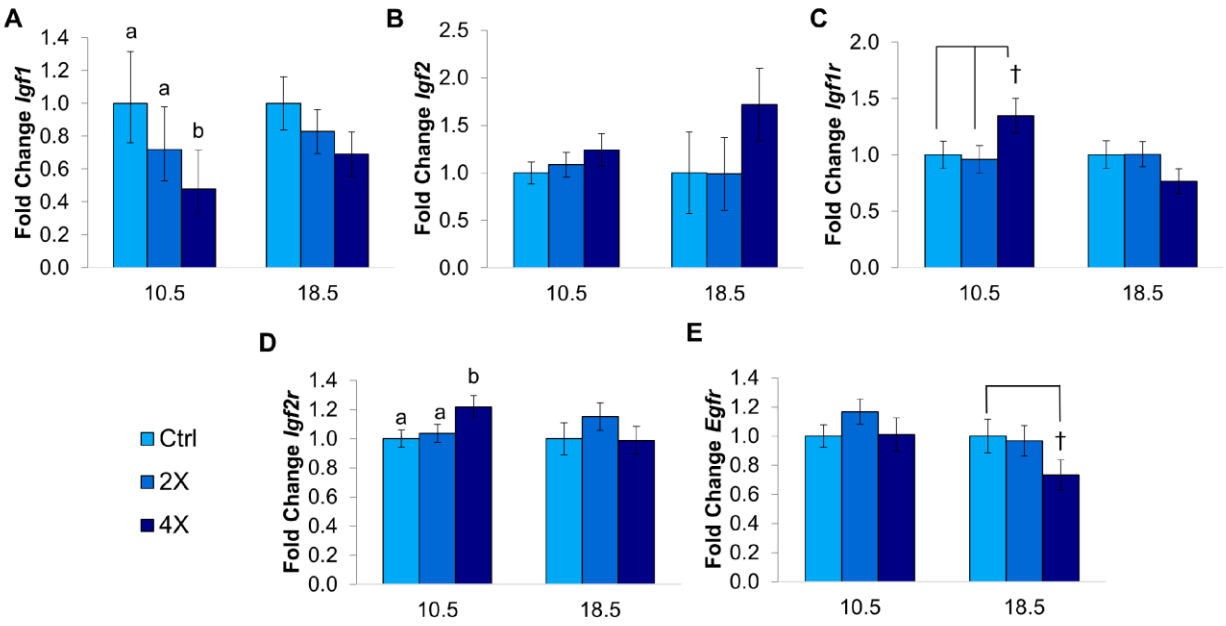


Figure 2. mRNA abundance of (A) *Igf1*, (B) *Igf2*, (C) *Igf1r*, (D) *Igf2r*, and (E) *Egfr* at E10.5 and E18.5 by maternal choline treatment (1X control, 2X, and 4X) in *Dlx3*+/− placentas. Fold changes are expressed relative to the housekeeping gene *Tbp*, with the 1X control group normalized to 1.0. Data were analyzed using mixed linear models controlling for maternal ID, fetal sex, and litter size. Log-transformed data (*Igf1* at E10.5) is represented by back-transformed means and 95% confidence intervals. All other values are presented as mean ± SEM. Differing letters denote $p \leq 0.05$. † denotes $p < 0.10$. n = 20 placentas per treatment per time point (two to three per dam).

Effects of *Dlx3* Genotype

Fetal and Placental Growth

No significant differences in embryo weight, placenta weight, crown rump length, or placental efficiency were detected between wildtype and heterozygote pups at E10.5, 12.5, or 15.5. At E18.5, heterozygous pups weighed ~2% less ($p = 0.044$) and tended to have ~6% lower placental efficiency than wildtypes ($p = 0.077$) (Supplementary Figure S1A,B). Homozygous null pups weighed ~30% less than wildtypes and heterozygotes at E10.5 ($p < 0.001$) and ~59% less at E12.5 ($p < 0.001$) (Supplementary Figure S1C). They also had ~7% to 8% shorter crown rump lengths than wildtypes and heterozygotes at E10.5 ($p < 0.001$) and were ~23% to 25% shorter by E12.5 ($p < 0.001$) (Supplementary Figure S1D). Their placentas were ~31% to 32% less efficient than wildtypes and heterozygotes at E10.5 ($p < 0.002$) and ~52% to 53% less efficient at E12.5 ($p < 0.001$) (Supplementary Figure S1E). Homozygous null placentas did not differ in weight compared to wildtypes and heterozygotes at E10.5 (Supplementary Figure S1F). However, by E12.5, homozygous null placentas weighed ~17% less than those of wildtypes and heterozygotes ($p = 0.027$ and 0.019, respectively) (Supplementary Figure S1F).

Growth Factor Gene Expression in the Placenta

Since we surprisingly observed similar fetal weights in *Dlx3* wildtype and heterozygous embryos, we also measured growth factor gene expression in E10.5 wildtype placentas to examine whether this could have resulted from compensatory changes in gene expression in *Dlx3*+/− placentas. Heterozygous pups overall had

~75% higher expression of *Igf1* compared to wildtypes (p = 0.002) (Supplementary Figure S2A). *Igf2*, *Igf1r*, and *Igf2r* expression did not differ between wildtype and heterozygous pups (not shown). Heterozygous placentas had ~43% higher expression of *Egfr* at E10.5 compared to wildtypes (p = 0.003) (Supplementary Figure S2B).

Determinants of Fetal Growth

Choline Metabolites

To determine whether choline metabolites were associated with fetal growth, we performed regression analyses of placental metabolites and fetal and placental growth parameters, adjusting for choline treatment group, fetal genotype and sex, litter size, and maternal identifier (Table 3, highly significant relationships between metabolites and growth outcomes shown in Figure 3). Placental betaine at E10.5 was positively associated with embryo weight (p = 0.0082, Figure 3A) and crown rump length (p = 0.011) and tended to be positively associated with placental efficiency (p = 0.08). At E15.5, betaine was positively associated with embryo weight and placenta weight (p = 0.04, 0.0013, respectively). At E18.5, betaine was positively associated with placenta weight (p = 0.0015, Figure 3B) and negatively associated with placental efficiency (p = 0.01).

At E12.5, choline was positively associated with crown rump length (p = 0.041) and placental efficiency (p = 0.002, Figure 3C) and tended to be positively associated with embryo weight (p = 0.065) while being negatively associated with placenta weight (p = 0.0025). At E15.5, choline was negatively associated with

embryo weight (p = 0.021) and placenta weight (p = 0.000013, Figure 3D). Choline tended to positively associate with crown rump length at E18.5 (p = 0.073).

At E12.5, methionine was negatively associated with crown rump length (p = 0.01) and placental efficiency (p = 0.018) and tended to be negatively associated with embryo weight (p = 0.09).

Table 3. Placental concentrations of choline metabolites as determinants of placental and fetal growth characteristics at E10.5, E12.5, E15.5, and E18.5. Data were analyzed using individual mixed linear models with metabolite, treatment, genotype, and sex as fixed effects and maternal ID as a random effect. # $p < 0.1$, * $p \leq 0.05$.

	E10.5		**E12.5**		**E15.5**		**E18.5**	
	β	**P value**	**β**	**P value**	**β**	**P value**	**β**	**P value**
Embryo weight								
Choline	0.0051	*0.37*	0.040	*0.065#*	-0.171	*0.021**	-0.025	*0.71*
Betaine	0.0019	*0.0082**	-0.0011	*0.61*	0.018	*0.040**	0.0095	*0.42*
Methionine	-0.0032	*0.75*	-0.080	*0.090#*	-0.45	*0.11*	0.018	*0.95*
Placenta weight								
Choline	-0.0055	*0.64*	-0.031	*0.0025**	-0.068	*0.000013**	-0.00058	*0.94*
Betaine	-0.00053	*0.721*	-0.00092	*0.37*	0.0059	*0.0013**	0.0040	*0.0015**
Methionine	-0.018	*0.36*	0.022	*0.35*	-0.072	*0.25*	-0.052	*0.10*
Crown rump length								
Choline	0.731	*0.38*	2.3	*0.041**	-0.65	*0.53*	2.1	*0.073#*
Betaine	0.262	*0.011**	0.063	*0.60*	0.17	*0.18*	-0.064	*0.76*
Methionine	-0.012	*0.99*	-6.3	*0.010**	-3.1	*0.43*	2.7	*0.60*
Placental efficiency								
Choline	0.19	*0.279*	1.3	*0.0020**	0.33	*0.63*	-0.018	*0.86*
Betaine	0.042	*0.080#*	-0.045	*0.30*	-0.017	*0.84*	-0.43	*0.010**
Methionine	-0.039	*0.90*	-0.23	*0.018**	-1.8	*0.49*	6.4	*0.13*

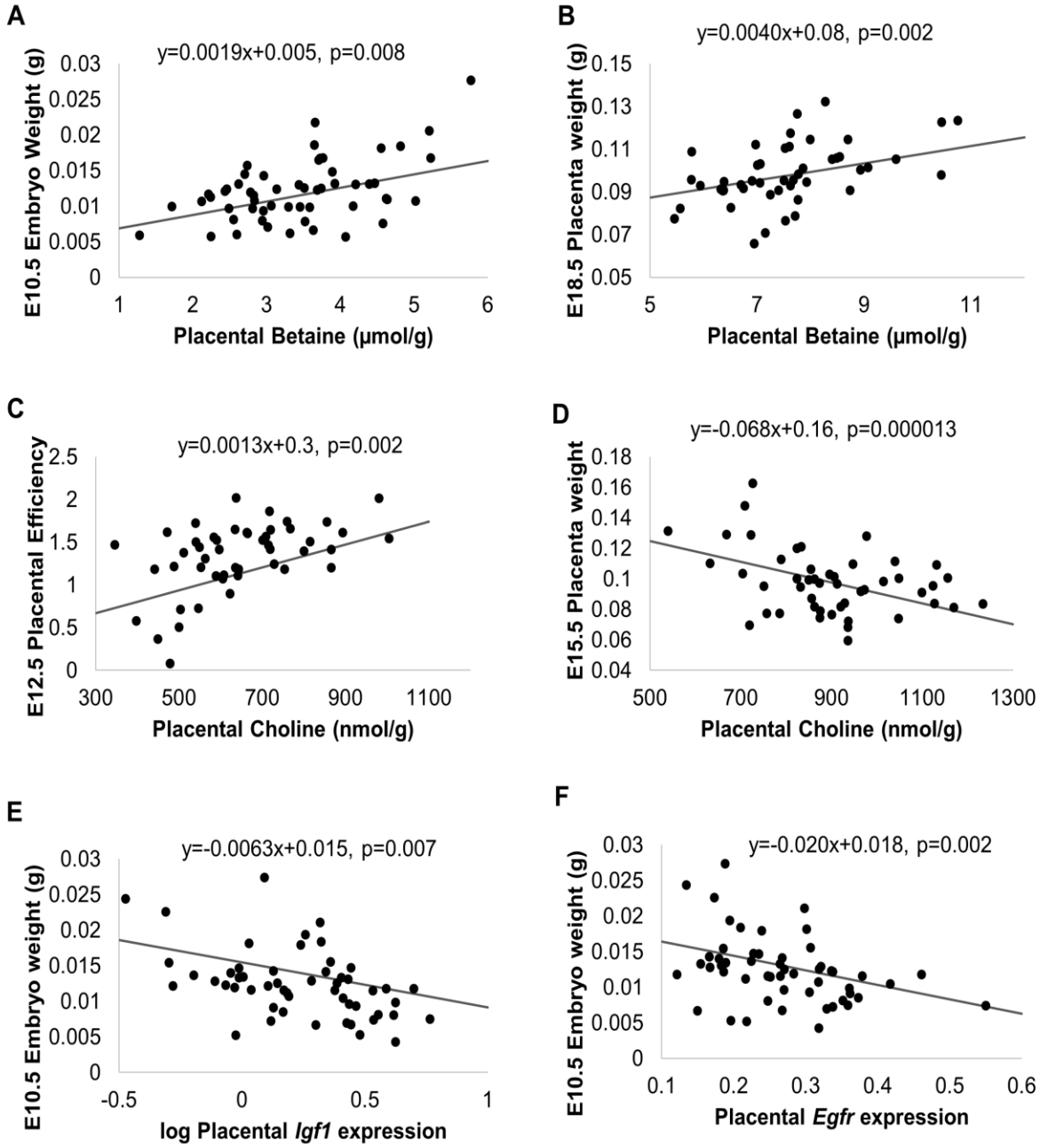


Figure 3. Relationships between placental choline metabolites, placental gene expression, and fetal and placental growth characteristics. Graphs present data points and regression lines obtained from linear mixed models adjusting for the mother's ID, fetal genotype and sex, and litter size, with the intercept set at average litter size. (A) Placental betaine concentrations and embryo weight at E10.5; (B) Placental betaine and placental weight at E18.5; (C) Placental choline and placental efficiency at E12.5; (D) Placental choline and placental weight at E15.5; (E) Placental *Igf1* expression and embryo weight at E10.5; (F) Placental *Egfr* expression and embryo weight at E10.5.

Expression of Growth Factor Genes

We performed regression analyses to determine whether the expression of these growth factor genes was associated with our fetal and placental growth data, adjusting for choline treatment group, fetal genotype and sex, litter size, and maternal identifier (Table 4, highly significant relationships shown in Figure 3). At E10.5, placental *Igf1* expression was negatively associated with embryo weight and crown rump length ($p = 0.007$ and 0.043 respectively, Figure 3E). Similarly, *Egfr* expression was negatively associated with embryo weight, crown rump length, and placental efficiency ($p \leq 0.033$ for all, Figure 3F). At E18.5, *Egfr* expression tended to be positively associated with embryo weight ($p = 0.077$), and *Igf2r* expression tended to be negatively associated with crown rump length ($p = 0.099$). Placental *Igf2* and *Igf1r* expression were not significantly associated with growth outcomes at either time point.

Table 4. Placental expression of growth-related genes as determinants of placental and fetal growth characteristics at E10.5 and E18.5. Data were analyzed using individual mixed linear models with gene fold change, treatment, genotype (for E10.5), and sex as fixed effects and maternal ID as a random effect. # $p < 0.1$, * $p \leq 0.05$.

	E10.5		E18.5	
	β	**P value**	**β**	**P value**
Embryo weight				
Igf1	-0.0063	*0.0072**	-0.045	*0.15*
Igf2	0.00012	*0.26*	-0.00015	*0.26*
Igf1r	0.0021	*0.55*	0.072	*0.12*
Igf2r	-0.00025	*0.84*	-0.12	*0.11*
Egfr	-0.020	*0.0019**	0.14	*0.077#*
Placenta weight				
Igf1	-0.0043	*0.33*	-0.00093	*0.82*
Igf2	0.070	*0.71*	0.000013	*0.43*
Igf1r	0.0084	*0.20*	-0.00033	*0.59*
Igf2r	-0.0014	*0.51*	-0.0014	*0.14*
Egfr	0.00065	*0.096*	-0.000080	*0.99*
Crown rump length				
Igf1	-0.64	*0.043**	-0.62	*0.20*
Igf2	-0.18	*0.21*	-0.0013	*0.54*
Igf1r	0.072	*0.88*	0.80	*0.28*
Igf2r	-0.17	*0.33*	-0.20	*0.099#*
Egfr	-1.8	*0.033**	2.0	*0.13*
Placental efficiency				
Igf1	-0.88	*0.24*	-0.29	*0.57*
Igf2	0.0028	*0.39*	-0.00019	*0.93*
Igf1r	-0.10	*0.36*	1.1	*0.17*
Igf2r	-0.020	*0.60*	0.018	*0.89*
Egfr	-0.57	*0.0041**	1.3	*0.36*

Discussion

In this study, we show for the first time that maternal choline supplementation modulates fetal growth in a model of placental insufficiency, the *Dlx3*+/− mouse.

Supplementing the maternal diet with extra choline accelerates fetal growth during the first half of pregnancy

Administering choline at 4X the recommended choline intake throughout pregnancy significantly increased embryo weight by mid-gestation (E10.5). This was accompanied by significant increases in placental weight, crown rump length, and placental efficiency in *Dlx3*+/− embryos and similar trends in *Dlx3*+/+ and −/− embryos. Notably, homozygous null fetuses at E10.5 in the 4X choline group achieved fetal weights and lengths similar to, or greater than, wildtype control diet embryos, suggesting a temporary rescue of the *Dlx3*−/− IUGR phenotype.

This acceleration in fetal growth during early pregnancy does not result in overgrowth

By E12.5 and E15.5, no differences in placental or fetal size by choline treatment group were detected, suggesting that compensatory mechanisms had been engaged to attenuate the early acceleration in fetal growth. By late gestation (E18.5), a small (~12%) but significant decrease in embryo weight and placental efficiency was observed among the 4X (versus 1X) choline group. It is unlikely that the slightly smaller weight of 4X embryos compared to controls in the current study would have negative health consequences as this decrease did not coincide with any changes in

body composition (fat or protein percentage). Additionally, the 4X embryo weights at E18.5 were similar to the control group embryo weights at E18.5 in a parallel study using a wildtype mouse with the same strain background under identical study conditions [9].

Supplementing the maternal diet with extra choline may uniquely benefit pregnancies characterized by placental insufficiency

The acceleration in growth during early pregnancy in our *Dlx3* mice was not observed in a parallel study conducted by our research group in mice without placental insufficiencies. Maternal choline supplementation did not significantly affect embryo weight at any gestational time point in wildtype mice [9]. This suggests that providing extra choline may have a unique effect in mothers with compromised pregnancies who are at risk of IUGR. Since a growth-promoting effect was seen in both wildtype and *Dlx3*+/− fetuses, it appears that the mother's genotype, rather than that of the fetus, is responsible. This may reflect the fact that circulating factors produced in dysfunctional placentas, such as sFlt-1, are capable of influencing their wildtype littermates.

The main metabolic fate of the supplemental choline in our study was betaine

Mothers in both choline groups had higher hepatic betaine levels at all four gestational time points. In control mothers, betaine was lower at E18.5 compared to all three earlier time points, suggesting a depletion of hepatic stores, which was prevented in the choline treatment groups. Placental betaine concentrations were also increased by 4X choline at E10.5 and E12.5. The positive association between placental betaine

and embryo weight at E10.5, even controlling for choline treatment group, supports the role of betaine in promoting early fetal growth in the *Dlx3*+/− mouse. Notably, the placenta does not express the enzyme BHMT (betaine-homocysteine S-methyltransferase) and therefore cannot use betaine as a methyl donor. However, BHMT is expressed in the early embryo [25] and in fetal livers [26]. Thus, a possible explanation for improved fetal growth in early gestation may be the enhanced transfer of betaine from the placenta to the fetus, allowing its use of the methyl groups. Betaine may also reduce osmotic stress [27], which could have contributed to the observed improvements in placental efficiency. Nonetheless, inverse associations between circulating concentrations of betaine and birth weight have been reported in human pregnancy. Higher maternal circulating concentrations of betaine and higher umbilical cord betaine and choline have shown possible associations with lower birth weights [28,29]. However, one study also found a positive association between cord blood dimethylglycine, produced when betaine acts as a methyl donor, and birth weight [29]. This suggests that reduced flux through choline metabolic pathways in smaller babies may have resulted in higher circulating levels.

Maternal choline supplementation also altered hepatic phosphocholine and GPC levels

Glycerophosphocholine (GPC) is an intermediate in the breakdown of phosphatidylcholine (PC); it has been shown to increase when demand for methyl groups is high, as in the case of folate deficiency [30]. At E12.5, mothers in both choline treatment groups had lower hepatic GPC levels, suggesting a reduced turnover

of PC due to higher choline availability. GPC levels have been shown to be higher in preeclamptic placentas and to positively correlate with the preeclampsia risk factor sFLT1 [31], indicating that high PC turnover to regenerate methyl groups may be a characteristic of this disorder. Phosphocholine is an intermediate in the CDP-choline (cytidine diphosphate-choline) pathway; its conversion to CDP-choline is the rate-limiting step in PC synthesis [5]. At E15.5, 4X mothers had higher hepatic phosphocholine concentrations, potentially due to a larger supply of choline being directed into the CDP-choline pathway.

Enhanced early growth led to placental downregulation of Igf1

The growth promoting role of IGF1 has been demonstrated in knockout mouse models [32] and in mouse models of IUGR, whereby *Igf1* overexpression corrected the placental insufficiency and normalized fetal weight [33]. Human studies of IUGR pregnancies show a mixed relationship with IGF1, with some studies reporting higher maternal levels of IGF1 in IUGR pregnancies, suggesting compensatory upregulation in response to placental insufficiency [14]. Surprisingly, we found lower expression of *Igf1* in 4X choline placentas at E10.5. In addition, *Igf1* expression was inversely associated with embryo weight. The expression of *Egfr*, although not significantly altered by choline treatment, was also negatively associated with embryo weight, which was unexpected given that a lack of *Egfr* induces an IUGR phenotype [34]. These findings suggest that the observed downregulations of *Igf1* and *Egfr* are compensatory mechanisms that occurred in larger embryos to protect against excessive growth. A bidirectional relationship between placental *Igf1* expression and

birth weight has been reported, whereby they are positively correlated in low and normal birth weights but inversely correlated in large babies [35]. In this way, *Igf1* may function to prevent extreme fetal weight in both directions. We also detected a slight but significant upregulation of *Igf2r* in 4X placentas at E10.5. As IGF2R regulates IGF2 availability by binding it and preventing signaling via IGF1R [36], this may represent an additional attempt by the placenta to restrain growth.

With compensatory mechanisms already occurring at E10.5, it is likely that the mechanisms resulting in increased early growth occur before this time. IGF2 controls cell proliferation from E9 to E10, resulting in restricted growth by ~E11 if IGF2 is disrupted [37]. Therefore, it is possible that choline increased the expression of *Igf2* before E10.5. Although we did not detect significant upregulation of *Igf2* at E10.5, higher betaine availability combined with the activation of *Igf2* by methylation [38] supports this gene as a plausible candidate for early growth promotion. Alternatively, since the fetus, but not the placenta, is able to use betaine's methyl groups, early modulation of the IGF axis may occur at the level of the fetus. Future studies examining gene expression in fetal tissues could assess this hypothesis.

Dlx3+/− placentas had higher expression of growth factor genes

Interestingly, *Dlx3*+/− placentas had higher expression of *Igf1* and *Egfr* at E10.5, a possible explanation for the similar weights of wildtype and *Dlx3*+/− embryos, in contrast to a previous study, which found reduced weights in heterozygotes compared to wildtypes [11]. Similar compensatory mechanisms via IGF1 have been reported in IUGR pregnancies [39,40]. Another possible explanation

for the different study findings is the use of a purified AIN-93 diet in the current study (in contrast to a standard chow diet), which is specially formulated to support growth during pregnancy and may have allowed *Dlx3*+/− embryos to achieve normal weights. The survival of a subset of *Dlx3*−/− embryos at E12.5 in the 1X control group was unexpected and may be another reflection of the quality of the diet. Previously, *Dlx3*−/− embryos were detected at E12.5 only after treatment with TEMPOL, a strong antioxidant [11].

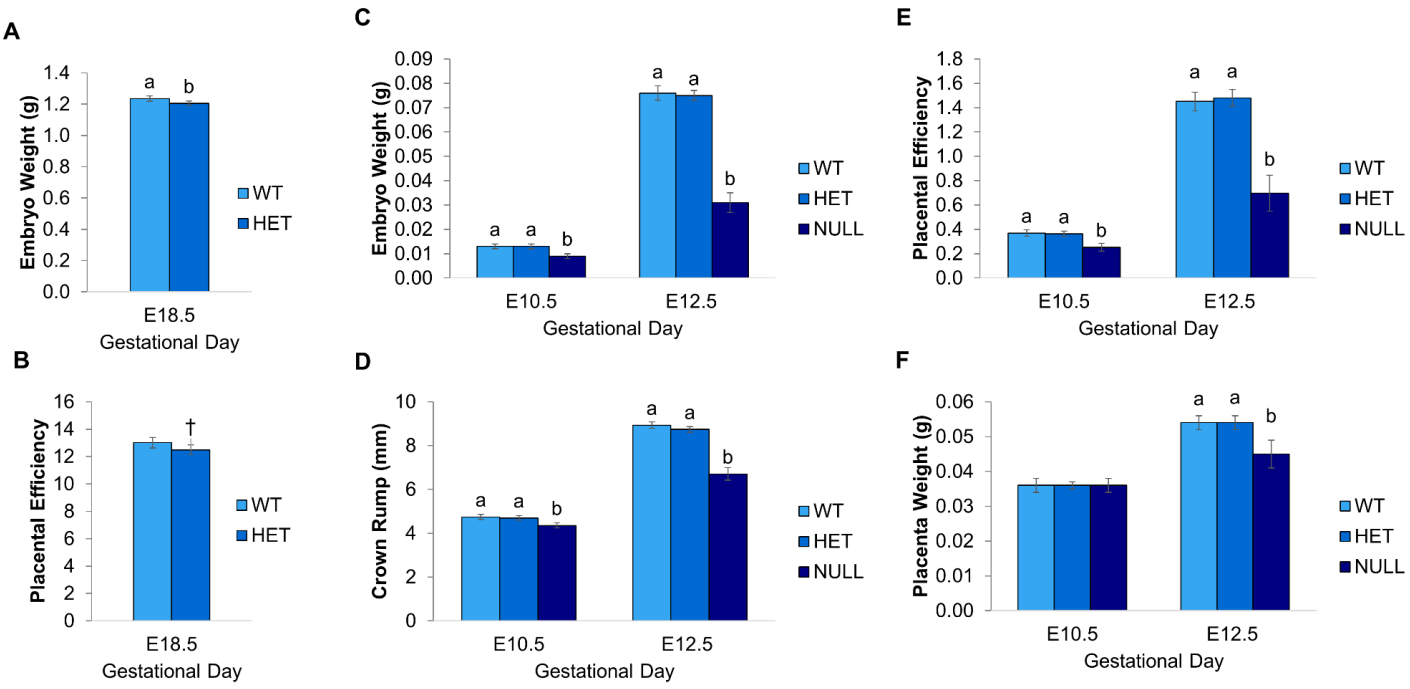
Strengths and Limitations

The strengths of our study include the use of four gestational time points, allowing a unique view of changes throughout pregnancy, and two supplemental doses, allowing the detection of dose-dependent effects. The use of an outbred mouse model displaying substantial variation between individuals limited our statistical ability to detect significant differences; however, it provided a wide range of responses to treatments, which is more similar to a human clinical trial and potentially leads to more generalizable findings. A disadvantage of using a mouse model to investigate pregnancy is that mouse fetuses are born at a comparatively underdeveloped stage compared to humans; thus, direct comparisons to pregnancy complications that occur late in gestation are difficult [41]. Our study was limited by the lack of a time point prior to E10.5, reducing our ability to determine mechanisms that increased early embryo growth. Additionally, our study was not powered to account for the effect of fetal sex; however, after determining that it was a source of variation in many outcomes, we included it in our statistical model. We were also limited by the high

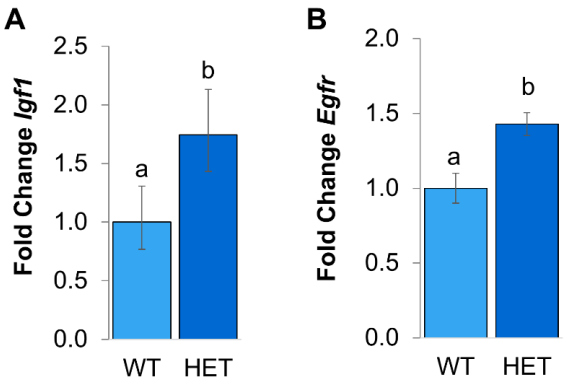
resorption rate and small litter sizes of *Dlx3*+/− mothers, which restricted our ability to perform further assays to assess protein expression and downstream signaling in the same cohort.

Conclusions

In conclusion, maternal choline supplementation increased fetal and placental growth during early-mid gestation in a mouse model of placental insufficiency, resulting in compensatory changes to slow growth. The marked increase in embryo weight at mid gestation with choline treatment provides support for the potential use of choline as an intervention for IUGR, small for gestational age, and placental insufficiency. Since many pregnancy complications such as IUGR and preeclampsia increase the risk of early delivery [42], increasing fetal growth by mid gestation may avoid negative health outcomes for the fetus. In order to understand the future potential of maternal choline supplementation as a treatment in humans, further research is warranted to assess the effects in early gestation and determine the mechanisms by which choline and betaine impact fetal growth. Overall, these data show that, in this mouse model of placental insufficiency, supplementing the maternal diet with extra choline enhances fetal and placental growth in early-mid gestation without leading to major differences in later gestation.



Supplementary Figure S1. Fetal and placental growth characteristics differ by *Dlx3* genotype. Embryo weight (A) and placental efficiency (B) at E18.5. Embryo weight (C), Crown rump length (D), placental efficiency (E), and placental weight (F) at E10.5 and E12.5. Placental efficiency defined as embryo weight/placental weight. Data were analyzed using mixed linear models controlling for choline treatment, maternal ID, fetal sex, and litter size. Values are presented as mean ± SEM. Differing letters denotes $P \leq 0.05$. † denotes $P<0.10$.



Supplementary Figure 2. Placental mRNA abundance of (A) *Igf1* and (B) *Egfr* at E10.5 by *Dlx3* genotype. Fold changes are expressed relative to the housekeeping gene *Tbp* with wildtypes normalized to 1. Data analyzed using mixed linear models controlling for choline treatment, maternal ID, fetal sex, and litter size. Log-transformed data (*Igf1*) is represented by back-transformed means and 95% confidence intervals. All other values are presented as mean ± SEM. Differing letters denotes $P \leq 0.05$.

Supplementary Table 1. Primers for genotyping and RT-qPCR.

Gene	*Name*	*Reference Sequence*	*Primer Sequences*	*Annealing Temperature*
Dlx3	Distal-less homeobox 3	NC_000077.6	F: 5’ GTGAACGGCAAGCCCAAA 3’ R (Wild type allele): 5’ CTCTGTGACACGCCATACACAGTT 3’ R (Knockout allele): 5’ AAAGGCCCGGAGATGAGGAAGAG 3’	Touchdown from 63° to 49°
Sry	Sex determining region Y	NC_000087.7	F: 5’ TGGGACTGGTGACAATTGTC 3’ R: 5’ GAGTACAGGTGTGCAGCTCT 3’	60°C
Igf1	Insulin-like growth factor 1	NM_010512.5	F: 5' GACCGAGGGGCTTTTACTTC 3' R: 5' CATCCACAATGCCTGTCTGA 3'	63°C
Igf2	Insulin-like growth factor 2	NM_010514.3	F: 5' CGCTTCAGTTTGTCTGTTCG 3' R: 5' GCAGCACTCTTCCACGATG 3'	63°C
Igf1r	Insulin-like growth factor receptor 1	NM_010513.2	F: 5' GCTTCGTTATCCACGACGATG 3' R: 5' GAATGGCGGATCTTCACGTAG 3'	63°C
Igf2r	Insulin-like growth factor receptor 2	NM_010515.2	F: 5' TCTGTGTTGGCTCGTCACTC 3' R: 5' CCGGTGACAGACGTTGATGA 3’	63°C
Egfr	Epidermal growth factor receptor	NM_207655.2	F: 5' GGACTGTGTCTCCTGCCAGAAT 3' R: 5' GGCAGACATTCTGGATGGCACT 3’	63°C
Tbp	TATA-binding protein	NM_013684.3	F: 5’ AGGAGCCAAGAGTGAAGAACAA 3’ R: 5’ AACTTCACATCACAGCTCCCC 3’	60°C

Table S2. Litter size, resorptions, total implantations and fetal body composition in litters born to *Dlx3+/-* dams in response to three different maternal choline treatments (1X control, 2X and 4X) at E10.5, E12.5, E15.5 and E18.5. For litter size, implantations and % resorptions, data were analyzed using ANOVA. For body composition, data was analyzed using mixed linear models controlling for fetal genotype, maternal ID, fetal sex, and litter size and represented as % of total body weight. Values are presented as mean ± SEM.

Time point	Diet	Implantations	% Resorptions	Litter size	Fetal water content (%)	Fetal lipid content (%)	Fetal protein content (%)
E10.5	1X	12.3 ± 0.8	43.6 ± 5.7	7.2 ± 1.1			
	2X	12.4 ± 1.0	34.6 ± 6.9	8.3 ± 1.3	-	-	-
	4X	13.2 ± 0.9	47.6 ± 6.0	7.0 ± 1.1			
P (Treatment)		*0.722*	*0.731*	*0.371*			
E12.5	1X	10.6 ± 1.0	55.6 ± 6.3	5.1 ± 1.0			
	2X	10.2 ± 1.1	50.2 ± 6.9	5.3 ± 1.1	-	-	-
	4X	12.7 ± 1.0	50.6 ± 6.3	6.3 ± 1.0			
P (Treatment)		*0.631*	*0.198*	*0.804*			
E15.5	1X	11.9 ± 1.2	58.0 ± 5.1	4.9 ± 0.6			
	2X	10.6 ± 1.2	60.4 ± 5.1	3.8 ± 0.6	-	-	-
	4X	12.4 ± 1.2	51.9 ± 5.1	5.8 ± 0.6			
P (Treatment)		*0.100*	*0.549*	*0.491*			
E18.5	1X	12.8 ± 0.9	52.9 ± 7.8	5.9 ± 0.9	83.8 ± 0.35	0.91 ± 0.8	10.5 ± 1.2
	2X	11.0 ± 0.9	53.4 ± 7.4	4.8 ± 0.8	83.8 ± 0.33	1.87 ± 0.7	10.7 ± 1.1
	4X	12.1 ± 0.9	52.0 ± 7.8	5.6 ± 0.9	84.1 ± 0.34	1.27 ± 0.8	11.7 ± 1.2
P (Treatment)		*0.644*	*0.359*	*0.991*	*0.552*	*0.377*	*0.437*

Table S3. Genotype distributions in offspring born to *Dlx3+/-* dams in response to three different maternal choline treatments (control, 2X and 4X) at E10.5, E12.5, E15.5 and E18.5. 2-tailed P values; Fisher's exact test.

			Embryo Genotype (%)			
Time point	**Diet**		**WT**	**HET**	**NULL**	**P value (vs. Ctrl)**
E10.5	1X		14 (21.5%)	35 (53.8%)	16 (24.6%)	
	2X		13 (25.0%)	33 (63.5%)	6 (11.5%)	0.20
	4X		10 (17.2%)	33 (56.9%)	15 (25.9%)	0.86
		Expected	*25%*	*50%*	*25%*	
E12.5	1X		23 (38.3%)	34 (56.7%)	3 (5.0%)	
	2X		21 (40.4%)	31 (59.5%)	0 (0.0%)	0.35
	4X		18 (24.3%)	49 (66.2%)	7 (9.5%)	0.17
		Expected	*33%*	*67%*	*0%*	
E15.5	1X		22 (40.0%)	33 (60.0%)	-	
	2X		15 (39.5%)	23 (60.5%)	-	1.0
	4X		24 (39.3%)	37 (60.7%)	-	1.0
		Expected	*33%*	*67%*	*0%*	
E18.5	1X		18 (34.0%)	35 (66.0%)	-	
	2X		22 (37.9%)	36 (62.1%)	-	0.70
	4X		15 (30.0%)	35 (70.0%)	-	0.68
		Expected	*33%*	*67%*	*0%*	

Table S4.

Embryo weight, placental weight, crown rump length, and placental efficiency by *Dlx3* genotype at E12.5 and E15.5. Data were analyzed using mixed linear models controlling for maternal ID, fetal sex, and litter size. Values are presented as mean ± SEM. #$P < 0.1$ vs. 1X controls. *$P < 0.05$ vs. 2X. n=7-10 dams per treatment, per time point. Values are presented as mean ± SEM.

Time point	Genotype	Diet	Embryo Weight	Placenta Weight	Crown Rump Length	Placental Efficiency
E12.5	**WT**	**1X**	0.080 ± 0.004	0.058 ± 0.003	9.00 ± 0.2	1.48 ± 0.1
		2X	0.076 ± 0.004	0.054 ± 0.003	8.69 ± 0.3	1.39 ± 0.1
		4X	0.071 ± 0.004	0.050 ± 0.003#	9.13 ± 0.2	1.49 ± 0.1
	HET	**1X**	0.076 ± 0.004	0.057 ± 0.003	8.89 ± 0.2	1.49 ± 0.1
		2X	0.083 ± 0.004	0.053 ± 0.003	8.76 ± 0.2	1.67 ± 0.2
		4X	0.068 ± 0.004*	0.052 ± 0.003	8.65 ± 0.2	1.35 ± 0.1
	NULL	**1X**	0.020 ± 0.007	0.037 ± 0.007	5.82 ± 1.0	0.68 ± 0.2
		2X	-	-	-	-
		4X	0.023 ± 0.004	0.047 ± 0.004	6.64 ± 0.4	0.50 ± 0.1
E15.5	**WT**	**1X**	*0.39* ± 0.02	0.098 ± 0.005	14.87 ± 0.4	4.14 ± 0.4
		2X	0.40 ± 0.03	0.084 ± 0.006	15.18 ± 0.3	4.84 ± 0.4
		4X	0.39 ± 0.03	0.095 ± 0.005	14.88 ± 0.3	4.23 ± 0.4
	HET	**1X**	*0.38* ± 0.02	0.094 ± 0.005	14.65 ± 0.2	4.11 ± 0.2
		2X	0.42 ± 0.03	0.087 ± 0.006	14.58 ± 0.3	4.80 ± 0.3#
		4X	0.39 ± 0.02	0.091 ± 0.005	14.91 ± 0.2	4.44 ± 0.2

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CHAPTER 2

MATERNAL CHOLINE SUPPLEMENTATION MODULATES PLACENTAL MARKERS OF INFLAMMATION, ANGIOGENESIS, AND APOPTOSIS IN A MOUSE MODEL OF PLACENTAL INSUFFICIENCY*

*King JH, Kwan ST(C), Yan J, Fomin VG, Levine SP, Wei E, Jiang X, Roberson MS, and Caudill MA. Maternal choline supplementation modulates placental markers of inflammation, angiogenesis, and apoptosis in a mouse model of placental insufficiency, *To be submitted.*

Abstract

Introduction: A properly functioning placenta allows efficient transfer of nutrients from mother to fetus and promotes fetal growth. Decreased placental efficiency is commonly associated with intrauterine growth restriction, preeclampsia, and spontaneous abortion. Choline is an essential nutrient that is a precursor for several molecules with crucial roles in fetal development: betaine, an osmolyte and methyl donor; acetylcholine, a neurotransmitter and signaling molecule; and phosphatidylcholine, the most prevalent membrane phospholipid. We previously showed that maternal choline supplementation in the *Dlx3*+/− mouse, a model of placental insufficiency, increases fetal and placental growth in mid-gestation.

Methods: We measured markers of angiogenesis, inflammation, apoptosis, and placental structure and development using real-time quantitative PCR, liquid chromatography-tandem mass spectrometry, and immunohistochemistry in the placentas of *Dlx3*+/− female mice consuming 1X (control), 2X or 4X the recommended intake levels of choline during gestation. Dams were sacrificed at embryonic days E10.5, E12.5, E15.5, and E18.5 and placentas were harvested and genotyped for sex and *Dlx3* genotype.

Results: 4X choline increased ($P<0.05$) placental labyrinth size at E10.5 and decreased ($P<0.05$) placental apoptosis in a fetal sex-dependent manner at E10.5 and E12.5. Choline supplementation, particularly at the 2X dose, decreased ($P<0.05$) expression of pro-angiogenic genes *Eng* (E10.5, E12.5, and E15.5) and *Vegf* (E12.5,

E15.5) and pro-inflammatory genes *Il1b* (at E15.5 and 18.5), *Tnfα* (at E12.5) and *Nfκb* (at E15.5).

Discussion: Maternal choline supplementation beneficially modulates markers of placental function in a mouse model of placental insufficiency, providing evidence that choline may be a useful therapy during pregnancy to mitigate development of placental disorders.

Introduction

Abnormal placental development underlies many pathologies of pregnancy including preeclampsia, intrauterine growth restriction (IUGR), and spontaneous abortion [1]. These conditions have serious consequences for the mother and fetus and few treatments are currently available for their prevention or treatment.

Although the etiology of placental insufficiency is not fully understood, an imbalance of pro- and anti-angiogenic and inflammatory factors may contribute [1,2]. The development of the placenta requires extensive angiogenesis and vasculogenesis, and the maternal uterine spiral arteries must be invaded and remodeled by cytotrophoblasts to allow for increased perfusion to the fetus [3]. This process requires adequate expression of angiogenic and remodeling genes including the vascular endothelial growth factor (VEGF) family. Inadequate trophoblast invasion is a characteristic of many placental pathologies. When this process is incomplete, blood flow to the placenta is compromised, and oxygen supply may be sporadic, leading to placental hypoxic injury. Oxidative stress, excessive apoptosis and inflammation result, compromising trophoblast function and preventing efficient transfer of nutrients [4]. Anti-angiogenic factors in the maternal circulation, including soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), may contribute to the pathogenesis of preeclampsia symptoms including hypertension and proteinuria, and can be used as biomarkers or predictors of risk when measured in early gestation [5,6]. Together these dysregulated processes interact to contribute to the pathogenesis of preeclampsia and IUGR.

The essential nutrient choline is required for synthesis of the neurotransmitter acetylcholine, the membrane phospholipid phosphatidylcholine, and the methyl donor and osmolyte betaine [7]. These molecules have crucial roles supporting pregnancy through cell growth, DNA methylation, and cellular signaling [8]. Choline has previously been shown to reduce levels of sFlt-1 in a randomized, controlled feeding trial of maternal choline supplementation during the third trimester of pregnancy in healthy women [9]. In addition, choline has been shown to modulate inflammation and angiogenesis of placental trophoblast cells in culture [10] and wild type mouse placenta [11]. Therefore, we hypothesized that choline supplementation would be beneficial for pregnancies complicated by placental insufficiency.

Dlx3 (distal-less homeobox 3) insufficiency in mice has been shown to result in placental insufficiency [12]. This homeodomain-containing transcription factor is required for the development of the maternal-fetal interface [13]. Placentas lacking one copy of this gene display inadequate vascularization and abnormal development of the placental labyrinth, which is the area of nutrient exchange between the mother and fetus [12]. Heterozygous embryos are viable but their placentas display abnormalities including reduced invasion of trophoblasts and impaired remodeling of maternal spiral arteries as well as increased placental oxidative stress and apoptosis [12]. In mice that are homozygous null for *Dlx3*, placental failure occurs between embryonic days 9.5-12.5 resulting in extremely restricted fetal growth and subsequently death [13]. Notably, we have previously shown that maternal choline supplementation in *Dlx3*+/- mice increases fetal and placental weights during mid-gestation in heterozygous embryos [14].

In the present study, we sought to use the *Dlx3*+/- mouse model of placental insufficiency to investigate the effects of maternal choline supplementation on markers of placental function during placental insufficiency and explore how these effects progressed throughout pregnancy.

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. *Dlx3*+/- mice were genotyped using tail DNA with three-primer duplex PCR (Supplemental Table 1). Primers were designed to amplify a wild type band, a knockout band, or both (indicating a heterozygote). Mice were housed in microisolator cages (Ancare) in an environmentally-controlled room (22-25°C and 70% humidity) with a 12-hour light-dark cycle. During breeding, mice were given *ad libitum* access to commercial rodent chow (Teklad) and water. *Dlx3*+/- females were mated with *Dlx3*+/- males and their offspring were genotyped at time of weaning (3 weeks of age). Heterozygous pups were given *ad libitum* access to the 1X choline control diet, AIN-93G purified rodent diet, (Dyets #103345) containing 1.4g choline chloride/kg diet. *Dlx3*+/- female mice were randomized five days before mating with *Dlx3*+/- male mice to receive either the 1X choline control diet, the 2X choline diet containing 2.8g choline chloride/kg (Dyets #103346), or the 4X choline diet containing 5.6g choline chloride/kg (Dyets #103347). Embryonic (E) day 0.5 was designated by presence of a vaginal plug. Pregnant mice were euthanized at four different gestational time points; E10.5, E12.5, E15.5 and E18.5.

Tissue Collection and Processing

After pregnant dams were sacrificed, embryos and placentas were carefully dissected with minimal decidual contamination and weighed. For approximately 1/3 of the litter, the fetus was extracted from the implantation site and the remainder was fixed in 10% formalin for histological analyses. For the remaining placentas, at gestational time points E12.5, E15.5 and E18.5, placentas were bisected across the chorionic plate; one half was stabilized in RNA*later* for mRNA analysis while the remaining half was immediately frozen in liquid nitrogen and stored at -80°C for metabolite analysis. Due to smaller tissue size, E10.5 placentas were designated for mRNA analysis or metabolite analysis, alternately. Fetal DNA was extracted for *Dlx3* genotyping and sex determination using a commercial kit (Qiagen). Sex genotyping was performed by PCR for the *Sry* gene with a commercial kit (Qiagen). Primers are listed in Supplementary Table 1.

Quantitative real-time RT-PCR

RNA was extracted from placentas maintained in RNA*later* using TRIzol reagent (Invitrogen). 2-3 placentas per dam were randomly selected for extraction. RNA concentration and quality were assessed with a NanoDrop ND-1000 instrument (Thermo), and samples with a A260/A280 ratio above 1.8 were used for quantification. Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega). Quantitative PCR was performed using SYBR® Green in a Roche LightCycler480. All primers for the targeted genes (*Vegfa, Pgf, Eng, MMP14, Tnfα, NfκB, Il1b*) were designed using NCBI Primer-BLAST (Supplemental

Table 1). Reaction conditions were as follows: 95°C for 5 minutes, followed by 40 cycles with 15 seconds at 95°C, 30 seconds annealing (see Supplementary Table 1 for annealing temperatures), and 30 seconds at 72°C. PCR product specificity was monitored using melting curve analysis at the end of the amplification cycles. Fold changes were calculated by the ΔC_t method [15] normalized to the expression level of housekeeping gene *Tbp* (TATA box binding protein), which has previously been shown to be stable in placental tissue [16] and in response to varying choline supply [17]. At E10.5, due to limited tissue availability, both wildtype and heterozygous placentas were used and genotype was included in the statistical model.

LC-MS/MS

Concentrations of acetylcholine were measured in the placenta by LC-MS/MS according to the method of Holm et al [18] with modifications based on our equipment [19].

Placental Morphometry

Placental tissues fixed in 10% formalin were paraffin embedded and sectioned at 10μm. Immunohistochemistry was performed on formalin-fixed sections as described previously [20]. For analysis of maternal spiral artery areas, placental sections were incubated with smooth muscle actin (SMA) antibody (1:50, DakoCytomatin, Glostrup, Denmark), followed by secondary antibody. Slides were imaged using an Aperio Scanscope (Vista, CA). Maternal spiral arteries were manually defined based on staining location and the presence of non-nucleated red

blood cells and Aperio ImageScope software was used to quantify area. Data are presented as the ratio of artery luminal area to total arterial area. For analysis of placental labyrinth area, placental sections were incubated with biotinylated GSL 1-isolectin B4 (1:100, Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole (AEC; Invitrogen, Carlsbad, CA) and counterstained with hematoxylin. Isolectin is a marker of endothelial cells and has been used previously to stain vasculature in other mouse tissues. The placental labyrinth compartment was defined manually based on staining location and area was calculated using Aperio ImageScope software. Data are expressed as mm^2 of cross-sectional labyrinth area.

Placental Apoptosis

Placental apoptosis was assessed using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. A commercial kit (Millipore, Billerica, MA) was used according to the manufacturer's instructions. Sections were imaged using an Aperio ScanScope and number of TUNEL-positive cells was determined in the decidua and labyrinth by the average number of TUNEL-positive cells in several randomly selected fields. Field sizes were as follows: for E10.5, five fields of 250x250 μm^2, for E12.5, five fields of 350x350 μm^2, for E15.5 and E18.5, ten fields of 500x500 μm^2.

Statistical Analysis

For all placental measurements, data were analyzed separately for each gestational day using a linear mixed model. The model included choline treatment,

fetal genotype, fetal sex, and litter size as fixed effects and maternal identifier as a random effect. Fetal sex and fetal genotype were removed from the model when data was stratified by sex or genotype. Data is presented as combined sexes (as noted in the text) when the same effect was seen in male and female placentas. Data were assessed for normality by assessing the distribution of residuals. Corrections for multiple analyses were not performed due to the hypothesis-driven nature of the study and the relatively limited sample size. Data are presented as means ± SEM. $P \leq 0.05$ was considered statistically significant and $0.05 < P < 0.10$ was considered to indicate trends. All analyses were performed using SPSS software, Version 23 (IBM).

Results

Placental morphometry

As we previously reported [14], maternal choline supplementation increased placental and fetal weights at E10.5 in offspring of *Dlx3+/-* mice. Because the *Dlx3* model is associated with labyrinth abnormalities, we sought to determine whether choline treatment increases placental weight by increasing the size of the labyrinth. At E10.5, 4X choline heterozygous placentas had 73% larger labyrinth area vs 2X choline (P=0.014), and tended to be ~46% larger than 1X control placentas (P=0.092). At E12.5 and 15.5, there were no significant differences in labyrinth size by choline treatment. At E18.5, 2X placentas were ~25% smaller compared to 1X controls (P=0.037) and tended to be ~20% smaller than 4X choline placentas (P=0.081, Fig. 1A).

Dlx3-/- placentas had ~40% smaller labyrinths at E10.5 versus heterozygous placentas (P=0.04). At E12.5, homozygous null placentas were ~59% smaller vs heterozygotes (P=0.012) and ~55% smaller vs wildtypes (P=0.019). Wildtype and heterozygous placentas did not differ in labyrinth size at any gestational time point; however, at E18.5 heterozygous placenta labyrinths tended to be ~14% larger than wildtypes (P=0.052, Fig. 1B).

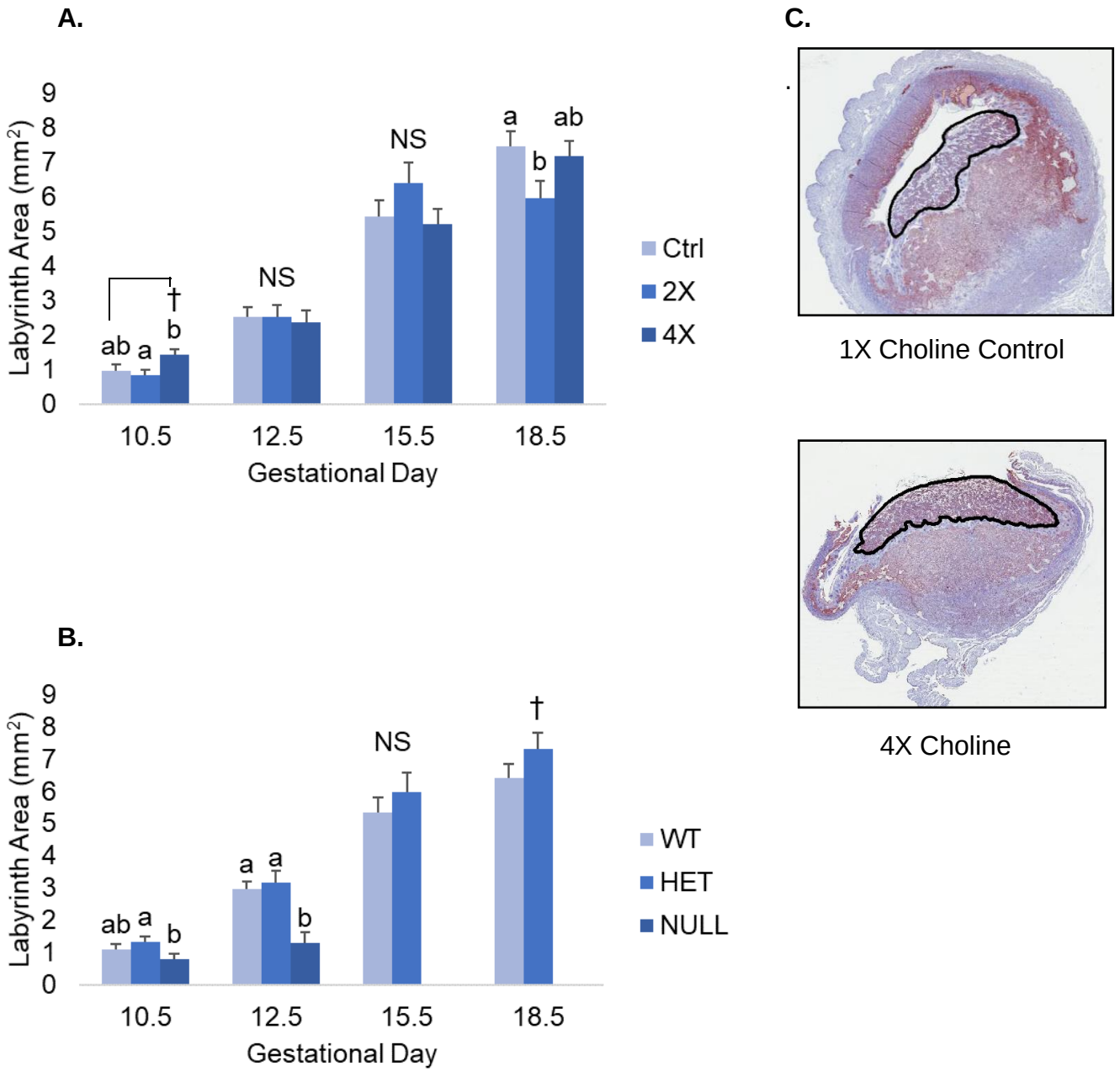

Figure 1. Placental labyrinth area at gestational days 10.5, 12.5, 15.5, and 18.5, by choline treatment (A) and fetal *Dlx3* genotype (B). Representative images of placental labyrinths at E10.5 are shown in (C). Data were analyzed using mixed linear models controlling for maternal ID, fetal sex, fetal genotype (for (A)) and litter size. Values are presented as mean ± SEM. Differing letters denote $P \leq 0.05$. † denotes P<0.1.

Placental apoptosis

We performed the terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL) to assess placental levels of apoptosis at all four gestational time points. At E10.5, choline treatment tended to result in ~96% lower TUNEL scores (average # TUNEL-positive cells per field) in the 4X choline placentas (P=0.053, versus 1X controls) (Fig. 2A). When split by fetal sex, in both female choline treatment groups TUNEL scores were lower vs 1X controls (~56% for 2X, P=0.043 and ~65% for 4X, P=0.027) (Fig 2B).

At E12.5, 4X choline placentas had ~58% lower TUNEL scores (P=0.048 versus 1X controls) (Fig. 2A). When split by fetal sex, the effect was significant in males (P=0.031, 4X versus 1X) but not females, although a similar non-significant trend was seen (Fig. 2C).

At E15.5 and E18.5, no differences in TUNEL score were detected between choline treatment groups (Fig. 2A).

Placental artery remodeling

Because we had previously found an effect of maternal choline supplementation on spiral artery remodeling in wildtype mice [11], we investigated whether it would have a similar function in a model of placental insufficiency. We assessed the luminal ratio of maternal spiral arteries, defined as (luminal area)/total arterial area. Luminal ratio did not differ by choline treatment or *Dlx3* genotype at any gestational time point (data not shown).

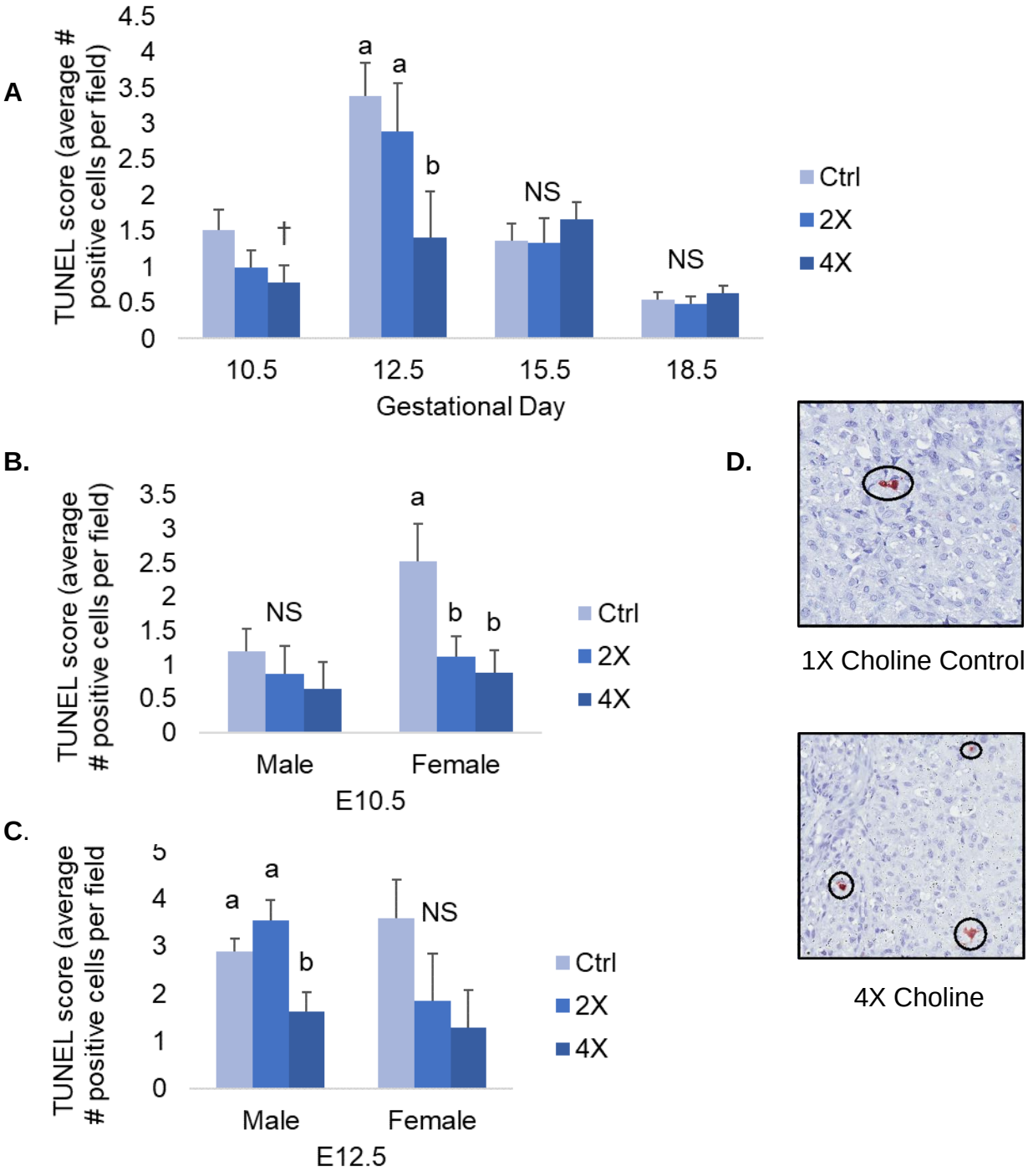

Figure 2. TUNEL score (calculated as the average number of TUNEL-positive cells per field) at E10.5, 12.5, 15.5, and 18.5 by maternal choline treatment (A) and stratified by fetal sex at E10.5 (B) and E12.5 (C). Representative images of TUNEL staining at E12.5 are shown in (D). Data were analyzed using mixed linear models controlling for maternal ID, fetal genotype and sex (for (A)) and litter size. Values are presented as mean ± SEM. Differing letters denote $P \leq 0.05$. † denotes P<0.1.

Placental expression of angiogenic genes

Because altered levels of pro-angiogenic factors have been implicated in the development of preeclampsia and other pregnancy disorders, we measured mRNA expression of several key modulators of placental angiogenesis throughout pregnancy (Fig. 3 A-D). We report the results according to fetal sex, or in all placentas combined when the effect was found in both sexes.

At E10.5, expression of *Eng* was ~31% lower in the female 2X choline pups versus 1X controls (P=0.029) (Fig. 3A). There were no significant differences in *Vegfa*, *Pgf*, or *Mmp14* expression at E10.5 (data not shown).

At E12.5, 2X and 4X choline males had lower expression of *Eng* vs 1X controls (~64% and ~43%, respectively, P=0.005, 0.013). Similarly, male 2X choline placentas had ~44% lower expression of *Vegfa* (P=0.026 versus 1X) (Fig 3B). There were no choline treatment differences in *Pgf* or *MMP14* expression at E12.5.

At E15.5, expression of *Eng* in female placentas was ~38% lower in the 2X choline group (P=0.002 vs 1X). Similarly, 2X choline female placentas had ~17% lower expression of *Vegfa* vs 1X controls (P=0.031) (Fig. 3C). Expression of *Mmp14* was ~42% lower in female 2X choline placentas (P=0.019, vs 1X) and ~41% lower in 4X choline placentas (P=0.015, vs 1X).

At E18.5, male placentas in the 2X choline group had ~35% higher expression of *Vegfa* versus 1X controls and 4X choline groups (P=0.011 and 0.01, respectively). Similarly, expression of *Pgf* tended to be ~29% higher in 2X choline males vs 1X control males (P=0.091) and was ~42% higher versus 4X choline (P=0.091). Male 2X

choline placentas also had ~62% higher expression of *Mmp14* vs 1X placentas (P=0.006) (Fig 3D). Expression of *Eng* did not differ by choline treatment at E18.5.

Placental expression of inflammatory genes

Abnormal regulation of inflammation has been shown in various placental pathologies; therefore, we also measured mRNA expression of several major inflammatory regulators in the placenta (Fig. 4 A-C).

At E10.5, there were no significant differences in *Tnfα, Nfκb*, or *Il1b* expression between choline treatment groups (data not shown).

At E12.5, 2X choline male placentas had lower expression of *Tnfα* (~57% versus 1X) and tended to have lower expression of *Nfκb* (~50%, P=0.096 versus 1X) (Fig. 4A). Expression of *Il1b* did not differ by treatment (data not shown).

At E15.5, female 2X choline placentas had ~33% lower expression of *Nfκb* vs 1X controls (P=0.020). Expression of *Il1b* was ~36% lower in 2X choline placentas of both sexes vs 1X controls (P=0.034) and also in males alone (P=0.031) (Fig 4B).

At E18.5, expression of *Il1b* was ~32% lower in 4X choline pups of male and female placentas combined (P=0.02). Female 4X choline placentas tended to have ~39% lower expression of *Nfκb* vs 1X control females (P=0.058) (Fig. 4C). No differences in *Tnfα* expression were seen between choline groups at E15.5 or E18.5 (data not shown).

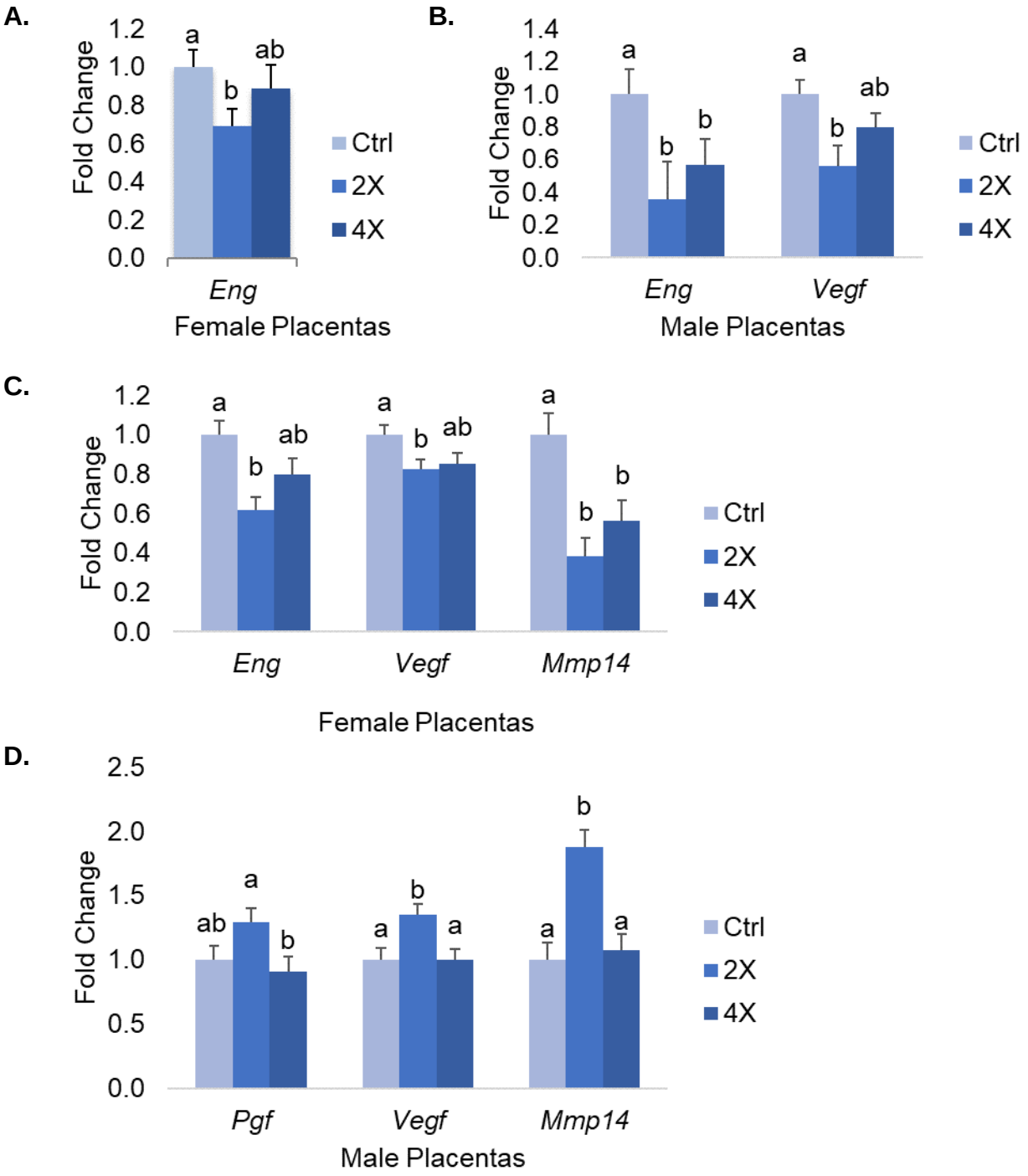

Figure 3. Placental mRNA abundance of angiogenic genes at (A) E10.5, (B) E12.5, (C) E15.5, and (D) E18.5 by maternal choline treatment. Data are expressed as fold-change relative to the housekeeping gene *Tbp*. Data analyzed using mixed linear models controlling for fetal genotype (for E10.5), maternal ID, and litter size. Values are presented as mean ± SEM. Differing letters denotes $P \leq 0.05$. n=20 placentas per treatment, per time point.

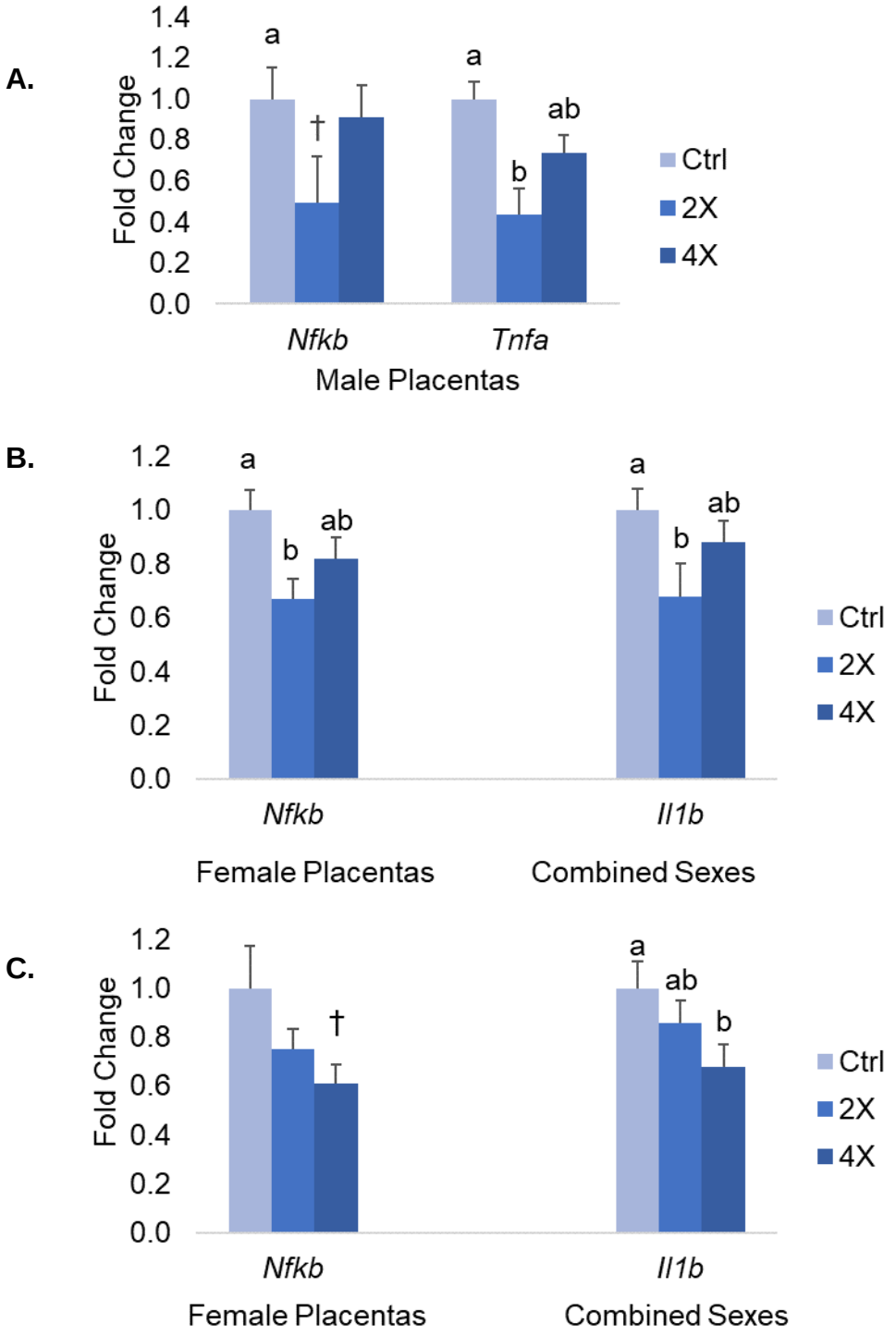

Figure 4. Placental mRNA abundance of inflammatory genes at (A) E12.5, (B) E15.5, and (C) E18.5 by maternal choline treatment. Data are expressed as fold-change relative to the housekeeping gene *Tbp*. Data analyzed using mixed linear models controlling for fetal genotype (for E10.5), maternal ID, and litter size. Values are presented as mean ± SEM. Differing letters denotes $P \leq 0.05$. † denotes P<0.10. n=20 placentas per treatment, per time point.

Placental acetylcholine

Because acetylcholine has been linked to angiogenic and inflammatory signaling in the placenta [21,22], we measured concentration of placental acetylcholine throughout gestation. At E12.5, 2X choline placentas had ~136% higher concentrations of acetylcholine vs 1X controls (P=0.011) and ~107% higher concentrations vs 4X choline placentas (P=0.020). At E10.5, E15.5, and E18.5, acetylcholine concentrations did not differ by choline treatment (Fig. 6).

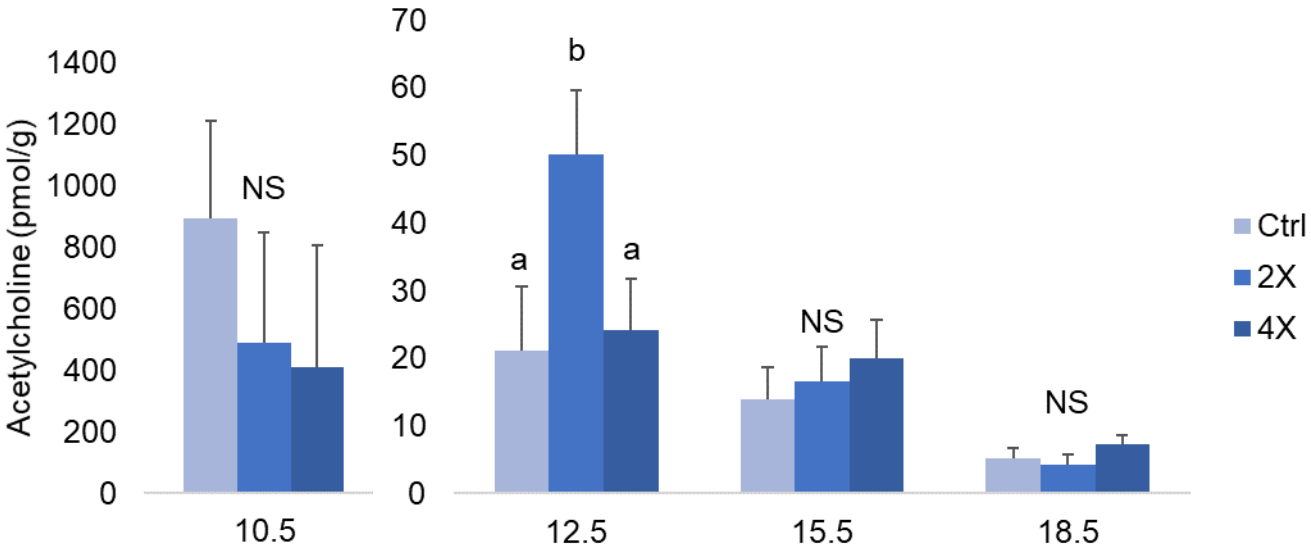

Figure 6. Placental acetylcholine concentrations (pmol/gram tissue) at E10.5, E12.5, 15.5, and 18.5 by maternal choline treatment (1X, 2X and 4X choline). Data analyzed using mixed linear models controlling for fetal genotype, fetal sex, maternal ID, and litter size. Values are presented as mean ± SEM. Differing letters denotes $P \leq 0.05$.

Discussion

In this study, we show for the first time that maternal choline supplementation can modulate angiogenesis, inflammation, and placental development in a mouse model of placental insufficiency, the *Dlx3*+/- mouse.

Maternal choline supplementation in the Dlx3+/- mouse increases placental labyrinth size and decreases placental apoptosis in mid-gestation

Because we had previously reported a higher placental weight at E10.5 with maternal choline supplementation in *Dlx3*+/- dams [14], we sought to determine whether this effect could be explained by alterations in the size of the placental labyrinth or apoptosis levels. Development of the labyrinth region, which facilitates nutrient transfer between mother and fetus, is defective in *Dlx3*-/- animals [13]. This was confirmed in the current study, where we found *Dlx3*-/- placental labyrinths to be significantly smaller at E10.5 and E12.5. At E10.5, a high dose of choline (4X the recommended intake) resulted in a larger labyrinth size, compared to a 2X choline dose. This increase in surface area for nutrient transfer could contribute to the larger embryo weights we previously reported in offspring of *Dlx3*+/- mothers that received 4X choline supplementation [14].

An additional explanation for the larger placental weights could be the decreased levels of apoptosis we found in both choline groups (particularly the 4X group), at E10.5 and E12.5. This effect was most pronounced in female placentas but a similar non-significant effect in the same direction was observed in males. These findings concur with previous results showing reductions in apoptosis with choline

supplementation in trophoblast cells and wildtype mice [10,11]. The elevated levels of apoptosis in *Dlx3*+/- placentas had previously been rescued using a strong antioxidant, TEMPOL [12]. Although apoptosis is required for normal placental development, elevated levels of apoptosis have been reported in preeclamptic and IUGR placentas [23–25]. Therefore, a reduction in placental apoptosis in mid-gestation may have beneficial effects in reducing risk of developing later placental complications.

Maternal choline supplementation did not alter maternal spiral artery remodeling in the Dlx3+/- mouse

In a preliminary histological examination of spiral artery remodeling, we did not find evidence that maternal choline supplementation altered arterial luminal area percentage in the *Dlx3*+/- mouse. This is in contrast to previous findings in wildtype mice, where 4X choline significantly increased luminal area percentage at E10.5, 12.5, 15.5, and 18.5 [11]. It is possible that the pathological phenotype of spiral artery remodeling in *Dlx3*+/- and *Dlx3*-/- placentas was too severe to be ameliorated by choline supplementation. However, due to the small size of our histology cohort this finding should be confirmed in a larger study.

Surprisingly, we did not detect any differences in labyrinth size, apoptosis levels, or spiral artery remodeling between *Dlx3*+/+ and *Dlx3*+/- placentas. We previously found that *Dlx3*+/- fetuses had higher expression of growth factor genes but comparable weights versus their *Dlx3*+/+ littermates when their mothers consumed a high quality pregnancy diet [14], suggesting that the *Dlx3*+/- placenta may activate compensatory mechanisms that lead to results similar to wildtype placentas for certain

outcomes.

Effects of maternal choline supplementation on placental angiogenic and inflammatory gene expression vary by gestational time point and fetal sex

Angiogenesis and inflammation have been shown to be dysregulated in pregnancy complications including preeclampsia, IUGR, and spontaneous abortion [1,26,27]. Because of this, we measured placental mRNA expression of major modulators of these processes to determine whether choline can modulate their placental expression in the *Dlx3*+/- mouse.

Overall trends seen included the tendency for pro-angiogenic and pro-inflammatory markers to be downregulated, and the 2X dose frequently having a larger effect than that of 4X. 2X maternal choline supplementation reduced expression of *Tnfα, Nfκb*, and *Il1b* at different gestational time points of pregnancy. Choline deficiency has been previously shown to lead to increased expression of pro-inflammatory cytokines in a placental trophoblast cell culture model [10] and maternal choline supplementation has been shown to reduce inflammatory response in pregnant rats challenged with lipopolysaccharide [28]. The results seen in the current study with a 2X choline dose provide additional support for the role of choline in mitigating inflammation. The lack of effect seen with the 4X treatment may reflect a dose response curve whereby maternal choline supplementation has an anti-inflammatory effect up to a certain concentration beyond which the effect is reduced. One possible explanation for this phenomenon could be an increase in production of trimethylamine N-oxide, a gut-derived metabolite produced from choline that can induce

inflammatory gene expression [29]. Alternatively, since we previously detected a strong growth-promoting effect of 4X choline supplementation in *Dlx3*+/- which subsequently resulted in compensatory mechanisms to reduce growth, these compensatory mechanisms may have counteracted the effects of choline in this group.

The soluble VEGF receptor endoglin (sEng) has been investigated as a predictor or clinical indicator of preeclampsia. It has been implicated in the pathological development of the disease as an anti-angiogenic factor that prevents VEGF from acting on target tissues [5]. Maternal choline supplementation at 2X or 4X lowered placental expression of endoglin at E10.5 and E15.5 in female placentas and E12.5 in males. These effects were mirrored by similar downregulations of *Vegfa*, which has been shown to be elevated or decreased in preeclamptic patients depending on the study [30–32]. It is possible that the larger labyrinth and placenta size at E10.5 resulted in a decreased need for placental angiogenesis, resulting in downregulation of pro-angiogenic factors. Expression of *Mmp14*, the matrix metalloproteinase that cleaves endoglin into its pathogenic soluble form [33], was also significantly lower at E15.5 in females but unexpectedly higher at E18.5 in males with 2X choline supplementation. A corresponding upregulation of *Pgf* and *Vegfa* expression was also detected in E18.5 2X choline males, which may have a beneficial role in preventing preterm birth as VEGF signaling in late gestation regulates timing of parturition [34,35].

The effects of maternal choline supplementation on placental gene expression were sex specific, as previously reported in wildtype mouse pregnancy [11]. Differential expression of angiogenic factors according to fetal sex has previously

been reported in both normal [36] and preeclamptic [37] human pregnancies. Although the mechanisms remain unclear, sex-specific differences in epigenetic regulation and hormone environment have been suggested to contribute [38,39]. These results add to the body of evidence demonstrating that fetal sex should be accounted for when examining placental and fetal outcomes during pregnancy.

Maternal choline supplementation increased placental acetylcholine in a non-dose-dependent manner only at E12.5

Because acetylcholine has been reported to play a role in angiogenic [22] and inflammatory [21] signaling in the placenta, we hypothesized that choline may be modulating gene expression by altering acetylcholine levels. Surprisingly, we did not detect an increase in placental acetylcholine concentrations in response to maternal choline supplementation at E10.5, E15.5 or E18.5. At E12.5, acetylcholine levels were higher in the 2X but not 4X choline group. Previously, we had shown that 2X maternal choline supplementation in healthy third trimester women resulted in increased acetylcholine concentrations in term placentas, and that 4X choline increased acetylcholine concentrations in the fetal brain of wildtype mice [40]. In the current study the 2X choline group at E12.5 was influenced by two outliers with extremely elevated acetylcholine levels, and therefore this finding should be confirmed in further studies.

Strengths and Limitations

The high degree of variability found in placentas from *Dlx3*+/- mothers resulted in difficulty achieving our planned statistical power levels. Because of this, our histological analyses are included as a hypothesis-generating examination of choline's effects in compromised pregnancies which should be confirmed in larger cohorts. We decided to control for the effect of fetal sex after determining that it had a strong effect many placental outcomes; however, our study was not originally powered to account for this. An additional limitation of the current study was the analyses of mRNA content only without concurrent analyses of secreted pro- and anti-angiogenic peptides. Our original design sought to examine mRNA expression levels as a guide to future studies examining secretion profiles of candidate factors. Our study had several considerable strengths, including the use of a standardized diet and two different supplemental doses of choline. Examining four gestational time points throughout pregnancy allows a more dynamic view of the changes that occur in the placenta. Using the mouse as a model of compromised pregnancy has several advantages, including the availability of genetic knockout models and the hemochorionic structure of the placenta which has similarities to the primate placenta; however, because of differences in vascular structure and gestational length, findings should be confirmed in larger mammals and humans in the future.

Conclusion

In conclusion, maternal choline supplementation modulated placental development and function in a mouse model of placental insufficiency, providing

evidence that choline may be a useful therapy during pregnancy to prevent or overcome development of placental disorders that impact fetal and maternal health. Decreases in pro-angiogenic and pro-inflammatory marker expression and placental apoptosis are promising findings that align with previous results showing similar effects of choline in different experimental models. Since many pregnant women do not currently meet the recommended intake levels of choline [41], further research is warranted to investigate beneficial effects of choline on human placental function. Overall, these data show that supplementing the maternal diet with choline in a mouse model of placental insufficiency reduces markers of placental dysfunction, and contribute to the body of literature demonstrating the benefits of choline during pregnancy.

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Supplementary Table 1. Primers for genotyping and RT-qPCR.

Gene	*Name*	*Reference Sequence*	*Primer Sequences*	*Annealing Temperature*
Dlx3	Distal-less homeobox 3	NC_000077.6	F: 5’ GTGAACGGCAAGCCCAAA 3’ R (Wild type allele): 5’ CTCTGTGACACGCCATACACAGTT 3’ R (Knockout allele): 5’ AAAGGCCCGGAGATGAGGAAGAG 3’	Touchdown from 63° to 49°
Sry	Sex determining region Y	NC_000087.7	F: 5’ TGGGACTGGTGACAATTGTC 3’ R: 5’ GAGTACAGGTGTGCAGCTCT 3’	60°C
Vegfa	Vascular endothelial growth factor A	NM_001025250.3	F: 5' CACTGGACCCTGGCTTTACT 3’ R: 5' ACTTGATCACTTCATGGGACTTCT 3’	63°C
Pgf (Plgf)	Placental growth factor	NM_008827.3	F: 5' TGTGCCGATAAAGACAGCCA 3' R: 5' TCGTCTCCAGAATAGGTCTGC 3'	63°C
Eng	Endoglin	NM_007932.2	F: 5' ATCAGTTTCCCGTCAGGCTC 3' R: 5' GTTCGATGGTGTTGGATGCC 3'	63°C
MMP14	Matrix metalloproteinase 14	NM_008608.4	F: 5' GCCCTCTGTCCCAGATAAGC 3' R: 5' TTGGTTATTCCTCACCCGCC 3’	63°C
NfκB	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	NM_008689.2	F: 5' AGCAACCAAAACAGAGGGGA 3' R: 5' TTTGCAGGCCCCACATAGTT 3’	60°C
Il1b	Interleukin 1 beta	NM_008361.4	F: 5' TGCCACCTTTTGACAGTGATG 3' R: 5' GCTCTTGTTGATGTGCTGCT 3’	60°C
Tnfα	Tumor necrosis factor alpha	NM_013693.3	F: 5’ AAGTTCCCAAATGGCCTCCC 3’ R: 5’ TGGTGGTTTGCTACGACGTG 3’	60°C

CHAPTER 3

MATERNAL CHOLINE SUPPLEMENTATION ALTERS VITAMIN B-12 STATUS MARKERS IN HUMAN AND MURINE PREGNANCY*

*King JH, Kwan ST(C), Bae S, Klatt KC, Yan J, Malysheva OV, Jiang X, Roberson MS, and Caudill MA. Maternal choline supplementation alters vitamin B-12 status markers in human and murine pregnancy. *To be submitted.*

Abstract

Background: Despite participation in overlapping metabolic pathways, the relationship between choline and vitamin B-12 has not been well characterized especially during pregnancy under conditions of controlled nutrient intake.

Objective: We sought to determine the effects of maternal choline supplementation on vitamin B-12 status markers in human pregnancy and in a mouse model of placental dysfunction. In humans, associations between common genetic variants in choline-metabolizing genes on vitamin B-12 status markers were also explored.

Methods: Pregnant women (n=26) consumed either 480 (approximately 1X the adequate intake) or 930 mg choline/day (approximately 2X the adequate intake) for 12 weeks while consuming a controlled diet. Wildtype and *Dlx3*+/- mice consumed either 1X, 2X or 4X choline diets and were sacrificed at gestational days 15.5 and 18.5. Serum vitamin B-12, methylmalonic acid (MMA), and homocysteine were measured in all samples; holotranscobalamin (in humans) and hepatic vitamin B-12 (in mice) were also measured.

Results: 2X maternal choline supplementation for 12 weeks in third trimester pregnant women resulted in higher levels of the active form of vitamin B-12, holotranscobalamin (~24%, P=0.01). Women with genetic variants in choline dehydrogenase (CHDH) and betaine-homocysteine S-methyltransferase (BHMT) had higher serum MMA concentrations (~31%, P=0.03) and lower holotranscobalamin

concentrations (~34%, P=0.03), respectively. A 4X dose of choline decreased serum homocysteine concentrations in wildtype mice and in those with placental insufficiency (~36% and ~43% respectively, P=0.006, 0.015).

Conclusion: Maternal choline supplementation and genetic variation in choline-metabolizing genes modulate vitamin B-12 status during pregnancy. A high dose of choline can lower homocysteine in mice with placental dysfunction. These findings support an interconnected relationship between choline and vitamin B-12 in pregnancy and warrant further investigation into functional consequences of these changes.

Introduction

The interconnected metabolic network referred to as one-carbon metabolism is essential for many vital biochemical processes including nucleotide synthesis, DNA methylation, and amino acid metabolism [1]. Demand for one-carbon (methyl) nutrients, including choline and vitamin B-12, is particularly high during pregnancy when cells of the placenta and fetus are undergoing rapid division [2].

The essential nutrient choline is a precursor for three metabolites that have crucial roles during pregnancy; the neurotransmitter acetylcholine, the membrane phospholipid phosphatidylcholine, and the methyl donor and osmolyte betaine [3]. Vitamin B-12 acts as a co-factor for two enzymes; methylmalonyl-CoA mutase, which converts methylmalonyl-CoA to succinyl-CoA, and methionine synthase, which remethylates homocysteine to form methionine. Choline and vitamin B12 are thus metabolically interrelated as they are both used to process homocysteine. Specifically, the remethylation of homocysteine to methionine is carried out by two enzymes: betaine-homocysteine S-methyltransferase (BHMT), which uses the choline metabolite betaine as a methyl donor, and methionine synthase (MTR), which uses methylcobalamin as a co-factor [1].

Notably, a few studies have reported associations between choline and vitamin B-12 status. Pregnant women deficient in vitamin B-12 have lower plasma choline concentrations [4], while patients receiving total parenteral nutrition exhibit an inverse relationship between plasma choline and methylmalonic acid concentrations [5]. Further, animal studies have reported a vitamin B-12-sparing effect of choline

supplementation [6,7]. However, less is known about the effects of supplemental choline on biomarkers of vitamin B-12 status during pregnancy.

Many pregnant women do not meet the adequate intake for choline of 450 mg/ day [8]. Additionally, several common genetic variants in choline-metabolizing genes can modulate choline metabolism and increase the requirement for this nutrient and folate, a related one-carbon B-vitamin [9–11]. These genes include *CHDH* (choline dehydrogenase), which synthesizes betaine from choline; *PCYT1* (phosphocholine cytidylyltransferase), the rate-limiting enzyme in phosphatidylcholine synthesis from exogenous choline; *PEMT* (phosphatidylethanolamine N-methyltransferase), which produces endogenous phosphatidylcholine and choline, and *BHMT*.

We sought to determine whether maternal choline supplementation or genetic variation in choline-metabolizing genes would alter vitamin B-12 status in pregnant women. In addition, because lower maternal vitamin B-12 status and higher homocysteine levels are risk factors for several placenta-derived pregnancy complications [12–16], we explored the relationship between maternal choline supplementation and vitamin B-12 status in a mouse model of placental insufficiency, the *Dlx3*+/- mouse, which shares several common features with placental pathologies in humans [17].

Materials and Methods

Human Feeding Study

Participants and study design

The human component of the present study included twenty-six healthy, third-trimester pregnant women who had participated in a 12-wk randomized choline intervention feeding study in 2009-2010 as described in Yan et al [18]. Eligible women (26-29 wk gestation, age 21-40) were randomly assigned to consume either 480 or 930 mg choline per day (380 mg/day from diet, 100 or 550 mg/day provided as supplemental choline) for 12 weeks. All meals and beverages (except water) were provided throughout the study and women consumed 1 meal per day at the study site throughout the work week. The vitamin B-12 content of the study diet was ~6 μg/day and participants also consumed a daily prenatal multivitamin containing 2.6 μg of vitamin B-12, for a total daily intake of ~8.6 μg/day [19], approximately 3 times the RDA for pregnant women. The study protocol was approved by the Institutional Review Board for Human Study Participant Use at Cornell University (Ithaca, NY, USA) and Cayuga Medical Center (Ithaca, NY, USA) and was registered at clinicaltrials.gov as NCT01127022. All participants provided informed consent.

Sample collection and processing

Blood and urine samples were obtained from participants at study baseline (week 0) and study-end (week 12) as previously described [18]. Serum was obtained from fasting (10 hr) blood samples, separated by clotting and centrifugation and stored

at -80°C until analysis. Urine samples from a 24-hour period were pooled and the total volume was recorded before storage at -80°C.

Biomarker measurements

Serum vitamin B-12, serum methylmalonic acid, serum and urinary homocysteine, and serum holotranscobalamin were measured as described in Bae et al [19]. Briefly, serum vitamin B-12 was measured with an automated chemiluminescence immunoassay using an Immulite 2000 instrument (Siemens Medical Solutions Diagnostics); serum MMA and homocysteine and urinary homocysteine were measured using gas chromatography-mass spectrometry; serum holotranscobalamin was measured by the Axis-Shield Active-B12 EIA (Axis-Shield Diagnostics) and urinary creatinine was measured with the Dimension Xpand Clinical Chemistry system (Siemens Healthcare Diagnostics).

Genotyping

PEMT rs7946, *CHDH* rs12676, *PCYT1* rs 939883 *and BHMT* rs3733890 SNPs were genotyped using DNA extracted from buffy coat with the DNeasy Blood and Tissue kit (Qiagen). Endpoint genotyping was performed using Applied Biosystems TaqMan Genotyping Master Mix and Assay Mix (Thermo Fisher) on a LightCycler 480 (Roche) as described previously [20].

Mice Feeding Study

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. *Dlx3*+/- mice were a generous gift from Dr. Maria Morasso (NIH/NIAMS). Wildtype mice of the same genetic background (non-swiss albino, NSA/CF-1) were purchased from Harlan (Indianapolis, IN). *Dlx3*+/- mice were genotyped using tail DNA with three-primer duplex PCR (Supplemental Table 1). Primers were designed to amplify a wildtype band, a knockout band, or both (indicating a heterozygote). Mice were housed in microisolator cages (Ancare) in an environmentally-controlled room (22-25°C and 70% humidity) with a 12-hour light-dark cycle. During breeding, mice were given *ad libitum* access to commercial rodent chow (Teklad) and water. NSA females were mated with NSA males, *Dlx3*+/- females were mated with *Dlx3*+/- males and their offspring were genotyped at time of weaning (3 weeks of age). NSA and *Dlx3* heterozygous pups were given *ad libitum* access to the 1X choline control diet, AIN-93G purified rodent diet, (Dyets #103345) containing 1.4g choline chloride/kg diet. NSA and *Dlx3*+/- female mice were randomized five days before mating with male mice of the same genotype to receive either the 1X choline control diet, the 2X choline diet containing 2.8g choline chloride/kg (Dyets #103346), or the 4X choline diet containing 5.6g choline chloride/kg (Dyets #103347). Embryonic (E) day 0.5 was designated by presence of a vaginal plug. Pregnant mice (n=7-9 per treatment, per

genotype) were sacrificed at 2 different gestational time points corresponding to late gestation; E15.5 and E18.5.

Sample Collection and Processing

Maternal blood was collected by cardiac puncture into microtainer collection tubes with clot activator and SST gel (Becton Dickinson), and was allowed to clot at room temperature for one hour. After centrifuging at 14,000 rpm for 6 minutes, the serum was dispensed into a separate tube and stored at -80°C. Maternal liver was removed and bisected; one half was immediately frozen in liquid nitrogen and stored at -80°C for metabolite analysis while the other half was preserved in RNA*later* for RNA extraction.

Quantitation of hepatic vitamin B-12 gene expression via real-time RT-PCR

RNA was extracted from livers fixed in RNA*later* using TRIzol reagent (Invitrogen). RNA concentration and quality were assessed with a NanoDrop ND-1000 instrument (Thermo), and samples with a A260/A280 ratio above 1.8 were used for quantification. Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega). Quantitative PCR was performed using SYBR® Green in a LightCycler480 (Roche). Primer sequences for the targeted genes (*Bhmt, Ahcy, Mtr, Mtrr, Mut*, and *Mat1a*) were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/, Supplemental Table 1). Reaction conditions were as follows: 95°C for 5 minutes, followed by 40 cycles with 15 seconds at 95°C, 30 seconds annealing (see Supplementary Table 1 for annealing

temperatures), and 30 seconds at 72°C. All PCR products were confirmed to be specific using melting curve analysis at the end of the amplification cycles. Fold changes were calculated by the ΔC_t method [21] normalized to the expression level of housekeeping gene *Tbp* (TATA box binding protein), which has previously been shown to be stable in response to different choline treatments [22].

Biomarker measurements

Serum homocysteine and methylmalonic acid were measured using a TSQ Quantum Access LC-MS system (Thermo Fisher Scientific), adapted from the methods of Turgeon et al. and Mineva et al. [23,24]. Serum (50 µL) was incubated with internal standards DL-homocystine-d_8 (CDN Isotopes) and methyl-d_3-malonic acid (Toronto Research Chemicals) and dithiothreitol followed by protein precipitation with 0.4% formic acid in acetonitrile. The sample was then centrifuged after which the supernatant was dried in a SpeedVac concentrator (Savant). After drying, the pellet was derivatized for 30 minutes at 60°C in n-butanol with 3N hydrochloric acid. After a second drying, the resulting pellet was resuspended in mobile phase (45% acetonitrile, 55% formic acid (0.1%) in H_2O). Extracts (10 µl) were injected into a Hypersil Gold C18 column (100mm x 2.1mm, 3 µm, Thermo Fisher) and separated by isocratic elution in 45% acetonitrile and 55% formic acid (0.1%) in H_2O at a flow rate of 400 µl/min. Monitored MRM transitions in positive mode were: m/z 192- 90, 118 for homocysteine; m/z 196- 94, 122 for homocysteine d_4; m/z 231- 119, 174 for MMA; m/z 234- 122, 178 for MMA d_3. Quantification was performed using XCalibur software (Thermo Fisher Scientific).

Serum vitamin B-12 was measured with an automated chemiluminescence immunoassay by Immulite 2000 according to the manufacturer's instructions (Siemens Medical Solutions Diagnostics).

Hepatic vitamin B-12 was measured using an UltiMate 3000 ultra-high performance LC (UHPLC) and Q Exactive Quadrupole-Orbitrap MS (Thermo Fisher Scientific). Extraction of vitamin B-12 from liver was adapted from the method of Oosterink et al. [25]. Briefly, ~0.05 g of liver tissue was weighed and homogenized in dithiothreitol, H_2O, and internal standard (Biotin-d_2 purchased from IsoSciences was used as internal standard as in Reuter et al. [26] since isotopically labelled cyanocobalamin is not widely available). The extracts were autoclaved at 121°C for 15 minutes in the presence of sodium cyanide to release the vitamin from tissue and convert all forms of vitamin B-12 to cyanocobalamin, which is the most stable form of vitamin B-12. Lipids were removed with hexane followed by centrifugation. Proteins were precipitated with 10% TCA. Following centrifugation, the supernatant was diluted with mobile phase (0.1% formic acid in water) before injecting (10 µl) into an XBridge C18 column (50mm x 2.1mm, 2.5 µm, Thermo Fisher). An elution gradient of 0.1% formic acid in H_2O and methanol at 0.2mL/min was run as follows: 0 min, 10% methanol; 4 min, 60% methanol; 10 min; 100% methanol; 13 min, 10% methanol. MS was operating in positive mode with following settings: sheath gas pressure was set at 40; auxillary gas 15; spray voltage 5kV; capillary temperature 380°C; S-lens RF level 40; heater temperature 100°C. MS2 mode was used for detection and quantification of cyanocobalamin, where resolution was set at 35,000; AGC target 5e4; isolation window 1 m/z; HCD 25. Inclusion list contained m/z 678.29

which represented the doubly charged cyanocobalamin molecular ion, m/z 790.34 for doubly charged adenosylcobalamin, and m/z 247.1 for biotin d_2. For quantification, the most abundant ion was used (m/z 147.09 for cyanocobalamin and m/z 229.1 for biotin d_2). Quantification was performed using XCalibur software (Thermo Fisher Scientific).

Statistical Analysis

For human outcomes, general linear models (GLM) were used for analysis; baseline values, choline treatment and genotypes were included in the model where specified. Due to limited numbers of homozygous variant individuals, women with either 1 or 2 copies of the variant allele were grouped for analysis except for rs 7946, where 0 and 1 copy were grouped due to a small number of women (n=2) without the variant. Age and BMI were investigated as potential confounders but were not retained as predictors ($P>0.1$ in all models). For murine outcomes, data were analyzed using general linear models (GLM) with choline treatment and litter size as independent variables. Partial correlations controlling for litter size were also tested among vitamin B-12 biomarkers and between gene expression fold changes and vitamin B-12 biomarkers. Partial correlations with $P<0.05$ are reported.

Data were assessed for normality by assessing the distribution of residuals. All human serum/urinary vitamin B-12 biomarkers were natural log transformed due to deviation from normality or homogeneity of variance and values are reported as backtransformed (geometric) means and 95% confidence intervals.

Corrections for multiple analyses were not performed due to the hypothesis-driven nature of the study and the relatively limited sample size. Data are presented as means ± SEM or back-transformed (geometric) means and 95% CIs. $P \leq 0.05$ was considered statistically significant and $0.05 < P < 0.10$ was considered to indicate trends. All analyses were performed using SPSS software, Version 23 (IBM).

Results

Vitamin B-12 status after choline supplementation in third trimester pregnant women

To determine whether a higher maternal choline intake would alter vitamin B-12 status in pregnant women, we analyzed status markers by choline treatment group, controlling for baseline concentrations of the vitamin B-12 metabolite. Baseline concentrations by choline treatment group are listed in Supplementary Table 2. At baseline, women in the 930 mg choline/day group had ~26% lower serum MMA levels (P=0.039) and tended to have lower serum homocysteine levels (P=0.053). Concentrations of serum vitamin B-12, serum holoTC, serum holoTC:vitamin B-12 ratio, and urinary homocysteine did not differ by treatment group at baseline (Supplementary Table 2).

At study-end, women in the 930 mg choline/day group had ~24% higher serum holotranscobalamin concentrations (P=0.01) and ~22% higher holoTC:vitamin B-12 ratio (P=0.03) versus women in the 480 mg/day group. Urinary homocysteine tended to be higher (~51%, P=0.09) in the 930 mg/day group. Serum vitamin B-12, serum MMA, and serum homocysteine did not differ by choline treatment (Table 1).

Table 1. Biomarkers of vitamin B-12 status at study end (week 12) by choline treatment in third trimester pregnant women with equivalent vitamin B-12 intakes under controlled feeding conditions. Data are derived from general linear models controlling for baseline status. Values are presented as backtransformed (geometric) means and 95% confidence intervals. * denotes a significance difference between treatment groups (P<0.05). + denotes a trend (P<0.1). n=12 per treatment group. holoTC, holotranscobalamin; MMA, methylmalonic acid.

	480 mg choline / day	**930 mg choline / day**	***P***
Serum vitamin B-12, pmol/L	428 (374, 492)	445 (385, 513)	0.70
Serum MMA, nmol/L	206 (182, 234)	197 (172, 225)	0.62
Serum holoTC, pmol/L	82 (73, 91)	101 (90, 113)	0.01*
Serum homocysteine, µmol/L	4.2 (3.8, 4.7)	4.4 (4.0, 5.0)	0.58
Serum holoTC:vitamin B-12 ratio	0.19 (0.17, 0.21)	0.24 (0.21, 0.27)	0.03*
Urinary homocysteine, µg/mg creatinine	3.2 (2.3, 4.5)	4.9 (3.4, 6.9)	0.09^{+}

Influence of genetic variation on vitamin B-12 status in third trimester pregnant women

To investigate whether genetic variants in choline-metabolizing genes (*BHMT, CHDH, PEMT, and PCYT1*) that modulate choline metabolism would influence vitamin B-12 status in pregnancy, we analyzed status markers by maternal genotype. After 12 weeks of controlled vitamin B-12 intake, women with 1 or 2 copies of the *CHDH* rs12676 variant had ~31% higher serum MMA levels compared to women without the variant (P=0.03, Table 2). Women with 2 copies of the *PEMT* rs7946 variant tended to have ~57% higher urinary homocysteine levels versus women with 0 or 1 copy (P=0.09). Women with 1 or 2 copies of the *BHMT* rs3733890 variant had ~34% lower concentrations of serum holotranscobalamin (P=0.03). The *PCYT1* rs939883 variant did not significantly affect any markers of vitamin B-12 status.

Table 2. Biomarkers of vitamin B-12 status by maternal genotype in third trimester pregnant women after 12 weeks of equivalent vitamin B-12 intakes under controlled feeding conditions. Data are derived from general linear models controlling for choline treatment. Values are presented as backtransformed (geometric) means and 95% confidence intervals. * denotes a significance difference between genotypes (P<0.05). + denotes a trend (P<0.1). BHMT, Betaine-homocysteine S-methyltransferase 1; CHDH, choline dehydrogenase; PCYT1, phosphocholine cytidylyltransferase; PEMT, phosphatidylethanolamine N-methyltransferase; holoTC, holotranscobalamin; MMA, methylmalonic acid.

		Serum vitamin B-12, pmol/L	Serum MMA, nmol/L	Serum holoTC, pmol/L	Serum homocysteine µmol/L	Serum holoTC:vitamin B-12 ratio	Urinary homocysteine, µg/mg creatinine
CHDH rs12676	**WT**	459 (390, 541)	176 (150, 208)	97 (78, 122)	4.4 (3.9, 5.0)	0.21(0.17, 0.27)	3.8 (2.6, 5.5)
	Variant	406 (339, 486)	232 (195, 276)	84 (65, 107)	4.3 (3.7, 4.9)	0.21 (0.17, 0.27)	4.3 (2.8, 6.6)
	P	0.3	0.03*	0.36	0.74	0.99	0.65
PEMT rs7946	**WT**	423 (345, 520)	214 (177, 258)	94 (73, 123)	4.4 (3.9, 5.0)	0.24 (0.18, 0.31)	3.2 (2.2, 4.6)
	Variant	441 (378, 515)	189 (158, 226)	88 (71, 110)	4.3 (3.8, 4.9)	0.20 (0.16, 0.24)	5.0 (3.4, 7.2)
	P	0.74	0.34	0.69	0.75	0.26	0.09+
PCYT1 rs939883	**WT**	401 (328, 491)	225 (184, 277)	78 (60, 103)	4.3 (3.7, 5.0)	0.19 (0.15, 0.25)	4.4 (2.7, 7.0)
	Variant	457 (390, 535)	186 (159, 219)	100 (81, 123)	4.4 (3.9, 4.9)	0.23 (0.19, 0.28)	3.8 (2.6, 5.4)
	P	0.32	0.15	0.17	0.84	0.31	0.61
BHMT rs3733890	**WT**	460 (391, 540)	191 (162, 225)	103 (84, 127)	4.5 (3.9, 5.1)	0.23 (0.18, 0.29)	4.2 (2.9, 6.2)
	Variant	367 (286, 472)	230 (185, 287)	68 (50, 93)	4.4 (3.7, 5.2)	0.19 (0.13, 0.27)	2.8 (1.7, 4.5)
	P	0.13	0.17	0.03*	0.94	0.38	0.16

Vitamin B-12 status after choline supplementation in wildtype and *Dlx3*+/- mouse pregnancy

To compare the effects of 2X and 4X choline supplementation, and determine whether placental insufficiency would influence this effect, we measured vitamin B-12 biomarkers in pregnant mice fed either 1X control, 2X choline, or 4X choline diets.

Serum homocysteine

At E15.5, *Dlx3*+/- 4X choline dams had ~37% lower concentrations of serum homocysteine versus 1X controls (P=0.015); no differences were detected between 2X and 4X or 1X and 2X choline groups (P>0.05). Wildtype dams did not differ by choline treatment group at E15.5. At E18.5, both wildtype and *Dlx3*+/- 4X choline dams had lower homocysteine concentrations versus 1X choline (~36% and ~43% respectively, P=0.006, 0.015) (Figure 1A); no differences were detected between 2X and 4X or 1X and 2X choline groups. Serum homocysteine concentrations did not differ between wildtype and Dlx3+/- dams at either gestational time point (Fig. 1C).

Serum methylmalonic acid

At E15.5, 4X choline dams of both genotypes had higher serum MMA levels versus 2X choline dams (~18% and ~28%, respectively, P=0.039, 0.037). Neither choline treatment group differed versus 1X controls (P<0.05). At E18.5, no differences in MMA concentration were detected by choline treatment (Fig. 1B). *Dlx3*+/- dams had ~19% higher serum concentrations of MMA versus wildtypes at E18.5 (P=0.019) but did not differ from wildtypes at E15.5 (Fig. 1C).

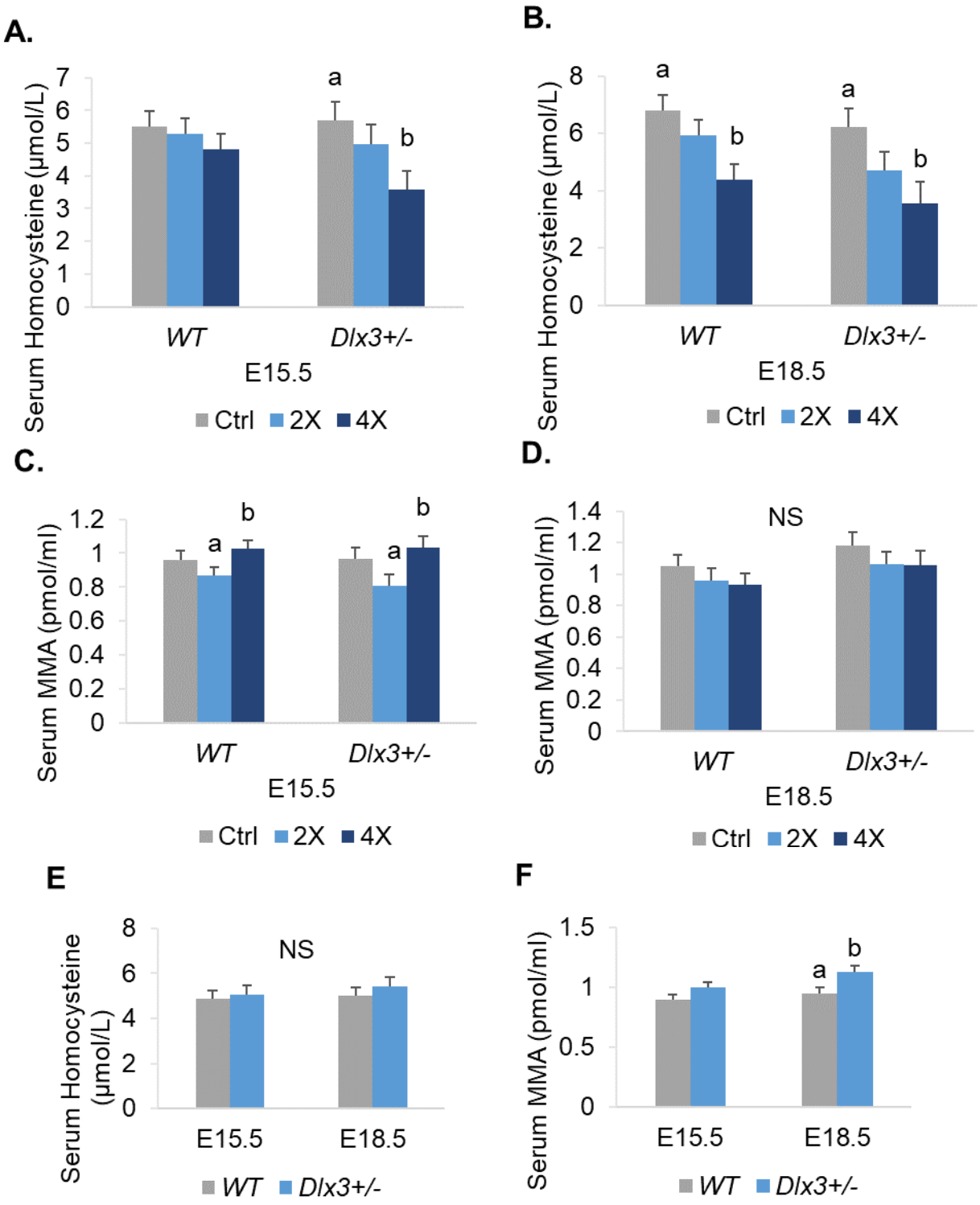

Figure 1. Functional biomarkers of vitamin B-12 status in pregnant wildtype (WT) and *Dlx3*+/- mice at gestational days 15.5 and 18.5 by choline treatment (control, 2X, or 4X). Serum homocysteine at (A) E15.5 and (B) E18.5. Serum MMA at (C) E15.5 and (D) E18.5. Serum homocysteine (E) and MMA (F) at E15.5 and E18.5 by dam genotype. n=7-9 per treatment, per time point. Differing letters denote $P<0.05$.

Serum vitamin B-12

At E15.5, 2X choline wildtype dams had ~33% lower serum vitamin B-12 versus 1X controls (P=0.038). 4X choline dams did not differ versus 1X or 2X choline dams (P>0.05). Serum vitamin B-12 did not differ by treatment in wildtype dams at E18.5 or at either time point in *Dlx3*+/- dams (Fig. 2A). There was an extremely strong effect of litter size on serum vitamin B-12 (P<0.001). With adjustment for litter size, *Dlx3*+/- dams had ~38% lower serum vitamin B-12 (P=0.011) versus wildtype dams (Fig. 2C). Without adjusting for litter size, serum vitamin B-12 in wildtype and *Dlx3*+/- dams did not differ. We confirmed the well-established inverse relationship between MMA and serum vitamin B-12 in our mouse cohort; there was a moderate, negative partial correlation between these biomarkers (r=-0.471, P=0.001).

Hepatic vitamin B-12

Hepatic vitamin B-12 concentrations did not differ by choline treatment group or dam genotype at either gestational time point (Fig. 2B, C). As expected, a moderate positive partial correlation between serum vitamin B-12 and hepatic vitamin B-12 at E18.5 (r=0.330, P=0.029) as well as a moderate negative partial correlation between serum MMA and hepatic vitamin B-12 (r=-0.381, P=0.011).

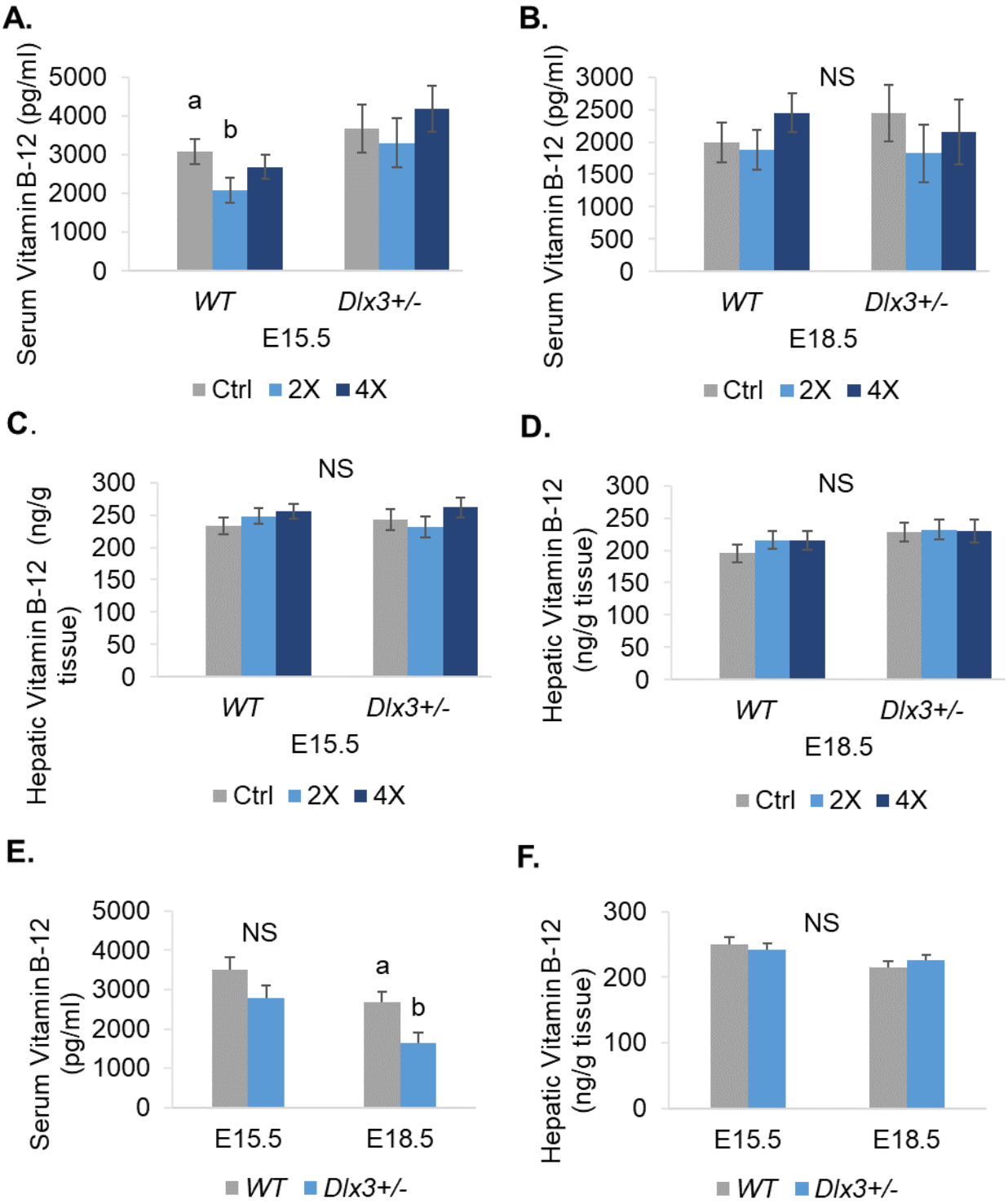

Figure 2. Serum and hepatic concentrations of vitamin B-12 in pregnant wildtype (WT) and *Dlx3*+/- mice at gestational days 15.5 and 18.5 by choline treatment (control, 2X, or 4X). Serum vitamin B-12 at (A) E15.5 and (B) E18.5. Hepatic vitamin B-12 at (C) E15.5 and (D). E18.5. Serum (E) and hepatic (F) vitamin B-12 at E15.5 and E18.5 by dam genotype. n=7-9 per treatment, per time point. Differing letters denotes $P<0.05$.

Maternal hepatic gene expression after choline supplementation in wildtype and *Dlx3*+/- mouse pregnancy

To determine whether changes in hepatic gene expression were responsible for changes in vitamin B-12 biomarker status, we measured the expression of genes of the methionine cycle (*Ahcy*, *Bhmt*, *Mtr*, *Mtrr*, and *Mat1a*) as well as *Mut*, which metabolizes MMA.

There were no differences in mRNA transcript abundance between choline treatment groups in either dam genotype group at either time point (data not shown). Compared to wildtype dams, *Dlx3*+/- dams had lower expression of *Ahcy* at E18.5 (~26%, P=0.035) and tended to have lower expression of *Mut* (~27%, P=0.095) at E15.5 (data not shown).

To determine whether hepatic transcript abundance mirrored serum status, correlations between transcript abundance and biomarkers of vitamin B-12 were explored. At 18.5, there were significant positive partial correlations of *Ahcy* expression with serum vitamin B-12 (r=0.356, P=0.024) and hepatic vitamin B-12 (r=0.334, P=0.035). In addition, *Bhmt* expression was positively correlated with serum vitamin B-12 at E18.5 (r=0.408, P=0.009) and *Mat1a* was positively correlated with hepatic vitamin B-12 (r=0.317, P=0.044).

Discussion

In this study, we show for the first time that maternal choline supplementation during pregnancy modifies markers of vitamin B-12 status in both mice and humans. Additionally, we report the effects of choline-related single nucleotide polymorphisms on vitamin B-12 status in third trimester pregnant women.

Maternal choline supplementation alters markers of vitamin B-12 status in third trimester pregnant women

A higher maternal choline intake (~2X the adequate intake level) resulted in higher serum holotranscobalamin and a higher ratio of serum holotranscobalamin to vitamin B-12. Holotranscobalamin is the active form of vitamin B-12 and its ratio to vitamin B-12 in serum has been reported to reflect tissue availability [27]. As such, choline supplementation may provide increased supply of vitamin B-12 for the mother and fetus. A tendency toward higher urinary excretion of homocysteine in the 2X choline group was also observed. This may reflect a greater use of choline-derived S-adenosylmethionine (SAM) in cellular methylation reactions which yield S-adenosylhomocysteine (SAH) and homocysteine. Additionally, this may result from a higher level of PEMT activity in response to choline supplementation, since this reaction produces 3 molecules of S-adenosylhomocysteine.

Studies investigating vitamin B-12 status in pregnant women have found significant rates of insufficiency that increase throughout gestation, with a recent meta-analysis estimating third trimester insufficiency at 29% of pregnant women [28]. Because vitamin B-12 is found solely in animal source foods, vegetarian and vegan

pregnant women may be at higher risk of inadequacy of this nutrient [29]. These findings collectively suggest that a higher intake of choline or its metabolite betaine, which is prevalent in plant foods, may be an additional strategy to improve vitamin B-12 status during pregnancy.

Genetic variants in choline-metabolizing enzymes alter vitamin B-12 status in third trimester pregnant women

Genetic variants in choline-metabolizing genes have previously been shown to modulate choline metabolism and alter susceptibility to choline deficiency [10,11]. However, their influence on vitamin B12 status has not been investigated. We found that the *CHDH* rs12676 variant, which can increase susceptibility of choline deficiency [10], led to higher levels of serum MMA, a functional indicator of vitamin B-12 status. CHDH is responsible for producing betaine from choline; thus a reduction in activity of this enzyme may reduce the amount of betaine available for remethylation of homocysteine. Consequently, more vitamin B-12 may be needed for methionine synthase activity, at the expense of MMA metabolism. Additionally, women with 1 or 2 copies of the *BHMT* rs3733890 variant had lower serum holoTC concentrations. This variant has been reported to increase BHMT affinity for betaine [30] and decrease turnover of choline to betaine [11]. Since pregnancy itself is another factor that decreases the partitioning of choline for betaine synthesis [31], it is possible that the partitioning of less choline towards betaine results in an inadequate betaine supply for homocysteine remethylation during this reproductive state, thereby increasing vitamin B-12 use for methionine synthase activity and reducing circulating

serum holoTC.

Maternal choline supplementation alters markers of vitamin B-12 status in pregnant wildtype mice and mice with placental insufficiency

Because low vitamin B-12 status has been associated with placenta-derived pregnancy complications, we sought to determine whether the effect of maternal choline supplementation on vitamin B-12 status would differ in mice with placental insufficiency. Additionally, we sought to compare the effects of a higher 4X choline dose to that of a 2X dose.

Maternal choline supplementation at 4X the recommended intake level reduced serum homocysteine concentrations in wildtype mice at E18.5 and *Dlx3*+/- mice at E15.5 and E18.5. A 2X choline dose did not result in lower serum homocysteine concentrations. Notably, given that the mice in our study were not fasted at time of blood collection, the magnitude of the reduction we detected in the 4X group was striking and suggests that choline may modulate postprandial homocysteine response at high doses.

Surprisingly, we observed slightly (but significantly) higher serum MMA in the 4X choline group relative to the 2X group. However, serum MMA was quite low (~1 nmol/L) in all choline treatment groups. Previous studies in non-pregnant wildtype mice also observed negligible levels of MMA, whereas mice with methylmalonic acidemia displayed concentrations that were 500x higher [32,33]. Therefore, it is unlikely that the slight differences between 2X and 4X choline groups would have clinical significance. Nevertheless, despite the low levels we were still able to detect

negative partial correlations between MMA and serum and hepatic vitamin B-12, consistent with the expected relationship between these biomarkers.

We did not detect an effect of maternal choline supplementation on serum or hepatic levels of vitamin B-12 at E18.5; whereas at E15.5 2X choline dams had lower serum vitamin B-12 versus 1X controls. The placenta accumulates vitamin B-12; concentrations are higher than those in the maternal circulation [34]. Therefore, the lower serum vitamin B-12 in the 2X choline group at E15.5 in wildtype mice, and the lack of differences in hepatic vitamin B-12 levels, may result from greater partitioning to the placenta and fetus under conditions of greater availability.

Surprisingly, we did not detect any differences by choline treatment in expression of *Mut* or genes of the methionine cycle (*Mat1a*, *Ahcy*, *Bhmt*, *Mtr*, *Mtrr*), despite choline's effects on status markers. It is possible that the changes we observed in response to choline are regulated post-transcriptionally, reflect changes in non-hepatic tissue metabolism and gene transcription, or that our sample size was not large enough to detect differences in these outcomes. However, the finding that *Ahcy*, *Bhmt*, and *Mat1a* were positively correlated with markers of vitamin B-12 status may indicate that higher hepatic methionine cycle activity irrespective of choline treatment influenced serum vitamin B-12 biomarkers.

Mice with placental insufficiency have higher serum MMA and lower serum vitamin B-12 in late pregnancy

Dlx3+/- mice displayed differences in vitamin B-12 status compared with wildtype mice at E18.5, suggesting possible effects of the placental insufficiency

phenotype on one-carbon metabolism. Lower serum vitamin B-12 combined with higher MMA levels both suggest reduced vitamin B-12 availability, which may result from wide-spread transcriptional changes induced by *Dlx3* haploinsufficiency [35].

Unexpectedly, we did not detect higher homocysteine levels in mice with placental insufficiency. Additionally, *Dlx3*+/- mice had lower hepatic expression of *Ahcy* whereas under conditions of hyperhomocysteinemia this enzyme increases in activity to carry out the reverse reaction (conversion of homocysteine to S-adenosylhomocysteine) [36]. This suggests that elevated homocysteine, which is commonly observed in women with preeclampsia, is not a feature in the placental etiology of the *Dlx3*+/- placental insufficiency phenotype.

Strengths and Limitations

Strengths of the current study include the controlled feeding study design which allowed us to assess the effect of maternal choline intake on vitamin B-12 status without confounding by vitamin B-12 intake. However, the fact that all women consumed adequate intake of vitamin B-12 and achieved vitamin B-12 sufficiency means that we cannot extrapolate the effect of choline supplementation to vitamin B-12 deficient individuals. The greatest effect of choline supplementation in humans was on holotranscobalamin status, for which there is currently no available assay for use with murine samples. However; assessing effects in mouse pregnancy in addition to that of humans allowed us to measure hepatic levels and use a dose of choline not previously used in human pregnancy, as well as examine multiple time points of pregnancy. The use of the *Dlx3*+/- model of placental insufficiency further allowed us

to explore the relationship between choline and vitamin B-12 in a compromised gestational environment.

Conclusions

In conclusion, 2X maternal choline supplementation for 12 weeks in third trimester pregnant women resulted in higher levels of the active form of vitamin B-12, holotranscobalamin, which would be expected to increase vitamin B-12 supply to tissues including the placenta and fetus. Additionally, genetic variants in several choline-metabolizing genes were found to impact vitamin B-12 status in pregnant women. A 4X dose of choline decreased homocysteine levels in wildtype mice and those with placental insufficiency. These findings support an interconnected relationship between choline and vitamin B-12 and a contribution of choline to the methionine cycle even in folate and vitamin B-12 replete individuals. As such, future observational and intervention studies may need to consider dietary choline and betaine intakes when assessing vitamin B-12 status. Further research is warranted to verify these effects in larger populations and those with vitamin B-12 deficiency.

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Supplementary Table 1. Primers for genotyping and RT-qPCR.

Gene	*Name*	*Reference Sequence*	*Primer Sequences*	*Annealing Temperature*
Dlx3	Distal-less homeobox 3	NC_000077.6	F: 5’ GTGAACGGCAAGCCCAAA 3’ R (Wild type allele): 5’ CTCTGTGACACGCCATACACAGTT 3’ R (Knockout allele): 5’ AAAGGCCCGGAGATGAGGAAGAG 3’	Touchdown from 63° to 49°
Ahcy	Adenosylhomocysteinase	NM_016661	F: 5’ CCCTACAAAGTCGCGGACATC 3’ R: 5’ GAGGCTGAGTACATCTCCCG 3’	63°C
Bhmt	Betaine-homocysteine S-methyltransferase 1	NM_016668	F: 5' TTAGAACGCTTAAATGCCGGAG 3’ R: 5' GATGAAGCTGACGAACTGCCT 3’	63°C
Mat1a	Methionine adenosyltransferase 1 alpha	NM_133653	F: 5' GTGCTGGATGCTCACCTCAAG 3' R: 5' CCACCCGCTGGTAATCAACC 3'	63°C
Mtr	5-methyltetrahydrofolate-homocysteine methyltransferase	NM_001081128	F: 5' GCTGGTGGACTACATTGACTGGA 3' R: 5' TTCTGGCTTCTTCACCTACTGC 3'	63°C
Mtrr	5-Methyltetrahydrofolate-homocysteine methyltransferase reductase	NM_172480	F: 5' GGACAGGCAAAGGCCATAG 3' R: 5' ACCCGTGGTAGATACAACCAT 3’	63°C
Mut	Methylmalonyl-CoA mutase	NM_008650	F: 5' TTTTTGCTATCGCCCCATTACC 3' R: 5' CCTCTGGGTTTTTGCCTTTCAG 3’	63°C

Supplementary Table 2. Vitamin B-12 status markers at baseline (week 0) of pregnant women receiving 480 mg or 930 mg choline per day. Differences between groups analyzed using general linear models. Values represent geometric means and 95% confidence intervals. * denotes P<0.05. + denote P<0.1.

	480 mg choline / day	**930 mg choline / day**	***P***
Serum vitamin B-12, pmol/L	436 (373, 509)	432 (370, 505)	0.95
Serum MMA, nmol/L	156 (127, 191)	115 (94, 141)	0.039*
Serum holoTC, pmol/L	75 (60, 94)	78 (62, 97)	0.82
Serum homocysteine, µmol/L	4.1 (3.6, 4.6)	3.5 (3.1, 3.9)	0.053+
Serum holoTC:vitamin B-12 ratio	0.17 (0.14, 0.22)	0.18 (0.14, 0.23)	0.85
Urinary homocysteine, µg/mg creatinine	3.0 (2.1, 4.2)	3.8 (2.7, 5.4)	0.33